Unravelling the Molecular and Cellular Mechanisms of Fetal B Cell Lymphopoiesis to Help Understand Childhood Leukaemia.

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To Alexander Thatte

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ABSTRACT

Unravelling the Molecular and Cellular Mechanisms of Fetal B Cell Lymphopoiesis to Help Understand Childhood Leukaemia.

Childhood Leukaemia accounts for nearly one in three of all childhood cancers, with approximately 400 cases diagnosed in the UK every year. Initial genetic lesions in paediatric and infant B cell leukaemias occur in utero in fetal haematopoietic progenitors. Despite the importance of understanding fetal B cell lymphopoiesis in relation to the aetiology of leukaemia, fetal B cell development remains poorly characterised. We hypothesised that by understanding and characterising murine fetal B cell lymphopoiesis, we would gain insights into the development of childhood leukaemias. In chapter III of this thesis I show that the first immune restricted cells with B cell potential in the mouse emerge in the E9.5 vascular plexus of the yolk sac. Subsequently, in chapter IV, I use a *Mb1*-cre fate mapping approach to show that progenitors which are entirely restricted to the B cell lineage can be detected as early as E12.5 in the fetal liver (FL), prior to the cell surface expression of CD19. In chapter V, I investigate B cell progenitors in the E14.5 FL, applying an advanced B cell staging originally developed for the adult bone marrow, modified to include the key lymphoid cytokine receptors IL-7Rα, KIT and FLT3. I identify and functionally and molecularly characterise a rare $CD19^+$ B cell progenitor that expresses FLT3 and peaks in frequency in late gestation embryos. I further show that $FLT3^+$ $CD19^+$ adult Pro B cells demonstrate an increased engraftment when transplanted into recipient mice compared to $CD19^+FLT3^-$ Pro B cells. Finally in chapter VI, I apply our new understanding of fetal B cell lymphopoiesis to characterise B cell progenitors in a mouse that expresses the TEL-AML1 translocation, an oncogene associated with 25% of paediatric B cell leukaemias. We find that the novel $FLT3^+$ $CD19^+$ ProB cell progenitor identified here is expanded in the fetal liver of E14.5 TEL-AML1 embryos, giving a new developmental insight into the functional impact of this oncogene.

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ABBREVIATIONS

Commonly used abbreviations

AID: Activation induced cytidine deaminase
AGM: Aorta-Gonad-Mesonephros
AML: Acute Myeloid Leukaemia
ALL: Acute Lymphoid Leukaemia
BCR: B Cell Receptor
BCP: B Cell Progenitor
BLP: A subset of the CLP that is primed towards B cell development
BM: Bone Marrow
CLP: Common lymphoid progenitor
CFU: colony forming unit
CMP: Common myeloid progenitor
CSR: Class Switch Recombination
CSC: Cancer Stem Cell
dHSC: Definitive Haematopoietic Stem Cells
E: Embryonic Day
ETP: Early thymic progenitor
FACS: Fluorescently Activated Cell Sorting
FCS: Foetal calf serum
FL: Fetal Liver
FLT3: Fms-like tyrosine kinase 3
FLT3L: Fms-like tyrosine kinase 3 ligand
FMO: Fluorescent Minus One
GMP: Granulocyte macrophage progenitor
HSC: Haematopoietic stem cell
Ig: Immunoglobulin
IL: interleukin
Lin: Lineage
LMPP: lymphoid primed multi potent progenitor
LSK: Lineage$^-$ SCA1$^+$ cKIT$^+$
MDS: Myelodysplastic Syndrome
ME: Megakaryocyte Erythroid Progenitor
Mk: Megakaryocyte
MkE: Megakaryocyte erythroid
MNC: Mononuclear Cells
MPP: Multi potent progenitor
MYP: *Mb1*-YFP B cell progenitor, proposed to be the first restricted B cell progenitor in the mouse embryo (CD45$^+$B220$^+$CD19$^-$*Mb1*-YFP$^+$KIT$^+$IL-7Rα$^+$FLT3$^+$)
MZ: Marginal Zone
NHEJ: Non homologous end joining
NK: Natural killer
NSG: An immuno-deficient mouse strain deficient for B cells, T cells and NK cells (NOD.Cg-Prkdcscid Il2 rgtm1Wjl/SzJ)

PAS: Para Aortic Splanchnopleura
PB: Peripheral Blood
PC: Peritoneal Cavity
Rag: Recombination activation gene
RSS: Recombination signal sequences
SLC: Surrogate Light Chain
SLAM: Signalling lymphocyte activation molecule
SCF: Stem cell factor
sp: Somite pair
TDT: Terminal Deoxynucleotidyl Transferase
TF: Transcription Factor
TSLP: Thymic stromal derived lymphopoietin
YFP: yellow fluorescent protein
YS: Yolk sac

Mouse Strain Abbreviations

ΔPax5: vavCre$^{tg/wt}$ *Pax5*$^{fl/f}$
Mb1-YFP: Mb1-cre $^{tg/wt}$ R26YFP$^{tg/tg}$
NSG: NOD.Cg-Prkdcscid Il2 rgtm1Wjl/SzJ
R26YFP: Rosa26 (R26R)$^{eYFP/eYFP}$
Rag1-GFP: *Rag1*-GFP$^{tg/wt}$
TA: *Etv6*$^{Runx1/wt}$

Haematopoietic Progenitor Populations

Abbreviation	Cell Surface Markers
Pre Pro B	Lin$^-$B220$^+$CD43$^+$CD19$^-$CD24$^-$AA4.1$^+$IL-7Rα$^+$KIT$^+$FLT3$^+$
Pro B FLT3$^+$	Lin$^-$B220$^+$CD43$^+$CD19$^+$CD24^{int}AA4.1$^+$IL-7Rα$^+$KIT$^+$FLT3$^+$
Pro B FLT3$^-$	Lin$^-$B220$^+$CD43$^+$CD19$^+$CD24^{int}AA4.1$^+$IL-7Rα$^+$KIT$^+$FLT3$^-$
Pro B KIT$^-$	Lin$^-$B220$^+$CD43$^+$CD19$^+$CD24^{int}AA4.1$^+$IL-7Rα$^+$KIT$^-$FLT3$^-$
MYP (*Mb1*-YFP Progenitor)	CD45$^+$B220$^+$CD19$^-$*Mb1*-YFP$^+$KIT$^+$IL-7Rα$^+$FLT3$^+$
LMPP	Lin$^-$SCA1$^+$KIT$^+$FLT3^{high}
HSC	Lin$^-$SCA1$^+$KIT$^+$FLT3$^-$CD48$^-$CD150$^+$
CLP	Lin$^-$FLT3$^+$IL-7Rα$^+$CD19$^-$B220$^-$KIT$^+$SCA1$^+$LY6D$^-$
BLP	Lin$^-$FLT3$^+$IL-7Rα$^+$CD19$^-$B220$^-$KIT$^+$SCA1$^+$LY6D$^+$

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CONTRIBUTIONS OF OTHERS

Chapter III

The experiments conducted in this chapter were done in relation to a project led by Charlotta Boiers (Boiers et al., 2013). I was involved in discussions and experiments focusing on when the first immune restricted progenitor emerges in the mouse embryo, due to its relevance to my interest in the emergence of the first B cell restricted progenitor. I led all the experiments included in this thesis, but the need for specialist techniques in early embryo dissection and imaging, meant when necessary experiments were performed in collaboration with Marella de Bruijn (dissection), Emanuele Azzoni (dissection and imaging) and Isabelle Godin (dissection).

Chapter IV

Stephen Loughran (SL), a previous post doctoral researcher in the Jacobsen lab, played an active role in the initiating the concept of *Mb1*-cre fate mapping to trace the earliest B lineage progenitors. The data included in figure 3C was performed by SL. The fluidigm data was performed in conjunction with SL. I performed all other experiments and analysed the data.

Chapter V

The data included in figure 7 was kindly collected by Alya Zriwil, in Lund, Sweden, due to availability of the mouse model. I analysed the data in Oxford.

The E14.5 Pro B single cell fluidigm (figure 8) was performed with the help of Tiago Luis.

RNA sequencing

After I sorted cells for global gene expression analysis, preparation of cDNA libraries was performed in the WIMM 'PCR product free' clean room facility by trained facilitators (Debbie Atkinson, Laura Stenson, Petter Woll, Tiphaine Bouriez Jones, Neil Ashley). RNA sequencing was performed at the Karolinska Institute, Stockholm. Bioinformatic analysis was performed with the assistance of the WIMM CBRG unit and Dr Nikolas Barkas.

CHAPTER I: INTRODUCTION

INTRODUCTION

The History of Haematopoietic Research

An adult human generates approximately 10^{12} blood cells every day, a number that comprises about 3% of all somatic cells in the body. How this process is regulated, sustained, and destabilised in malignancy underpins the field of haematopoietic cell biology. Haematopoietic stem cells (HSCs) are functionally defined as cells that are capable of in vivo long-term multi-lineage reconstitution when injected into gamma irradiated or cytotoxic drug treated adult recipients (Lemischka, 1991). The development of mature lineage-restricted progenitors from self renewing, multi-potent HSCs provides the paradigm for stem cell biology.

During World War Two, widespread bone marrow failure following atomic radiation accelerated stem cell research (Abdelhaleem, 2007), leading to the discovery that lethally irradiated mice could survive after receipt of bone marrow from a non-irradiated donor(Lorenz et al., 1951). Pioneering experiments demonstrated that after injection of bone marrow cells at limiting dilution into irradiated recipients, splenic nodules (Colony Forming Units-Spleen, CFU-S) developed. Consisting of multi-lineage clones (Till and Mc, 1961) these results demonstrated that bone marrow contains multi-potent cells.

During the 1970s and 1980s, the development of in vitro cell based assays led to the characterisation of the lineage potential of multi-potent cells and their

downstream progenitors and the hypothesis that blood cells develop in a hierarchical manner from the haematopoietic stem cell.

Purification of HSCs and Progenitors

Originally white blood cells were crudely purified based on their physical properties using techniques such as velocity sedimentation, with limited purity. A major break through was the development and application of flow cytometry in the 1960s and 1970s, which allowed cells to be separated based on the binding of fluorescently conjugated antibodies to cell surface markers (Shapiro, 2003). Although most cell surface markers are widely expressed, the combination of multiple markers permits the definition of highly purified cell populations that can be isolated and sorted. Flow cytometry is now a routine tool to define cell populations based on cell surface markers.

Cells that are negative for mature lineage markers and express SCA-1 and KIT (LSK), low levels of THY1 and are negative for CD34 have multi-lineage repopulation capacity (Morrison and Weissman, 1994; Osawa et al., 1996; Spangrude and Brooks, 1992). However, only 20% of these LSK $THY1^{lo}$ $CD34^{-}$ cells demonstrate long-term multi-lineage reconstitution. Accordingly the phenotypic definition of HSCs has been further refined by removing cells that express FLT3 (Adolfsson et al., 2001; Christensen and Weissman, 2001) with further subcategorising based on expression of the SLAM markers CD150 and CD48 (Kiel et al., 2005). Long term HSCs are now defined as LSK $FLT3^{-}$ $CD34^{-}$ $CD150^{+}$ $CD48^{-}$. Single cell transplantations and sequencing have allowed the functional and molecular characterisation of HSCs at high resolution (Kim et al.,

2006; Mansson et al., 2007; Osawa et al., 1996). Recently, the transcriptome and genome from a single haematopoietic cell have been sequenced in parallel (Macaulay et al., 2015) providing the opportunity for a further wealth of molecular data. However, as cell surface markers may be functionally irrelevant and not homogeneously expressed, HSC purity could remain as low as 50% (Purton and Scadden, 2007), with the only reliable assay being the demonstration of long term multi-lineage reconstitution in irradiated recipients. This lack of purity must be considered when interpreting the results of molecular and in vitro assays. Recently integration of gene expression and functional assays at the single cell level has helped link molecular signatures to functional activity, increasing the purity of true long term stem cells to around 70% and giving an insight into the key molecules that are associated with stem cell self-renewal (Wilson et al., 2015).

Human haematopoietic stem cells are enriched within the $CD34^{+}CD38^{-}CD90^{+}$ bone marrow population (Baum et al., 1992; Bhatia et al., 1997; Civin et al., 1984), although their purity is significantly lower than in mice and their population much more poorly defined. Furthermore, stem cell potential has additionally been reported in the $CD34^{-}$ fraction (Anjos-Afonso et al., 2013; Bhatia et al., 1998)

The Haematopoietic Tree

While it is undisputed that HSCs reside at the top of the haematopoietic hierarchy (Osawa et al., 1996), the exact 'road map' of cell differentiation into

lineage restricted, committed progenitors is being continuously 'ironed out'. Initially, HSCs lose the ability to self renew, but retain multi-potency, generating a 'multipotent progenitor' (MPP). The identification of a common lymphoid progenitor (CLP) (Kondo et al., 1997) and common myeloid progenitor (CMP) (Akashi et al., 2000; Arinobu et al., 2007b) indicated that the initial lineage restriction resulted in cells with mutually exclusive lymphoid or myeloid-erythroid potential (Figure 1.1) (Orkin and Zon, 2008). However, studies have demonstrated low levels of myeloid potential in the CLP (Mebius et al., 2001; Rumfelt et al., 2006a) and B cell potential in the CMP (Yang et al., 2007a). In addition, in vitro studies have failed to identify progenitors with only B and T cell potential (Kawamoto et al., 2000a). The in vitro characterisation of LSK cells that expressed high levels of FLT3 revealed a cell population that lacked significant megakaryocyte-erythroid (MkE) potential, but retained granulocyte-macrophage (GM) and B and T lymphocyte potential. These 'lymphoid primed multi-potent progenitors' (LMPPs**)** are not compatible with the classical haematopoietic hierarchy and thus led to the suggestion of an alternative road map for haematopoiesis (Figure 1.2)**.**

A number of independent studies have now confirmed the existence of LMPP-like progenitors in mice and humans (Doulatov et al., 2010; Goardon et al., 2011; Kohn et al., 2012), but the original suggestion of a lympho-myeloid pathway of lineage restriction was highly controversial. Fate mapping studies using *Il-7rα* or *Rag1* driven cre expression demonstrated limited labelling of the myeloid lineage questioning the physiological relevance of the LMPP to myeloid cells in the adult (Schlenner et al., 2010; Welner et al., 2009b). However as both *Il-7rα*

and *Rag1* are expressed at very low levels in the LMPP, this approach is likely to underestimate the contribution of the LMPP to the myeloid lineage. Furthermore, equivalent studies in the fetus where *Rag1* is much more highly expressed, reveals significant labelling of the myeloid compartment (Boiers et al., 2013). A second challenge to the LMPP came after studies transplanted a high number of LMPPs (>500) into recipient mice and demonstrated a contribution to the MkE lineage in vivo (Forsberg et al., 2006). It was later shown that most of this MkE potential can be removed by further defining the LMPP as expressing PU.1 (Arinobu et al., 2007a) and not expressing the thrombopoietin receptor 'MPL' (Luc et al., 2008). A study based on *Flt3* fate mapping also suggested the LMPP may contribute to the MkE lineage (Boyer et al., 2011), but as *Flt3* is expressed in progenitors upstream of LMPPs, including fully multi-potent haematopoietic progenitors (Buza-Vidas et al., 2011), *Flt3* fate mapping will not exclusively label the LMPP. It is not disputed that purified LMPPs may generate low levels of platelets, but this does not preclude the importance or the existence of a lympho-myeloid progenitor. Therefore the existence of the LMPP, which was originally highly contentious, is now largely accepted by the field supported by convincing functional and molecular data.

The haematopoietic road map as shown in figure 1.2 is far from definitive. Recently, a myeloid restricted progenitor with self-renewing activity has been identified (Yamamoto et al., 2013). This challenges the dogma that lineage restriction is coupled to loss of self-renewal and indicates that HSCs can divide asymmetrically to directly generate lineage restricted progenitors (Yamamoto et al., 2013). Furthermore, characterisation of individual HSCs has revealed

heterogeneity regarding self-renewal, differentiation and proliferation (Copley et al., 2012; Dykstra et al., 2007). Single cell analyses revealed a subset of HSCs, identified by expression of the *Von Willebrand* factor (*Vwf*), that are primed towards platelet reconstitution and have been shown to reside at the apex of the haematopoietic hierarchy (Sanjuan-Pla et al., 2013) are capable of generating platelet biased *Vwf*$^{+}$ stem cells and non platelet biased *Vwf*$^{-}$ stem cells. Furthermore, individual HSCs may have a lineage bias and not be 'balanced' in their differentiated cell output (Copley et al., 2012). The best known example of a lineage bias is the decreased lymphoid but increased myeloid ouput of HSCs with age (Beerman et al., 2010; Geiger et al., 2013). Recently the haematopoietic heirarchy has been further challenged by the suggestion that artificial in vitro assays and the stress and irradiation associated with in vivo transplantation may 'instruct' lineage potentials that are not reflective of steady state hematopoiesis. In the human (Notta et al., 2016), data suggests the majority of progenitors may in fact be unipotent, and in the mouse, in situ barcoding approaches suggest that steady state haematopoiesis is maintained by thousands of clones instead of a limited number of haematopoieitc stem cells (Sun et al., 2014).

Lineage Commitment or Lineage Restriction

The words 'commitment' and 'restriction' are often used interchangeably when referring to haematopoietic progenitors. However, I interpret the two words to mean subtle but critically different things. If a progenitor is restricted to a lineage, it cannot form alternative lineages. However, if a progenitor is 'committed,' 'biased' or has 'potential' for a lineage it still has residual capacity to generate other lineages, perhaps at low frequency. This concept is important for

this thesis where we investigate the emergence of B cell *potential*, and subsequently B cell *restriction* in fetal lymphopoiesis. Lineage restriction and potential are important concepts in understanding the origin of haematopoietic malignancies where leukaemias can express markers of multiple lineages, or even switch lineage between diagnosis and relapse.

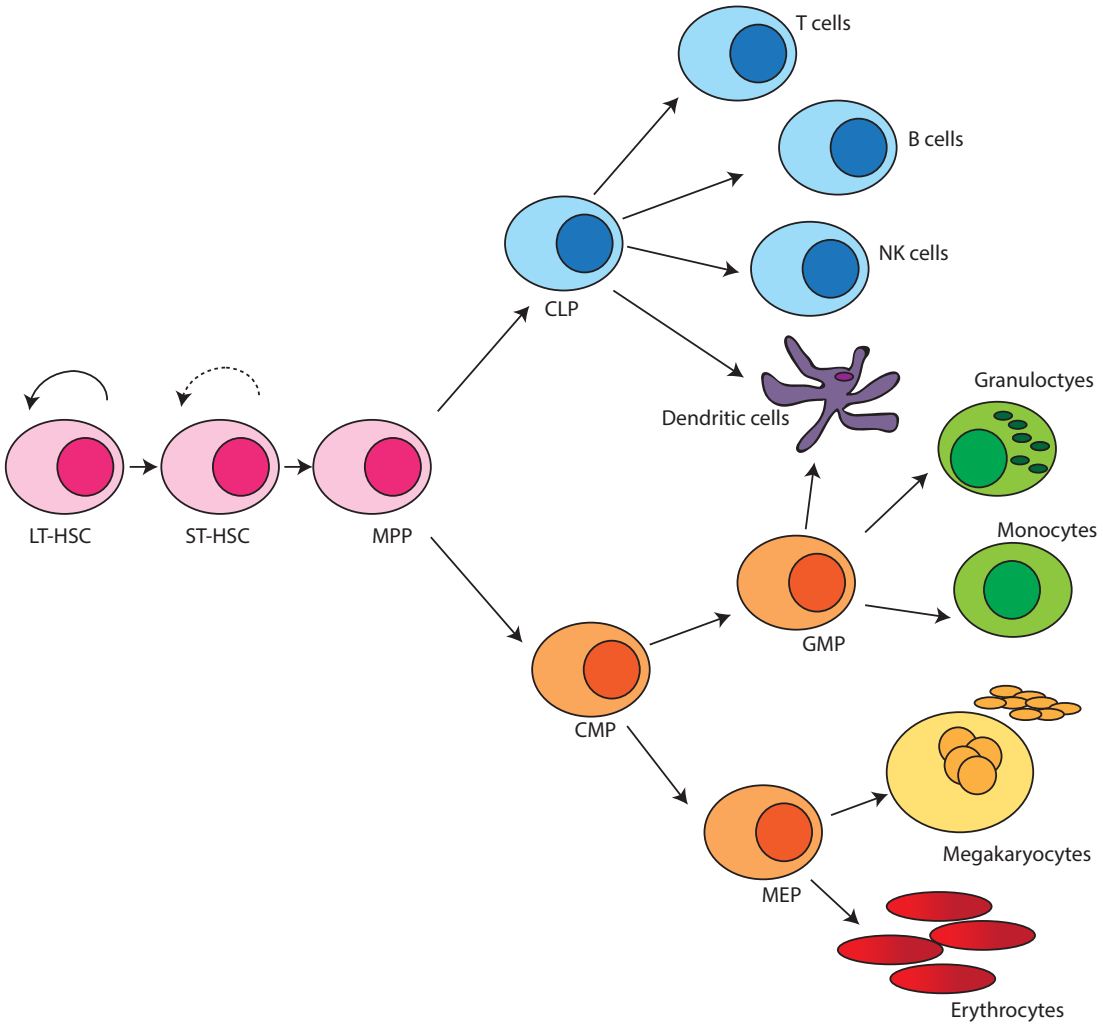


Figure 1.1: The classical haematopoietic cell tree
Long Term Haematopoietic Stem Cells (LT-HSCs) and Short Term Haematopoietic Stem cells (ST-HSCs) have prolonged engraftment potential and can self renew. They give rise to multi-potent progenitors (MPPs) that retain all lineage potentials but lack the ability to self renew and so exhaust. The first lineage restriction steps are a bifurcation into the lymphoid and myeloid lineages, by the generation of the Common Lymphoid Progenitor (CLP) and Common Myeloid Progenitor (CMP). Curved arrows indicate self renewal ability.

NK-Natural Killer. GMP-Granulocyte Macrophage Progenitor. MEP-Megakaryocyte Erythroid Progenitor.

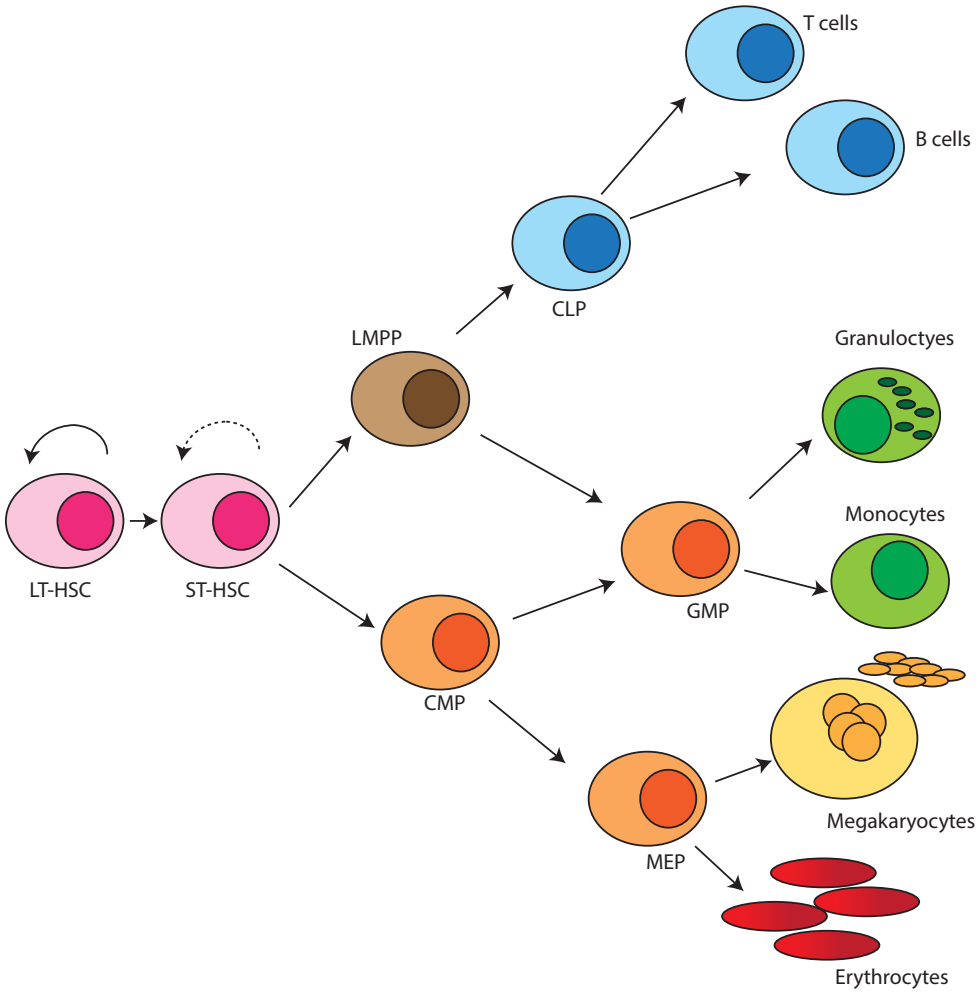


Figure 1.2: The 'alternative' haematopoietic cell tree

There is no strict separation between the lymphoid and myeloid lineages in the alternative model. The LMPP (lymphoid primed multi-potent progenitor) can give rise to the myeloid and lymphoid lineages, but lacks megakaryocyte and erythroid potential (Adolfsson et al., 2005). Curved arrows indicate self renewal.

Fetal Versus Adult Haematopoiesis

Comparison of fetal and adult HSCs has revealed marked differences in their properties, perhaps unsurprisingly given the different haematopoietic needs of embryos and adults. Transplantation studies highlighted that an intrinsically regulated switch of functional properties occurs between three and four weeks of age in the stem cell compartment in the mouse (Bowie et al., 2007). Following this, fetal HSCs that are actively cycling transition to largely quiescent adult HSCs (Bowie et al., 2006) that have reduced self renewal ability and generate daughter cells with slower kinetics (Rebel et al., 1996). These functional differences are coupled to changes in phenotypic surface markers. For example fetal stem cells lose CD34, AA4.1, VE-Cadherin and MAC-1 expression as they develop into adult stem cells during ontogeny ((Kim et al., 2005; Morrison et al., 1995; Petrenko et al., 1999) reviewed in Childhood Leukaemia, 2006, Cambridge university press pages 69-105).

Molecular studies have reinforced these functional and phenotypic differences, providing evidence of distinct regulation of fetal and adult haematopoiesis. *Sox17* was identified as a master regulator of fetal HSCs and is expressed in fetal and neonatal, but not adult, HSCs (Kim et al., 2007). Ectopic *Sox17* expression in adult HSCs results in recapitulation of fetal HSC properties, increasing their self renewal and reconstitution capacities (He et al., 2011).

During fetal development haematopoiesis generates different subsets of mature blood lineages. The lymphoid lineage produces innate γδ T cells and B1 cells, which are discussed in more detail later. Fetal erythrocytes are distinguished as

they start to express fetal haemoglobin (Wilber et al., 2011), and the embryo generates a unique fetal macrophage subset (Ginhoux and Jung, 2014). Fetal macrophages can develop independently of HSCs, in the absence of MYB (Schulz et al., 2012). Recently fate mapping studies have shown that the majority of adult tissue resident macrophages (Kupffer cells in the liver, microglia in the brain, Langerhans cells in the epidermis and alveolar macrophages in the lung) derive from E8.5 fetal YS progenitors, highlighting the ability of fetal haematopoietic cells to persist and contribute towards adult haematopoiesis (Gomez Perdiguero et al., 2015).

The *Lin28b-let7* pathway is associated with regulating the higher self renewal potential of fetal HSCs. Forced lentiviral expression of *Lin28b* conveys fetal-like properties upon adult HSCs, such as elevated self renewal and fetal lymphoid differentiation including generation of γδ T cells (Mold et al., 2010) and B1 innate immune cells (Yuan et al., 2012).

Fetal Haematopoiesis

Primitive haematopoiesis

In 1917, Francis Sabin directly visualised the emergence of haematopoietic cells in the chick yolk sac (YS). These primitive, nucleated erythrocytes which express embryonic globins emerge at E7.0-7.5 (E: embryonic days post conception) in mouse YS 'blood islands' at the same time as and physically adjacent to endothelial cells, resulting in the suggestion that both cells emerge from a common mesodermal precursor termed the 'haemangioblast' (Dzierzak and Speck, 2008). However, inter-species grafting of avian embryo bodies and YSs

demonstrated that adult haematopoietic cells in fact arise from within the embryo body (Dieterlen-Lievre, 1975). This finding was subsequently supported by similar findings in amphibian embryos (Turpen et al., 1981; Walmsley et al., 2002).

Between Primitive and Definitive Haematopoiesis

Between the waves of primitive haematopoiesis at E7.5 and emergence of definitive HSCs (dHSCs) at E10.5 numerous additional haematopoietic progenitors are detected in the mouse conceptus (figure 1.3). Erythro-myeloid progenitors are detected in the YS and allantois from E8.25, and CFU-S are detectable in the YS and AGM at E9.0 (Dzierzak and Speck, 2008). After four days of whole organ culture, the E8 PAS (0-5 somite pair stage (Tam, 1981)) can generate low levels of multi-lineage reconstitution in immune deficient recipients, indicating the early PAS has the potential to foster a lympho-myeloid (Cumano et al., 2001). HSCs capable of repopulating neonatal but not adult recipients are found in the post circulation E9 YS and AGM. The lineage potential and contribution of these progenitors toward adult haematopoiesis remains a lively area of research (Arora et al., 2014).

Endothelium to blood – definitive haematopoiesis

YS haematopoietic cells only transiently sustain haematopoiesis. dHSCs (i.e. capable of long term multi lineage reconstitution in recipient mice) emerge autonomously from specialised 'haemogenic endothelium' in the ventral region of the dorsal aorta in the E10.5 aorta-gonad-mesonephros (AGM) and vitelline

and umbilical arteries of the mouse embryo (de Bruijn et al., 2000; Medvinsky and Dzierzak, 1996; Muller et al., 1994). The endothelial to haematopoietic transition (EHT) is dependent on the transcription factor RUNX1. *Runx1* null embryos lack haematopoietic clusters in the dorsal aorta and die at E12.5 due to haematopoietic failure (North et al., 1999; Okuda et al., 1996; Wang et al., 1996b). dHSCs enter circulation and seed the FL by E11.5 where they expand, self renew and begin differentiating to generate mature lineages. Haematopoietic progenitors are detected in the fetal BM by E15.5 (Delassus and Cumano, 1996).

The placenta has also been shown to harbour HSCs, supporting definitive haematopoiesis during mid-gestation. HSCs are initially detected in the placenta slightly later than in the AGM at E11.5 and the population expands until by E12.5-E13.5 it contains fifteen times as many HSCS as in the AGM region (Gekas et al., 2005; Ottersbach and Dzierzak, 2005).

Despite the many anatomical locations of fetal haematopoietic cells, mouse lineage tracing studies suggest, all adult dHSCs are derived from fetal cells. A *Runx1-Cre-β-gal* mouse model showed significant contribution of cells labelled at E9.5 to haematopoietic cells 9-12months later (Samokhvalov et al., 2007). Furthermore a fate mapping approach which used *Scl-Cre*, a gene essential for the development of all haematopoietic lineages (Porcher et al., 1996), demonstrated that 10% of haematopoietic cells are labelled at E14.5, with the same proportion of cells labelled in adulthood. This indicated there is *de novo* generation of HSCs after E14.5 (Gothert et al., 2005) and that the contribution

and relevance of fetal haematopoiesis to the adult should not be underestimated.

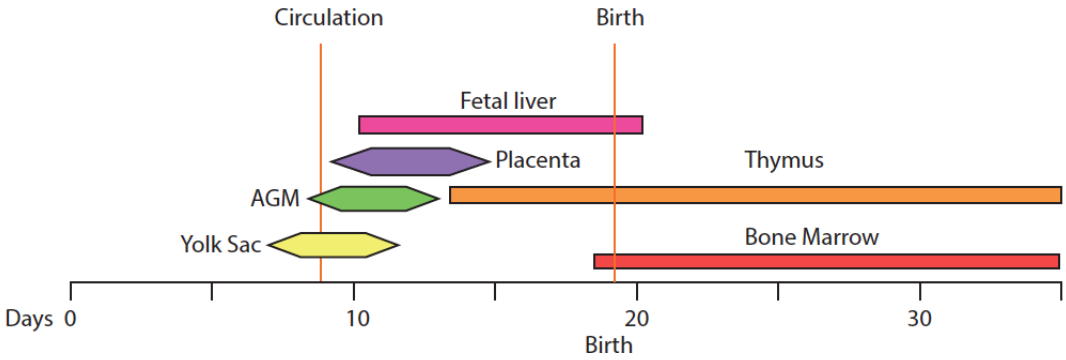


Figure 1.3: Sites of haematopoiesis. Adapted from (Orkin and Zon, 2008)
During mouse embryogenesis haematopoiesis occurs in multiple anatomical locations, shifting at different developmental time points.

B Cells

B cells are critically involved in raising an immune response against pathogens. Mature activated plasma B cells secrete a wide range of antibodies (immunoglobulins - Ig, figure 1.4), which function in three ways. Firstly the antibodies bind bacterial toxins and viruses, blocking their access to cells (neutralisation). Secondly they coat pathogens marking them for removal by phagocytosis (opsonisation) and thirdly they complement plasma proteins, leading to the destruction and removal of pathogens. Defects in B cell function result in immune deficiency, autoimmunity, malignancy and allergic responses.

B cell development can be divided into antigen independent and dependent phases. The antigen independent phase involves the progressive lineage restriction of HSCs into mature, naïve B cells that express a unique, functional B cell Receptor (BCR) consisting of immunoglobulin heavy and light chains**.** The antigen dependent phase involves the binding of the BCR to its cognate antigen.

Subsequently, in a T cell dependent or independent fashion, the B cell clone expands in a germinal centre reaction in the spleen where it undergoes affinity maturation and Ig class switching, before clonal expansion to generate a large number of antibody secreting plasma cells. This thesis is concerned with the antigen independent phase of B cell development.

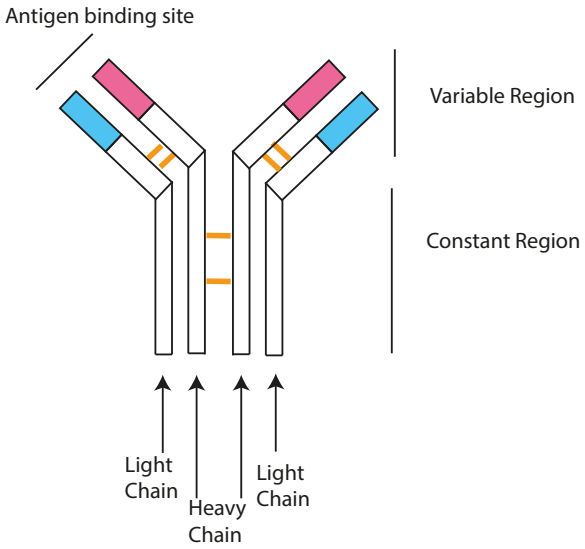


Figure 1.4: Structure of an antibody
Antibodies consist of four polypeptide chains, two heavy and two light chains held together by disulphide bond (yellow). Switching of the constant region alters the antibody subtype (IgM, IgA, IgE, IgG, IgD). The variable region forms the antigen-binding site and is generated from recombination of V, D and J segments. Antibodies may be either membrane bound, when held in place by a short hydrophobic trans-membrane domain as part of the BCR, or secreted.

B cell subsets

Mature B cells can be divided into three main subsets, 'B-1' B cells, Follicular B cells and Marginal Zone B cells (MZ). Follicular B cells repeatedly recirculate throughout the blood, lymph, Peyer's patches, and spleen, where they are required for T cell dependent immune responses to protein antigens. MZ B cells reside in the marginal zone of the spleen and are involved in T-independent

responses to antigens on blood borne pathogens and lipid antigens. Both MZ and follicular B cells are known as 'B2' cells. B1 B cells were first identified based on expression of CD5, a marker expressed by T cells, although now $CD5^+$ (B1a) and $CD5^-$ (B1b) subsets have been identified. B1 cells populate the pleural and peritoneal cavities and are also found in the spleen at low numbers. They demonstrate limited ability to switch antibody subclasses and therefore secrete high levels of IgM. B1a cells secrete 'Natural IgM,' a poly reactive antibody with weak affinity for bacterial carbohydrate antigens (Coutinho et al., 1995) that is critical in the initial response to encapsulated bacteria. B1b cells are required for long lasting immunity to pathogens such as *Streptococcus pneumonia* (Haas et al., 2005).

VDJ Recombination

The progression of a multi-potent haematopoietic progenitor into a lineage restricted B cell requires rearrangement of the immunoglobulin (Ig) heavy and light chain loci to generate a functional BCR. In utero B cell development occurs in the fetal liver, before migrating to the bone marrow late in embryogenesis at E17.5 (Mikkola and Orkin, 2006). The various stages and names of B cell development can be confusing, but each stage is defined based on the molecular rearrangement status of the immunoglobulin loci (figure 1.5).

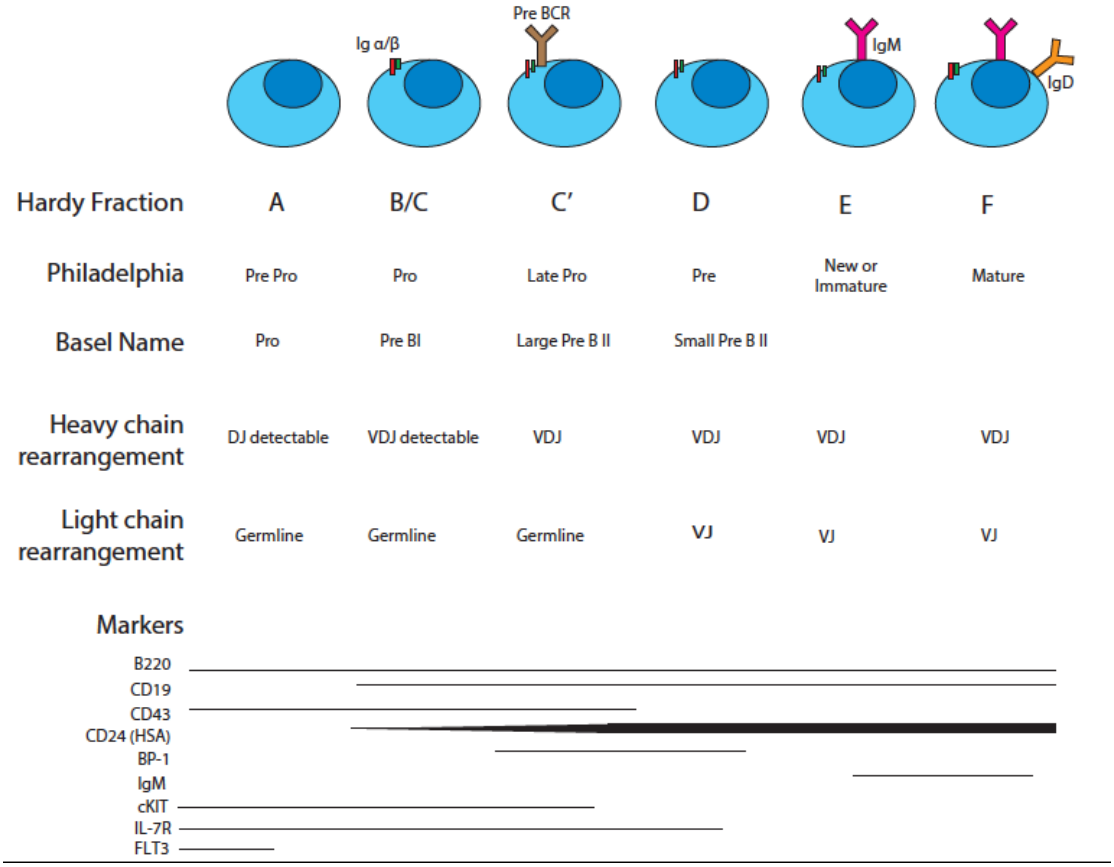


Figure 1.5: B cell staging in the adult bone marrow
Sequential development of early B cell progenitors to committed, mature B cells secreting functional immunoglobulins. The various nomenclatures for the corresponding staging, as determined by rearrangement status is shown. Only six cell surface markers are shown though many more markers are used to precisely determine each fraction. Marker selection adapted from (Hardy and Hayakawa, 2001). V Variable, D Diversity, J Joining.

Each heavy chain locus (IgH) consists of multiple constant (C), variable (V), diversity (D) and joining (J) segments (figure 1.6). The two light chain loci (IgLλ and IgLκ) contain C, V and J segments. The unique recombination of these segments mediated by the RAG-1 and RAG-2 enzymes, can generate 10^5-10^7 different possible antigen binding sites. Successful heavy chain recombination occurs first, which associates with the proteins λ5 and VPreB ('surrogate light chain' (SLC)) to assemble a 'pre BCR'. Signalling from the Pre BCR triggers clonal expansion and developmental progression. Subsequent recombination of the

light chain and association with the heavy chain results in formation of the BCR. Diversity in antigen binding stems from the combination of VDJ segments, the choice λ or κ light chain and the addition of non-template nucleotides at joining regions by Terminal Deoxynucleotidyl Transferase (TDT). Notably TDT is not expressed by fetal B cells resulting in reduced junctional diversity (Li et al., 1993a). The BCR associates with the CD79a/CD79b (Igα/Igβ) heterodimers, which transduce a tyrosine kinase signal inside the cell when the BCR binds antigen.

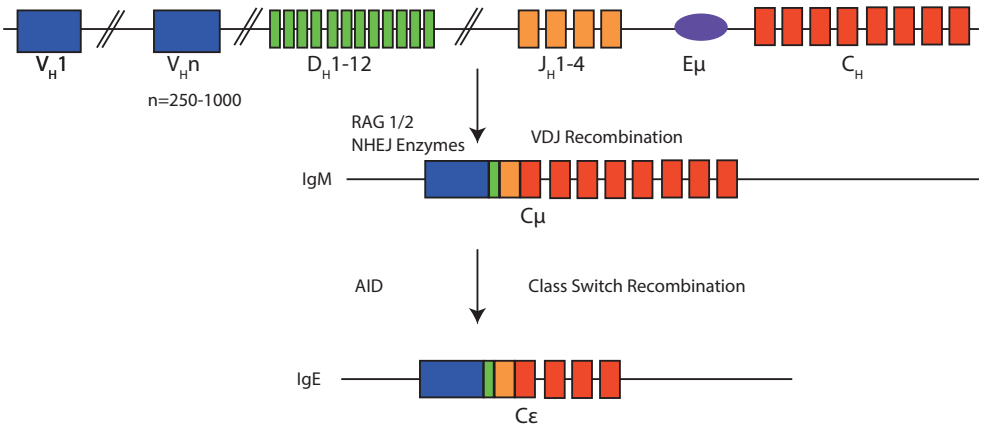


Figure 1.6: VDJ recombination at the immunoglobulin heavy chain locus.
Adapted from (Chaudhuri and Alt, 2004). The RAG-1/RAG-2 complex binds recombination signal sequences (RSS) that flank the rearranging gene segments and cleave the DNA to cause a double strand break. The DNA ends are joined using the non-homologous end joining (NHEJ) machinery. Cytokines stimulate class switch recombination (CSR), which exchanges the constant region of the Ig to generate alternative antibody subtypes (IgM to IgE is shown). CSR requires the enzyme activation-induced cytidine deaminase (AID).

B cell staging by flow cytometry

With the development of multi colour flow cytometry, surface markers were sought to identify B cell progenitor stages without the need for molecular

analysis of the Ig rearrangement status. Richard Hardy originally sub-divided B cell progenitors based on expression of B220, CD43, CD24, BP-1 and IgM (Hardy et al., 1991b) ("Philadelphia" B cell staging). Fritz Melchers, based in Basel, Switzerland, incorporated the tyrosine kinase receptor, KIT and CD25 into a B cell classification, and developed the "Basel" staging, which confusingly, has different nomenclature to the Philadelphia B cell staging (Rolink et al., 1994). More and more markers have been added to the B cell progenitor staging. Fritz Melchers describes B cell progenitor staging as 'a mess, but a beautiful mess' (Van Epps, 2006) and I will use the Hardy nomenclature in this thesis, though an overview of both the Hardy and Melchers staging is provided in figure 1.5.

Fetal B cell Lymphopoiesis

Differences between fetal and adult B cell lymphopoiesis

To cope with the different priorities and demands of the embryo and adult immune systems, fetal and adult lymphopoiesis differ in their regulation and antigen-binding repertoire. Initial studies using the New Zealand Black mouse strain observed B cell progenitors in young mice which were not sustained in adult haematopoiesis and rapidly declined with age (Jyonouchi et al., 1982). Subsequently, cell lines derived from fetal Pro B cells were observed to last several months, whereas adult B cell lines under the same culture conditions, ceased to proliferate after four to six weeks (ten Boekel et al., 1995).

A difference between fetal and adult lymphopoiesis was revealed upon transplantation of unfractionated FL and BM cells into irradiated recipients and comparison of the B cell subsets produced from the donor cells. Transplantation of unfractionated FL cells resulted in the generation of a novel $CD5^+$ B cell subset, now referred to as B1a cells (Hayakawa et al., 1985). However, transplantation of adult bone marrow cells resulted in the generation of follicular B2 cells. It is undisputed that B1 and B2 subsets exist with different functional properties in the mouse. However the different B cell subsets are often difficult to distinguish as there is no definitive marker of each subset, and instead identification is based on surface marker expression and anatomical location. Molecularly the populations are not well understood and throughout the field different groups use slightly different cell surface markers to identify B1 cells. Therefore definitively identifying a $CD19^+IgM^+$ B cell as B1 or B2 is not easy.

Characterisation of transgenic mouse models provided further evidence that fetal and adult B cell lymphopoiesis differ. Examination of the bone marrow B cell progenitors in mice engineered to lack the cytokine IL-7 demonstrated neonatal mice had detectable $CD19^+$ B cell progenitors, but that by 6 weeks of age $CD19^+$ B cell progenitors were absent from the BM. This suggested fetal but not adult B cell progenitors can function independently of IL-7 (Carvalho et al., 2001). As B cells are long lived, IgM^+ mature cells in the spleen look ostensibly normal in IL-7 deficient mice and detailed BM analysis is required to reveal the major defect in adult BM B cell progenitors. Additionally Thymic Stromal Derived Lymphopoietin (TSLP) which binds the IL-7Rα, stimulates robust fetal Pro B cell proliferation, but adult Pro B cells do not respond to TSLP (Vosshenrich et al., 2003). Furthermore, mice with *Pax5* deleted had a block in B cell development in the adult marrow at the Pro B cell stage whereas the block was reported to be before the emergence of $B220^+KIT^+$ cells in the FL (Nutt et al., 1997). However the B cell staging used was not of high resolution and this finding has not been corroborated (further discussed in chapter V).

Gene expression studies showed that Pro-B cells from the FL do not express TDT, an enzyme which adds nucleotides during VDJ recombination to generate further antigen binding diversity (Rolink et al., 1993), or regulatory myosin light chain (PLRLC) (Oltz et al., 1992) unlike their adult counterparts. Molecular analysis of rearranged Ig loci in the embryo showed preferential selection for V segments at the 3' end of the V cluster resulting in an altered antigen binding specificity of the immunoglobulin repertoire (Jeong and Teale, 1988; Perlmutter et al., 1985).

B1 cell progenitors

B1 cells were first identified when transplantations from fetal livers and newborn bone marrow generated CD5 expressing B cell subsets (Hayakawa et al., 1985). Transplantation of fetal splanchnopleura (Godin et al., 1993) revealed they predominantly generate $CD5^+$ B1 cells in the peritoneal cavity 6 months after transplantation. However, transplantation of the E13 and E14 FL resulted in the production of B1 and B2 cells (Kantor et al., 1992; Solvason et al., 1991). Adult BM can generate B2 cells and a limited number of B1b cells, but has not been shown to generate B1a cells to date (Kantor and Herzenberg, 1993; Kantor et al., 1992).

A major source of debate in the field is whether B1 and B2 cells originate from the same progenitor that develops into a B1 or B2 cell depending on antigen specificity of the BCR ('selection model' (Berland and Wortis, 2002; Haughton et al., 1993) or two separate progenitors that emerge at different times in development ('layered immune system hypothesis' (Herzenberg and Herzenberg, 1989). The functional differences between B1 and B2 cells support the layered immune system hypothesis as it can be argued that the two populations evolved successively to meet the increasing immunological demands of higher organisms (Herzenberg and Tung, 2006). Data supporting the layered immune system hypothesis originates from transplantation studies that show fetal tissue has a bias towards B1 cells. Additionally, experiments that depleted immunoglobulins in neonatal mice permanently altered the B1 cell compartment, suggesting the majority of B1 cells originate in fetal lymphopoiesis (Lalor et al., 1989). It has been proposed that a unique, self renewing B1 cell

progenitor emerges in fetal development and sustains the B1 cell pool in the adult bone marrow (Kantor and Herzenberg, 1993). This hypothesis is supported by studies showing the B1 pool can self replenish, as peritoneal IgM^+ $CD5^+$ B1a cells can reconstitute the B1a compartment in recipient mice (Hayakawa et al., 1986).

Support for the selection hypothesis also came from studies in transgenic mice engineered to artificially express antigen receptors associated with B1 cells during development. This resulted in a bias towards generating B1 cells (Arnold et al., 1994; Haughton et al., 1993), indicating antigen specificity drives the lineage towards a B1 or B2 cell fate.

The identification of a B1 specified progenitor ($Lin^-CD93^+B220^-CD19^+$) that peaked in frequency at E17 and could reconstitute B1a and B1b cells but not B2 cells, was argued as definitive support for the layered immune hypothesis, (Montecino-Rodriguez et al., 2006). The B1 progenitor has subsequently been identified and studied by multiple laboratories (Esplin et al., 2009a; Ghosn et al., 2011; Yoshimoto et al., 2011). However it has not been unequivocally accepted by the field due to limited in vivo data, making it hard to conclude that the progenitor is entirely restricted to generating B1 cells, with no residual B2 cell capacity. Whether the $Lin^-CD93^+B220^-CD19^+$ B1 progenitor is heterogeneous and contains separate B1a and B1b progenitors is unknown, as single cell analysis has not been performed.

Emergence of B cell restricted progenitors in the mouse embryo

B cell restriction is associated with surface expression of the maker CD19, which is under the control of the transcription factor PAX5. CD19 expression is detected in the FL between E13.5 and E14.5 in the mouse (Douagi et al., 2002; Kawamoto et al., 2000b) and cell surface IgM is found at E17 (Raff et al., 1976). B cell progenitors are detectable in the fetal bone marrow as early as day 15 (Delassus and Cumano, 1996). One study claimed to detect $CD19^+$ B cells in the E11.5 AGM and FL (de Andres et al., 2002) but no other publications support this finding and, more likely, the B cells detected originated from the mother and contaminated the embryonic tissues during dissection. In vitro cultures of Lin^- KIT^+ $CD45^+$ $IL\text{-}7R^+$ FL cells detect uni-potent B cell and T cell restricted progenitors emerging between E11 and E12. This study did not isolate any pure populations based on cell surface phenotype, and as many different progenitor populations exist within the Lin^- KIT^+ $CD45^+$ $IL\text{-}7R^+$ compartment, lineage capacity was not thoroughly assessed and conclusions were reliant solely on one in vitro assay (Kawamoto et al., 2000b). To conclude that a population is indeed lineage restricted, it must be prospectively purified and assessed in multiple optimised molecular and functional assays, with kinetics and conditions optimised to reveal all possible lineage potentials.

Emergence of B cell potential in the mouse embryo

B cell *restriction* implies cells have lost the ability to generate other lineages (uni-potent) and is a different concept to B cell *potential*. The first embryonic stem cell has B cell *potential,* but is not lineage restricted. However, multiple studies have focused on looking at what developmental age and location B cell potential

can be detected in assays. Interestingly, the progenitors identified do not fit in the classical haematopoietic hierarchy and studies have failed to identify cells with combined B and T cell potential without myeloid potential, (Katsura and Kawamoto, 2001; Kawamoto et al., 2000a), challenging the relevance of the CLP to fetal lymphopoiesis.

B cell potential has been detected as early as the E9.5 YS (Yoshimoto et al., 2011), but these data do not support that the cells are B lineage restricted and furthermore, in a separate publication, the same group show that same E9.5 YS cells also have T cell potential (Yoshimoto et al., 2011; Yoshimoto et al., 2012). However B cell potential arises independently of definitive HSCs indicating they may stem from an interim progenitor.

A progenitor within the $AA4.1^{+}$ $B220^{-}$ $MAC1^{-}$ $SCA1^{+}$ compartment that had B cell and macrophage potential, but not other myeloid cell potential, was identified in the E12.5 FL (Cumano et al., 1992). Given that both B cells and macrophages have shared properties such as MHC Class II antigen presentation, a common progenitor seems feasible. Critically however, this study did not probe T cell potential and a follow up study four years later reported using a different assay, that the same cells could indeed generate T cells, suggesting they are in fact multipotent (Delassus and Cumano, 1996) . The contribution of these early B cell progenitors to adult lymphopoiesis is unknown, although more recently a rare B/Macrophage progenitor has been proposed in the adult bone marrow (Montecino-Rodriguez et al., 2001). Furthermore, many leukaemias express cell surface antigens of both the B and myeloid lineage, and can even switch during

diagnosis and relapse within the same patient, providing a functional example of the relationships between the B cell and myeloid lineage (Dorantes-Acosta and Pelayo, 2012).

These data on emergence of B1 cell potential can be assimilated into the hypothesis that mouse B cells develop in three waves. The first wave occurs prior to the emergence of definitive HSCs in the PAS and YS, giving rise to B1 cells but not B2 cells. B1 cell contribution towards adult haematopoiesis is unknown. The second wave occurs during mid gestation from dHSCs in the FL and produces B cells that mostly develop into B1 cells. The third wave in late gestation is associated with a decline of B1 cells and the onset of B2 cells (Montecino-Rodriguez and Dorshkind, 2012). Given we know other lineages, such as erythrocytes, emerge in waves, the notion that B cells also emerge in waves is feasible. It may explain phenomena such as why infants show diminished response to vaccination (PrabhuDas et al., 2011), as agents designed to boost the B2 cell response would not be effective whilst B1 cells dominate in infancy. As FACS poorly defines B1 cells and functional transplantation assays with early fetal lymphoid progenitors are limited, this model of B cell development is far from definitive. Accordingly, whether the mature IgM^+ cells found late in gestation are fetal B1 cells is not known.

Cytokines and Transcription Factors in B cell Development

Cytokines

Cytokines are soluble proteins that regulate cell function locally or systemically. In haematopoiesis, cytokines bind to cell surface receptors and activate intracellular signalling pathways that regulate proliferation, self renewal, differentiation and survival.

IL-7 is secreted by immune cells and non-immune epithelial and stromal cells in the BM (Funk et al., 1995), FL and thymus (Tsuda et al., 1996) and binds to a dimer of the IL-7Rα chain and the common γ chain. IL-7 signalling is integral to B cell proliferation, survival and maturation (Corfe and Paige, 2012) and the IL-7R is up regulated at the CLP stage and expressed through to the Pre B cell stage. Adult B cell progenitors are dependent on IL-7 as adult IL-$7^{-/-}$ mice demonstrate a block in B cell development at the early Pro B cell stage. In contrast, $CD19^{+}$ B cell progenitors are detected in the neonatal bone marrow (Carvalho et al., 2001). Interestingly, the remaining B cells in IL-$7^{-/-}$ adult mice are B1 or MZ cells, indicating they may persist from fetal development. Excessive signalling from the IL-7 receptor can be inhibitory to B cell development, indicating the IL-7 dose is important (Jiang et al., 2005; Purohit et al., 2003). However IL-7 is not required for human B cell development, as X-Linked Severe Combined Immunodeficiency patients who harbour loss of function mutations in the IL-7 signalling pathway can sustain B cell but not T cell development in the peripheral blood (Kovanen and Leonard, 2004; Puel et al., 1998). The BM has never been analysed in these patients and it is possible that as in the mouse, the

early B cell progenitors are defective but the peripheral blood B cell compartment ostensibly normal due to persistence of long lived mature cells.

The cytokine TSLP was proposed to be responsible for fetal IL-7 independent lymphopoiesis, as fetal liver Pro B cells proliferated robustly with TSLP but adult Pro B cells did not (Vosshenrich et al., 2003). However, later work indicated that although fetal Pro B cells proliferate in response to TSLP, Il-7$^{-/-}$ TSLP$^{-/-}$ mice show no enhanced defects in fetal B cell development (Jensen et al., 2008; Jensen et al., 2007). Mice lacking FLT3 have a reduced number of B cell progenitors, but the number of mature B cells is unaffected (Mackarehtschian et al., 1995). FLT3L and IL-7 function synergistically, as addition of FLT3L and IL-7 to cultures increased the survival and proliferation of Pre Pro B cells (Funk et al., 1993). FLT3L is required for the IL-7 independent generation of fetal and adult B cells, as mice lacking IL-7Rα and FLT3 ligand completely lack all stages of fetal and adult B cell development (Jensen et al., 2008; Jensen et al., 2007; Sitnicka et al., 2003).

KIT, the receptor for stem cell factor (SCF), is widely expressed by haematopoietic progenitors of multiple lineages. Murine embryos deficient in KIT have reduced B cell progenitors (Waskow et al., 2002) and, similar to FLT3L, KIT works synergistically with IL-7 in vitro to increase the numbers of cultured Pro B cells (Zsebo et al., 1990).

Transcription Factors

The downstream output of cytokine signalling is mediated by transcription factors (TFs). Much work on the elucidation of transcriptional circuits has been performed on the immune system due to the ease of isolating, manipulating and characterising developmental intermediates. LMPPs can develop into B or Myeloid lineages in the bone marrow, and the cell fate choice requires regulation of a series of TFs. PU.1 and C/EBPα are TFs that drive development of the myeloid lineage, but low levels of PU.1 are also required for B cell development (Laslo et al., 2006; Scott et al., 1994), (DeKoter et al., 2002; Medina et al., 2004). Interestingly, deletion of PU.1 results in a block in BM B cell progenitors prior to the Pro B cell stage and a shift in mature lineages towards $IgM^{+}MAC1^{+}$ B1b like cells, highlighting the differences in progenitor regulation for B1 and B2 cells (Rosenbauer et al., 2006). The TF IKAROS is required to drive B lineage specification from early lymphoid progenitors, as Ikaros null mice lack B lineage cells (Wang et al., 1996a). Ikaros has been shown to drive the expression of B lineage genes such as *Tdt*, *Rag-1*, *VPreB* and *λ5*.

The TFs E2A (encoded by *Tcfe2a)* and EBF are required for specification and commitment of LMPPs to the B cell lineage by activating genes such as *CD79a*, *CD79b, VpreB1* and *λ5*, which encode functional B cell proteins required to form the BCR and activate enzymes involved in VDJ recombination (Lin and Grosschedl, 1995; Zhuang et al., 1994). Subsequently PAX5 is required for the maintenance of the B cell lineage (Mikkola et al., 2002). Defects in these TFs result in a block in B cell development (Hagman and Lukin, 2006; Lin and Grosschedl, 1995; Urbanek et al., 1994; Zhuang et al., 1994). E2A is additionally involved in chromatin remodelling of the Ig heavy chain locus to permit access

for the recombination machinery (Espinoza and Feeney, 2005). E2A, EBF and PAX5 are expressed in a specific order with E2A activating EBF expression, which subsequently activates PAX5 resulting in a hierarchical transcriptional network (Medina et al., 2004) (figure 1.7).

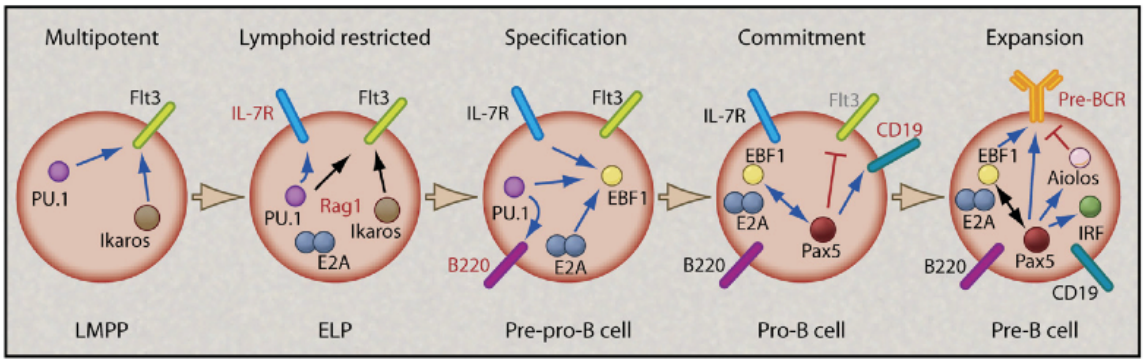


Figure 1.7: Transcriptional control of B cell development, adapted from (Nutt and Kee, 2007)

Progression of LMPPs through to Pre B cells. The developmental potential of each cell type is shown above the figure. Key TF, growth factor receptors and cell surface markers are shown. Blue arrows indicate the event is initiating at that particular stage. Rag1 expression starts in the ELP. ELP: Early Lymphoid Progenitor (*Rag1* expressing LMPP). IRF-Interferon Regulatory Factor.

In a series of seminal papers it was shown that commitment and maintenance of the B cell lineage could be attributed to a single TF, PAX5 (Mikkola et al., 2002; Nutt et al., 1999; Rolink et al., 1999). *Pax5* deletion in previously committed Pro B cells can result in their transdifferentiation into T cells in *Rag2*$^{-/-}$ mice and development into macrophages in vitro (Mikkola et al., 2002). This was the first demonstration that mature somatic cells can be manipulated to change lineage. Specifically for the B cell lineage, it revealed that PAX5 is not only required to specify B cell restriction but also to maintain B cell commitment (Cobaleda et al., 2007; Mikkola et al., 2002; Nutt et al., 1999; Rolink et al., 1999). More recent data now suggests that in addition to PAX5, EBF1 is also essential for B cell

maintenance and that it represses *Notch1* and *Csf1r*, key determinants of the T cell and myeloid cell lineages (Li et al., 2006; Radtke et al., 2013; Wiktor-Jedrzejczak et al., 1990). In fact in a similar fashion to PAX5, deletion of EBF1 in committed Pro B cells after transfer into alymphoid mice results in transdifferentiation of *Ebf1*$^{-/-}$ Pro B cells to T and innate lymphoid cells. Furthermore, EBF deficient, PAX5 expressing cells can generate T cells, suggesting PAX5 is not the sole B cell lineage locker (Nechanitzky et al., 2013).

Childhood Leukaemia

Aberrant production and regulation of haematopoietic lineages can result in malignancy. When malignant cells overcrowd the haematopoietic niche and disrupt the normal function of the blood system, serious immune disorders and death can occur. In children, leukaemia is the most common form of cancer, accounting for almost one in three cases of childhood cancer and with 400 cases diagnosed each year in the UK. The majority (75%) of childhood leukaemias affect the lymphoid compartment, with the remaining 25% of cases being myeloid leukaemias (American Cancer Society). Standard therapy for childhood ALL involves an intense one to two month treatment regime to induce remission, followed by up to two years of maintenance chemotherapy. More than 95% of children with ALL will enter remission after standard therapy, but treatment is associated with serious life threatening complications such as infections. Furthermore, patients can suffer from long term physical side effects such as increased risk of secondary cancers, heart and lung problems, stunted growth, fertility issues and osteoporosis coupled with possible psychological side effects (Dickerman, 2007). In one study of childhood cancer survivors, 41% reported

endocrine problems, 26% developed organ toxicity, and 15% were infertile (Stevens et al., 1998). Therefore understanding the biology of the disease in order to improve treatment regimes is an important area of research.

Some key research questions in malignancy probe the cancer's cell of origin and how, via accumulation of secondary mutations, pre cancerous clones progress to overt disease, as understanding these processes is the first step in designing successful therapies. Due to the heterogeneity of cancer, this process differs between diseases as well as patients. This thesis is focused on the better understanding of fetal B cell lymphopoiesis in order to gain an insight into the development of childhood ALL, specifically studying leukaemias associated with the TEL-AML1 oncoprotein.

Haematopoietic malignancies

Whether malignant haematopoiesis is hierarchical, with a 'cancer stem cell' (CSC) propagating disease or stochastic, with a wide variety of cells able to self renew and propagate the cancer, has been a controversial area in the field (Dick, 2008) (figure 1.8).

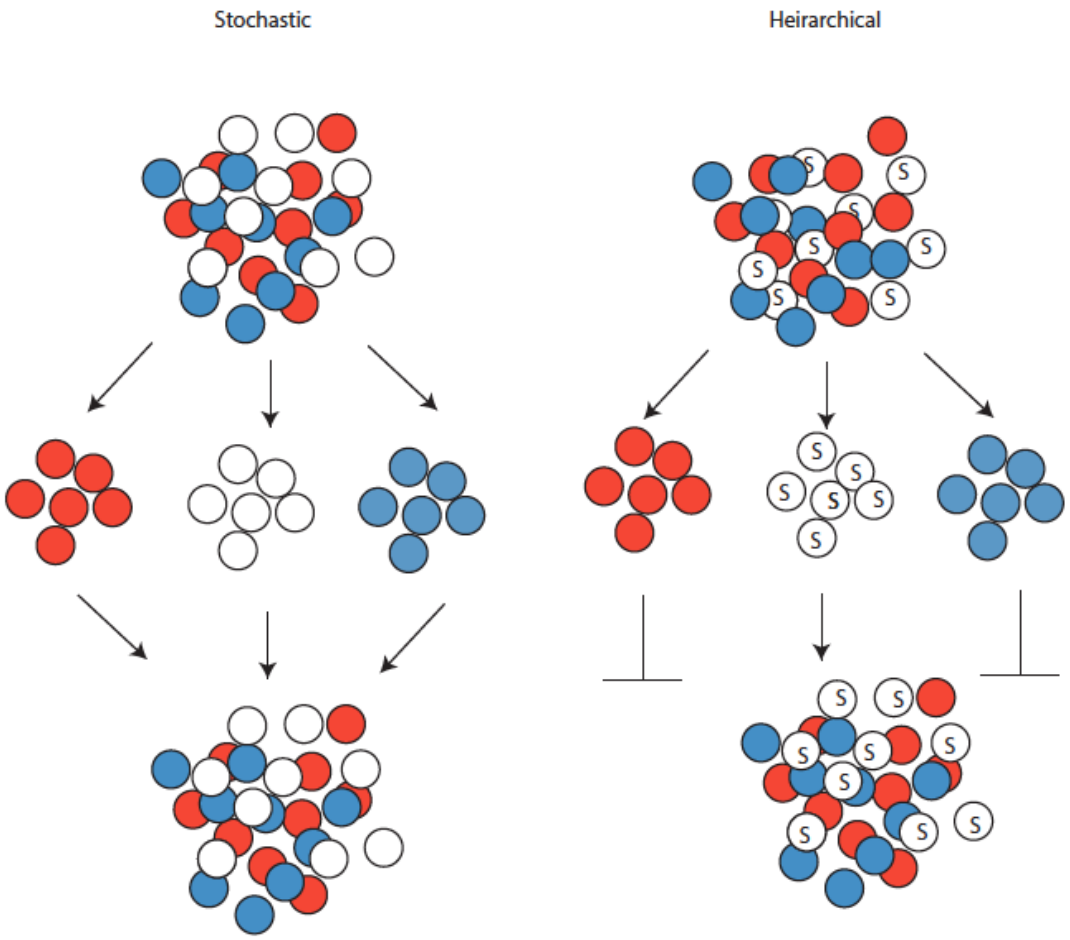


Figure 1.8: The cancer stem cell and stochastic model of cancer development. The stochastic model (left hand panel) proposes multiple cancer cell types can self renew and sustain the tumour. The hierarchical model (right hand panel) proposes that only rare cancer stem cells ('s') are capable of self renewal and the generation of the other cell types of the tumour. Coloured circles represent different cell populations.

The stochastic model proposes that any cell in the tumour is capable of self renewal and disease propagation. Random extrinsic and intrinsic signals determine whether a cell becomes a tumour driver. This model implies that it is impossible to isolate putative cancer driver cells, as they have no distinguishing markers and therefore therapies must eliminate all tumour cells.

In contrast, the hierarchical model proposes that a rare distinct population of cancer stem cells are alone capable of driving malignancy. The cancer stem cells self renew and can propagate all cell types in the tumour. Cancer stem cells do not have to arise from normal tissue stem cells, but can in theory come from committed progenitors that have re-acquired self renewal ability. Accordingly, this theory implies that successful therapy must eliminate the cancer stem cell.

John Dick's laboratory identified the first putative cancer stem cells when they showed in acute myeloid leukaemia (AML) patients that only the $CD34^{+}CD38^{-}$ bone marrow sub-fraction could reconstitute leukaemia in NOD/SCID mice (Bonnet and Dick, 1997). Controversially it emerged that the CD38 antibody may functionally disrupt the $CD38^{+}$ cells to prevent engraftment, questioning the cancer stem cell hypothesis (Taussig et al., 2008). However, since their identification in leukaemia, prospective cancer stem cells have been identified in brain, breast, colon and other tumour types (Al-Hajj et al., 2003; Bonnet and Dick, 1997; Dalerba et al., 2007; Lapidot et al., 1994; Singh et al., 2004; Visvader and Lindeman, 2008).

Cancer stem cells have been shown to exist in mouse genetic studies (Chen et al., 2012; Driessens et al., 2012; Schepers et al., 2012) for glioblastomas, squamous skin tumours and intestinal adenomas. However, it has been harder to definitively prove their existence in humans. Most human cancer stem cell assays are limited in value as they rely upon xenotransplantation assays, which generate an artificial non-physiological environment. Recently in intermediate risk myelodysplastic syndrome (MDS), rare multi-potent stem cells have been

identified and characterised, and tracked in patients at different time points by following genetic lesions using exome sequencing, providing the best evidence to date that human cancer stem cells exist at least in MDS, using assays that avoid the pitfalls of xeno-transplantation assays. (Woll et al., 2014).

TEL-AML1 and Childhood Acute Lymphoblastic Leukaemia

In 25% of childhood B precursor ALL, a t(12;21) translocation forms a TEL-AML1 (TA, ETV6-RUNX1) fusion gene (figure 1.9). TEL-AML1 positive leukaemias are associated with a good prognosis following standard therapy, although as for other childhood cancers long treatment regimes can be disabling with long term side effects such as endocrine and heart defects and increased incidence of secondary malignancies (Dickerman, 2007). TEL-AML1 is considerably less prevalent in adult ALLs and is found in only 3% of cases (Kwong and Wong, 1997; Zelent et al., 2004a). Detection of the TEL-AML1 transcript in 1% of neonatal blood spots and studies of twins with concordant ALL revealed clonotypically identical TEL-AML1 transcripts, proving that the translocation must have occurred in utero (Greaves et al., 2003; Greaves and Wiemels, 2003; Mori et al., 2002a). However, the high frequency of detection in the normal population (1% of all live births) has been challenged, suggesting the data may contain false positives, and that the actual incidence of TEL-AML1 in live births is much closer to disease frequency (Kusk et al., 2014; Lausten-Thomsen et al., 2011). If this is valid, TEL-AML1, thought to be a subtle leukaemic oncogene which requires additional secondary mutations for disease development, may in fact be more of an aggressive driver of disease, a notion that does not fit with the body of functional data.

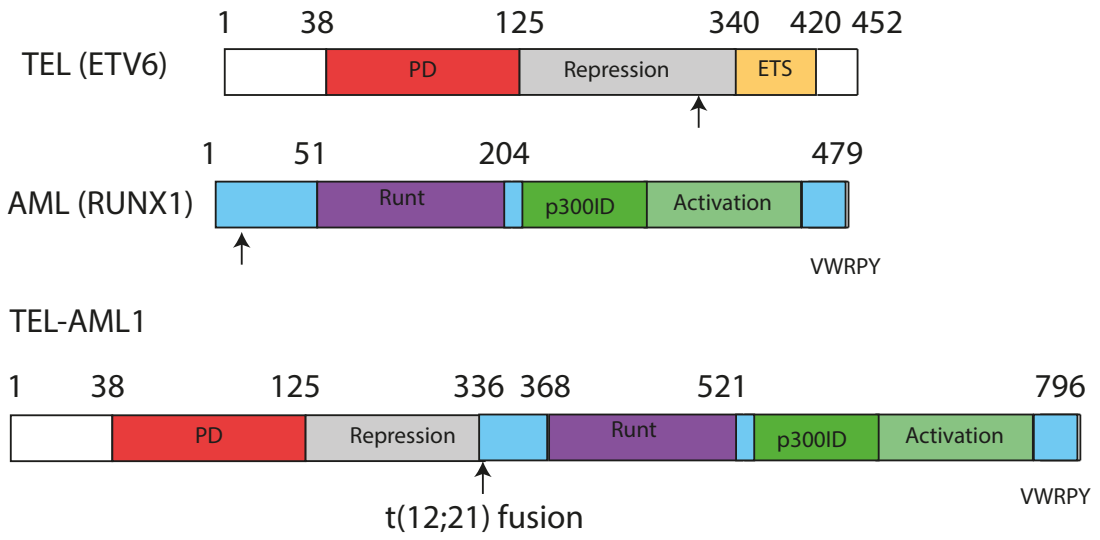


Figure 1.9: The domains of AML1, TEL and TEL-AML1 proteins
In AML, the Runt DNA binding domain and region that is reported to interact with the p300 HAT (p300 ID) are indicated along with the transcriptional activation domain (activation). In the TEL protein, the oligomerisation pointed domain (PD), central repression domain (repression) and ETS DNA binding domain (ETS) are indicated. Arrows indicate the break and fusion points. Adapted from (Zelent et al., 2004b).

Wild type function of TEL and AML1

Both TEL and AML1 are transcription factors (TFs) involved in normal HSC functioning. AML1 (RUNX1) encodes a transcriptional activator with a DNA binding domain with homology to *Drosophila* Runt. Homozygous loss of AML1 results in lack of fetal liver haematopoiesis and embryonic lethality at E12.5 (North et al., 1999; Okuda et al., 1996; Wang et al., 1996b; Wang et al., 1996c). TEL is a member of the ETS family of transcription factors and TEL knockout mice are embryonic lethal between E10.5 and E11.5, with defective yolk sack angiogenesis and apoptosis of mesenchymal and neural cells (Wang et al., 1997). Within the haematopoietic compartment, TEL is essential for establishing haematopoiesis in neonatal bone marrow and speculated to be required for

either the migration of haematopoietic progenitors to the bone marrow or their survival within the bone marrow niche (Waga et al., 2003). TEL has also been shown to be required for specifying the first haematopoietic stem cells in the dorsal aorta in *Xenpus laevis* (Ciau-Uitz et al., 2010) and knock down of *Tel* in zebrafish resulted in reduced erythroid and myeloid cells but enhanced lymphopoiesis (Rasighaemi et al., 2015). TEL expression is not required in most mature lineages except megakaryocytes but is required for the survival of adult HSCs (Hock et al., 2004).

TEL-AML1 and Disease

TEL-AML1 alone is insufficient for overt leukaemia, with a latency period of up to a decade before overt disease presenting with on average six secondary mutations (Mullighan et al., 2007). The additional mutations are secondary to the fusion of *Etv6* and *Runx1* supported by the differences in the mutation patterns found in twins and differences between diagnosis and relapse samples from the same patient (Anderson et al., 2011; Bateman et al.; Ford et al., 2001; Maia et al., 2001; Romana et al., 1996). Pre-leukaemic clones evolve in a Darwinian fashion with advantageous mutations resulting in positive selection (Greaves and Maley, 2012). The evolution of TEL-AML1 leukaemic clones has been beautifully tracked in patients using fluorescent in situ hybridisation revealing a dynamic sub clonal architecture that can change between diagnosis and relapse (Anderson et al., 2011).

The most common secondary mutations include loss of the remaining wild type *Etv6* allele and *Pax5* haploinsufficiency (Mullighan et al., 2007). These secondary mutations are likely driven by enzymes of the VDJ recombination machinery such as RAG mediated recombination at RSS motifs (Papaemmanuil et al., 2014), and AID (Activation-Induced Cytidine Deaminase), which is involved in somatic hyper mutation and class switch recombination (Swaminathan et al., 2015). RAG expression is first detected in the LMPP, and is found at higher levels in the fetus than the adult (Boiers et al., 2013).

These observations have led to a model wherein the TEL-AML1 translocation arises in a fetal haematopoietic stem or progenitor cell to generate a covert pre-leukaemic clone with a partially stalled passage through B cell development. This stalling extends RAG and AID activity and thus increases the likelihood of secondary mutations (Swaminathan et al., 2015). Epidemiological studies suggest that leukaemic development is promoted by abnormal cytokine signalling and repetitive inflammatory stimuli associated with chronic infections in childhood (Gilham et al., 2005; Greaves, 2006; Greaves and Maley, 2012). This is supported by studies that show early exposure to infections such as by attending day care reduces the risk of leukaemia (Gilham et al., 2005; Urayama et al., 2010). Introduction of vaccination programs during early childhood also reduces the incidence of ALL, by reducing the incidence of chronic infections and the subsequent immune responses they initiate (Greaves, 2009). This has been well reported for *Haemophilus influenzae* type B in three independent studies (Auvinen et al., 2000; Groves et al., 2001; Ma et al., 2005). These etiological studies, coupled with the data that show TEL-AML1 inhibits TGFβ signalling

(Ford, 2009), has led to the proposal that chronic childhood infections, followed by a deregulated quelling of the immune response due to failure to respond to TGFβ, may promote the expansion or persistence of pre-leukaemic clones, at a developmental stage where RAG and AID are active in B cell progenitors and capable of causing secondary mutations. However how TEL-AML1 stalls B cell differentiation is not understood at the molecular level or cellular level. TEL-AML1 has been shown to disrupt wild type AML1 function in a dominant negative fashion (Ichikawa et al., 2004), bind to SMAD3 and inhibit TGFβ signalling (Ford et al., 2009), increase signalling via the erythropoietin receptor (Torrano et al., 2011) and increase the generation of mutations caused by reactive oxygen species in cells (Kantner et al., 2013). How and if these mechanisms translate into altered B cell lymphopoiesis is not understood.

Modelling TEL-AML1 positive B cell progenitor ALL

The effect of the TEL-AML1 translocation on haematopoiesis has been studied in genetically modified zebrafish (Sabaawy et al., 2006) and mice (Schindler et al., 2009; van der Weyden et al., 2011). Unfortunately, there are no models that have faithfully reproduced a B cell ALL phenotype with mouse malignancies mainly of the T cell lineage (Schindler et al., 2009). This is discussed in more detail in chapter VI.

B cell progenitor ALLs have been modelled in mice with high penetrance by constitutive activation of STAT5 coupled with haploinsufficiency to the key B cell transcription factors PAX5 or EBF1 (Heltemes-Harris et al., 2011), indicating

specific inactivation of key genes known to be involved in B cell leukaemias is a plausible strategy by which to model ALL. Importantly restoration of PAX5 expression resulted in disease remission, indicating that the loss of PAX5 may be a driver in leukaemic development.

Cell of Origin of Childhood B cell ALL

Understanding the cell of origin of leukaemias can be subdivided into understanding firstly in which cell the initial TEL-AML1 translocation occurs, secondly the cell(s) in which subsequent hits are accumulated, and finally the cell which is functionally deregulated and drives leukaemic development. As TEL-AML1 B cell progenitor ALLs are predominately $CD19^{+}$ Pre B cells, the cell of origin must be somewhere between the HSC and Pre B cell stage (Golub et al., 1995), or in a later progenitor that can de-differentiate.

The cells in which these three processes occur may be the same or different. Genetic models all suggest that the initial TEL-AML1 translocation occurs in a multi-potent progenitor as expression in B cell restricted progenitors has so far failed to drive disease development (Sabaawy et al., 2006 Schindler et al., 2009 (Andreasson et al., 2001). More recent studies, suggesting that secondary mutations are driven by aberrant activity of the recombination machinery (Papaemmanuil et al., 2014; Swaminathan et al., 2015), suggest secondary mutations are accumulated in cells which contain active RAG and AID.

Interestingly, 42% of TEL-AML1 positive leukaemias and 67% of relapsed cases express the myeloid markers CD13 and CD33, higher than other subclasses of ALL (Shaker et al., 2001, (Abdelhaleem, 2007). This may indicate that the fetal cell of origin may have, or regained, myeloid potential. (Abdelhaleem, 2007).

A better understanding of the cell compartment in which these events occur, will help improve therapy design. Furthermore, understanding the functional and molecular differences between fetal and adult B cell progenitors may also help elucidate why children have such high rates of ALL compared to adults.

Thesis Overview and Aims

Despite the well-characterized staging of adult B cell lymphopoiesis, little has been done to understand B cell development in the embryo. There are conflicting reports on when and where during embryonic development lineage restricted B cell progenitors emerge, through what stages of B cell commitment they develop and how they are regulated. We hypothesize that to gain a better insight into B cell progenitor ALLs in children where the initial events often occur in utero, we must first characterize B cell development in the fetus. This thesis looks at the emergence of an immune restricted progenitor with B cell potential, prior to the emergence of definitive stem cells in the early embryo (chapter 3), then specifically focuses on the emergence of B lineage restricted progenitors (chapter 4), before characterizing and staging the progression of fetal B cell development and identifying a unique fetal Pro B committed progenitor (chapter 5). Finally, we examine these fetal B cell progenitors in a pre-leukaemic setting, by using a TEL-AML1 expressing mouse model (chapter 6).

This thesis aims to understand:

1. Where and when in the embryo the first fully B cell restricted progenitors emerge, and what functionally and molecularly characterizes these progenitors
2. Through what stages these early B cell progenitors progress during development and how these stages relate to their post-natal counterparts
3. How the TEL-AMl1 translocation, known to target a cell with B cell potential in utero, influences the early stages of B cell development in the mouse embryo

CHAPTER II: MATERIALS AND METHODS

MATERIALS AND METHODS

Mice

Husbandry

All animals were bred and maintained by Biomedical Services at the University of Oxford. Procedures were performed under the UK Home Office Animals Scientific Procedures Act (1986).

Strains

C57BL/6 (CD45.2), C57B6SJL CD45 (CD45.1) and NOD.Cg-Prkdc^{scid}IL2r ^{tm1Wjl}/Szj (NSG) mice were obtained from Biomedical Services at the University of Oxford or Harlan Animal Research Laboratories.

Mice with *Rag1* driven GFP expression were obtained from N. Sakaguchi (Department of Immunology, Kumamoto University School of Medicine, Japan) (Kuwata et al., 1999). For experiments the *Rag-1*-GFP was inherited from the male to ensure all GFP expressing cells were embryo derived and not due to maternal contamination.

Rosa26 (R26R)$^{eYFP/eYFP}$ (R26YFP) mice were generated by S. Srinivas (Department of Genetics and Development, Columbia University, New York, USA) (Srinivas et al., 2001)

Mb1-cre mice were obtained from A. Potocnik, The National Institute for Medical Research, The Ridgeway, Mill Hill, London, NW7 1AA (Hobeika et al., 2006b). For

fate mapping experiments the *Mb1*-cre allele was inherited from the male and YFP from the female. (Hobeika et al., 2006a)

TEL-AML1$^{wt/tg}$ (*Etv6*$^{Runx1/wt}$: 'TA') mice were obtained from the Wellcome Trust Sanger Institute on a mixed C57Bl/6-129vEvBrd background (van der Weyden et al., 2011) and backcrossed twice to C57Bl/6.

Pax5$^{tg/tg}$ (*Pax5Δ)* mice were generated on 129/Ola and obtained from M. Busslinger at the Research Institute of Molecular Pathology (Horcher et al., 2001), before back crossing to C57Bl/6.

*vav*Cre mice were obtained from the Department of Molecular Immunology, National Institute of Medical Research, London (Boer et al., 2003). They were generated on a CBA/Ca x C57F7BL/10 background and have been backcrossed to C57Bl/6 more than 10 times.

Obtaining Embryos

Timed over night mating was set up, confirmed by detection of a vaginal plug the following morning. This was set as embryonic day (E) 0.5.

Genotyping of mouse strains

Ear biopsies were taken from transgenic mice and the DNA extracted using either the Phire Animal Tissue Direct PCR Kit (ThermoFisher Scientific) or E.Z.N.A Tissue DNA Kit (Omega Bio-Tek). 1-2ul of pre-diluted genomic DNA was

then subjected to specific PCR reactions as outlined below. PCR reactions were performed using a thermal cycler (Tetrad II 96-well Alpha Unit, Bio-Rad). Products were separated by 2% agarose gel electrophoresis. DNA was visualized using ethidium bromide stain followed by imaging with an ultra-violet transilluminator.

Table 2.1: PCR protocol for genotyping

Strain	**Primers**	**Reagents**	**PCR cycle**
TEL-AML1	5'-CCAGAACAGCGGCAAAGGAC-3' 5'- CGTGCTTCACGGTTGGGATG-3' Product size: 323bp	FirePol	1. 98°C for 30secs 2. 98°C for 10secs 3. 60°C for 30secs 4. 72°C for 10secs step 2 x 35 5. 72°C for 10mins 6. 4°C forever
Mb1-Cre	5'-AGATGCCAGGACATCAGGAACCTG-3' 5'-AGATGCCAGGACATCAGGAACCTG-3'	Kappa Kit	1. 95°C for 3mins 2. 95°C for 15secs 3. 60°C for 15secs 4. 72°C for 1 sec/kb step 2 x 25 5. 72°C for 1min/kb
*vav*Cre	5'-AGATGCCAGGACATCAGGAACCTG-3' 5'-AGATGCCAGGACATCAGGAACCTG-3'	Kappa kit	1. 95°C for 3mins 2. 95°C for 15secs 3. 60°C for 15secs 4. 72°C for 1 sec/kb step 2 x 25 5. 72°C for 1min/kb
Pax5$^{tg/tg}$	5'-CCACAGCTACTTTGAATAGGGTGAT-3' 5'-GTGATCTCAGAGGGGTCTGG-3' Product size: WT 496bp, Floxed 693bp	Kappa kit	1. 95°C for 3mins 2. 95°C for 15secs 3. 60°C for 15secs 4. 72°C for 1 sec/kb step 2 x 25 5. 72°C for 1min/kb
Rosa 26 YFP	5'-AAA GTC GCT CTG AGT TGT TAT-3' 5'-AAA GTC GCT CTG AGT TGT TAT-3' 5'-GCG AAG AGT TTG TCC TCA ACC-3' Product size: WT 600bp, Knock in 300bp.	EMBL Taq	1. 94°C for 3mins 2. 94°C for 30secs 3. 54°C for 30secs 4. 72°C for 40secs step 2 x 35 5. 72°C for 5mins 6. 4°C forever
Rag-1 GFP	5'-AGG TAG CTT AGC CAA CAT GG-3' 5'-GCT CAG GTA GTG GTT GTC GG-3' 5'-CAA CAT CTG CTT CAC GTC GAT CC-3' Product size: WT 515bp, Knock in 625bp	Kappa kit	1. 94°C for 3mins 2. 94°C for 30secs 3. 54°C for 30secs 4. 72°C for 40secs step 2 x 35 5. 72°C for 5mins 6. 4°C forever

Phenotyping of mouse strains

YFP expressing tissues were phenotyped by mixing 20ul of single cell suspension with 80ul of 1% PBS/FCS before analysis by flow cytometry. Mono nuclear cells were identified by their FSC/SSC profile and YFP detected using the 488nm blue laser and 525/50 filters.

Preparation of murine haematopoietic cells

Single cell suspensions were prepared from haematopoietic tissues as outlined below. Cellularity was either counted using a Sysmex KX-21N or for low cell numbers in a Neubauer chamber after viability staining with trypan blue (Sigma). Cell suspensions were kept on ice at all times. Cells were spun down at 500g for 5 minutes, before suspension in PBS/FCS containing FcR (CD16/CD32) block and staining with antibody cocktails.

Bone Marrow

Femur, tibia and cristae were extracted, cleaned of associated tissues, crushed and re-suspended in 2% PBS/FCS before filtering through a 70uM filter.

Peripheral Blood

Mice were warmed up in a heat box before local sterilization of the tail and collection of 100-200ul of peripheral blood into an EDTA-coated collection tube. No more than 10% of total blood volume was sampled, and no more than 15% of total blood volume was taken within a 30 day period. Blood was treated with equal volumes of 2% dextran at 37°C for 20 minutes. The upper layer containing white blood cells was removed, and washed with 1ml of PBS/FCS before

treatment with ammonium chloride (Stem Cell Technologies) for 2 minutes to lyse erythrocytes. Peripheral blood was not routinely treated with FcγR block.

Spleen, liver and thymus

Whole spleen, liver and thymi were mashed through a cell strainer and cells were re-suspended in 2% PBS/FCS.

Immune cells of the peritoneal cavity

The peritoneum of sacrificed mice was washed by injection with 5ml of ice cold 5% PBS/FCS before massaging the stomach and re-aspirating the fluid.

Fetal haematopoietic tissues

E11.5 embryos were staged by counting somite pairs (sp) (Bee et al., 2010). Fetal livers were dissected before generation of a single cell suspension using a 27G needle. Fetal thymi were dissected by Tiago Luis before fine needle suspension. E9.5 Yolk sac (without vitelline and umbilical cord), PAS, Placenta and E8.5 whole concepti (yolk sac, allantois and embryo proper, without the ectoplacental cone) were dissected by Emanuele Azzoni, Gemma Swiers and Marella de Bruijn. Cell suspensions were prepared by 0.12% collagenase treatment for 20minutes at 37°C before gentle pipetting. All embryonic tissues were handled in 10% FCS/PBS.

In vivo assays

In vivo repopulation assay in adult mice

C57BL/6 or C57B6SJLCD45 recipient mice were typically conditioned with two doses of 450cGy irradiation from a cesium-137 source. Within 24 hours, FACS purified or unfractionated haematopoietic cells were intravenously injected into

the tail along with 200,000 congenic competitor bone marrow or fetal liver cells from either wild type, *Rag-1* deficient or NSG mice, in a total volume of 200µl. Recipient and donor cells were distinguishable based on CD45.1 or CD45.2 allotype expression using monoclonal antibodies followed by flow cytometry.

NSG recipient mice were sub-lethally conditioned with two doses of 100cGy irradiation and injected with FACS purified test cells without competitor support cells.

In vivo repopulation assay in newborn mice

1 to 3 days old newborn mouse pups (C57BL/6 or C57B6SJLCD45) were irradiated with 3.5Gy of irradiation from a cesium-137 source. 4 hours later 25µl of cell suspension was injected into the anterio-facial vein using a 30G needle. The pups were positioned over a light source to illuminate the vein before gentle insertion of the needle. Bleeding was stopped by gentle application of pressure with a cotton bud and pups were rubbed in bedding before returning to the mother (Park et al., 2008).

Peripheral blood sampling in reconstitution assays

Mice were warmed in a heat box to increase tail vein circulation before retention in a sterilized mouse restrainer. The tail was sterilized locally before generation of a small incision and collection of 100 to 200µl of peripheral blood in an EDTA coated collection tube (Sarstedt). Collection volume never exceeded 10% of total blood volume or 15% over a 30 day period.

Bone marrow sampling in reconstitution assays

Bone marrow was sampled from mice injected with test cells at 2 weeks and 6 weeks after injection. Mice were treated with vaporized iso-fluorane before subcutaneous injection of vetergesic. The leg joint was dry shaven then sterilized locally before angling the knee to allow access to the femur shaft. A 1ml insulin syringe with a 29.5 gauge containing 100µl of sterile, filtered PBS was used to gently drill into the bone. 50µl of sterile PBS was injected into the femur cavity, then the syringe drawn back to generate a vacuum and aspirate around 20-50µl of marrow. The mouse was removed from iso-flourane and on awakening examined to check for normal movement of the knee joint.

Flow cytometry analysis and cell sorting

Cell Staining for Flow Cytometry Analysis and Cell Sorting

FC block treated cells were stained for 15mins on ice with cell surface antibody cocktails detailed in table 2.2**.** Samples were stained typically in 25µl per 2.5 million cells then washed with 1ml of PBS with FCS before resuspension in PBS with FCS. For two step stainings involving biotin and streptavidin conjugates, after washing samples were resuspended in 1x dilution of the respective secondary antibody and incubated for 15mins on ice before washing and resuspension. Dead cells were excluded using 7AAD (Sigma) or DAPI (Invitrogen) before analysis on a BD LSRII SORP (table 2.3) or a BD LSR Fortessa. For all experiments the cytometer voltages and compensation was performed using single stained controls and fluorescent-minus-one (FMO) controls. Single stained controls were typically prepared with 1µl of antibody and one drop of species specific CompBeads and one drop of negative control beads before staining as for cells.

Enrichment of KIT expressing cells

When necessary bone marrow or fetal liver cells that express KIT were enriched by magnetic activated cell sorting according to manufacturer's instructions (MACS, Miltenyi Biotec). 2.5µl of KIT beads were added to 100million cells in 100ul and incubated for 20mins on ice before washing and resuspension in PBCS with FCS. The cells were filtered and added to a MACS LS column on a MACS separator. The column was washed before removal from the magnetic field and elution of the KIT enriched fraction by flushing with PBS with FCS.

Cell Sorting

Cells were prepared in a sterile fume hood. Cell sorts were performed on a BD FACSAriaII cell sorter (table 2.3). Sort purity was determined by test sorting and reanalysis prior to and after the sample sort and was typically over 95%.

Table 2.2: List of Antibodies used in Flow Cytometry

Marker	Conjugate	Clone	Company	Panel
AA4.1 (CD93)	APC	AA4.1	eBiosciences	Pro B
AA4.1 (CD93)	PeCy7	CD93	eBiosciences	Pre Pro B
B220	PeCy5	RA3-6B2	BioLegend	HSC, Bryder, ETP, IL-7R
B220	FITC	RA3-6B2	eBiosciences	OP9
B220	PECF594	RA3-6B2	BD	PC
B220	BV785	RA3-6B2	BioLegend	B1 Spleen
B220 (CD45R)	APCeF780	RA3-6B2	eBiosciences	Pre Pro B, Pro B, OP9GM, BMA, Basic B cell panel.
B220 (CD45R)	PECy7	RA3-6B2	BioLegend	B cell emergence, PB
BP-1	PE	6C3	eBiosciences	Basic B cell panel
CD105	Biotin	MJ7/18	BioLegend	Bryder
CD11b (Mac-1)*	PeCy5	M1/70	BioLegend	HSC
CD11c	PeCy5	N418	BioLegend	Pre Pro B, Pro B
CD150	PeCy7	TC15-	BioLegend	HSC

		12F12.2		
CD150	APC	TC15-12F12.2	BioLegend	Bryder
CD16/32	PE	93	eBiosciences	Bryder
CD19	PECF594	ID3	BD	Pre Pro B, Pro B, BMA, B1 spleen
CD19	APC	eBioID3	eBioscience	B cell emergence, PB
CD19	PeCy5	ID3	BD	ETP, IL-7R
CD19	PE	ID3	BD	OP9DL
CD19	PeCy7	ID3	eBiosciences	OP9, OP9GM, PC
CD1d	Biotin/SA BV605	1B1	BioLegend	B1 Spleen
CD21	PE	7G6	BD	B1 Spleen
CD23	PeCy7	B3B4	BioLegend	B1 Spleen
CD24	APC	30-F11	Novus Biologicals	Pre Pro B, Basic B cell panel.
CD24	PE	30-F1	eBiosciences	Pro B
CD25	PCPCy5.5	PC61	BioLegend	ETP, OP9DL
CD3e	PeCy5	145-2C11	eBiosciences	Pre Pro B, Pro B
CD4	PeCy5	RM4-5	BioLegend	HSC, Bryder
CD4	PeCy7	RM4-5	Becton Dickinson	ETP
CD4	APCeF780	RM4-5	eBiosciences	OP9DL
CD41	PeCy7	MWReg30	eBiosciences	Bryder, IL-7R
CD43	FITC	S7	BD	Pre Pro B, Pro B, Basic B cell panel.
CD43	APC	S7	BD	B1 Spleen
CD45	PO	30-F11	Caltag	OP9DL, OP9, OP9GM
CD45.1	FITC	A20	BioLegend	BMA, PC, PB
CD45.1	BV650	A20	BioLegend	B1 Spleen
CD45.2	AF700	104	BioLegend	B cell emergence, HSC, Bryder, ETP, BMA, PC, IL-7R, B1 Spleen, PB
CD48	APC	HM48-1	BioLegend	HSC
CD5	PeCy5	53-7.3	BioLegend	HSC, Bryder, ETP
CD5	BV421	53-7.3	BioLegend	PC, B1 Spleen
CD8	APC	53-6.7	eBioscience	ETP
CD8a	PeCy5	53-6.7	BD/BioLegend	HSC, Bryder
CD8a	PECy7	53-6.7	eBiosciences	OP9DL
KIT (CD117)	PE	2B8	eBiosciences	Pre Pro B
KIT (CD117)	PECy7	2B8	eBiosciences	Pro B, BMA
KIT (CD117)	APCeF780	2B8	eBioscience	B cell emergence, HSC, Bryder, ETP, IL-7R
F4/80	PeCy5	BM8	BioLegend	Pre Pro B, Pro B

F4/80	APC	BM8	Caltag	OP9GM
Flt3 (CD135)	Bi/SA BV605	A2F10	eBiosciences	Pre Pro B, Pro B
Flt3 (CD135)	PE	A2F10	BioLegend	B cell emergence, HSC, ETP, BMA
Gr-1 (Ly6C)	APC	RB6-8C5	BioLegend	OP9
Gr-1 (Ly6C)	PE	RB6-8C5	eBiosciences	OP9GM
Gr-1 (Ly6C)	PeCy5	RB6-8C5	BioLegend	Pre Pro B, Pro B, HSC, Bryder, ETP
Gr-1	PO	RB6-8C5	BioLegend	PB analysis
IgD	APC Cy7	11-26c.2a	BioLegend	PC, B1 Spleen
IgM	APC	II/41	BD	BMA
IgM	PE	R6-60.2	BD	PC, PB
IgM	FITC	R6-60.2	BD	B1 Spleen
IgM	Biotin	R6-60.2	BD	Basic B cell Panel
IL-7R (CD127)	PE	A7R34	eBiosciences	IL-7R
IL-7R (CD127)	Biotin	A7R34	eBioscience	B cell emergence
IL-7R (CD127)	BV421	A7R34	BioLegend	Pre Pro B, Pro B, BMA
Ly6C	AF700	Al-21	Becton Dickenson	Pre Pro B
Mac-1 (CD11b)	AF700	M1/70	eBiosciences	OP9, OP9GM
Mac-1 (CD11b)	APC	M1/70	BioLegend	PC
NK1.1	PeCy5	PK136	BioLegend	Pre Pro B, Pro B
NK1.1	PB	PK136	BioLegend	OP9DL, OP9, OP9GM
SCA-1 (Ly-6A/E)	FITC	E13-161.7	BioLegend	HSC, Bryder
Streptavidin	BV605	n/a	BioLegend	Pre Pro B, Pro B
Streptavidin	QD655	n/a	Invitrogen	B cell emergence
Streptavidin	PE Texas Red	n/a	BD/BioLegend	Bryder
Ter119 (Ly-76)	PeCy5	TER-119	BioLegend	Pre Pro B, Pro B, HSC, Bryder, ETP, IL-7R
VeCadherin (CD144)	APC	BV13	eBiosciences	IL-7R
Thy1.2 (CD90.2)	APC	30-H12	BioLegend	OP9DL

PB: Peripheral blood, BMA: Bone marrow aspiration, IL-7R: Fetal LMPP panel,

Table 2.3: Instrument configurations for BD LSRII SORP and BD FACSAriaII SORP

Laser	Wavelength	Filter settings
Green (150mW)	532	PE: 575/25 PE-Texas Red: 610/20 PECy5: 685/35 PECy5.5 (PerCPCy5.5): 710/40 PECy7: 780/60
Red (40mW)	638	APC: 670/14 Alexa Fluor 700: 730/45 APCeFluor780: 780/60
Blue (100mW)	488	SSC: 488/10 FITC: 525/50
Violet (50mW)	405	Pacific Blue and BV421: 450/50 Sytox Blue: 525/50 Pacific Orange: 585/42 QD605 and BV605: 610/20 QD655: 660/40 BV785:

In vitro assays

In vitro evaluation of single lymphoid potentials

For evaluation of B, T and myeloid potential, 1 to 7 haematopoietic stem or progenitor cells in a total volume of 100ul of OptiMEM supplemented with 10% FCS , 1% β-mercaptoethanol and 1% Penicillin/Streptomycin were manually seeded onto 96 well plates containing a confluent monolayer of OP9 (B and myeloid) or OP9DL (T cell, express the Notch delta like 1 ligand) stroma. Stroma was pre plated 24 hours in advance by seeding 2000 cells into each well in 100ul of OptiMEM supplemented with 10% FCS, 1% β-mercaptoethanol and 1% Penicillin/Streptomycin. On the day of seeding, cytokines listed in table 2.4 were added.

Culture media was typically changed once a week by exchanging 100µl of culture medium for fresh media and cytokines. After one week SCF was omitted from the OP9DL1 cultures. Cultures were evaluated after 14 to 28 days by flow cytometry. Clones were transferred to round bottom plates before spinning down and staining with cell surface antibodies.

Table 2.4: Cytokines used for OP9 co-culture assays

Stroma	Lineage	Cytokines	Concentration (ng/ml)
OP9	B	mSCF	10
		hFL	5
		hIL-7	10
OP9	GM (+B)	mSCF	10
		hFL	5
		M-CSF (hCsf-1)	10
		mGM-CSF	10
		mIL-3	20
		hG CSF	20
OP9DL1	T	mSCF	10
		hFL	10

In vitro evaluation of GM potential in liquid culture (terasaki assays)

Fetal liver or adult haematopoietic stem or progenitor cells were sorted into a predefined volume at a density of 1 cell per 20µl of culture media. 60 well terasaki plates (Nunc) were manually plated with 20µl per well. Culture media was X-Vivo15 with Gentamycin, supplemented with 10% FCS and 1% β-mercaptoethanol. The media was supplemented with the cytokines listed in table 2.5. Colonies were evaluated using an inverted microscope (Ix71, Olympus) and when necessary lineages confirmed by preparation of cytospins and May-Grunwald Giemsa staining.

Table 2.5: Cytokines used for liquid GM terasaki assays.

Cytokine	Concentration for adult cells (ng/ml)	Concentration for fetal cells (ng/ml)
mSCF	2	50
mGM-CSF	10	50
hTPO	5	50
hG-CSF	10	50
hFL	5	50
mIL3	5	20

Whole organ explant cultures

Pre circulation embryos (≤6 somite pairs) were dissected by Isabelle Godin to separate the YS and PAS (Boiers et al., 2013; Godin et al., 1995). Whole explants were cultured in 24 well plates or 8 well μ-Slides (Ibidi). The embryo remnants were used for genotyping. Explants were cultured for 48 hours in OptiMEM supplemented with 1% Penicillin/Streptomycin, 0.1-1% β-mercaptoethanol and 10% FCS. After culture organs were taken for quantitative PCR or whole mount immunostained.

Imaging

Whole mount immunostaining

Imaging was performed by Dr Emanuele Azzoni (Boiers et al., 2013). Embryos and YS were dissected and fixed in 4% paraformaldehyde solution for 30minutes at 4°C. Samples were treated with 0.2% Triton X-100 and 2% donkey serum to permeabilise and block then incubated overnight with primary antibodies (table 2.6). After washing and secondary antibody stain, YS were cleared in 50% glycerol/PBS then flat mounted on Superfrost microscope slides. Embryos were

treated with a tissue transparency treatment as described (Yokomizo et al., 2012) and imaged using a Zeiss 780 confocal microscope.

Table 2.6: Antibodies used for whole mount immunostaining

Antibody-conjugate	**Host**	**Supplier**
Anti-GFP	Rabbit	Molecular Probes
Anti-CD31	Goat	R and D systems
Anti-CD45 APC-Cy7	Rat	BD
Anti-Rabbit Alexa 488	Donkey	Molecular Probes
Anti-Goat Alexa 555	Donkey	Molecular Probes
Anti-Rat Alexa 647	Chicken	Molecular Probes

Gene expression analysis

Single cell and multiple cell quantitative PCR

Multiplex quantitative gene expression analysis was performed using the BioMark 48.48 dynamic array (Fluidigm) as described in (Boiers et al., 2013). 1-50 cells were directly sorted into 5µl CellsDirect 2x reaction mix (Invitrogen), 0.1 µl SUPERase 12-In RNAse inhibitor (Ambion), 1.2µl TE buffer (Sigma), 1.2µl CellsDirect RT/Taq mix (Invitrogen) and 2.5µl 0.2x gene specific TaqMan assay mix (Applied Biosystems) in a total volume of 10µl. Reverse transcription was performed on a thermocycler with the following protocol:

1. 50°C for 15mins
2. 95°C for 2 mins
3. 95°C for 15secs
4. 60°C for 4mins

Go to step 2 x 22

Preamplified cDNA was diluted 1:5 in TE buffer. TaqMan assay and sample mixes were loaded onto a 48.48 dynamic array (Fluidigm) according to manufacturer's instructions before performance of a quantitative PCR reaction. The data were analysed using the BioMark Real Time PCR analysis software and ΔC_t values calculated by normalization to *Hprt*.

RNA sequencing:

100 cells were sorted into lysis buffer with RNAse inhibitor then prepared using the SMARTer™ Ultra Low RNA kit for Illumina Sequencing (Clontech) as previously described (Boiers et al., 2013; Ramskold et al., 2012). Amplified cDNA libraries were validated with a distinct peak spanning 400 and 9000bp in size as measured by a high sensitive DNA kit (Agilent) on a Agilent 2100 BioAnalyzer. Samples were further processed for tagmentation and Nextera indexing (Illumina). 8-10 samples were pooled per lane before sequencing on a 50bp single end flow cell on a HiSeq2500 (Illumina). The raw sequence data was mapped to the mouse genome GRCm38 using STAR, an ultra fast universal RNA-seq aligner (Dobin et al., 2013). After removing reads that were not uniquely mapped we used edgeR to calculate the counts per million for each gene. We also used RNA-SeQc to assess the quality of the data (DeLuca et al., 2012).

Quantitative real time PCR of dissected embryonic tissue or sorted cells

Single cell suspensions were dissolved into buffer-RLT with 1% 2-mercaptoethanol and frozen at −80°C. RNA extraction and DNase treatment was performed with the RNeasy Micro kit (Qiagen). Eluted RNA samples were

reverse transcribed using SuperScript II and random hexamers (Invitrogen) according to the manufacturer´s protocol. PCR reactions were performed by mixing 2 x TaqMan universal PCR master mix, 20 x Assays-on-Demand (primer/MGB probemix) (table 2.7), RNase-free H_2O and cDNA. Reactions were run on a 7500 Fast Real Time qPCR machine on the 'standard' speed setting for 50 cycles (Applied Biosystems). Signals were only deemed genuine if they differed from controls prepared without the addition of reverse transcriptase by more than 5 cycles.

Table 2.7: Probes used for q-RT PCR of embryonic tissue or multiplex quantitative PCR

Gene symbol	**Gene name**	**Assay ID**
Actb	actin, beta, cytoplasmic	Mm00607939_s1
B2m	beta-2 microglobulin	Mm00437762_m1
Cd19	CD19 antigen	Mm00515420_m1
Cd3e	CD3 antigen, epsilon polypeptide	Mm00599683_m1
Cd3g	CD3 antigen, gamma polypeptide	Mm00438095_m1
Cd79a	CD79A antigen (immunoglobulin-associated alpha)	Mm00432423_m1
Cd79b	CD79B antigen	Mm00434143_m1
Cebpa	CCAAT/enhancer binding protein (C/EBP), alpha	Mm00514283_s1
Crlf2	cytokine receptor-like factor 2	Mm00497362_m1
Csf1r	colony stimulating factor 1 receptor	Mm00432689_m1
Csf2ra	colony stimulating factor 2 receptor, alpha, low-affinity (granulocyte-macrophage)	Mm00438331_g1
Csf2rb	colony stimulating factor 2 receptor, beta, low-affinity (granulocyte-macrophage)	Mm00655745_m1
Csf3r	colony stimulating factor 3 receptor (granulocyte)	Mm00432735_m1
Dntt	deoxynucleotidyltransferase, terminal	Mm00493500_m

		1
Ebf1	early B-cell factor 1	Mm00395519_m1
Epor	erythropoietin receptor	Mm00438760_m1
Fli1	Friend leukaemia integration 1	Mm00484410_m1
Flt3	FMS-like tyrosine kinase 3	Mm00439016_m1
Gata1	GATA binding protein 1	Mm00484678_m1
Gata2	GATA binding protein 2	Mm00492300_m1
Gata3	GATA binding protein 3	Mm00484683_m1
Hes1	hairy and enhancer of split 1 (Drosophila)	Mm01342805_m1
Hprt1	hypoxanthine guanine phosphoribosyl transferase 1	Mm00446968_m1
Id2	inhibitor of DNA binding 2	Mm00711781_m1
Ikzf1	IKAROS family zinc finger 1	Mm00456421_m1
Il-7r	interleukin 7 receptor	Mm00434295_m1
Itgam	integrin alpha M	Mm00434455_m1
Kit	kit oncogene	Mm00445212_m1
Klf1	Kruppel-like factor 1 (erythroid)	Mm00516096_m1
LAMBDA 5	Lambda 5	LAMBDA5-EX12
Lat	linker for activation of T cells	Mm00456761_m1
Lck	lymphocyte protein tyrosine kinase	Mm00802897_m1
Ly6d	lymphocyte antigen 6 complex, locus D	Mm00521959_m1
Mafb	v-maf musculoaponeurotic fibrosarcoma oncogene family, protein B (avian)	Mm00627481_s1
Mpl	myeloproliferative leukaemia virus oncogene	Mm00440310_m1
Mpo	myeloperoxidase	Mm00447886_m1
Pax5	paired box gene 5	Mm00435501_m1
Ptcra	pre T-cell antigen receptor alpha	Mm00478361_m

		1
Rag1	recombination activating gene 1	RAG-1-C
Rag2	recombination activating gene 2	Mm00501300_m1
Runx1	runt related transcription factor 1	Mm01213405_m1
Sfpi1	SFFV proviral integration 1	Mm00488140_m1
Sterile Igh	Sterile IgH	STERILEIGH-B
Tcfe2a	transcription factor E2a	Mm01175588_m1
Thy1	thymus cell antigen 1, theta	Mm00493681_m1
Vpreb1	pre-B lymphocyte gene 1	Mm00785614_sH
Vwf	Von Willebrand factor homolog	Mm00550376_m1

Data analysis

Flow cytometry data was collected using FACS DIVA and analysed using FlowJo 887 (TreeStar). Graph pad prism was used for generation of tables and statistical analysis.

Chapter III:
Investigating the origin of a lympho-myeloid immune restricted progenitor in the early mouse embryo

INVESTIGATING THE ORIGIN OF A LYMPHO-MYELOID IMMUNE RESTRICTED PROGENITOR IN THE EARLY MOUSE EMBRYO

Introduction

The initial events of many infant and childhood leukaemias occur in utero, potentially in unique fetal progenitors. Related to our interest in fetal B cell lymphopoiesis, we first investigated where and when the first immune restricted progenitor cells with B cell, T cell and Myeloid potential (lympho-myeloid) could be detected in the embryo.

The haematopoietic hierarchy provides a paradigm for the stepwise lineage restriction of multi-potent HSCs into uni-potent progenitors. However, the precise roadmap of lineage restriction remains disputed. The division into the lymphoid and myeloid lineages was thought to be an early fate decision supported by the identification of common myeloid and common lymphoid progenitors (Akashi et al., 2000; Arinobu et al., 2007b; Kondo et al., 1997). However the characterisation of $Lin^{-}SCA1^{+}KIT^{+}FLT3^{high}$ cells demonstrated a population that retained granulocyte macrophage (GM), B cell and T cell potential but had no Megakaryocyte Erythroid (MkE) potential (Adolfsson et al., 2005). These cells were coined lymphoid primed multi-potent progenitors (LMPPs) and indicated that the initial lineage restriction in haematopoiesis may not involve segregation of the lymphoid and myeloid lineage but instead result in the separation of the MkE and lympho-myeloid lineages. Supporting the relevance of LMPPs, mixed lineage leukaemias occur almost exclusively between the GM/B or GM/T lineages, but rarely between the MkE and B or T cell lineage,

indicating that the separation of the MkE lineage from the lympho-myeloid lineages is an early step in HSC differentiation (Matutes et al., 1997; Weir et al., 2007).

The physiological relevance of the LMPP has been challenged, and the field does not unequivocally accept it. Firstly fate-mapping studies where cre expression is driven by either *Il-7rα* or *Rag1*, genes both expressed by the LMPP, demonstrate a limited labelling of myeloid cells (Schlenner et al., 2010; Welner et al., 2009b). This questions the physiological relevance of the LMPP to the myeloid lineage in the adult. As not all LMPPs express *Il-7rα* or *Rag1* this approach likely underestimates the myeloid contribution of the LMPP in the adult. In contrast in the fetus, where proportionately more LMPPs express *Rag1*, fate mapping shows a more significant labelling of the myeloid compartment (Boiers et al., 2013). Secondly studies transplanting high numbers of LMPPs (>500) in vivo were able to detect MkE potential *(*Forsberg et al., 2006), although it was later shown that the majority of this MkE potential could be removed when Lin-SCA1^{+}KIT^{+}FLT3^{high} LMPPs were further defined as expressing PU.1 (Arinobu et al., 2007a) and not expressing the thrombopoietin receptor 'MPL' (Luc et al., 2008). Thirdly, *Flt3*-cre fate mapping studies claimed all haematopoietic lineages, including the MkE lineage, have progressed through a 'FLT3^{+}' progenitor stage, further challenging the loss of MkE potential by the LMPP (Boyer et al., 2011). However, this study itself shows as many as 74% of short term HSCs with 'intermediate' expression of FLT3 are labelled by fate mapping and, as the LMPP is found only within LSK cells that express the highest levels of FLT3, labelling cells based on *Flt3* expression will not exclusively label LMPPs.

Furthermore it has been shown that even fully multi-potent haematopoietic progenitors are labelled by *Flt3*-cre fate mapping (Buza-Vidas et al., 2011), meaning it is difficult to draw clear conclusions about the contribution of the LMPP to the MkE lineage based on *Flt3* fate mapping.

Definitive HSCs that sit at the apex of the haematopoietic tree emerge at E10.5 in the AGM region (Dzierzak and Speck, 2008). Prior to this the haematopoietic needs of the growing embryo are met by primitive waves of haematopoiesis. Initially primitive erythrocytes arise from blood islands in the YS, followed by a second wave of YS derived haematopoietic cells consisting of erythroid, megakaryocyte myeloid and multi-potent progenitors (EMPs). The contribution of these cells towards adult haematopoiesis is poorly characterised.

These primitive waves of haematopoiesis are not thought to generate lymphocytes. Lymphopoiesis is thought to initiate later in embryonic development, after the establishment of dHSCs in the E10.5 FL, however, some studies are compatible with lymphoid commitment initiating earlier. Firstly cells possessing multi-lineage potentials have been identified prior to the emergence of dHSCs (Kieusseian et al., 2012; Rybtsov et al., 2011) and secondly the genes *Rag1* and *Il-7rα*, which are restricted to the B and T cell lineages (Kawamoto et al., 2000a), have been detected in the FL as early as E10.5-E11 (Kawamoto et al., 2000b; Yokota et al., 2006). Furthermore, progenitors with B and T cell potential have been found in the E9 YS (Yoshimoto et al., 2011; Yoshimoto et al., 2012) and B, M and T cell potential has been found in the YS of 10 somite pair (sp) embryos, prior to the seeding of the FL at 28-32sps (Godin et al., 1995).

Interestingly, in vitro studies identified cells in the E12.5 FL with combined B cell and macrophage potential (Cumano et al., 1992), suggesting a fetal progenitor that does not concur with the classical haematopoietic hierarchy, although lack of T cell potential by these cells was not conclusively shown. Additionally, studies of early haematopoiesis fail to find a 'CLP' like progenitor with combined B and T cell potential, instead finding progenitors which either retain all of B, T and myeloid potential or are restricted to only one lineage, further suggesting the classical hierarchy does not encompass all early embryonic haematopoietic waves (Kawamoto et al., 2000b)

Recently, cells characterised as $Lin^{-}KIT^{+}FLT3^{+}IL\text{-}7R\alpha^{+}$ have been identified in the E11.5 FL. At the single cell level these cells have been shown to have GM, B and T cell, but no MkE potential in vitro, suggesting they represent an early embryonic equivalent of the LMPP. Gene expression analysis supported the in vitro data and demonstrated myeloid and lymphoid priming within single cells, but no expression of committed B cell or T cell genes (Boiers et al., 2013).

Early embryo tissues such as the YS require collagenase treatment to generate a single cell suspension for analyses by flow cytometry. Unfortunately collagenase breaks down the FLT3 receptor so it cannot be reliably detected on early haematopoietic tissue, making characterisation of $Lin^{-}KIT^{+}FLT3^{+}IL\text{-}7R\alpha^{+}$ cells prior to E10.5 difficult. As it has been shown that FLT3 and *Rag1-GFP* expression highly overlap (Boiers et al., 2013), using a *Rag1*-GFP reporter $Lin^{-}KIT^{+}\underline{FLT3^{+}}IL\text{-}$

7Rα$^+$ cells can be defined instead as Lin$^-$KIT$^+$*Rag1*-GFP$^+$IL-7Rα$^+$ cells. This therefore allows the progenitor cells to be isolated in the early embryo after collagenase treatment, independently of cell surface FLT3 expression.

We wanted to address where and when in the embryo the Lin$^-$KIT$^+$*Rag1*-GFP$^+$IL-7Rα$^+$ 'fetal LMPP' emerges. In this chapter by analysing cell surface markers, gene expression and using imaging analysis, we show that this newly characterised Lin$^-$KIT$^+$*Rag1*-GFP$^+$IL-7Rα$^+$ 'Fetal LMPP,' emerges in the E9.5 YS, prior to the colonisation of the FL by dHSCs.

Results

The fetal LMPP is readily detectable in the E10.5 FL (Boiers et al., 2013). We decided to analyse early embryo tissue, digested with collagenase, for *Rag1*-GFP expression by flow cytometry. Whole concepti from E8.5 (4-7 somite pairs, sp) *Rag1*-GFP embryos were analysed by flow cytometry. Very few if any *Rag1*-GFP IL-7Rα$^+$ cells were found (figure 3.1A) in the CD45$^-$ or small CD45$^+$ fractions. Therefore we proceeded to look one day later and dissected and analysed the YS, placenta and PAS of the E9.5 embryo. At this developmental age we identified a small, but distinct Lin$^-$KIT$^+$*Rag1*-GFP$^+$ IL-7Rα$^+$ population in the E9.5 YS (30 ± 4 cells/YS) (figure 3.1B). Interestingly, most cells in the E9.5 YS did not express CD45 but the majority of the Lin$^-$KIT$^+$*Rag1*-GFP$^+$ IL-7Rα$^+$ population expressed CD45, and additionally expressed CD41. A small number of *Rag1*-GFP$^+$ cells also expressed the endothelial cell marker VE-Cadherin (figure 3.1C).

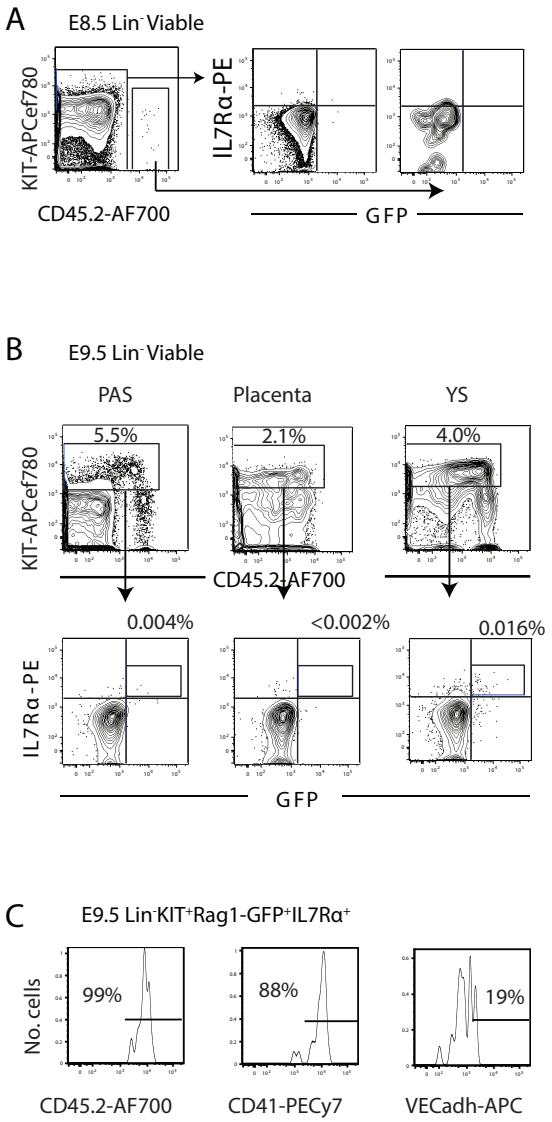


Figure 3.1: Expression of cell surface markers in E8.5 and E9.5 Rag1-GFP⁺ Embryos

A. Lack of expression of IL-7Rα and *Rag1*-GFP in E8.5 (4-7sp) concepti. Viable cells are gated negative for mature lineage markers. The middle and right panels show expression analysis on CD45⁻ and CD45⁺ cells respectively.
B: Co-expression of IL-7Rα and *Rag1*-GFP in different tissues at E9.5 (14-26sp); PAS (Para Aortic Splanchnopleura, left panel), placenta (middle panel) and YS (right panel). Viable cells were gated negative for mature lineage markers and then as depicted in the panels. Percentages are mean of total cells (3 experiments).
C: Expression of CD45, CD41 and VE-Cadherin within the E9.5 YS Lin⁻KIT⁺*Rag1*-GFP⁺IL-7Rα⁺ population. Mean percentages of parent gate (3 experiments). Lineage cocktail included antibodies against TER119, B220 and CD19.

To confirm these observations, whole mount imaging was performed on E9.5 *Rag1*-GFP$^+$ embryos. In agreement with the flow cytometry data, *Rag1*-GFP$^+$ cells were detected in the YS of 14/14 embryos investigated (figure 3.2A, 3.2B 3.2C). Imaging of the PAS (Para Aortic Splanchnopleura) failed to detect *Rag1*-GFP$^+$ cells. The YS *Rag1*-GFP$^+$ cells co-expressed the pan haematopoietic marker CD45 and the endothelial marker CD31 (figure 3.2A and 3.2B). Notably, the majority of the *Rag1*-GFP$^+$ cells were located proximal to the primitive vascular plexus (figure 3.2 D), and not in the large vessels of the YS, allowing tentative speculation that these cells are more likely to emerge from the vascular plexus.

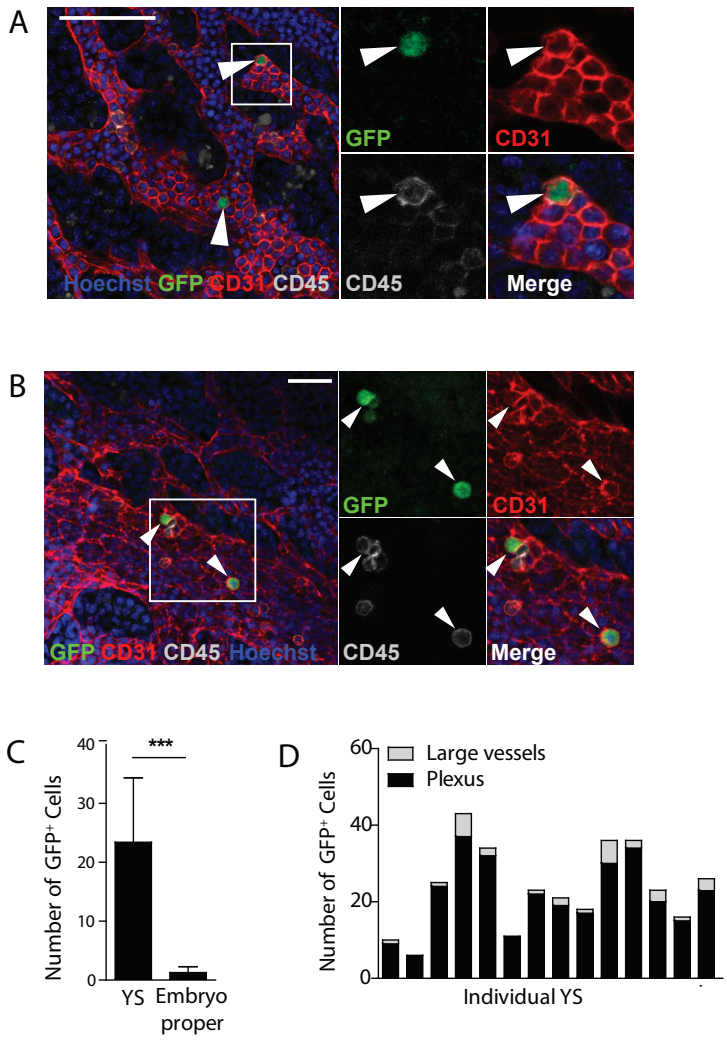


Figure 3.2: E9.5 Immuno-labelling detects Rag1-GFP⁺ in the YS Plexus

A and B: Confocal image of whole mount immune-labelled E9.5 (23sp) *Rag1*-GFP YS. Arrows indicate GFP⁺ cells in the vascular plexus co-expression CD31 and CD45. One of these (boxed area) is enlarged to show fluorescence in the individual channels. In 'B' the GFP⁺ cell in the top left hand corner is within a CD45⁺ cluster. Original magnification of 250x. Scale bar represents 100цM (A) and 50μM (B)

C. The number of GFP⁺ cells per E9.5 YS and whole embryo proper respectively (mean ± SD, 11-14 embryos, *** $p<0.001$)

D. The distribution of GFP⁺ cells within individual YS, divided as cells located in the vascular plexus or larger vessels.

We next performed qPCR analysis of E8.5 whole concepti, E9.5 PAS and E9.5 YS to look for lymphoid gene expression. As we were seeking to detect very low levels of gene expression from a few cells, multiple controls to exclude background noise were included such as samples in which no reverse transcriptase has been added to control for contamination with genomic DNA. After excluding genes that were not detected at least five cycles before the controls, we observed low but significant expression of *Il-7r* and *Rag2* in the E9.5 YS, but not E8.5 or E9.5 PAS region from equivalent embryos (figure 3.3).

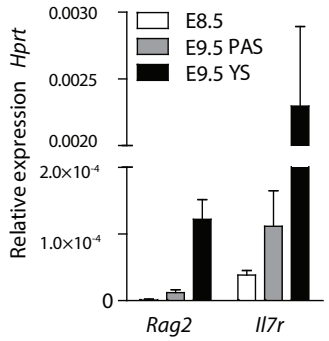


Figure 3.3: Lymphoid gene expression is detected in the E9.5 YS
Analysis of whole E8.5 embryos, and PAS and YS from E9.5 embryos for *Rag2* and *Il-7r* expression by qPCR. Mean expression ±SD, normalized to *Hprt,* with 2-4 biological replicates.

The combination of analysis of cell surface markers, gene expression and imaging data are compatible with the emergence of Lin$^-$KIT$^+$*Rag1*-GFP$^+$ IL-7Rα$^+$ fetal LMPP cells in the E9.5 YS. However by E9.5 circulation has already been established and the YS and intra-embryonic blood vessels have been connected for around 1 day. It is therefore possible that the fetal LMPP cells emerge elsewhere in the embryo before circulating via the blood vessels to the YS, which is extensively vascularised. We therefore decided to dissect pre-circulation embryos (E8, ≤6sp) and culture whole organ explants of the YS and PAS for 48

hours, before performing whole mount imaging and gene expression analysis (figure 3.4) (Cumano et al., 2001).

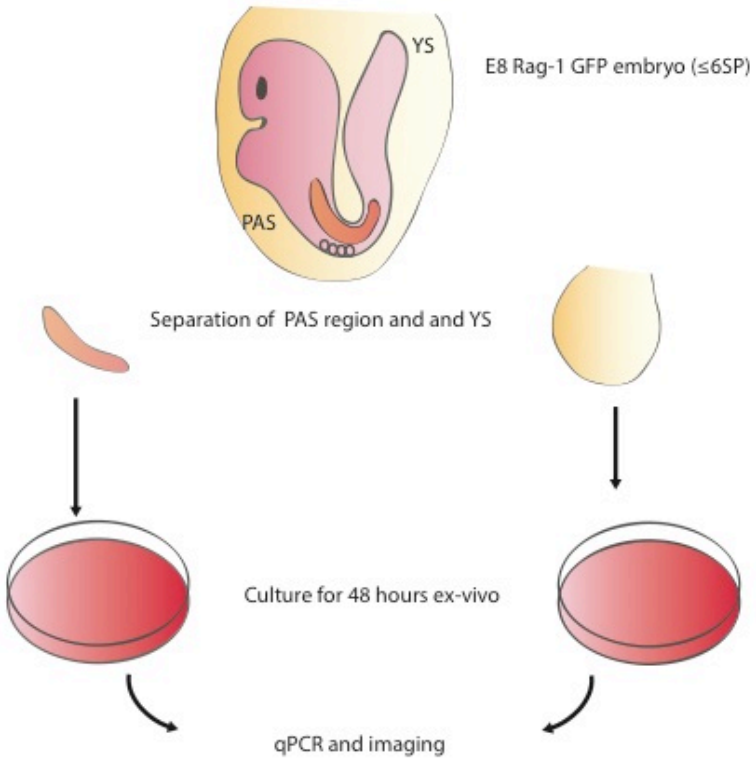


Figure 3.4: Schematic of explant cultures on pre circulation (≤6sps) embryos
Pre-circulation embryos ≤6sp were dissected to separate the PAS and the YS prior to culture for 48hrs then immune-labelled or analysed by qPCR

After 48 hours, whole mount imaging detected *Rag1*-GFP$^+$ cells in 6/10 of the YS explants. The *Rag1*-GFP$^+$ cells co-expressed CD45 and CD31 (figure 3.5A and 3.5B). Notably, none of the 7 pre-circulation PAS explants contained detectable *Rag1*-GFP$^+$ cells. Expression of *sIgH* was detectable in the YS explant cultures (figure 3.5C), but not the PAS, supporting the hypothesis that the YS is the site of *de novo* generation of Lin$^-$KIT$^+$*Rag1*-GFP$^+$ IL-7Rα$^+$ fetal LMPP progenitors.

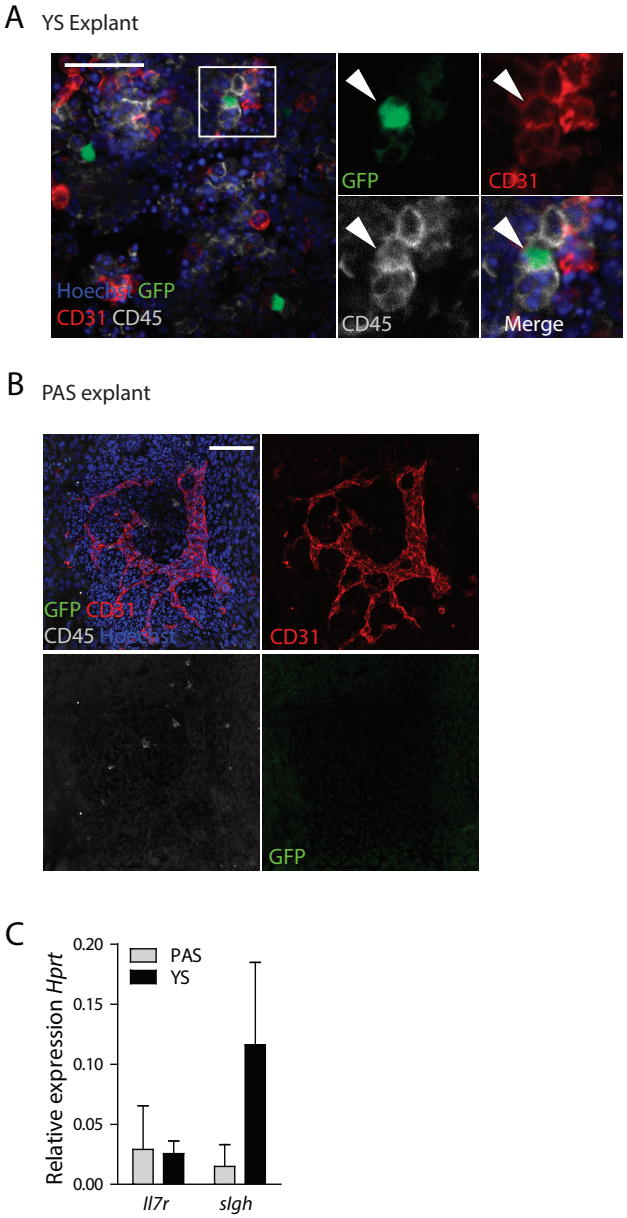


Figure 3.5 – Pre circulation explant cultures detect Rag1-GFP and lymphoid genes in the YS but not the PAS

A. Explant culture of pre circulation embryos were immune-labelled to detect the presence of *Rag1*-GFP⁺cells. Representative confocal images of a YS explant from 1sp stage. The boxed area is enlarged and individual channels are shown for GFP⁺ cells co-expressing CD31 and CD45. Original magnification 250x. Scale bar represents 50µm

B. Representative confocal images of an explant cultured PAS from 1sp stage. CD31 and CD45 expressing cells were apparent, but no GFP expression was seen, characteristic of all 7 PAS investigated, none of which produced GFP⁺ cells within the 48 hours culture time. Original magnification 250x. Tile scan re-constructed image. Scale bar 200µm

C. Pre-circulation PAS and YS were analysed after 48 hours of explant culture for *Il-7r* and *Sterile IgH* expression by qPCR. Mean expression ± SD, normalized to *Hprt.* 3 biological replicates.

Discussion

In this chapter we have further characterised the emergence of an immune restricted, Lin$^-$IL-7Rα$^+$KIT$^+$*Rag1*-GFP$^+$ fetal LMPP progenitor that has lymphoid and myeloid potential but no detectable Megakaryocyte-Erythroid potential (Boiers et al., 2013), with an emphasis on the developmental stage and location at which the progenitor emerges. We show that this novel fetal LMPP emerges between E8.5 and E9.5 in the mouse YS. The emergence of these Lin$^-$IL-7Rα$^+$KIT$^+$*Rag1*-GFP$^+$ cells already by E9.5 demonstrates they emerge independently of definitive HSCs, which are not found until E10.5 in the AGM region.

It has been shown previously that lympho-myeloid progenitors exist in haematopoiesis in the E12 FL (Cumano et al., 1992; Katsura and Kawamoto, 2001; Kawamoto et al., 2000b; Kawamoto et al., 1997), and LMPPs have been identified and characterised in the adult bone marrow and E14.5 FL (Adolfsson et al., 2005; Arinobu et al., 2007b; Mansson et al., 2007). However, this is after the emergence of dHSCs at E10.5. B cell potential has been detected as early as E8.5 (10sps) in the PAS and YS after culture on irradiated S17 stromal cells with the addition of the cytokines IL-7, IL-3 and KIT Ligand. These cells were shown to be multipotent with granulocyte, macrophage, megakaryocyte and T cell potential, and no prospective progenitors were purified (Godin et al., 1995). Further pre-circulation followed by organ explant experiments suggested that these cells arose de novo in the PAS and not the YS (Cumano et al., 1996; Cumano et al., 2001). However, the cell lineages produced were analysed after 20 days in culture, by which time we would expect dHSCs to have emerged from the PAS

region and be producing a robust source of hematopoietic cells, whereas it is possible the lower level of YS output was missed.

There has been some indication that cells with lymphoid and myeloid *potential* may exist prior to dHSCs in the E9.5 YS based on in vitro cultures and in vivo assays. These studies demonstrate cells exist in the E9.5 YS with lympho-myeloid potential but do not show whether the progenitors are *restricted* to specific lineages (Yoshimoto et al., 2011; Yoshimoto et al., 2012). The authors of these studies claim to identify T lineage restricted cells in the YS (Yoshimoto et al., 2012) but in their second study they show the same cells have B cell potential in different assays, demonstrating the progenitors are not in fact T lineage restricted (Yoshimoto et al., 2011). Therefore the YS progenitors identified by Yoshimoto et al., may originate from a progenitor with lympho-myeloid *potential*, such as the one identified herein, but not uni-potent restricted progenitors.

It is unknown whether the YS progenitors arise directly from the haemogenic endothelium, or from upstream progenitors that retain additional MkE potential. A recent study claims that E9.5 YS progenitors with lymphoid potential may derive from haemogenic endothelium in the E9.5 YS (Yoshimoto et al., 2012). This is compatible with our data that detects *Rag1*-GFP^{+} cells in the YS and the co-expression of *Rag1*-GFP^{+} with the endothelial cell marker CD31, with some cells even expressing VE-Cadherin (figures 3.2A and 3.2B).

As the YS is connected to the intra-embryonic body by E8.5, we performed pre-circulation explant cultures of the YS and PAS regions and found that the Lin$^-$IL-7Rα$^+$Kit$^+$*Rag1*-GFP$^+$ progenitors can emerge *de novo* in the YS. These data could be further supported by additional analysis of *Ncx* knockout mice models, bred to the *Rag1*-GFP reporter. *Ncx* null mice lack the high capacity sodium-calcium exchanger, fail to initiate a heartbeat, lack circulation and die by E11. These mice have been used by other groups to investigate the location of the emergence of haematopoietic progenitors in the early embryo (Yoshimoto et al., 2011). Analysis of the E9.5 YS could be performed without the complication of dissection and explant culture, with no caveat of contaminating circulating cells to thoroughly address at what anatomical site the fetal LMPP emerges. However, these studies have limitations. Firstly shear forces from circulatory liquids have been shown to be required for *Runx1* expression and the early differentiation of haematopoietic cells (Adamo et al., 2009) therefore the absence of circulation may disrupt normal haematopoietic development. Secondly, despite the absence of a heartbeat driving circulation, the vessels are intact in *Ncx* mice and cells could potentially migrate around the vasculature via other mechanisms such as using cilia.

The contribution of lympho-myeloid restricted progenitors to myelopoiesis under physiological circumstances has been challenged. In the adult, *Il-7r* and *Rag1* fate mapping showed limited contribution of the LMPP to the myeloid lineage (Schlenner et al., 2010; Welner et al., 2009a). However only a small fraction of adult LMPPs express *Il-7r* or *Rag1*, and the *Rag1* expressing LMPPs have reduced myeloid potential compared to the *Rag1*$^-$ LMPPs (Adolfsson et al.,

2005; Luc et al., 2008), suggesting that these studies are likely to underestimate the myeloid contribution of adult LMPPs. *Rag1* fate mapping in the embryo demonstrates that the Lin$^-$IL-7Rα$^+$KIT$^+$*Rag1*-GFP$^+$ progenitor cells contribute significantly to fetal myelopoiesis with 36% of the GM lineage labelled at E11.5, but no evidence of contribution to the MkE lineage (Boiers et al., 2013). The greater proportion of fetal labelling is most likely reflective of fetal LMPPs expressing higher levels of *Rag1* than adult LMPPs, highlighting how different genetic tools are required to optimally fate map different developmental ages. The Lin$^-$IL-7Rα$^+$KIT$^+$*Rag1*-GFP$^+$ early YS cells may via self-renewal or de novo generation maintain a minor role in the adult immune system, although this has not been definitively investigated (Boiers et al., 2013). Accordingly, to establish the contribution to adult lympho-myelopoiesis an inducible *Rag1*-cre mouse model would be needed that would label progenitors in a small embryonic time window. Alternatively, we could perform transplantations directly into fetal livers in utero (Liuba et al., 2009), although technical challenges make this approach difficult and an inducible fate mapping strategy is more likely to give robust data.

Despite major progress in the elucidation of the haematopoietic hierarchy, the field has been fraught with controversies over whether the lineage commitment of HSCs to uni-potent progenitors progresses via the 'classical' hierarchy with strictly segregated lymphoid and myelo-erythroid lineages (Orkin and Zon, 2008) or via an 'alternative' hierarchy with early segregation between the lympho-myeloid and MkE lineages (Adolfsson et al., 2005). As lineage restrictions involve 'loss of function,' researchers are faced with the impossible

task of proving a negative, ensuring the controversy continues! However, the demonstration that this Lin$^-$IL-7Rα$^+$KIT$^+$*Rag1*-GFP$^+$ fetal LMPP sustains lympho-myeloid but not MkE potential in the fetus adds support for the 'alternative' pathway for lineage restriction. Furthermore, this pathway has been supported by the identification of LMPPs in the E14.5 FL (Mansson et al., 2007) and adult BM (Adolfsson et al., 2005), albeit the progenitor herein emerges prior to dHSCs. More recently, the entire dogma of the haematopoietic hierarchy has been challenged with the suggestion that the majority of progenitors are in fact uni-potent (Notta et al., 2016), with the assays instructing lineages that would not normally develop. This challenges the relevance of the haematopoietic tree, instead suggesting it is merely a reflection of the assays used to assess lineage potential.

The initial events of childhood ALLs often occur *in utero* in unidentified progenitors (Greaves and Wiemels, 2003). The robust B cell potential of the Lin$^-$IL-7Rα$^+$KIT$^+$*Rag1*-GFP$^+$ and their contribution towards embryonic lymphopoiesis (Boiers et al., 2013) indicate it may be a relevant cell for pre-leukaemic events in B cell ALLs. Interestingly, a number of childhood leukaemias, classed as 'bi-phenotypic' express both B and Myeloid lineage markers on their cell surface. The combined lymphoid and myeloid potential of the Lin$^-$IL-7Rα$^+$KIT$^+$*Rag1*-GFP$^+$ cells indicate they may be relevant to bi-phenotypic leukaemia development.

Having identified the first murine embryonic progenitors that have B cell potential, in the next chapter I look to identify and characterise the first cells that

are *restricted* to the B cell lineage, and have lost the ability to generate T cells and myeloid cells.

Chapter IV: Identification of the earliest characterized B lineage restricted progenitors in the mouse embryo

IDENTIFICATION OF THE EARLIEST CHARACTERIZED B LINEAGE RESTRICTED PROGENITORS IN THE MOUSE EMBRYO

Introduction

The stage-wise progression of multi-potent progenitors to committed B cells has been characterized molecularly and functionally in the adult mouse (Rumfelt et al., 2006a). Whether the same functional stages of lineage commitment apply to B cells developing during embryogenesis, is largely unstudied. A better understanding of the earliest stages of fetal B cell development is of relevance to childhood B cell leukaemias, as the initial genetic lesions can occur in utero. In chapter III I showed that the earliest known immune restricted progenitor with B, T and Myeloid cell potential emerges in the E9.5 YS and I next wanted to address when and where in the embryo the first progenitors entirely restricted to the B cell lineage could be detected.

Previous limited dilution studies have detected around 60 cells with B cell potential in the E8.5 YS (Cumano et al., 1993) although these studies involved culture of cells in vitro for 10-14 days prior to lineage analysis. Therefore no conclusions can be drawn about whether the progenitors are restricted only to the B cell lineage, and at precisely what developmental age immune restricted B cell progenitors emerge. The B cell genes *Ebf* and *Pax5* have been detected in the E14.5 FL (Kawamoto et al., 2000b) and cell surface IgM expression is detected at very low levels (0.15% of fetal liver cells) at E17.5. The same study reports intracellular IgM 5 days earlier at E12, but the two replicates reported only 0% and 0.03% of fetal liver cells expressing intracellular IgM, with similar low

percentages seen throughout development until E16, when a ten fold increase to 0.3% of fetal liver cells is seen (Raff et al., 1976). This suggests the earlier intracellular IgM may be a background signal, and intracellular IgM is only truly found at E16, one day prior to its detection on the cell surface. Successful VDJ rearrangement is required for the production of immunoglobulins at both the immunoglobulin heavy and light chain loci, a process that is only completed relatively late in B cell development and therefore detectable IgM is a clear indicator of a restricted B cell. Studies have detected VDJ rearrangement in the E17 FL (Feeney, 1990), with limited studies focusing on younger embryos. DJ rearrangement has been suggested to initiate in the E12 FL (Cumano and Paige, 1992), but DJ rearrangement is insufficient to mark B lineage restriction as DJ rearrangement is detected in cells that are not committed to the B cell lineage (Li et al., 1993a; Rumfelt et al., 2006a). One study cultured embryonic $Lin^-KIT^+CD45^+IL\text{-}7R\alpha^+$ FL cells and found at a very low frequency, cells which could generate only B lineage cells in culture, suggesting B cell restricted progenitors exist at E12.5, although they have not to date been isolated and characterized (Kawamoto et al., 2000a). Without prospective purification it is impossible to thoroughly probe lineage potentials at the functional and molecular level. These data cumulatively indicate that B cell restricted progenitors may be present in the E12 FL but no putative cells have been isolated or characterized based on expression of cell surface markers.

Originally the expression of B220, an isoform of CD45R, on the cell surface was suggested to be the first expressed marker that correlated with B lineage restriction (Allman et al., 1999; Hardy et al., 1991b). It is now known that $B220^+$

Pre Pro B cells, even though 90% have undergone DJ recombination at the IgH locus, still retain T cell and NK cell potential (Huntington et al., 2007; Kouro et al., 2002; Rumfelt et al., 2006a). Definitive B cell commitment is now associated with the expression of the TF *Pax5* which subsequently drives cell surface expression of CD19. One study indicates that a small number of $CD19^+$ cells are capable of generating dendritic cells in culture, indicating CD19 is still not a definitive marker for B cell lineage restriction (Bjorck and Kincade, 1998). However, this study has not been corroborated by others and the purity of the cell sort is not convincingly shown. CD19 expression is reported between E13.5 and E14.5 in the mouse fetal liver (Douagi et al., 2002; Kawamoto et al., 2000a; Ogawa et al., 2000) and B cell progenitors are detected in the fetal bone marrow by E15.5 (Delassus and Cumano, 1996).

B cell commitment can occur in the adult BM prior to CD19 expression (Allman et al., 1999), suggesting that if we can find appropriate markers then it may be possible to isolate B cell restricted progenitors prior to CD19 expression during embryonic development. The Pre Pro B cell fraction, or 'Hardy Fraction A', which does not express CD19 (Hardy et al., 1991b) has now be shown to contain cells that are not B lineage restricted (Rumfelt et al., 2006a). It has proved historically challenging to isolate the true $B220^+CD19^-$ B cell progenitors within the Pre Pro B cell fraction based on cell surface markers. IL-7 signalling is critical for B cell specification, commitment, proliferation and survival (Corfe and Paige, 2012) and B cell progenitors are found to be enriched within the $IL\text{-}7R^+$ fraction of E12 FL cells (Kawamoto et al., 2000b), but since we and others have shown that IL-7Rα is also expressed on earlier immune-restricted progenitors with B, T and

myeloid cell potential (Boiers et al., 2013), this marker alone, like B220, is insufficient to identify B cell restricted progenitors. Though IL-7R is still a useful 'enriching' marker as all B cell progenitors express the IL-7R, and the receptor is not widely expressed in haematopoiesis.

In the adult BM, a transgenic reporter under the control of the λ5 promoter, the latter a protein that combines with VPreB to form the surrogate light chain, identified a small (4.5%) sub-fraction of the CLP, which was enriched for B cell potential. Up to 10% of the cells retained detectable T cell potential, indicating even though B cell potential was enriched, the population was not entirely B lineage restricted (Mansson et al., 2008). Further studies showed that the marker LY6D was expressed by 50% of CLP cells, marking a population that only contributed to the B lineage upon transplantation into irradiated mice (Inlay et al., 2009b). However the study failed to investigate lineage potentials thoroughly in vitro and therefore T or Myeloid cell development may have been missed.

In this chapter we first investigate the expression of the B cell restricted marker CD19 throughout haematopoietic sites in the early embryo. Previous studies that have attempted to purify B cell progenitors prior to CD19 expression have relied principally on expression of cell surface markers. Herein, we use a fate mapping approach that employs knowledge of B cell specific genes to subfractionate the Pre Pro B cell compartment in the E12.5 fetal liver and isolate and characterize a putative B cell restricted progenitor prior to CD19 expression. *Mb1* (*CD79a*) encodes the immunoglobulin-α receptor, which is involved in signalling from the BCR. In the adult BM *Mb1* is expressed prior to CD19 (Dworzak et al., 1998).

Whether *Mb1* is expressed exclusively in progenitors that are entirely restricted to the B cell lineage has not been definitively shown. The proximal enhancer of *Mb1* is regulated in combination by E2A, EBF1, PAX5, ETS1 and RUNX1 (Hagman and Lukin, 2005). Importantly *Mb1* is expressed at high levels early in B cell development, prior to the completion of productive VDJ rearrangement in B cell progenitors (Li et al., 1993a). We find prior to CD19 expression in Mb1-cre$^{tg/wt}$ ('*Mb1*-cre') Rosa26-YFP$^{eYFP/wt}$ ('R26-YFP') animals, a CD19^{-}B220^{+}KIT^{+}IL-7Rα^{+}FLT3^{+} B cell progenitor emerges in the E12.5 FL that expresses detectable YFP. Henceforth YFP expressing cells in Mb1-cre$^{tg/wt}$ Rosa26 YFP$^{eYFP/wt}$ mice will be referred to as '*Mb1*-YFP'. This CD19^{-}B220^{+}KIT^{+}IL-7Rα^{+}FLT3^{+} *Mb1*-YFP progenitor has robust B cell potential in vitro but no detectable T or myeloid cell potential, suggesting it could be the earliest characterized fetal B cell restricted progenitor.

Results

CD19 is largely accepted as a B cell specific surface marker in the adult BM. We investigated where we could first detect cell surface expression of CD19 and B220 in the FL, the main haematopoietic organ in the embryo after the emergence of dHSCs. In the E11.5 FL (~40sp) we could not find any cells that expressed B220 or CD19, but by E12.5 B220 expression on the cell surface was detected (0.036 ± 0.007% of total MNCs, figure 4.1). At E13.5 a low number of cells expressing both B220 and CD19 were detected (0.013 ± 0.005% of total MNCs, 161±79 per FL) and by E16.5 this had grown to a significant cell population (1.32% ± 0.53% of total MNCs, 200,000 cells ± 98,000 per FL, figure 4.1). In the E13.5 FL we also detected cells that expressed CD19 but did not express B220, an unusual population that has been characterized by others as a putative B1 cell progenitor in late gestation (Kobayashi et al., 2014; Montecino-Rodriguez et al., 2006). The earliest time point that this B1 progenitor has been characterized previously is the E15.5 FL, with one study showing E9.5 YS cultured for 8 days having the potential to produce $B220^{-}CD19^{+}$ cells (therefore effectively E17.5) (Yoshimoto et al., 2011), but the B1 progenitor has not been analysed in the E13.5 FL thus far.

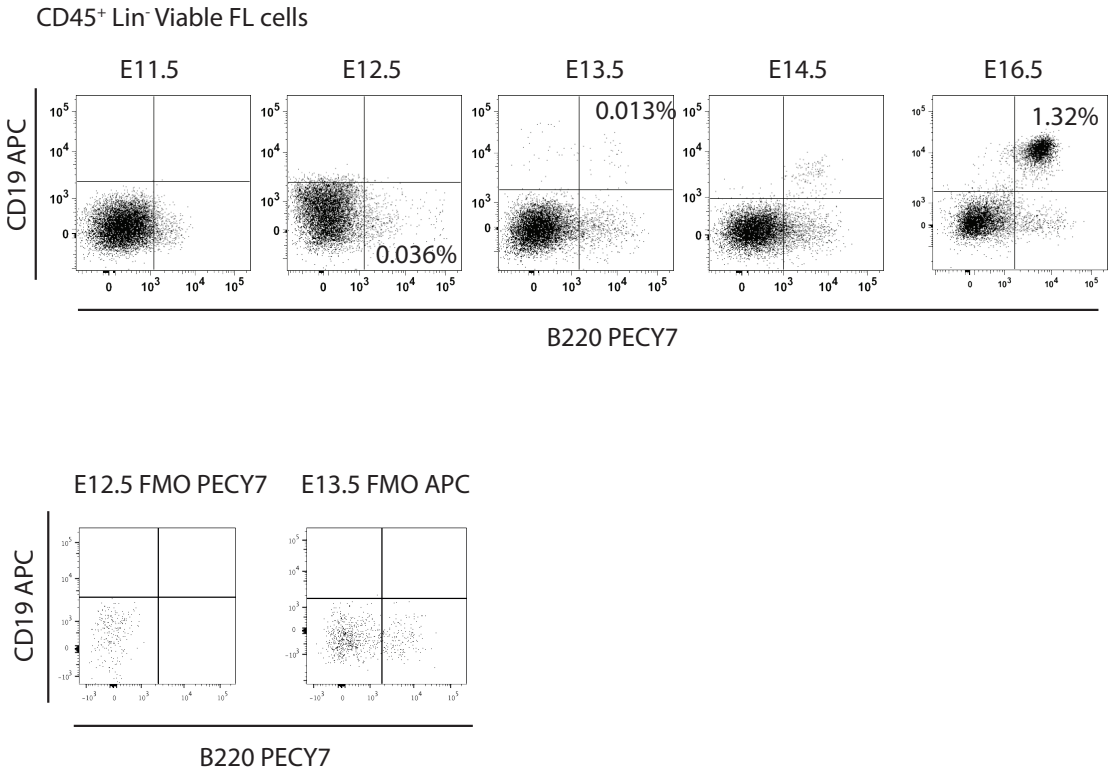


Figure 4.1: CD19 expression is first detected in the E13.5 FL
Co-expression of CD19 and B220 on Lin$^-$CD45$^+$ FL cells. Each plot is representative of >5 FLs. Lower panels show fluorescent minus one controls for APC and PECY7. Percentages are of total MNCs. Lineage cocktail included antibodies against GR-1, CD3, NK1.1, TER119 and CD11c.

We next analysed the YS, AGM and placenta to investigate whether CD19$^+$ cells could be detected at other haematopoietic sites in the embryo prior to E13.5. To avoid potential contamination from maternal blood cells, CD45.1 males were mated with CD45.2 females, and only cells that co-expressed both CD45.1 and CD45.2 were analysed (figure 4.2). In 45sp embryos (E11.5), CD19$^+$ B220$^+$ and CD19$^+$ B220$^-$ cells could not be detected in the AGM, fetal liver or YS, although unlike early E11 embryos, B220 expression was detected, indicating B220 expression commences between E11.5 and E12.

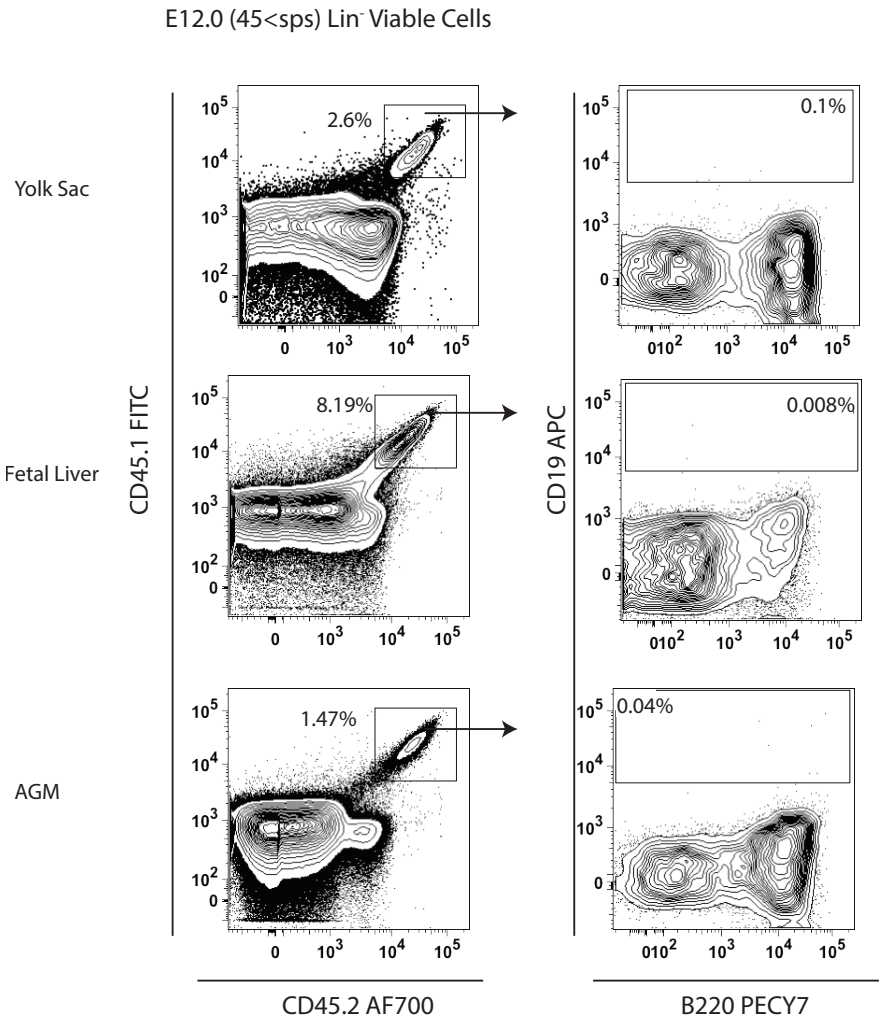


Figure 4.2: No CD19⁺ B cell restricted progenitors are found in the YS, AGM and FL of 45SP embryos

Co-expression of surface CD19 and B220 on E12.0 YS, FL and AGM cells from CD45.2 females plugged by CD45.1 males. FACS plots are representative of four biological replicates, each containing 1-2 organs. Percentages are of total MNCs.

Having established that CD19 expression is first seen in the E13.5 FL, we next wanted to establish whether any B cell restricted progenitors could be identified prior to CD19 expression. In vitro culture studies have indicated B cell restricted progenitors in the E12.5 FL (Kawamoto et al., 2000a) indicating the first B cell lineage restricted progenitors may be found at this time point, one day prior to when we first detected CD19 expression in the E13.5 FL. However, these studies

notably failed to prospectively purify cell populations and thoroughly assess lineage potentials in optimized functional and molecular assays.

We therefore decided to use a fate-mapping approach whereby gene expression drives expression of a fluorescent protein. Thereby intracellular proteins that cannot be detected by flow cytometry can be used to characterize and isolate cell populations. The *Mb1* (*CD79a*) gene encodes a component of the immunoglobulin-α receptor, a B lineage restricted protein involved in signalling from the BCR. In the adult BM *Mb1* is expressed prior to CD19, in progenitors that also co-express *TdT* (Dworzak et al., 1998). We crossed Mb1-cre $^{tg/wt}$ ('*Mb1*-cre') animals with a Rosa26 YFP$^{eYFP/eYFP}$ ('R26YFP') reporter line, generating a system whereby cells in which *Mb1* is or has been expressed during ontogeny are marked by YFP expression. The genetic knock in of the *Cre* allele disrupts the *Mb1* allele function, and mice with a homozygous deletion of *Mb1* have a total block in B cell development. One *Mb1* allele has been shown to be sufficient for normal B cell lymphopoiesis (Kraus et al., 2004) and so all animals used in experiments were heterozygous for *Mb1*-cre expression. We initially validated this model in the adult BM and E14.5 FL and thymi of *Mb1*-cre$^{tg/+}$ R26R$^{eYFP/+}$ (*Mb1*-YFP) animals. We found over 97% of CD19^{+} B cells were marked by YFP in both the fetus and the adult (figure 4.3). However, prior to CD19 expression, 4.16% of B220^{+}CD43^{+}CD19^{-}CD24^{-} Pre Pro B cells in the adult BM were found to express YFP, confirming M*b1* is expressed prior to CD19 (Dworzak et al., 1998) and can sub-fractionate the Pre Pro B cell compartment (data not shown).

Only 0.27% of adult $CD4^+CD8^+$ cells were YFP^+ and only 0.01% of fetal early thymic progenitors (ETPs) were marked by YFP. 0.20% of $MAC1^+GR\text{-}1^+$ adult BM cells expressed YFP and 1.06% of fetal liver $MAC1^+GR\text{-}1^+$ cells expressed YFP (figure 4.3). Despite the low level of YFP expression by the T and Myeloid lineages indicating *Mb1*-cre faithfully fate maps the B cell lineage, 5 times as many $MAC1^+GR1^+$ fetal myeloid cells were labelled by *Mb1* cre in the E14.5 FL than the adult bone marrow ($p=0.008$). Additionally we examined upstream progenitors in the adult BM and found YFP undetectable in the LSK compartment, expressed in 2.78% of $B220^+CD43^+CD19^-CD24^-$ Pre Pro B cells and 99.8% of Pro B cells (figure 4.3C).

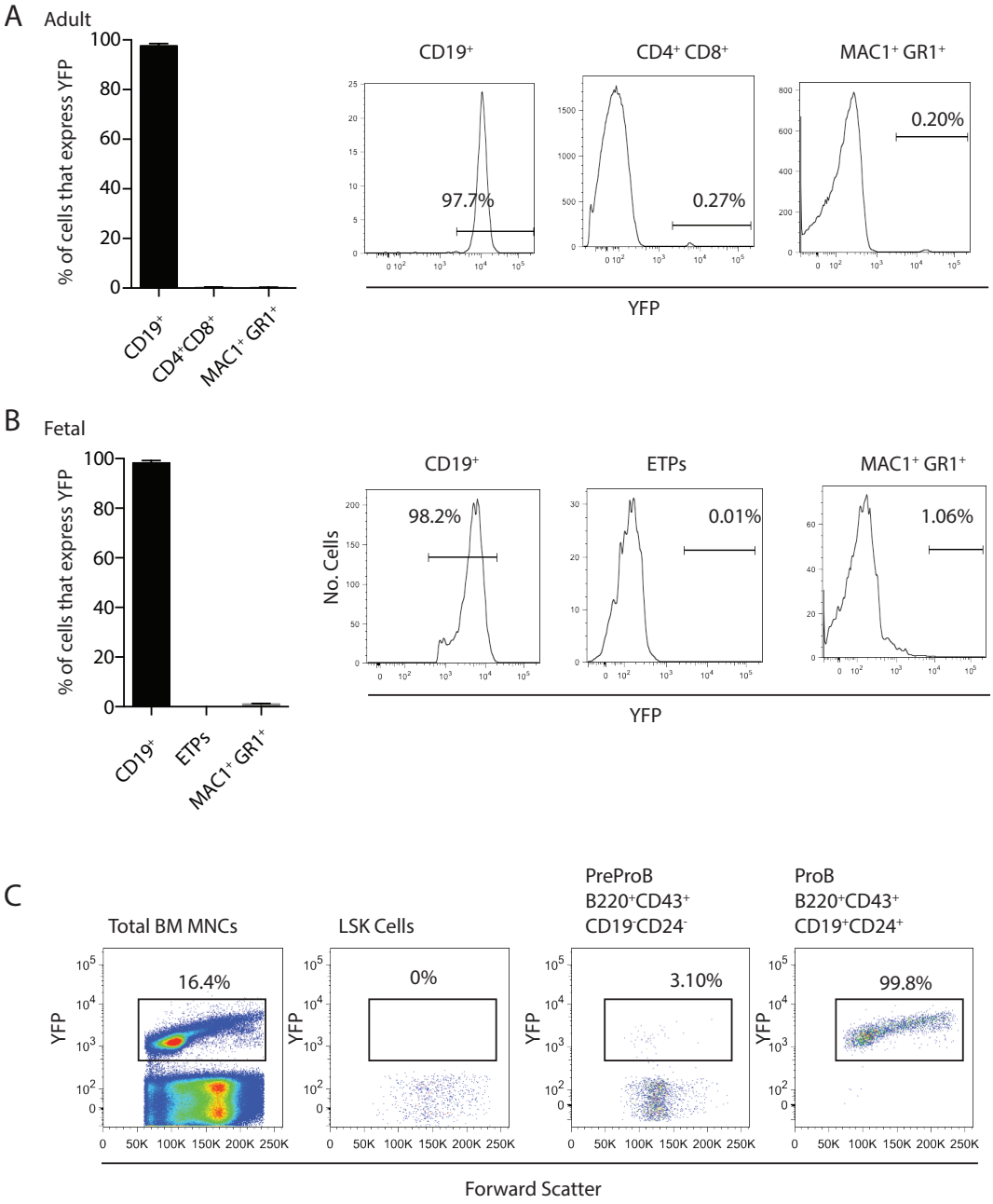


Figure 4.3: Mb1-cre faithfully and efficiently marks the B cell lineage, but not the T or myeloid cell lineages in the BM and FL

A: % of cells expressing *Mb1*-YFP within CD19$^+$ BM B cells, CD4$^+$CD8$^+$ thymic T cells or MAC1$^+$GR1$^+$ BM Myeloid cells (6-8 weeks old). *Mb1*-cre$^{tg/wt}$ R26YFP$^{tg/wt}$ animals.

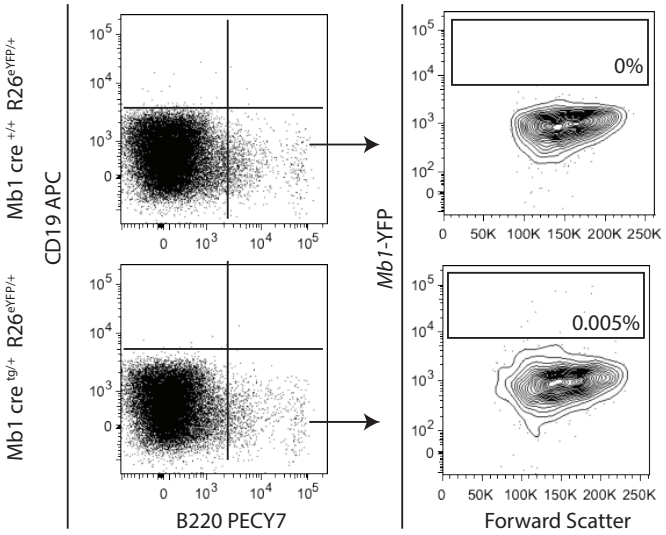
B: % of cells expressing *Mb1*-YFP within CD19$^+$ FL B cells, Lin$^-$CD4$^-$CD8$^-$KIT$^+$CD25$^-$FLT3$^+$ ETPs in the fetal thymus or MAC1$^+$GR1$^+$ myeloid cells in the FL of E14.5 of *Mb1*-cre$^{tg/wt}$R26YFP$^{tg/wt}$ animals

Right hand plots are representative of each animal. Left hand bar graph displays mean. Myeloid cell 3 replicates, B cells 6-9 replicates, T cells 3-6 replicates.

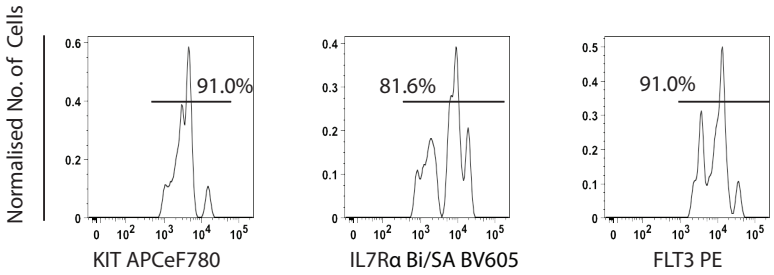
C: Analysis of adult BM in *Mb1*-cre$^{tg/wt}$ R26YFP$^{tg/wt}$ animals. Representative of 2 mice analysed. Percentages shown of YFP$^+$ cells within the given cell populations.

We proceeded to investigate the $CD19^-$ cell population in the E12.5 FL. To increase specificity we looked for the co-expression of B220 with *Mb1*-YFP. A small, but significant $CD45^+B220^+CD19^-$*Mb1*-YFP^+ population was detected in the E12.5 FL of *Mb1*-$cre^{tg/+}$ $R26R^{eYFP/+}$ animals (0.005 ± 0.002% of viable MNCs) (figure 4.4A), which also homogeneously expressed the key lymphoid cytokine receptors KIT, IL-7Rα and FLT3 (figure 4.4B). Importantly, by crossing *Mb1*-$cre^{tg/+}$ males with $R26R^{eYFP/eYFP}$ females, expression of YFP marked cells that either currently or historically expressed *Mb1,* but also confirmed that the cells were of fetal origin and not maternal contaminants, as only fetal cells will contain both the cre and fluorescent protein alleles. Cells from *Mb1*-$cre^{tg/+}$ $R26R^{eYFP/+}$ animals that express YFP are henceforth referred to as *Mb1*-YFP^+. $CD45^+B220^+CD19^-$*Mb1*-$YFP^+KIT^+IL\text{-}7R\alpha^+FLT3^+$ cells (MYP '*Mb1*-YFP Progenitor') from the E12.5 and E13.5 FL were sorted to a high purity (<95%) and reanalysis is shown in figure 4.4C. In the E13.5 FL, 11.1% of $CD19^-B220^+$ cells were *Mb1*-YFP^+, showing this population forms a minority of the classically defined Pre Pro B cell compartment (figure 4.4D). When analysing the E12.5 FL, we concurrently dissected and analysed the YS from the same embryos, and found no significant MYP cells in the YS at this time point (1 cell or less/250,000 YS cells, figure 4.4E).

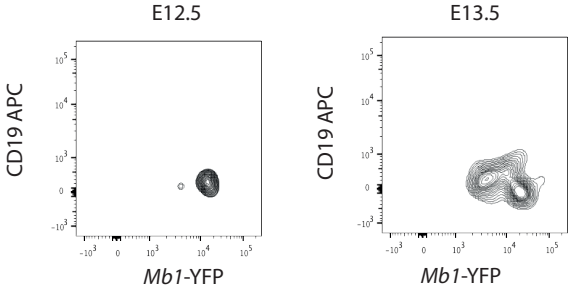
A E12.5 FL CD45$^+$ Viable Cells



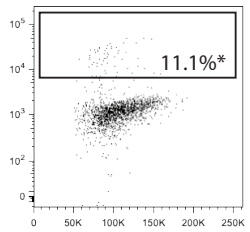
B CD45$^+$ B220$^+$CD19$^-$*Mb1* YFP$^+$ cells



C CD45$^+$cKIT$^+$IL7R$^+$FLt3$^+$B220$^+$CD19$^-$*Mb1*-YFP$^+$ (MYP) Reanalysis

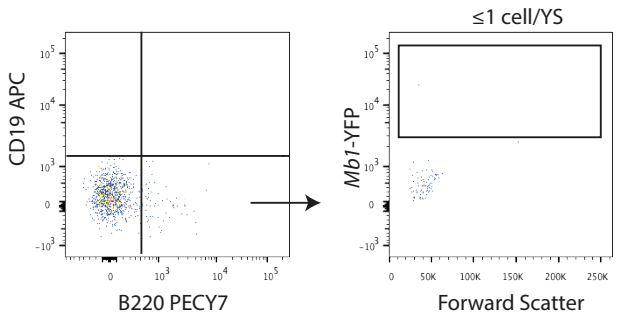


D E13.5 FL CD45$^+$ B220$^+$CD19$^-$ cells



*Percentage of B220$^+$CD19$^-$ cells

E E12.5 **YS** Cells
Viable Lin$^-$CD45$^+$



Viable Lin$^-$CD45$^+$B220$^+$ CD19$^-$
E12.5 FL Cells

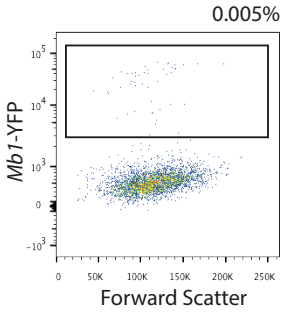


Figure 4.4: A rare CD45$^+$B220$^+$CD19$^-$ progenitor that is Mb1-YFP$^+$ in the E12.5 FL cells

A: Co-expression of surface B220, CD19 and *Mb1*-YFP in the E12.5 FL. Plots are representative of 5+ experiments and gating as outlined in the figure. Percentages of total FL MNCs.

B: Co-expression of KIT, IL-7Rα and FLT3 on CD45$^+$ B220$^+$CD19$^-$ *Mb1*-YFP$^+$ cells from the E12.5 FL. Representative plots of 5+ experiments. Percentage is of total CD45$^+$ B220$^+$CD19$^-$ *Mb1*-YFP$^+$ cells, mean of 5 biological replicates.

C: Purity reanalysis of sorted MYP (CD45$^+$B220$^+$CD19$^-$*Mb1*-YFP$^+$FLT3$^+$KIT$^+$IL-7Rα$^+$) cells from the E12.5 (left) and E13.5 FL (right)

D: Percentage of CD45$^+$B220$^+$CD19$^-$ cells in the E13.5 FL that express *Mb1*-YFP

E: Co-expression of B220, CD19 and *Mb1*-YFP in the E12.5 YS. Plots are representative of 2 YSs. A FL sample from the same embryo is shown in the right hand panel as a positive control for YFP expression. Percentages are of total FL MNCs.

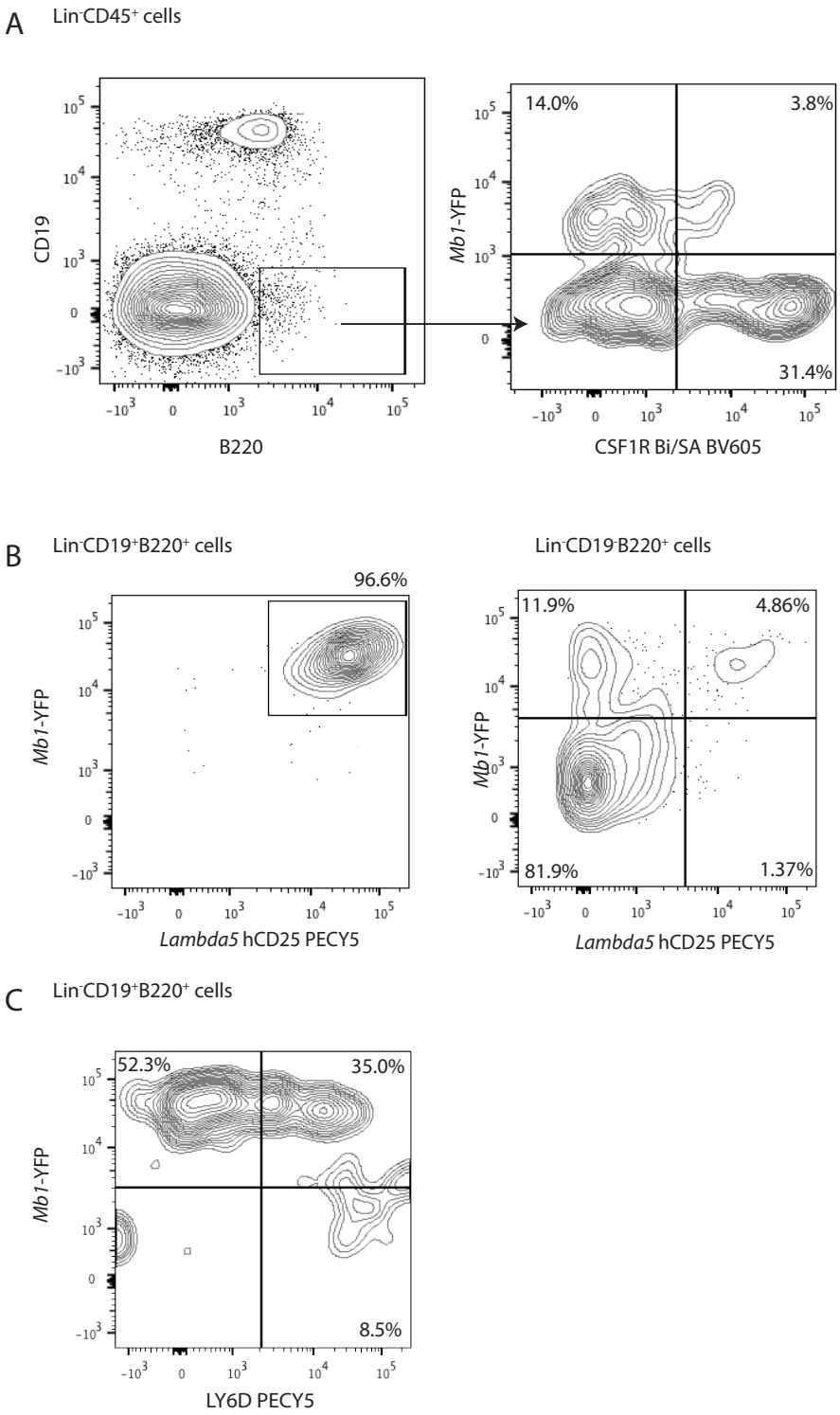


Figure 4.5: Co-expression of Mb1-YFP with CSF1R, λ5-hCD25 and LY6D.

A: Co-expression of CSF1R and *Mb1*-YFP within E14.5 B220$^+$CD19$^-$ FL cells. Percentages show frequency of the B220$^+$ CD19$^-$ population expressing markers, average of 6 FLs.

B: Co-expression of *Mb1*-YFP and *λ5*-hCD25 by B220$^+$CD19$^+$ cells (left hand panel E13.5) and B220$^+$CD19$^-$ cells (right hand panel) in E15.5 embryos. 1-2 embryos.

C: Co-expression of *Mb1*-YFP and LY6D by B220$^+$CD19$^+$ E13.5 FL cells. Percentages show frequency of the B220$^+$ CD19$^+$ cells, average of 4 FLs.

Next we wanted to investigate the co-expression of *Mb1*-YFP with other markers associated with B cell restriction. CSF1R is a cytokine receptor that promotes monocyte and macrophage development (Dai et al., 2002) and has been shown to dissect the heterogeneous $CD19^-$ pre pro B cell fraction into $CSF1R^+$ cells that have myeloid potential and $CSF1R^-$ cells that are more B lineage restricted (Zriwil A, Sitnicka E and Jacobsen SE, unpublished data). We found that within the $CD19^-B220^+$ cell population, 31.4% of cells expressed CSF1R and 14% of cells expressed *Mb1*-YFP, but only 3.8% of cells co-expressed both CSF1R and *Mb1*-YFP (figure 4.5A).

In the adult BM, expression of λ5, a component of the surrogate light chain, identifies a small population of the CLP that is enriched for the B cell lineage (Mansson et al., 2008). Additionally, in 1 week old neonatal mice, λ5 expression has been shown to be expressed prior to CD19 expression in B cell progenitors (Ogawa et al., 2000). We used a mouse model where the λ5 promoter drives expression of human CD25 (hCD25) on the cell surface (Martensson et al., 1997), to investigate the co-expression of *λ5*-hCD25 and *Mb1*-YFP on fetal B cell progenitors. We found that in the embryo $CD19^+$ cells co-expressed both *Mb1*-YFP and *λ5*-hCD25, but within the $CD19^-B220^+$ population 16.8% of cells expressed *Mb1*-YFP and only 4.9% co-expressed both *Mb1*-YFP and *λ5*-hCD25. Very few cells (1.37%) expressed *λ5*-hCD25 without *Mb1*-YFP (figure 4.5B) suggesting that *Mb1*-YFP expression is expressed earlier than *λ5*-hCD25 in the FL.

In the adult BM, LY6D expression bifurcates the CLP into a LY6D$^+$ CLP, which is primed for B cell development, and a LY6D$^-$ CLP, which is primed for T cell development (Inlay et al., 2009b). We hypothesized that surface LY6D expression may help reveal B lineage restricted progenitors within the heterogeneous Pre Pro B cell compartment so included antibodies against LY6D in our B cell progenitor analysis. We found FL LY6D expression did not correlate with CD19 expression. Only 43% of CD19$^+$ fetal cells co-expressed LY6D, indicating it is not a useful marker in the embryo to mark B cell restricted progenitors within CD19$^-$ Pre Pro B cells (figure 4.5C). In fact, when we analysed CD19$^+$B220$^+$ cells in the 7-8 week old adult BM, only 52.7 ± 5.8% of cells expressed LY6D (appendix 1, mean ± SEM, average of 5 mice). Therefore despite being a useful marker to enrich B primed cells within the adult CLP (Inlay et al., 2009b), LY6D is not expressed by all the earliest CD19$^+$ B cell progenitors.

Next we wanted to investigate the lineage potential and gene expression properties of MYP cells. B cell progenitors from *Mb1*-cre$^{tg/+}$ R26R$^{eYFP/+}$ (*Mb1*-YFP) FLs were analysed and sorted as depicted in figure 4.6 for gene expression and in vitro culture assays. At E12.5, CD19$^+$ cells are yet to emerge, so CD19$^+$ fractions were sorted from E13.5 embryos (figure 4.6B). Interestingly at E13.5, we again saw the putative B1 cell progenitor that is B220$^-$CD19$^+$ (CD45$^+$KIT$^+$IL-7Rα$^+$B220$^-$CD19$^+$), but only 66.8% of these cells expressed YFP (figure 4.6).

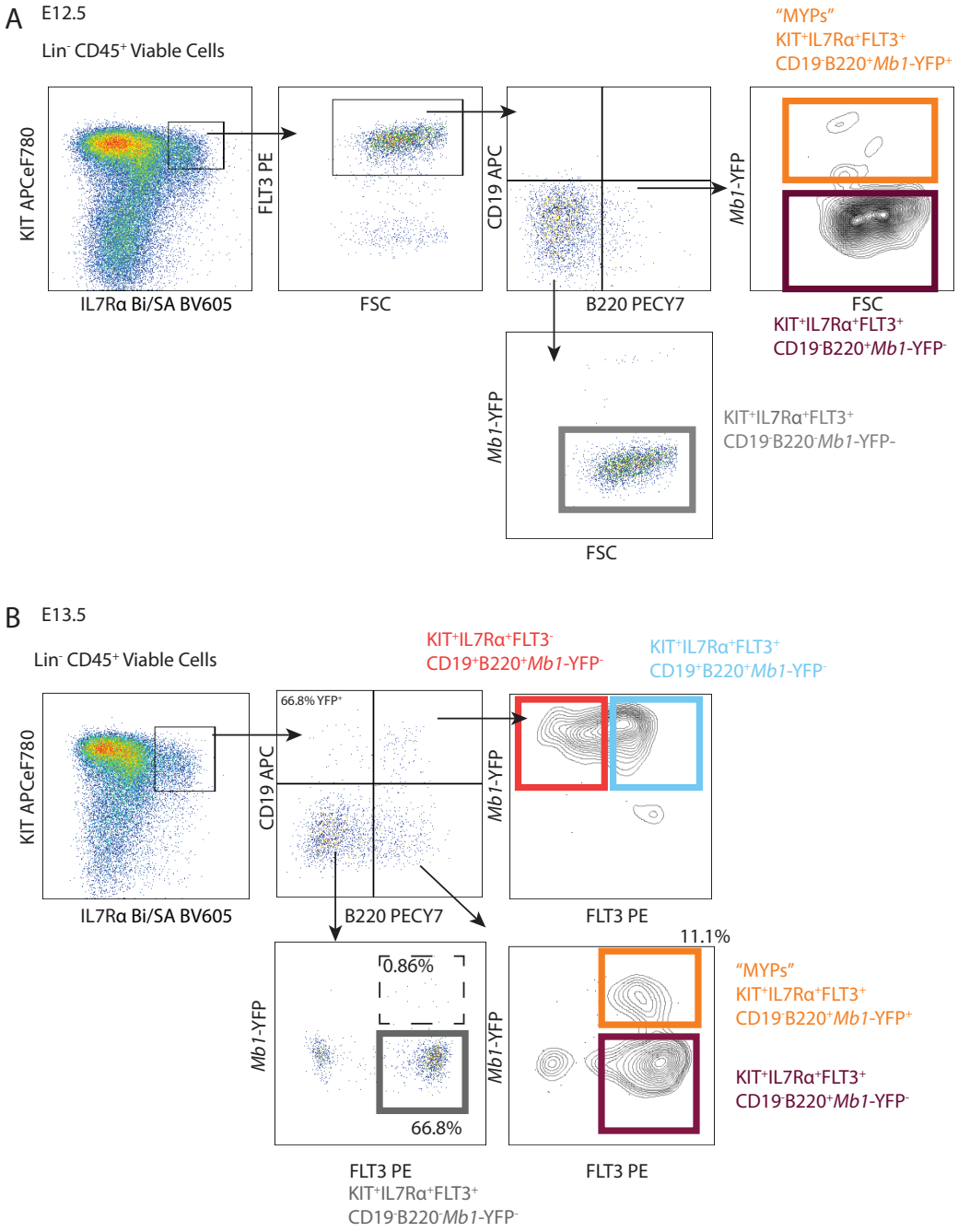


Figure 4.6: Gating strategy for sorting of E12.5 and E13.5 FL cells
Gating strategy for sorting cells from E12.5 (A) and E13.5 FL (B) for multiplex qPCR and in vitro culture analysis. Percentages shown in lower figure are of parent population. Population in dashed box was not sorted, but shown to give indication of YFP+ cells that do not express CD19 or B220. Of the B220-CD19+ population, 66.8% also expressed YFP. All percentages are mean of 5 animals.

E14.5 FL MYP cells readily grew into B220+CD19+ B cell clones in vitro, but failed to generate any T cells (figure 4.7A, 167 cells plated). However, cells that expressed B220, but not *Mb1*-YFP (CD45+B220+CD19-*Mb1*-YFP-KIT+IL-

7Rα^{+}FLT3^{+}) could readily develop into T cell clones and are therefore not B lineage restricted (figure 4.7B). This is compatible with the hypothesis that onset of *Mb1*-YFP expression correlates with loss of T cell potential and agrees with the literature reporting B220 expression alone is insufficient to isolate B cell lineage restricted progenitors (Rumfelt et al., 2006a).

Several in vitro studies are compatible with a fetal progenitor retaining B cell and Myeloid cell potential (Cumano et al., 1992; Katsura and Kawamoto, 2001; Ohmura et al., 1999) although the assays were limited by failing to isolate and characterize prospectively pure populations. We decided to investigate whether MYP cells had myeloid potential in vitro. We found no significant myeloid potential when MYP cells were cultured in liquid terasaki assays (figure 4.7C) or on OP9 stroma supplemented with GM cytokines (figure 4.7D). Despite the presence of cytokines promoting the myeloid lineage, cells plated on OP9 stroma still could develop into B cells (figure 4.7D) demonstrating the strong B lineage bias of this progenitor.

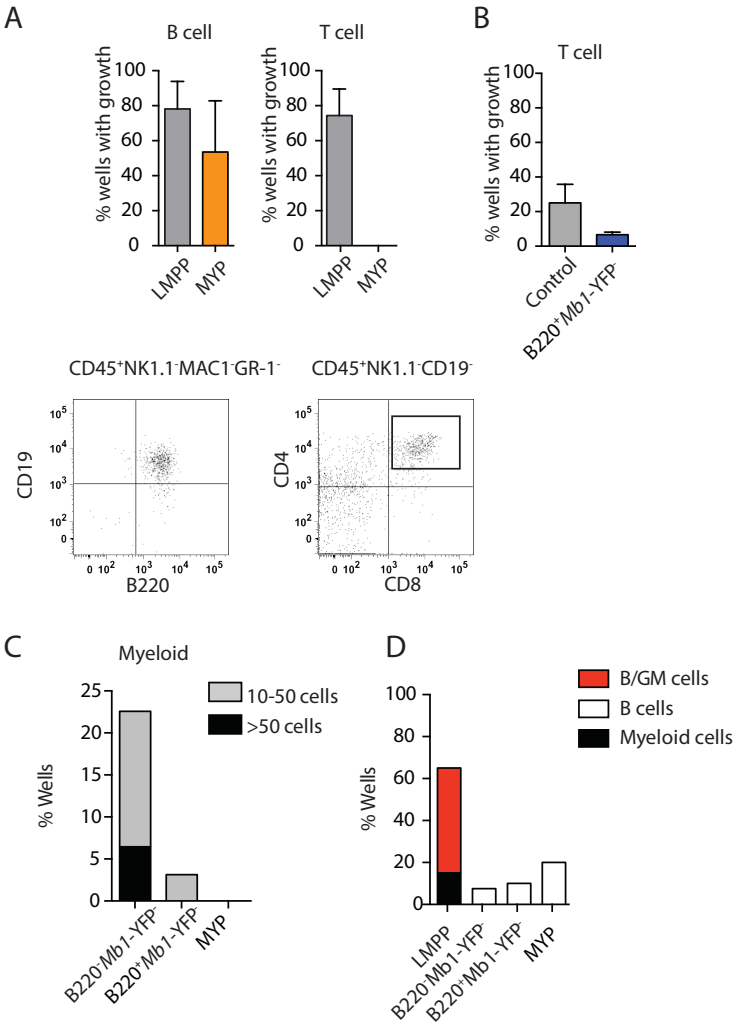


Figure 4.7: FL MYP Progenitors are B lineage restricted in vitro

A: E14.5 FL MYPs (CD45$^+$B220$^+$CD19$^-$*Mb1*-YFP$^+$FLT3$^+$KIT$^+$IL-7Rα$^+$) (7 cells per well) and LMPP cells (Lin$^-$SCA-1$^+$KIT$^+$FLT3$^+$) (1 cell/well) were plated onto OP9 stroma (B cells) or OP9 DL stroma (T cells). Cultured cells were analysed for lineages after 14 (B: NK1.1-CD19+B220+) or 21 (T: NK1.1$^-$CD4$^+$CD8$^+$ or NK1.1$^-$ Thy1.2$^+$CD25$^+$) days. 4 biological replicates, 4-8 wells per replicate. Mean ± SD.
Panels below depict representative flow cytometry analysis of cultured wells.
B: E13.5 Control cells (CD45$^+$B220$^-$CD19$^-$*Mb1*-YFP$^-$FLT3$^+$KIT$^+$IL-7Rα$^+$) and CD45$^+$B220$^+$CD19$^-$*Mb1*-YFP$^-$FLT3$^+$KIT$^+$IL-7Rα$^+$ were plated on OP9 DL stroma at single cell density and analysed for the T cell lineage after 21 days by flow cytometry. 3-4 experiments, 20 wells/experiment. Mean ± SD
C: E13.5 FL cells were seeded at single cell density in liquid terasaki assays with GM cytokines. Colonies were analysed by light microscopy after 7 days and scored as in the figure. One experiment, 30 wells/experiment.
D: E13.5 Cells were plated on OP9 stroma at single cell density with cytokines to promote GM cell development and analysed on day 12-13. 40 wells plated, two biological replicates.
LMPP: Lin$^-$SCA-1$^+$KIT$^+$FLT3$^+$
B220$^-$*Mb1*-YFP$^-$: CD45$^+$B220$^-$CD19$^-$*Mb1*-YFP$^-$FLT3$^+$KIT$^+$IL-7Rα$^+$
B220$^+$*Mb1*-YFP$^-$: CD45$^+$B220$^+$CD19$^-$*Mb1*-YFP$^-$FLT3$^+$KIT$^+$IL-7Rα$^+$
B220$^+$*Mb1*-YFP$^+$: CD45$^+$B220$^+$CD19$^-$*Mb1*-YFP$^+$FLT3$^+$KIT$^+$IL-7Rα$^+$ (MYPs)

Cells were sorted from the E12.5FL for gene expression analysis, using the gating strategy outlined in figure 4.6A. As there is no CD19 expression at E12.5, equivalent populations were sorted from the E13.5 FL as outlined in figure 4.6B. As B cell commitment regulated by PAX5 results in both *Cd19* transcription and *Flt3* repression (Holmes et al., 2006), $CD19^+$ cells could be divided into $FLT3^+$ and $FLT3^-$ populations, and were sorted accordingly. These $FLT3^+$ and $FLT3^-$ Pro B cell populations are further discussed and analysed in the following chapter, but the gene expression analysis included here in suggests that the $FLT3^+$ Pro B cells are an upstream progenitor of $FLT3^-$ Pro B, as indicated by the increased expression of B cell genes such as *Cd19, VPreB* and *Lambda5* by $FLT3^-$ Pro B cells. Notably gene expression analysis confirmed the flow cytometry analysis with detectable expression of the cytokine receptors *Kit*, *IL-7rα* and *Flt3* and furthermore *Mb1* expression was detected in YFP^+ cells (figure 4.8). *Mb1*-YFP expression correlated with up-regulation of *Rag1,* which is required for DJ rearrangement and expressed prior to B cell lineage commitment (figure 1.7). Importantly, MYPs up-regulated B lineage specific genes such as *Pax5*, *CD19* and *Lamda5* and down regulated the T cell genes *Lat* and *Gata3*. The myeloid genes *Mpl* and *Mpo* were down regulated with the expression of *B220* (figure 4.8). In the E13.5 FL, a similar pattern was seen in that the MYP cells showed marked up-regulation of B cell genes such as *Pax5* and *CD19* and down regulation of genes of the T cell and myeloid lineages (figure 4.9). However, myeloid lineage genes were not completely absent. *Sfpi1* was expressed in MYPs at E12.5 and E13.5. *Sfpi1* encodes the TF PU.1, which is involved in early B/Myeloid fate decisions, in that high levels of expression drive myeloid differentiation but moderate levels are required for B cell differentiation (DeKoter and Singh, 2000; Mak et al.,

2011). *Runx1,* which is best known for its role in the emergence of dHSCs and involvement in genetic lesions, was found to be generally expressed across the B cell progenitors, including MYPs. Interestingly, *Csf1r* appeared to be up-regulated in E12.5 MYPs but not E13.5 or adult MYPs.

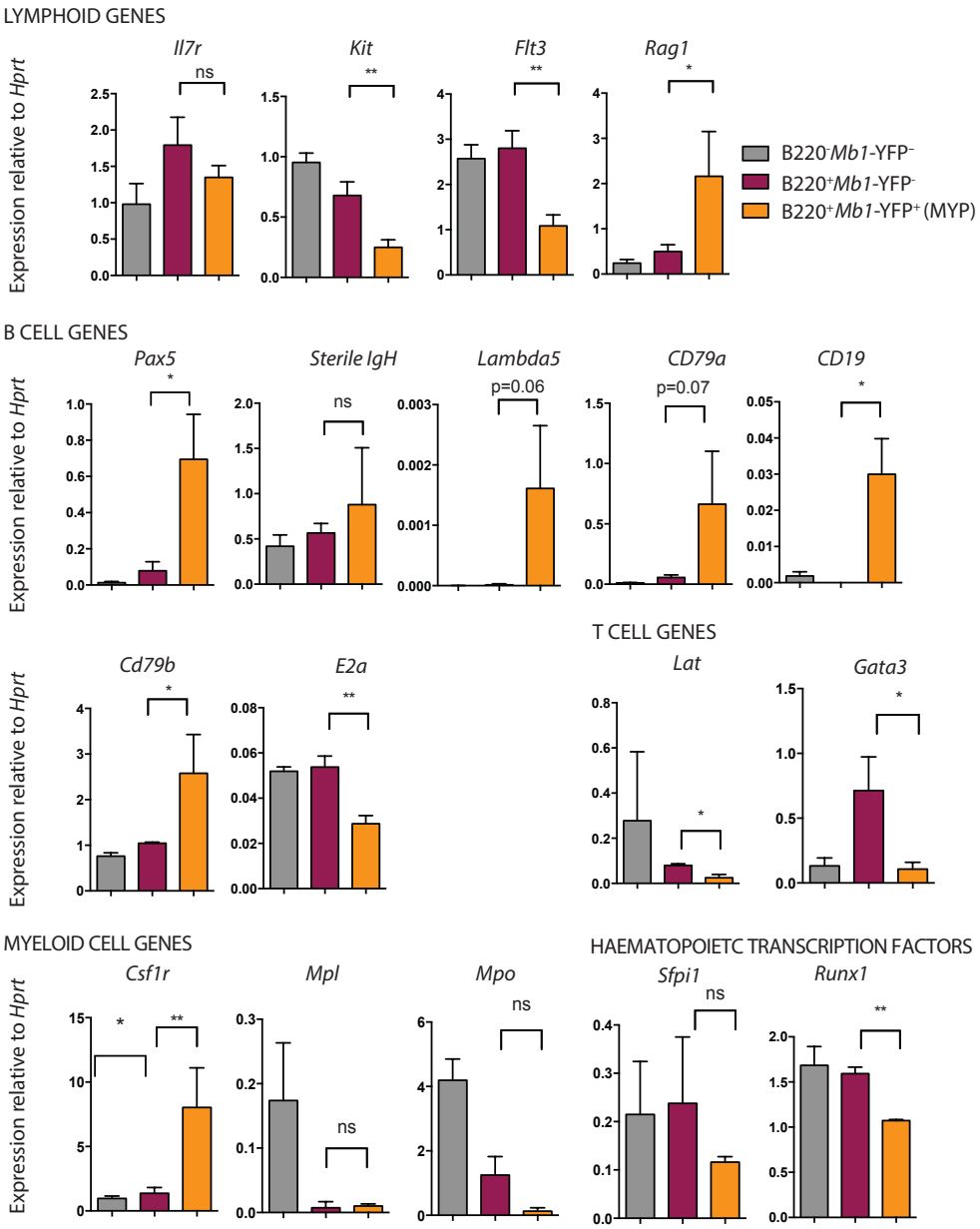


Figure 4.8: E12.5 MYP progenitors up-regulate B cell genes and down regulate T cells and myeloid lineage genes

Quantitative gene-expression analysis of 25 sorted E12.5 FL cells of specified populations. Expression normalized to *Hprt*. 2-4 replicates per population. Error bars show mean ± SD.

B220^{-}*Mb1*-YFP^{-}: CD45^{+}B220^{-}CD19^{-}*Mb1*-YFP^{-}FLT3^{+}KIT^{+}IL-7Rα^{+}
B220^{+}*Mb1*-YFP^{-}: CD45^{+}B220^{+}CD19^{-}*Mb1*-YFP^{-}FLT3^{+}KIT^{+}IL-7Rα^{+}
B220^{+}*Mb1*-YFP^{+}: CD45^{+}B220^{+}CD19^{-}*Mb1*-YFP^{+}FLT3^{+}KIT^{+}IL-7Rα^{+} (MYPs)

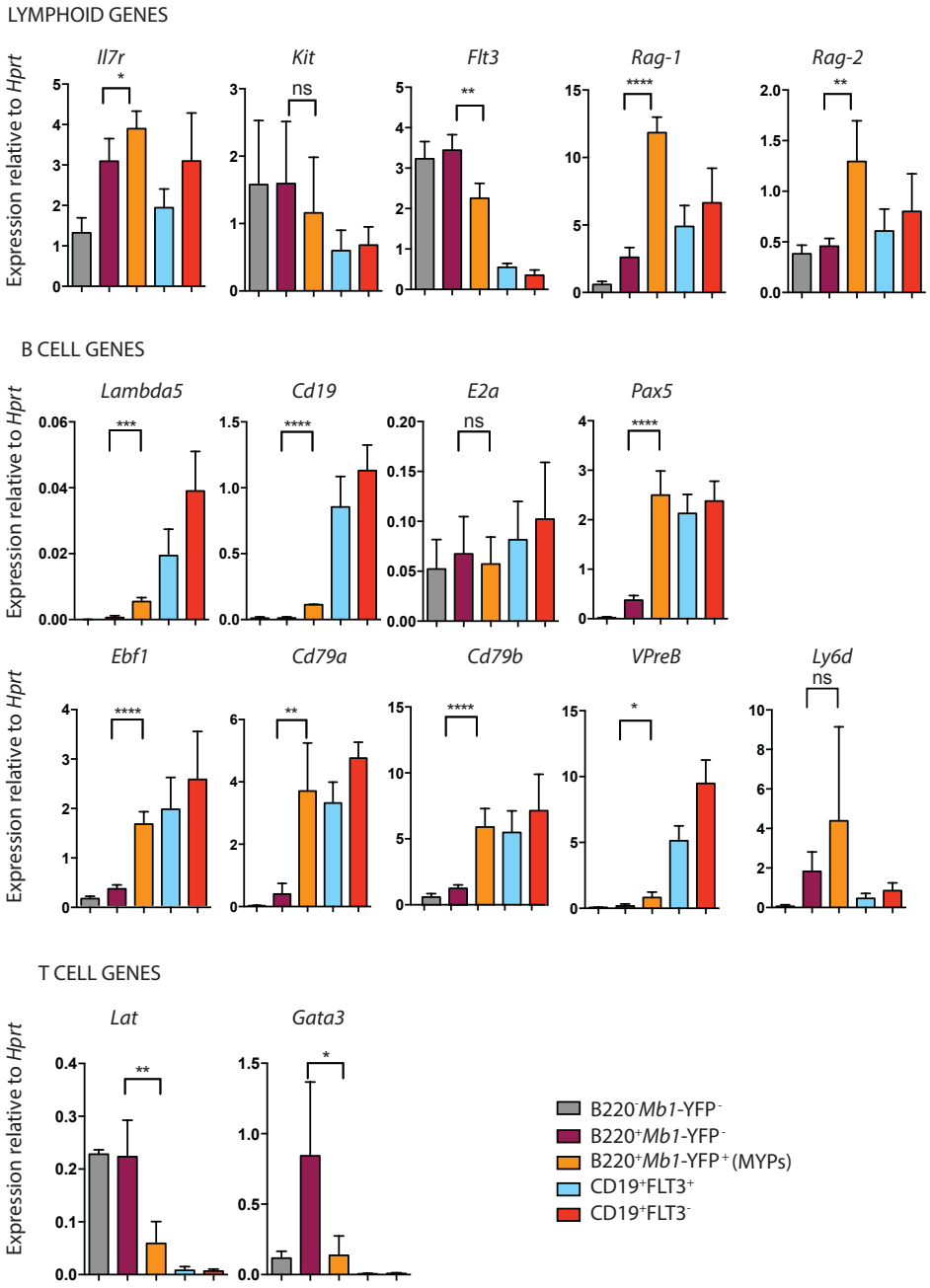


Figure 4.9 (page 1 of 2): E13.5 MYP progenitors up-regulate B cell genes and down regulate T and myeloid lineage genes

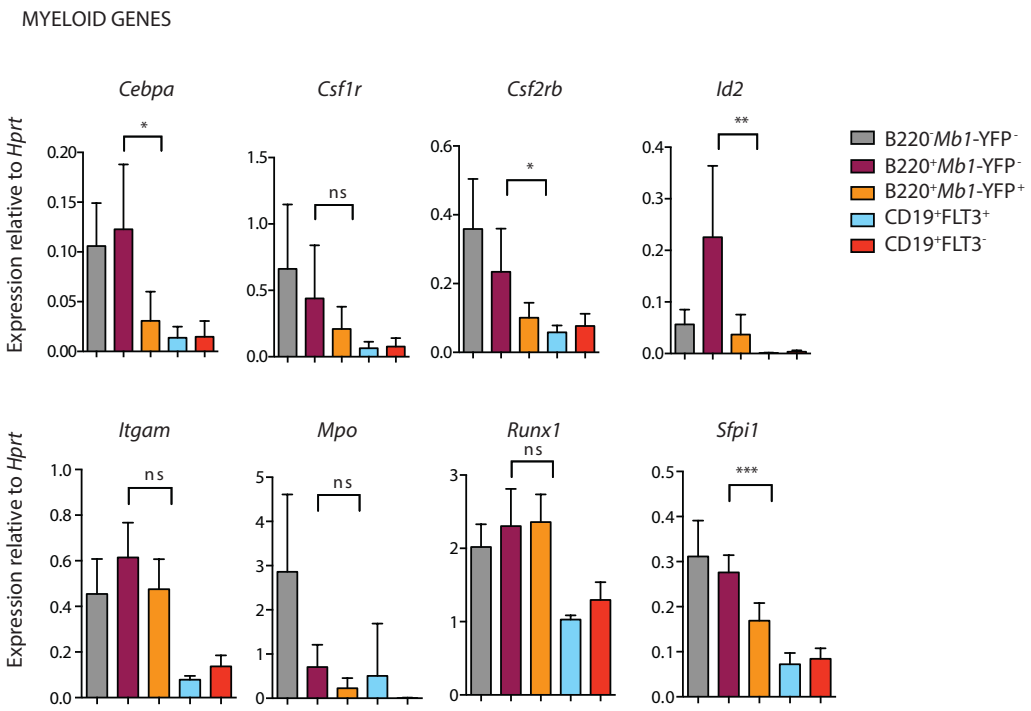


Figure 4.9: E13.5 MYP progenitors up-regulate B cell genes and down regulate T and myeloid lineage genes

Quantitative gene-expression analysis of 25 sorted E13.5 FL cells. Expression normalized to *Hprt*. 4-5 replicates per population. Error bars show mean +/- SD.

B220^{-}*Mb1*-YFP^{-}: CD45^{+}B220^{-}CD19^{-}*Mb1*-YFP^{-}FLT3^{+}KIT^{+}IL-7Rα^{+}
B220^{+}*Mb1*-YFP^{-}: CD45^{+}B220^{+}CD19^{-}*Mb1*-YFP^{-}FLT3^{+}KIT^{+}IL-7Rα^{+}
B220^{+}*Mb1*-YFP^{+}: CD45^{+}B220^{+}CD19^{-}*Mb1*-YFP^{+}FLT3^{+}KIT^{+}IL-7Rα^{+} (MYPs)
CD19^{+}FLT3^{+}: CD45^{+}B220^{+}CD19^{+}*Mb1*-YFP^{+}FLT3^{+}KIT^{+}IL-7Rα^{+}
CD19^{+}FLT3^{-}: CD45^{+}B220^{+}CD19^{+}*Mb1*-YFP^{+}FLT3^{-}KIT^{+}IL-7Rα^{+}

We next wanted to investigate if the *Mb1*-cre$^{tg/+}$ R26R$^{eYFP/+}$ reporter system could be used to identify equivalent B cell restricted populations prior to CD19 expression in the adult BM. MYP cells were present in 6-week-old BM, but at a much lower frequency, only making up 0.00055 ± 0.00027% of total BM MNCs, (figure 4.10). As in the FL, rare adult MYP cells readily developed in culture into B cells but not myeloid or T cells (figure 4.11). Gene expression analysis supported the in vitro data and demonstrated up regulation of B cell genes such as *Pax5*, *Lambda5* and *Cd19* and down regulation of genes associated with the T and myeloid cell lineages such as *Lat, Lck* and *Mpo* (figure 4.12).

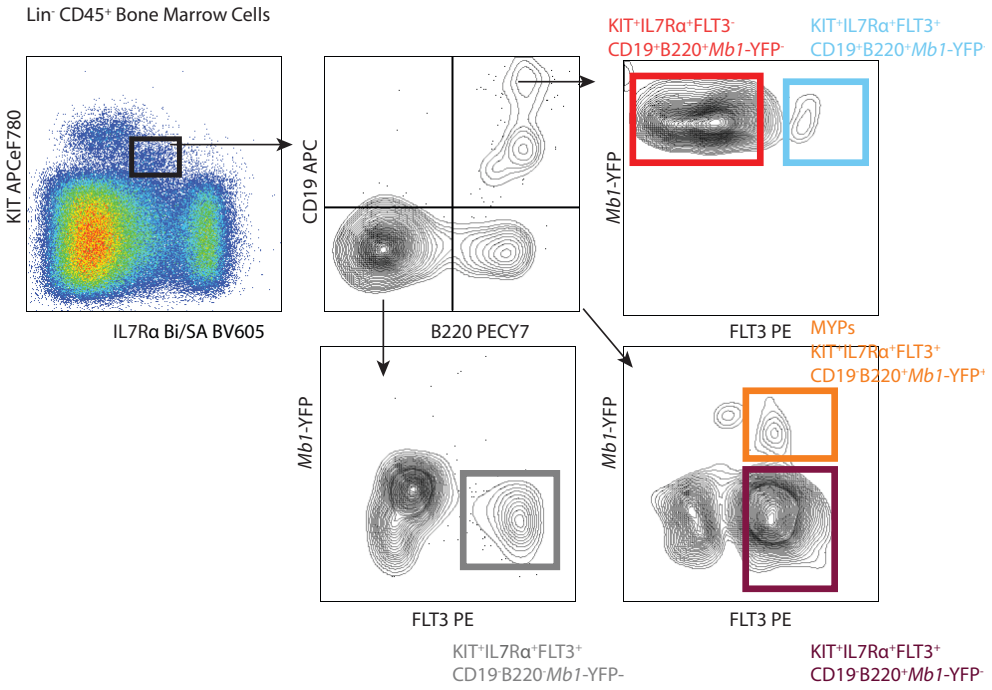


Figure 4.10: Gating strategies for sorting adult BM B cell progenitors

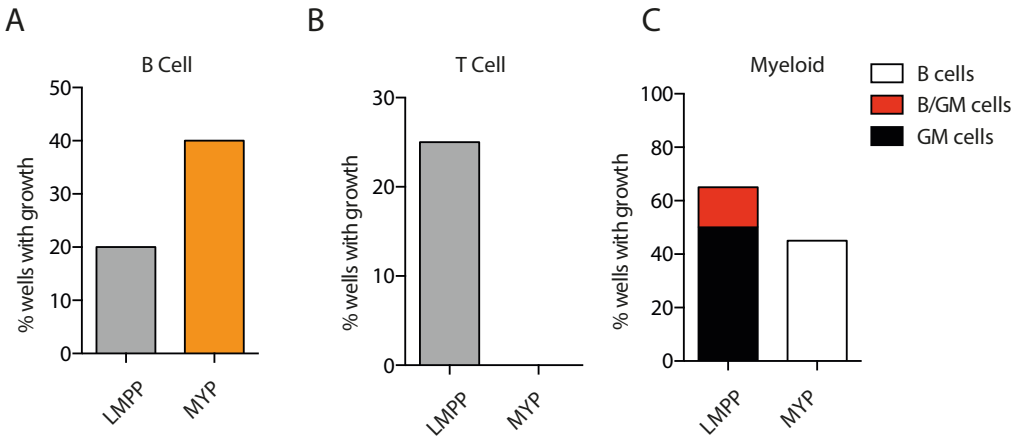


Figure 4.11: Adult MYP progenitors are B lineage restricted in vitro

CD45+B220+CD19-*Mb1*-YFP+FLT3+KIT+IL-7Rα+ ('MYP') cells and Lin-SCA-1+KIT+FLT3+ control cells ('LMPPs') were sorted from 9 week old BM and plated at single cell density on OP9 stroma with cytokines to investigate B (A) or GM cell potential (C) , and OP9 DL stroma (B) to investigate T cell potential. Cultured cells were analysed for lineages after 16 (B: NK1.1-CD19+B220+), 28 (T: NK1.1-CD4+CD8+ or NK1.1-Thy1.2+CD25+) or 12 (M: MAC1+GR1+) days. 1 biological replicate, 20 wells/replicate. One experiment, 20 wells plated.

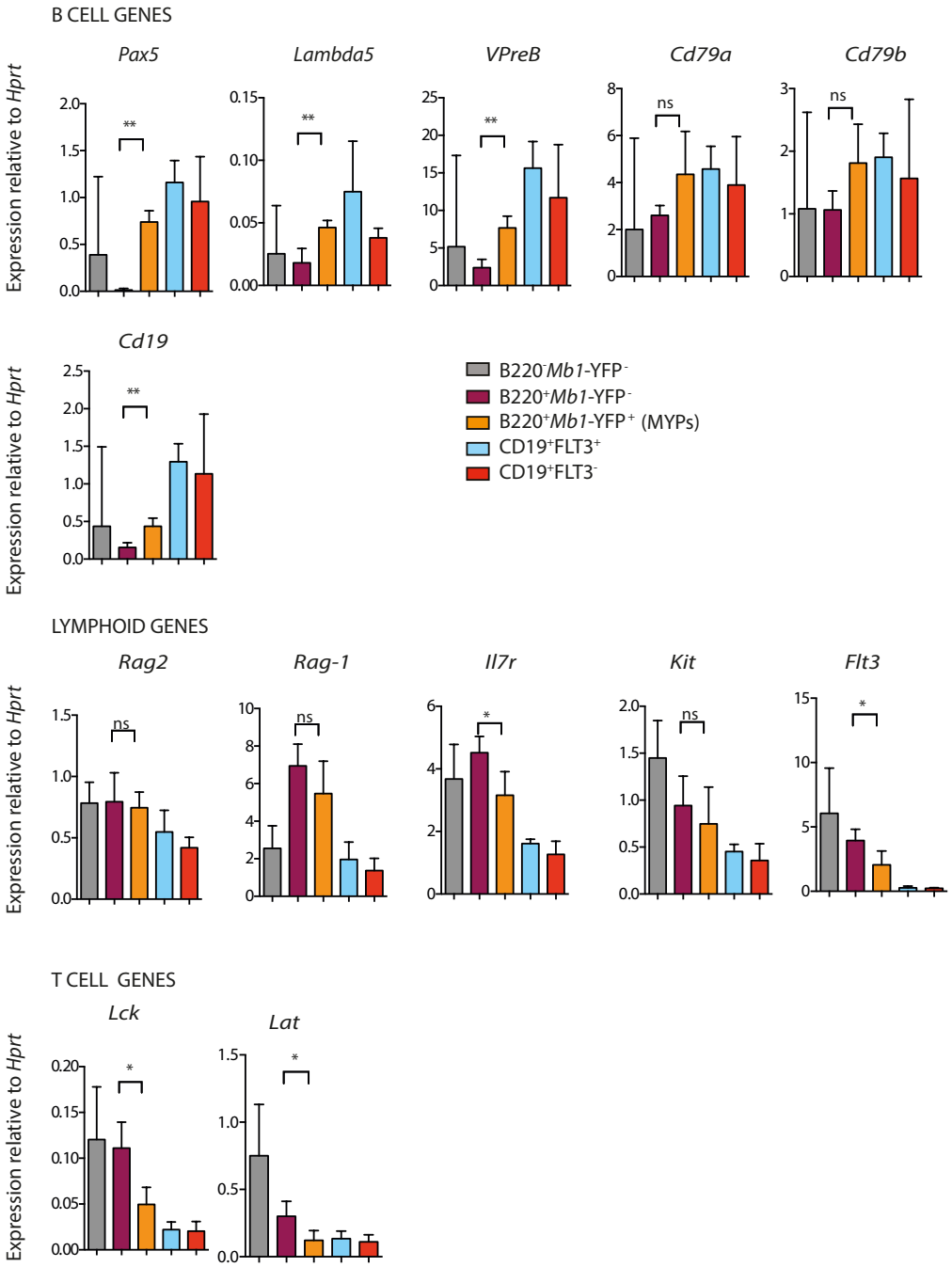


Figure 4.12 (page 1 of 2): Adult BM MYP progenitors up-regulate B cell genes and down regulate T and myeloid lineage genes

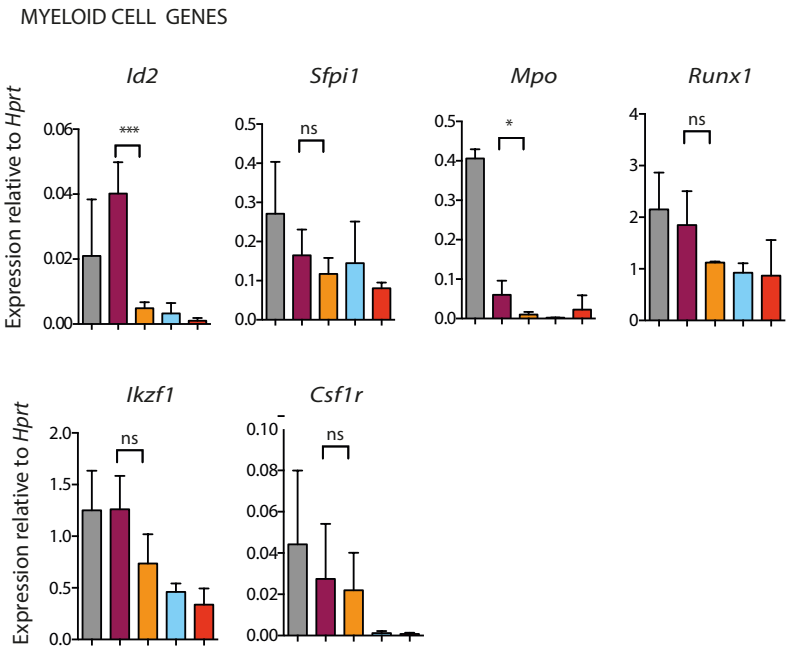


Figure 4.12: Adult BM MYP progenitors up-regulate B cell genes and down regulate T and myeloid lineage genes

Quantitative gene-expression analysis of 25 sorted Adult BM cells of specified populations. Expression normalized to *Hprt*. 4-5 replicates per population. Error bars show mean ± SD.

B220^{-}*Mb1*-YFP^{-}: CD45^{+}B220^{-}CD19^{-}*Mb1*-YFP^{-}FLT3^{+}KIT^{+}IL-7Rα^{+}
B220^{+}*Mb1*-YFP^{-}: CD45^{+}B220^{+}CD19^{-}*Mb1*-YFP^{-}FLT3^{+}KIT^{+}IL-7Rα^{+}
B220^{+}*Mb1*-YFP^{+}: CD45^{+}B220^{+}CD19^{-}*Mb1*-YFP^{+}FLT3^{+}KIT^{+}IL-7Rα^{+} (MYPs)
CD19^{+}FLT3^{+}: CD45^{+}B220^{+}CD19^{+}*Mb1*-YFP^{+}FLT3^{+}KIT^{+}IL-7Rα^{+}
CD19^{+}FLT3^{-}: CD45^{+}B220^{+}CD19^{+}*Mb1*-YFP^{+}FLT3^{-}KIT^{+}IL-7Rα^{+}

Discussion

Herein we used an *Mb1*-YFP fate mapping approach to identify a CD45$^+$B220$^+$CD19$^-$*Mb1*-YFP$^+$KIT$^+$IL-7Rα$^+$FLT3$^+$ (MYP) population in the E12.5 FL that is B cell restricted in culture, prior to the detection of cell surface CD19. These data are compatible with an in vitro study that suggested B cell restricted progenitors are present in the E12.5 FL within the IL-7Rα$^+$ sub-fraction (Kawamoto et al., 2000a), but no progenitors were prospectively purified and characterized. However, as CD19, the traditional marker of B cell restriction, is not expressed until E13.5 on the cell surface, as shown herein and by others (Douagi et al., 2002; Kawamoto et al., 2000a), these fetal CD19$^-$ B cell restricted progenitor cells have not been phenotypically identified and purified until now.

A fetal B/Myeloid progenitor?

We demonstrate that MYP cells could not generate T or myeloid cells in vitro, although it is possible that MYPs harbour residual lineage potentials that we failed to detect in our assays. For example we plated 167 MYPs under conditions that favour T cell growth but if T cell potential were retained at a very low frequency we would miss this lineage output. However, gene expression analysis supports that MYPs are B cell lineage restricted as they markedly up-regulate the B cell genes *CD19, Pax5, λ5* and *Cd79b* whilst down regulating T cell and myeloid cell genes. The detection of the *Cd19* and *Pax5* transcripts in the E12.5 FL suggests we have isolated a cell population just prior to expression of CD19 on the cell surface. Interestingly, the MYP contains detectable *Flt3* and *Pax5* transcripts, indicating that both these gene transcripts can persist in cells

concurrently, even though it has been previously reported that PAX5 represses *Flt3* expression (Holmes et al., 2006). In the future, analysis of VDJ rearrangement of the Ig loci in MYP cells, which we have shown to express the recombination gene *Rag1*, would strongly support the suggestion that MYPs are B lineage restricted.

We detected moderate expression of *Sfpi*1 in MYPs, which encodes PU.1, a TF that drives myeloid differentiation when expressed at high levels and B Cell differentiation when expressed at lower levels (DeKoter and Singh, 2000; Mak et al., 2011). Interestingly myeloid lineage genes although reduced were not totally absent in MYPs, and significant expression of the myeloid lineage gene *Csf1r*, was seen in the E12.5 FL MYPs, but expression was much lower in E13.5 and adult BM MYPs. Furthermore, 5 times as many $MAC1^{+}GR1^{+}$ myeloid cells were labelled by *Mb1* cre in the E14.5 FL than the adult BM (1.06% versus 0.20%, p=0.008). Whether this is due to increased 'leakiness' of cre expression in the fetal liver, or the labelling is specific and the earliest B cell progenitors in the fetal liver retain a residual myeloid programme is not known. Other studies have used in vitro assays to suggest that the E12 FL contains progenitors with combined B and myeloid cell potential, but no T cell potential (Cumano et al., 1992; Katsura and Kawamoto, 2001; Ohmura et al., 1999) but thus far phenotypic characterization of this cell has eluded the field. The experiments herein were not designed to probe combined B and Myeloid cell potential, therefore it is possible that the MYP may have residual myeloid potential which we failed to detect especially as, due to the short lived nature of many myeloid cells, the kinetics and time points analysed must be optimized.

Even though expression of *Mb1*-YFP and CSF1R are largely mutually exclusive, a small but significant fraction of FL MYPs co-expressed the myeloid antigen CSF1R on the cell surface (figure 4.5A). It would be interesting to further sub-fraction the MYP and isolate and characterize the rare cells that co-express both CSF1R and MYP. We can speculate that the B220$^+$ 'Pre Pro B' cell compartment, which has been shown to have B, T and myeloid cell potential (Rumfelt et al., 2006b), could be divided into CSF1R$^-$*Mb1*-YFP$^+$ B cell restricted progenitors, *Mb1*-YFP$^-$CSF1R$^+$ myeloid progenitors, *Mb1*-YFP$^+$CSF1R$^+$ combined B/GM progenitors, with T cell potential retained in the *Mb1*-YFP$^-$CSF1R$^-$ compartment.

The presentation of childhood leukaemias that express both B and myeloid lineage antigens, as well as the observation that leukaemias switch between the B and myeloid lineages much more frequently than between the B and T lineages highlights the relationship between the B and myeloid lineage in humans (Dorantes-Acosta and Pelayo, 2012). Furthermore, B cells can be readily converted into macrophages in culture by forced expression of C/EBPα (Bussmann et al., 2009). The relationship between the B and myeloid lineages is further explored in the general discussion in chapter VII.

B cell restriction prior to CD19 expression

The classical CD19$^-$ Pre Pro B cell (Hardy et al., 1991b) is now known to contain progenitors with multiple lineage potentials and is not B cell lineage restricted (Rumfelt et al., 2006a). Studies in the adult bone marrow support that B cell restricted progenitors may exist prior to CD19 expression (Mansson et al., 2008).

We examined co-expression of the markers *λ5* and LY6D by early B cell progenitors in the embryo, which have been associated with B cell restriction in CD19$^-$ bone marrow cells (Inlay et al., 2009b; Mansson et al., 2008). Fetal CD19$^+$ cells co-express *Mb1*-YFP and *λ5*-hCD25, but approximately only 1/3 of the CD45$^+$B220$^+$CD19$^-$ *Mb1*-YFP$^+$ express *λ5*-hCD25, whereas very few *λ5*-hCD25$^-$ *Mb1*-YFP$^+$ cells are detectable and these cells do not look like a clear population (figure 4.5B, right hand panel), suggesting that expression of *Mb1*-YFP$^+$ identifies B cell lineage progenitors earlier than *λ5*-hCD25 could. This is compatible with studies in the adult bone marrow where gene expression analysis indicated both *Mb1* and *λ5* are expressed in early Pro B cells. The authors commented on the unusually high expression of *Mb1*, perhaps explaining why fate mapping with *Mb1*-cre identifies B cell progenitors prior to a *λ5* reporter (Li et al., 1993b). Our data suggest in the embryo and adult BM, LY6D expression is not useful in identifying B cell restricted progenitors within the Pre Pro B fraction as only 35% of fetal and 53% of committed adult CD19$^+$ cells co-express LY6D.

The MYP only accounts for 11.1% of B220$^+$CD19$^-$ cells in the E13.5 FL (figure 4.4D) and it will be interesting to establish whether the MYP is the only B cell lineage restricted population within the heterogeneous Pre Pro B cell compartment. It will be important to try and identify the MYP based on cell surface marker expression, as currently identification is reliant on the availability of the relevant mouse lines. The MYP not only marks a true B lineage restricted population within the Pre Pro B cell compartment, but it is also the first B cell restricted progenitor to emerge in development. Therefore it could be a relevant cell population in the development of childhood and paediatric ALLs

and modelling the effect of pre-leukaemic genetic lesions on the MYP may give an insight into the aetiology of disease.

Limitations and caveats of using cre lines

A limitation of this study is that the majority of the in vitro data were generated from E14.5 MYP cells, and ideally we would like to characterize the in vitro potential of E12.5 MYPs, but the scarcity of cells and increasing difficulty in culturing cells from younger embryos, made this experiment too difficult. We therefore would want to confirm the relationship between E12.5 and E14.5 MYPs at the molecular level by performing single cell gene expression analysis, which requires fewer cells than for in vitro cultures.

The co-expression of *Mb1*-YFP with the B cell associated marker B220 increases the confidence in the specificity of the staining and reduces the chance of identifying 'false positives' due to leakiness of the cre. However, caution should be applied when interpreting data based on cre expression as cre lines can be associated with unexpected expression patterns, varied recombination efficiency and toxicity (Schmidt-Supprian and Rajewsky, 2007). As we are using *Mb1*cre to fate map rather than deleting a *LoxP* flanked allele, most of these caveats are not relevant, but none the less the use of cre lines as a genetic tool has limitations. There will be a delay between *Mb1*-cre expression and production and detection of YFP. As development progresses rapidly in the embryo, the time required for recombination and protein synthesis may mean progenitors are detected slightly later than their true emergence time.

By discriminating based on CD45 allotypes, or *Mb1*-YFP$^+$ expression, we ensured the MYP B cell progenitors examined herein were derived from the fetus and were not due to maternal contaminants. One study reported a CD19$^+$ B cell population in the E11.5 AGM and FL (de Andres et al., 2002), but the experimental set up could not exclude maternal contamination and all other published work and our data cannot detect CD19 expression until at least two days later in the E13.5 FL (Kawamoto et al., 2000a).

In addition to maternal contamination, non-specific antibody binding, auto-fluorescence (especially from the YS) and low levels of 'leaking' from cre alleles could result in false positives when looking for rare fetal progenitors. We increased assurance by purity reanalysis and by ensuring co-expression of multiple markers associated with the B and lymphoid lineages such as KIT and B220. Rare cells that demonstrated unexpected CD19 or *Mb1*-YFP were often excluded as they counter intuitively co-expressed high levels of the T cell proteins CD4 and CD8 indicating auto-fluorescence, non specific binding or another flow cytometry artefact. However, whether *Mb1* is truly expressed should be confirmed by quantitative PCR. Certain populations would be interesting to characterize such as the B220$^-$CD19$^+$ cells that express *Mb1*-YFP in the E13.5 FL (figure 4.6), previously suggested to be a putative B1 cell progenitor (Montecino-Rodriguez et al., 2006).

Possible upstream progenitors of the MYP

In the adult, both the classical and alternative haematopoietic hierarchies suggest B cell restricted progenitors develop from dHSCs that undergo stepwise lineage commitment, with some argument about whether they progress through a LMPP or CLP stage. In the early embryo, the multiple waves of haematopoiesis complicate the issue. The MYP B cell restricted progenitors identified could arise from dHSCs that emerge in the AGM at E10.5 and start to seed the FL at E11.5 (de Bruijn et al., 2000; Dzierzak and Speck, 2008; Medvinsky and Dzierzak, 1996). In theory, MYPs could also arise from earlier haematopoietic waves, such as the Lin$^-$IL-7Rα$^+$Kit$^+$*Rag1*-GFP$^+$ cells that emerge in the E9.5 YS, identified in chapter III. MYP cells are first detected in the FL but this is not definitive proof that this is where they first emerge as circulation has been established for 4 days by E12.5 and it is possible, although perhaps unlikely, that B cell restricted progenitors emerge at a low frequency, below the limits of detection at other haematopoietic sites before migration to, and expansion in the FL. However it seems most plausible that MYPs develop from definitive haematopoietic progenitors in the FL.

Cytokine receptor expression in early lymphoid progenitors

The expression of IL-7Rα by B cell restricted MYP cells is compatible with other studies that indicate the earliest B cell progenitor should express IL-7R. IL-7 has been shown to be required for the development of CLPs into B cells with IL-7Rα$^{-/-}$ mice lacking early B cell progenitors (Miller et al., 2002a). Furthermore single

E12.5 IL-7Rα$^+$ FL cells were found to contain uni-potent B cell progenitors in vitro. It has been shown that mice deficient for both FLT3L and IL-7Rα have a total lack of fetal and adult B1 and B2 cells (Sitnicka et al., 2007) and that mice lacking *KIT* have reduced numbers of B cell progenitors (Landreth et al., 1984). Therefore it is noteworthy that almost all MYP cells homogeneously expressed IL-7Rα, FLT3 and KIT, consistent with the importance of these lymphoid cytokine receptors in the development of B cell progenitors.

Herein we have shown that MYP cells are restricted to the B cell lineage in vitro. As it has been suggested that *Pax5* expression is required and sufficient for B cell lineage restriction in adult haematopoiesis (Mikkola et al., 2002; Nutt et al., 1999), it would be interesting to investigate whether E12.5 MYP cells that lack *Pax5* expression regain the potential to develop into other lineages in culture.

Conclusions

For the first time we have developed a strategy to identify the earliest B cell restricted progenitors in the mouse embryo by using a fate mapping approach in combination with cell surface markers. How fetal B cell progenitors molecularly and functionally compare to phenotypically matched B cell stages from the adult BM remains unknown.

In order to better understand childhood B cell leukaemias and how they respond to therapy, we next wanted to investigate later stages of fetal B cell development

and subsequently compare the fetal and adult $CD19^{+}$ Pro B cell compartments (chapter V).

Chapter V:
Staging CD19^{+} B cell restricted progenitors in the mouse fetal liver: characterization of a novel CD19^{+} FLT3^{+} Pro B cell

STAGING CD19^{+} B CELL RESTRICTED PROGENITORS IN THE MOUSE FETAL LIVER: CHARACTERIZATION OF A NOVEL CD19^{+} FLT3^{+} PRO B CELL

Introduction

When children present with B cell ALL, white blood cell analysis reveals that as few as 1 but on average 6, genetic lesions have occurred (Mullighan et al., 2007). For leukaemias that express the TEL-AML1 oncoprotein, the initial translocation occurs in utero (Mori et al., 2002b), but the precise lineage restriction and developmental stage of the fetal progenitor in which the translocation occurs and subsequently influences lymphopoiesis is unknown.

In chapter III, we identified an early mouse fetal immune restricted progenitor with B cell, T cell and GM cell potential. Subsequently in chapter IV in the E12.5 FL we identified the earliest characterized immune cells that are entirely restricted to the B cell lineage. The next logical step is to identify and characterize the fetal Pro B cells that develop from the E12.5 MYP cells in the FL. We wanted to directly compare the similarities and differences of fetal Pro B cells with their well characterized adult counterparts. Hence my objective was to investigate and complete another piece in the intriguing puzzle of fetal B cell lymphopoiesis, and take a step towards understanding its deregulation in childhood B cell ALL.

A myriad of B cell markers have been applied to adult bone marrow cells to phenotypically separate B cell progenitors (Hardy and Hayakawa, 2001). The 12-colour flow cytometry approach published by Richard Hardy's group in 2006 (Rumfelt et al., 2006a) resulted in a high-resolution definition of $CD19^+$ and $CD19^-$ B cell progenitors in the adult bone marrow. In comparison, very little work has been published on B cell progenitor staining and characterization in the fetus, and it has been largely assumed that the same phenotypic markers will apply in the fetal liver as for the adult bone marrow (Dorshkind and Montecino-Rodriguez, 2007b).

There are known functional differences between fetal and adult B cell lymphopoiesis. Firstly, the embryo and neonate but not the adult can sustain $CD19^+$ B cell progenitors in the absence of IL-7 (Carvalho et al., 2001). Secondly, fetal liver progenitors have a bias towards generating the B1 cell subset whereas the adult BM has a bias towards generating B2 cells (Hayakawa et al., 1985). Recently it has been suggested that B1a cells are generated independently of HSCs in the embryo (Ghosn et al., 2016). We wanted to directly compare fetal and adult B cell progenitors and see if we could identify specific functional and molecular differences between B cell lymphopoiesis in the adult bone marrow and fetal liver.

PAX5 is a key TF involved in B cell lineage restriction and maintenance (Medvedovic et al., 2011). Loss of one PAX5 allele is a common secondary mutation in childhood leukaemias that express the TEL-AML1 oncoprotein (Mullighan et al., 2007). Despite acceptance that PAX5 is essential for

commitment and maintenance of B cells in the adult bone marrow (Mikkola et al., 2002; Nutt et al., 1999; Rolink et al., 1999), the role of PAX5 in fetal liver B cell lymphopoiesis has not been studied, although it has been reported that complete absence of PAX5 in the embryo results in a very early block in B cell development, prior to the emergence of $B220^{+}$ Pre Pro B cells (Nutt et al., 1997).

Herein we develop a high resolution, multi colour flow cytometry panel to characterize Pre Pro B cells and Pro B cells in WT and subsequently PAX5 deficient mice. We first validated the B cell staging in the adult bone marrow then isolated FL B cell progenitors for functional and molecular characterization. We identified a rare Pro B cell fraction expressing the cytokine receptor FLT3 that emerges during embryonic development and peaks in frequency in late gestation, but is rare in adult bone marrow. This $FLT3^{+}$ Pro B cell has increased engraftment in vivo compared to $FLT3^{-}$ Pro B cells.

Results

We first developed a high-resolution Pre Pro B and Pro B cell-staining panel, based on published work by Richard Hardy's laboratory in the adult bone marrow. We included the B cell markers B220, CD43, CD19, CD24 (HSA), CD93 (AA4.1) and KIT (Rumfelt et al., 2006a). Given that 91% of the $CD45^{+}B220^{+}CD19^{-}$*Mb1*-$YFP^{+}KIT^{+}IL\text{-}7R\alpha^{+}$ earliest B cell restricted progenitors identified in Chapter IV express the cytokine receptor FLT3 (figure 4.4B), and that $FLT3^{-/-}$ $IL\text{-}7^{-/-}$ mice show complete lack of B cells (Jensen et al., 2008; Sitnicka et al., 2003) we decided to also include antibodies against the FLT3 receptor in our analysis of B cell progenitors. See appendix 2 for the spectral analysis of the Pre Pro B and Pro B panels.

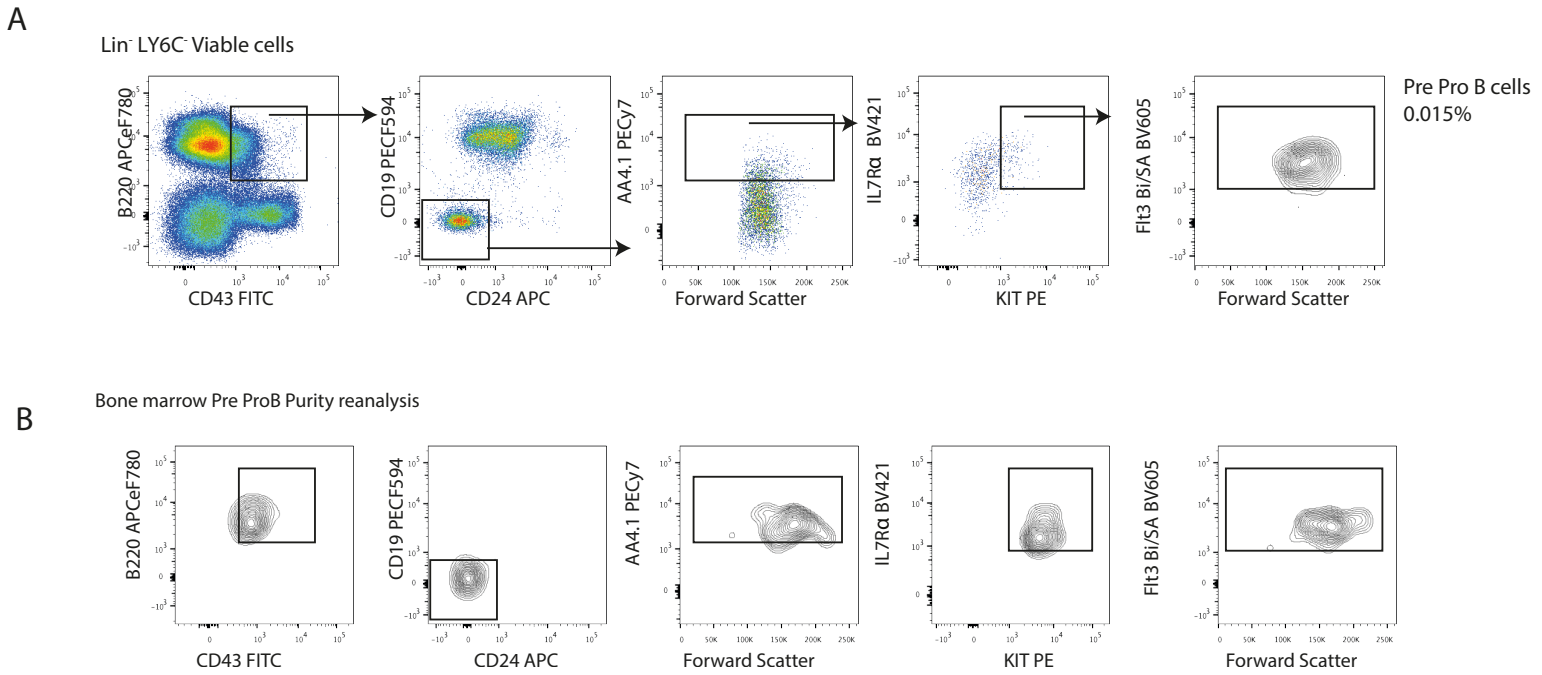


Figure 5.1 Gating strategy for the identification of adult BM Pre Pro B cells

A: BM was stained using 10 fluorescently conjugated antibodies and analysed by flow cytometry. B: Purity reanalysis of Pre Pro B cells. Data shown are representative of more than 10 independent analysis using 6-8 week C57 Bl/6 mice. % of total MNCs. Lineage cocktail included antibodies against GR-1, CD3, NK1.1, TER119 and CD11c and F4/80.

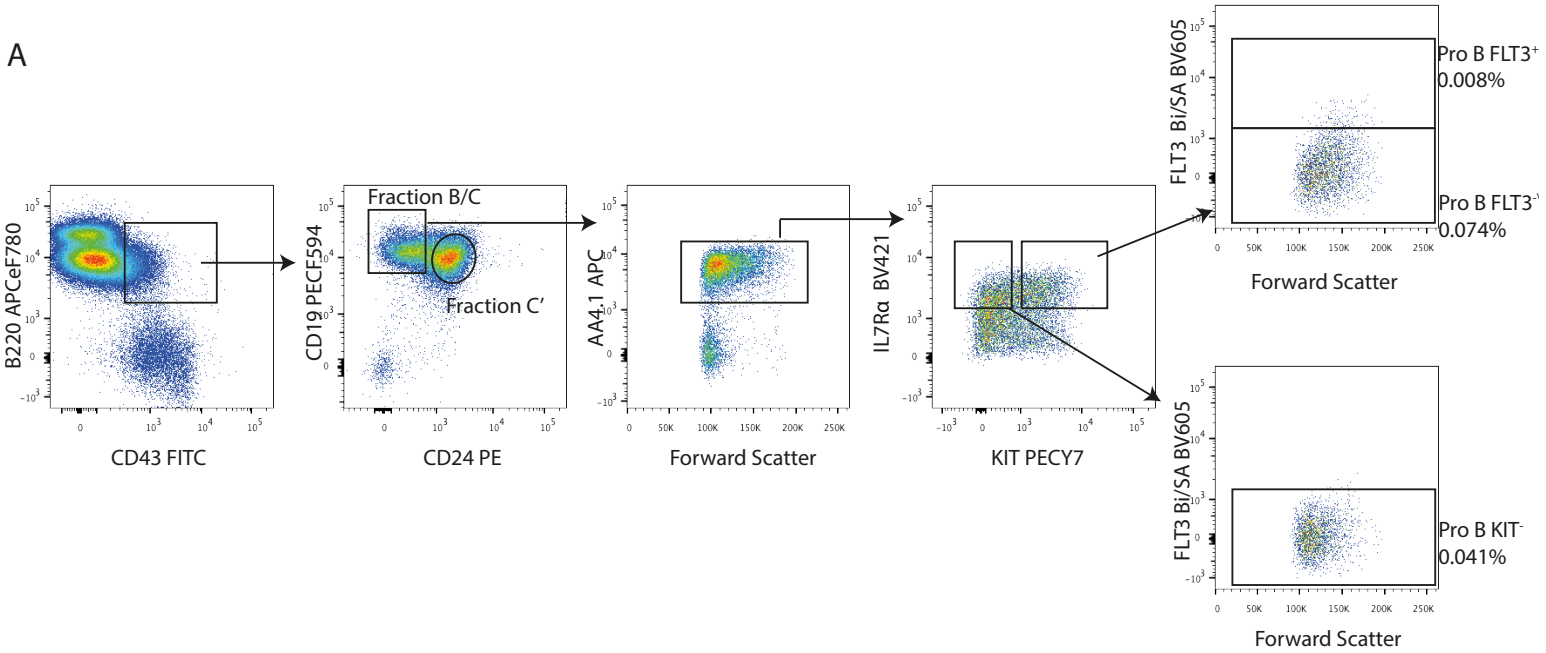


Figure 5.2 Gating strategy for the identification of adult BM Pro B cell sub-fractions

A: BM was stained using 9 fluorescently conjugated antibodies and analysed by flow cytometry. Early Pro B cells (fraction B/C) were defined as expressing intermediate levels of CD24 (Rumfelt et al., 2006a), and further gated as in the figure. Pro B cells expressing high levels of CD24 form the late Pro B fraction 'C'. IL-7R^{-} cells were not analysed further. % of total MNCs.

B: (Following page) Purity Data showing reanalysis of sorted Lin^{-}CD43^{+}B220^{+}CD19^{-} CD24^{-} AA4.1^{+}cKIT^{+}IL-7Rα^{+}FLT3^{+} **(Pro B FLT3^{+}),** Lin^{-}CD43^{+}B220^{+}CD19^{-} CD24^{-} AA4.1^{+}cKIT^{+}IL-7Rα^{+}FLT3^{-} **(Pro B FLT3^{-})** and Lin^{-}CD43^{+}B220^{+}CD19^{-} CD24^{-} AA4.1^{+}cKIT^{-}IL-7Rα^{+}FLT3^{-} **(Pro B C KIT^{-})** cells. Representative of more than 10 independent analyses on 6-8 week C57 Bl/6 mice.

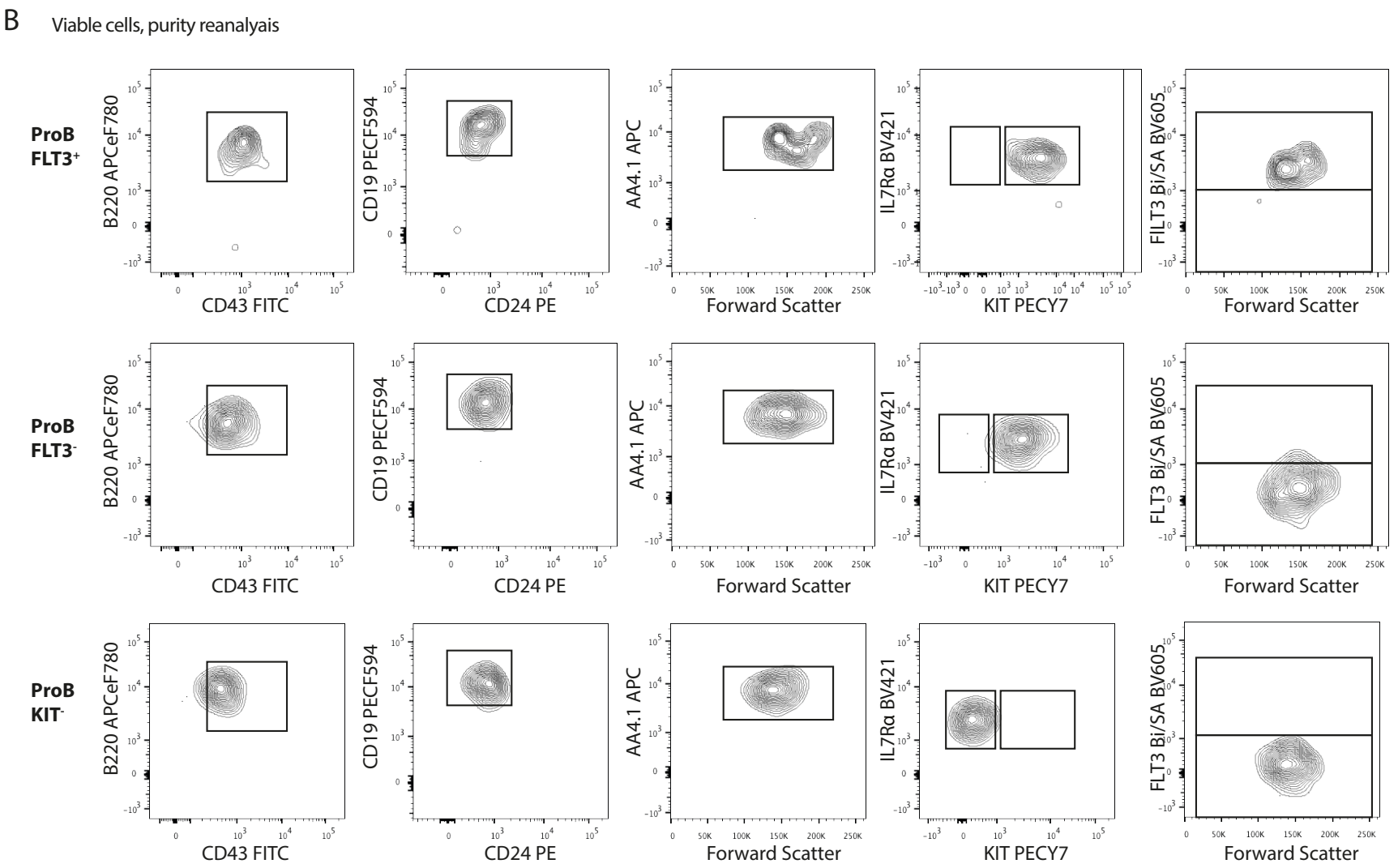


Figure 5.2 Gating strategy for the identification of adult BM Pro B cell sub-fractions (page 2 of 2)

We first gated to exclude erythroid, myeloid and T cells from the bone marrow and fetal liver using a mixture of lineage specific antibodies against GR-1, CD3, NK1.1, Ter119, CD11c and F4/80. We did not use antibodies against MAC-1 (CD11b) in the lineage exclusion cocktail due to data suggesting that fetal lymphoid progenitors may express MAC-1 (Yokota et al., 2003), and instead relied on GR-1 to exclude cells of the myeloid lineage. We also did not include antibodies against CD4 in the lineage depletion cocktail as it has been suggested B cell progenitors in the Pre Pro B compartment in the adult bone marrow may expresses CD4 (Allman et al., 1999), although this has not been followed up in further studies. Instead we excluded T cells based on CD3ε expression.

Subsequently we gated on key markers in B cell development, namely B220, CD19, AA4.1, CD24 and CD43. As the choice of antibody clone can affect staining profiles for some markers, we consistently used the S7 clone for detecting CD43 and 30-F1 clone for detecting CD24 (Hardy et al., 1991a; Rumfelt et al., 2006a). We specifically chose not to use the M1/69 clone, a commonly used CD24 antibody, as studies have reported that it also binds to $LY6C^+$ cells which do not give rise to mature B lineage cells (Tudor et al., 2000).

We next included antibodies against the cytokine receptors KIT, IL-7Rα and FLT3. Levels of KIT decline as cells develop from LMPPs to mature B cell progenitors in the adult bone marrow (Igarashi et al., 2002; Medina et al., 2001; Payne et al., 1999), which some early studies mistook for lack of KIT staining on early B cell progenitors (Allman et al., 1999), although later studies showed that KIT was expressed by immature B cell progenitors (Ogawa et al., 2000).

Additionally PAX5, whilst activating CD19 expression represses FLT3 expression (Holmes et al., 2006), and therefore levels of cell surface FLT3 were expected to be low on $CD19^{+}$ Pro B cells (Ogawa et al., 2000). In order to detect these 'dim' cytokine receptors we used the newly developed 'Brilliant Violet' (BV) conjugates (Chattopadhyay et al., 2012) which are excited by the violet laser (405nm) and emit a 10 fold brighter signal than standard dyes (De Rosa, 2012) (see appendix 3 for a spectral analysis of BV421).

We first analysed adult BM B cell progenitors using our optimized 10 colour Pre Pro B cell and 9 colour Pro B cell panels (figure 5.1A and 5.2A). The bright, phycoerythrin ('PE') fluorochrome conjugated to an antibody that binds CD24 was used in order to obtain high enough resolution to separate out multiple Pro B cell stages expressing varying levels of CD24. It has been shown that early Pro B cells express lower levels of CD24 (Fraction B/C ($CD24^{intermediate}$)), whereas late Pro B cells that have begun to cycle and develop into Pre B cells express higher levels of CD24 (Fraction C' ($CD24^{high}$) (figure 5.2A) (Rumfelt et al., 2006a). Historically, fractions B and C have been distinguished as only the latter expresses BP-1 (Hardy et al., 1991a). However following suit with other laboratories (Rumfelt et al., 2006b), we excluded antibodies against BP-1 due to inconsistent expression of BP-1 between mouse strains (David Allman, personal communication).

Based on previous studies demonstrating that AA4.1 (CD93) is expressed by HSCs through to immature B cells (Jordan et al., 1990; McKearn et al., 1984; McKearn et al., 1985), we selected $AA4.1^{high}$ cells. Within the Pre Pro B cell

compartment the bright PE conjugated antibodies were used to detect KIT that has been reportedly difficult to detect on Pre Pro B cells (Allman et al., 1999). Antibodies against LY6C were included in the Pre Pro B panel to exclude dendritic cells, but with the onset of $CD19^+$ expression, this was not deemed necessary for the Pro B cell panel, so this antibody was excluded to reduce the spectral overlap on the red laser (see appendix 2 for spectral plots).

We found that $Lin^-LY6C^-CD43^+B220^+CD19^-$ $CD24^-$ $AA4.1^+KIT^+IL\text{-}7R\alpha^+$ Pre Pro B cells all homogeneously expressed FLT3 (figure 5.2A), whereas $Lin^-CD43^+B220^+CD19^+CD24^{intermediate}$ $AA4.1^+$ Pro B cells could be subdivided into $KIT^+IL\text{-}7R\alpha^+FLT3^+$ ('Pro B $FLT3^+$'), $KIT^+IL\text{-}7R\alpha^+FLT3^-$ ('Pro B $FLT3^-$'), and $KIT^-IL\text{-}7R\alpha^+FLT3^-$ ('Pro B KIT^-') fractions. A summary of the B cell populations and their abbreviations used in this thesis is shown in table 5.1. As PAX5 is known to repress FLT3 expression (Holmes et al., 2006), the detection of a significant Pro B $FLT3^+$ fraction was unexpected. Back gating analysis revealed that all Pro B $FLT3^+$ cells expressed the cytokine receptors KIT and IL-7Rα (appendix 4). Purity reanalysis for sorted cell populations are shown in figure 5.1B and figure 5.2B. Gates were set on populations known to be positive and negative for specific markers (for example the Pro B cell FLT3 gate was set by comparison to Pre Pro B cells which express FLT3) with the aid of isotype controls and fluorescent minus one controls (figure 5.3). We used back gating analysis to assess whether we may have excluded significant populations of cells expressing the cell surface markers of interest.

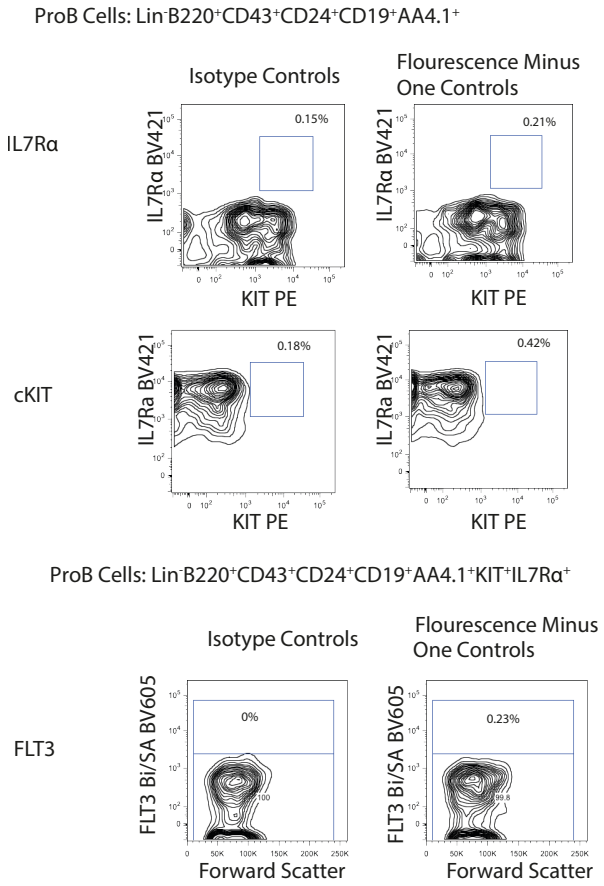


Figure 5.3: Setting the gates for cytokine receptors using FMO and IC controls
BM samples were stained and gated for Pro B cells as in Fig. 5.2. Where indicated isotype controls, or no antibody (fluorescence minus one) were added instead of the fluorescent cKIT, IL-7Ra or FLT3 antibodies. Percentages depict percentage of parent population.

Table 5.1: Cell surface markers used to define B cell progenitors

Tissue	Abbreviation	Cell Surface Markers
BM	Pre Pro B	Lin$^-$B220$^+$CD43$^+$CD19$^-$CD24$^-$AA4.1$^+$KIT$^+$IL-7Rα$^+$FLT3$^+$
BM	Pro B FLT3$^+$	Lin$^-$B220$^+$CD43$^+$ CD19$^+$CD24^{int}AA4.1$^+$IL-7Rα$^+$KIT$^+$FLT3+
BM	Pro B FLT3$^-$	Lin$^-$B220$^+$CD43$^+$ CD19$^+$CD24^{int}AA4.1$^+$IL-7Rα$^+$KIT$^+$FLT3$^-$
BM	Pro B KIT$^-$	Lin$^-$B220$^+$CD43$^+$CD19$^+$CD24^{int}AA4.1$^+$IL-7Rα$^+$KIT$^-$FLT3$^-$
FL	Pre Pro B	Lin$^-$B220$^+$CD43$^+$CD19$^-$CD24$^-$AA4.1$^+$KIT$^+$IL-7Rα$^+$FLT3$^+$
FL	Pro B FLT3$^+$	Lin$^-$B220$^+$CD43$^+$ CD19$^+$CD24^{int}AA4.1$^+$IL-7Rα$^+$KIT$^+$FLT3+
FL	Pro B FLT3$^-$	Lin$^-$B220$^+$CD43$^+$ CD19$^+$CD24^{int}AA4.1$^+$IL-7Rα$^+$KIT$^+$FLT3$^-$

Next, I investigated the capacity of adult B cell progenitors to generate B, T and myeloid cells in vitro (figure 5.4). All populations were found to readily develop into B cell clones on OP9 stroma in two different culture conditions, except Pro B KIT^- cells which demonstrated a 5 times lower cloning frequency than Pro B $FLT3^+$ and Pro B $FLT3^-$ cells (figure 5.4A). In agreement with published studies (Rumfelt et al., 2006a) Pre Pro B cells had the potential to generate T cells in culture (figure 5.4B) but we could not detect myeloid potential on OP9 stroma (figure 5.4C). 20% of Pre Pro B cells plated in liquid terasaki assays with GM cytokines demonstrated limited growth, but the cell number was too low to confirm the cells were of the myeloid lineage using morphology analysis.

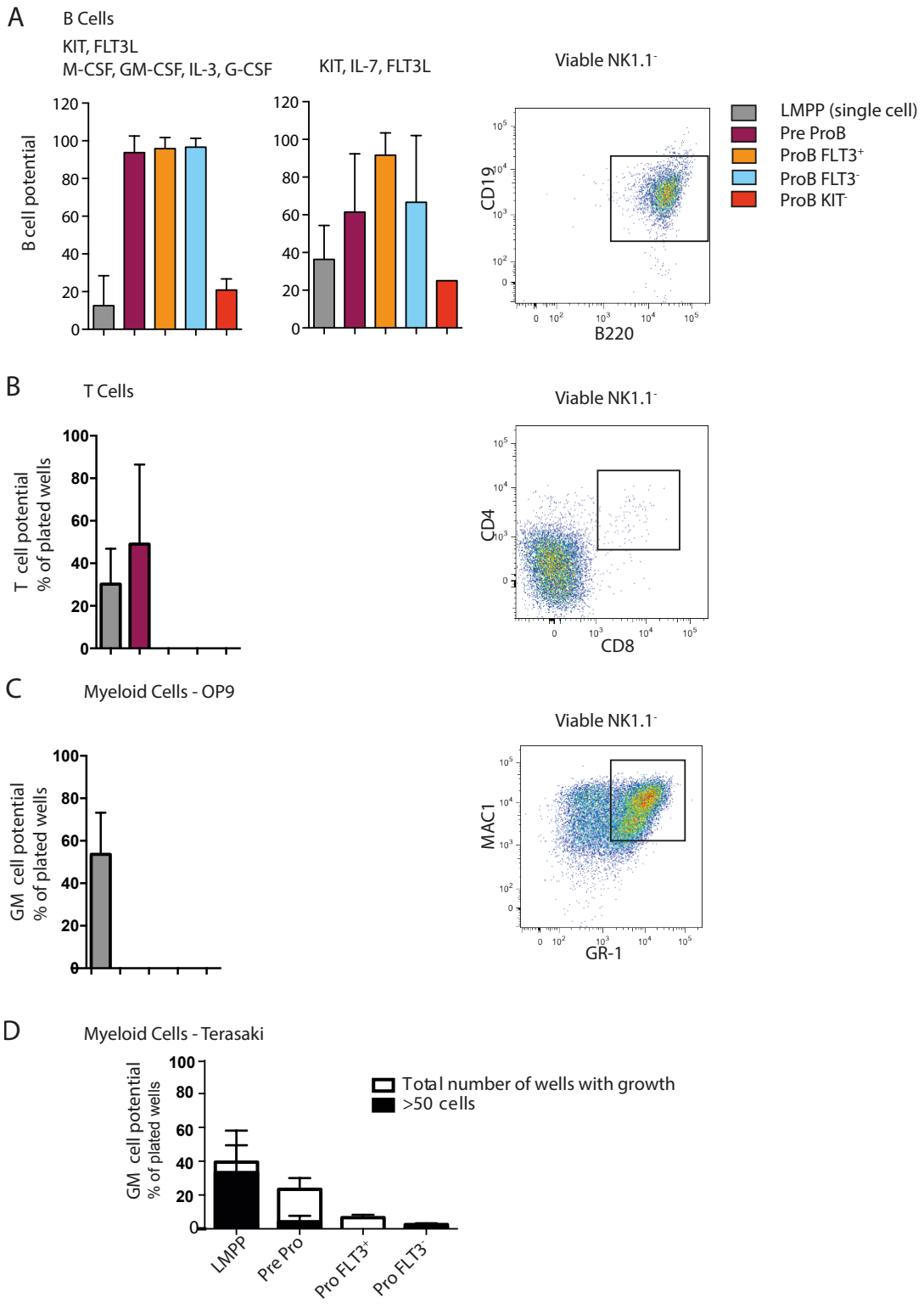


Figure 5.4: Adult Pre Pro B cells have T cell and B cell potential, but Pro B cells are B lineage restricted

A: B cell potential ($NK1.1^{-}CD19^{+}B220^{+}$) was analysed by sorting adult bone marrow cells and plating at 7 cells per well on OP9 stroma supplemented with mSCF, hFL, h-Csf1, mGM-CSF, mIL-3, hGSCF or just mSCF, hFL and IL-7 (right hand graph). Lineages were analysed after 10 days by flow cytometry. 24-55 wells plated.

B: T cell ($NK1.1^{-}THY1.2^{+}CD25^{+}$ or $NK1.1^{-}CD4^{+}CD8^{+}$) potential was analysed by plating 7 cells per well on OP9 DL stroma, supplemented with mSCF and hFL and lineages were analysed 28 days after culture. 24-54 wells plated.

C: Myeloid ($NK1.1^{-}MAC1^{+}GR\text{-}1^{+}$) potential was analysed by plating 7 cells per well on OP9 stroma supplemented with mSCF, hFL, h-Csf1, mGM-CSF, mIL-3, hGSCF (left hand panel) and lineages analysed after 10 days by flow cytometry. 24-30 wells plated. Right hand panels depict representative FACS plot of a clone that was scored positive for B, T or Myeloid cell potential respectively.

D: GM lineage potential in liquid terasaki assays was evaluated by plating single sorted cells with GM cytokines. White bars represent total cloning, black bars represent clones with >50 cells. Mean ± SD.

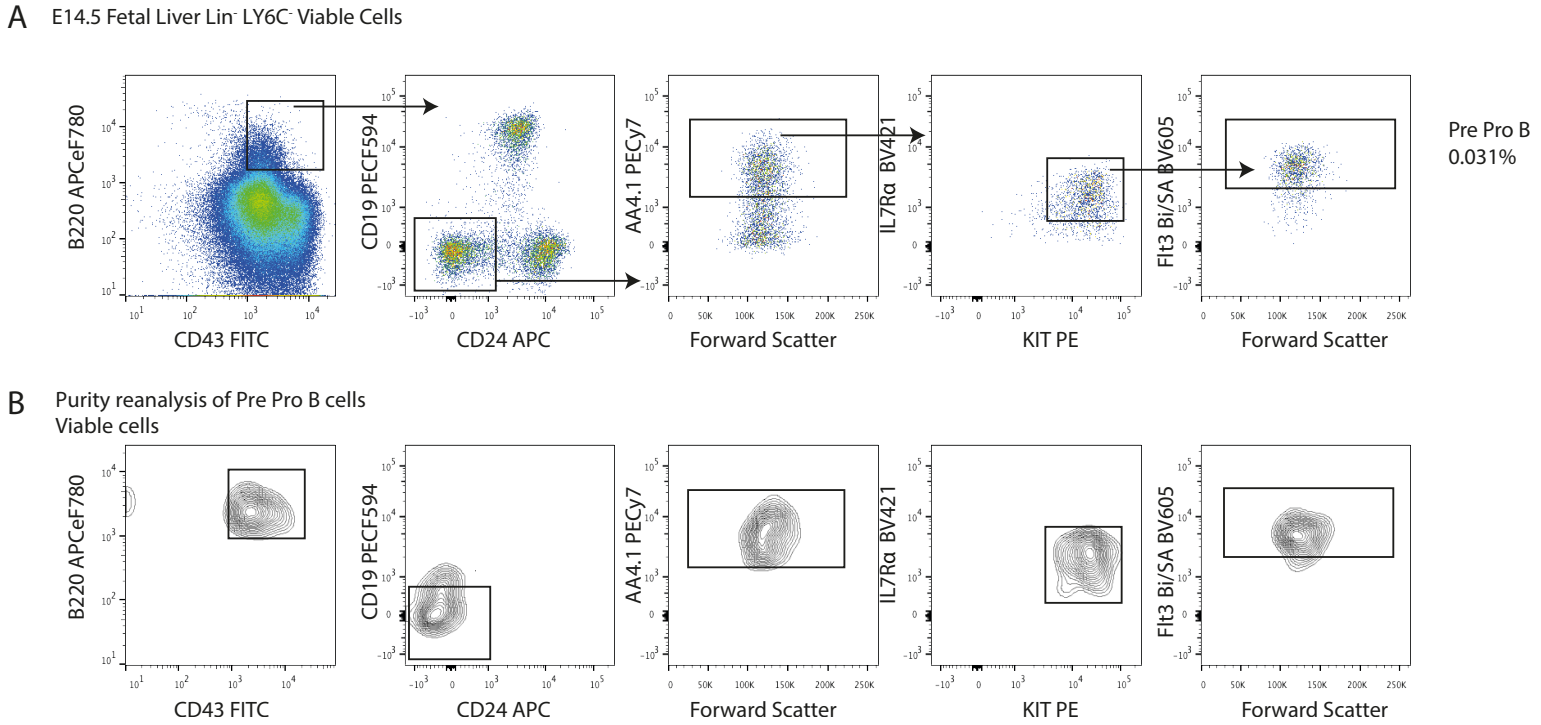


Figure 5.5: Gating strategy for the identification and characterization of E14.5 FL Pre Pro B cells

A: E14.5 FL cells were stained using 10 fluorescently conjugated antibodies and analysed by flow cytometry. B: Purity reanalysis of sorted populations.

Data shown are representative of more than 10 independent analyses in C57 BL/6 mice. % shown of MNCs.

Subsequently the phenotypes of the Pre Pro B cell (figure 5.5) and Pro B cell (figure 5.6) compartments in the E14.5 fetal liver were investigated, the first time point when we, and others (Hardy, 2012), detect a significant number of $CD19^+$ B cell progenitors (figure 4.1). $CD43^-$ Pre B cell progenitors have yet to develop in the E14.5 FL and so could not be further characterized. The majority of FL Pro B cells were found to express KIT and IL-7Rα, with no distinguishable Pro B KIT^- population as in the adult BM. 22% of fetal $KIT^+IL\text{-}7R\alpha^+$ Pro B cells expressed FLT3, compared to just 9% of cells from the adult bone marrow. The E14.5 FL Pro B cell compartment expressed homogeneous levels of CD24 with no distinguishable Fraction B/C or C' at this developmental age (figure 5.6).

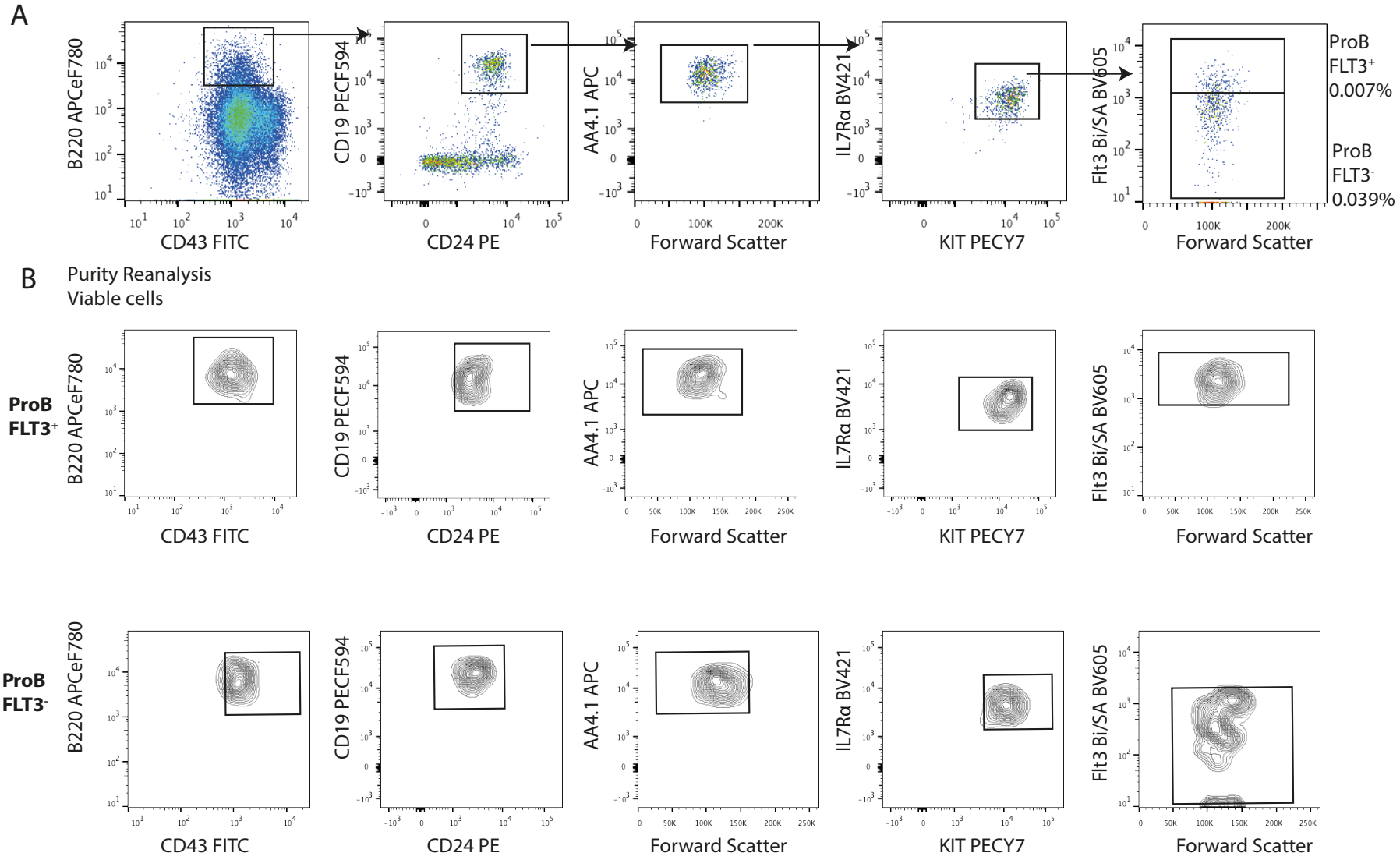


Figure 5.6: Gating strategy for the identification of E14.5 FL Pro B cells

A: FL cells were stained using 9 fluorescently conjugated antibodies and analysed by flow cytometry. B: Purity reanalysis of sorted populations. Data shown are representative of more than 10 independent analysis in C57 BL/6 mice. % shown of MNCs.

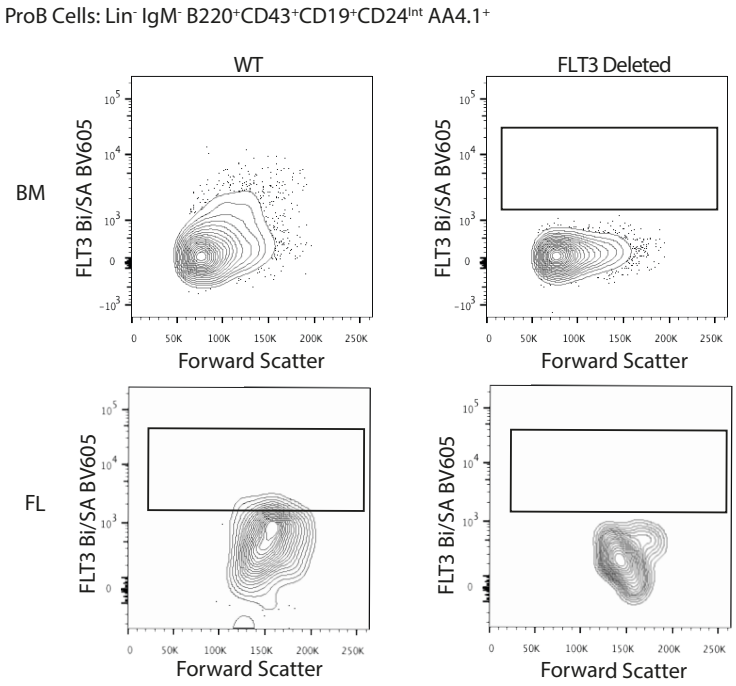


Figure 5.7: Analysis of mice lacking vavCre $^{tg/wt}$ Flt3$^{tg/tg}$ mice confirms a subfraction of Pro B cells express FLT3 on the cell surface

Pro B cells in 6-7 week adult BM (A) or E14.5 FL (B) were analysed in *vavCre* $^{tg/wt}$ *Flt3*$^{tg/tg}$ mice, in which the *Flt3* allele is flanked by LoxP sites and therefore in the presence of the vavCre not expressed in haematopoietic cells. Top panels show BM analysis and lower panels show FL analysis.

As PAX5 is known to repress FLT3 expression (Holmes et al., 2006), we wanted to further validate that CD19$^+$ Pro B cells had genuine FLT3 staining on the cell surface and that staining was not due to an experimental artefact. We therefore compared the cell surface expression of FLT3 protein on wild type animals with those that had been genetically modified to delete *Flt3* in the haematopoietic compartment (*vavCre*$^{tg/wt}$ *Flt3*$^{tg/tg}$, figure 5.7, unpublished mouse model) and confirmed surface FLT3 expression was significantly higher in the Pro B cell compartment of WT animals, compared to animals without *Flt3* expression. Subsequently, we detected *Flt3* transcripts in 29/39 (74%) single CD43$^+$B220$^+$CD19$^+$CD24^{int}AA4.1$^+$ KIT$^+$IL-7Rα$^+$ Pro B cells from the E14.5 FL,

analysed by qPCR (figure 5.8). Additionally, every cell had detectable expression of the B cell genes *Cd79a, Ebf1, Pax5, Cd19* and *VpreB*, whereas most T lineage and myeloid lineage genes such as CD3e and *Mpo* were undetectable (key genes shown in figure 5.8, see appendix 5 for full figure of genes analysed). Interestingly 8/39 fetal liver Pro B cells showed detectable expression of the myeloid gene *Csf1r*. It has been previously reported that PAX5 represses *Flt3* expression in the adult BM (Holmes et al., 2006). However, in the E14.5 FL, we did not notice a difference in *Pax5* expression between Pro B cells that expressed *Flt3* and those that did not.

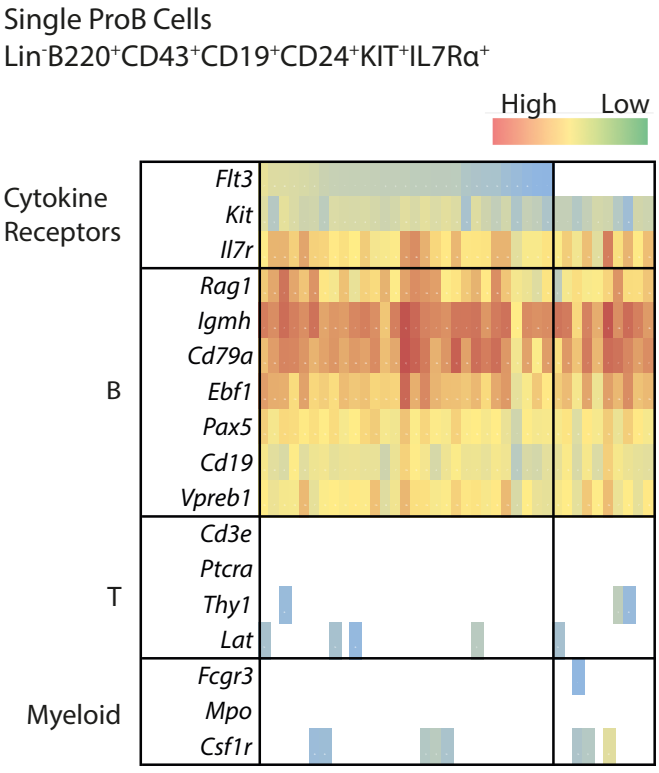


Figure 5.8: Gene expression analysis confirms Pro B cells express Flt3
Quantitative gene expression analysis of single Lin-CD43+B220+CD19+CD24+ AA4.1+cKIT+IL-7Rα+ Pro B cells from the E14.5 FL. Expression of genes are shown as Δ Ct values, each row represents a single cell (39 cells). Selected genes are shown here, with a full composite of genes shown in appendix 5.

Subsequently I investigated multiple developmental ages throughout fetal and adult life and discovered that FLT3^{+} and FLT3^{-} Pro B cells were most frequent in late gestation and in the neonatal bone marrow (figure 5.9) before declining in frequency in the adult bone marrow. At all time points the FLT3^{-} Pro B cell compartment was dominant and found at a higher frequency than FLT3^{+} Pro B cells.

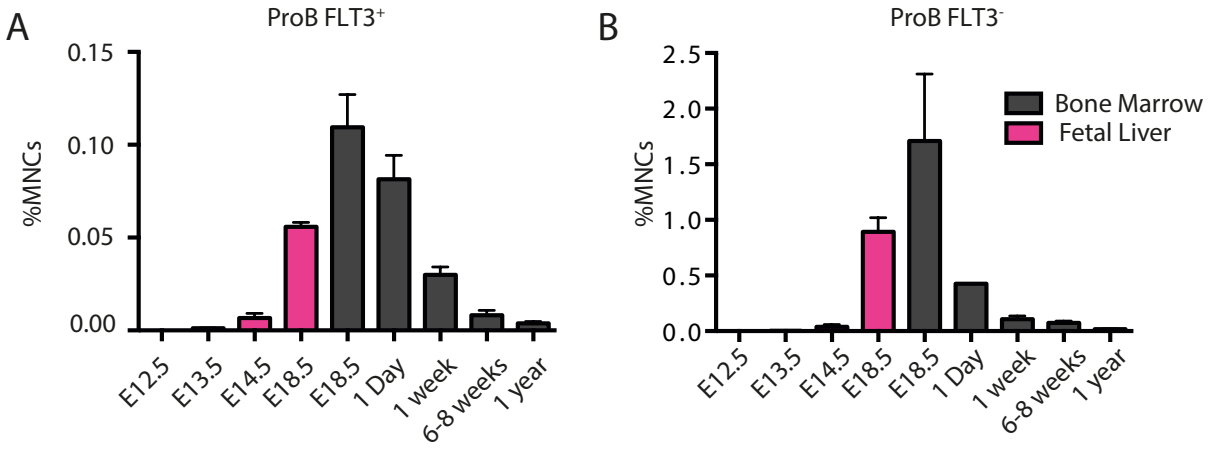


Figure 5.9: Frequency of Pro B cell progenitors at different developmental ages
Frequency of Pro B FLT3^{+} (A) and Pro B FLT3^{-} (B) cells within total MNC population in the FL (pink) or BM (grey) at different developmental ages. 5-10 replicates, except E12.5 FL, E18.5 BM and 1 day old BM (2 pooled replicates)

We next investigated the capacity of fetal B cell progenitors to generate B, T and myeloid cells in vitro. Similar to the adult bone marrow, the E14.5 FL Pre Pro B cell compartment readily developed into B cells (figure 5.10A), retained residual T cell potential (figure 5.10B), but had no significant myeloid cell potential (figure 5.10C and figure 5.10D). E14.5 FL Pro B cells developed readily into B cell clones, but not myeloid or T cell clones (figure 5.4A-D). Interestingly, when cytokines that were designed to stimulate the growth of myeloid clones were

added to the OP9 stroma co-culture, the fetal progenitors developed into B cells with increased frequency (figure 5.10A).

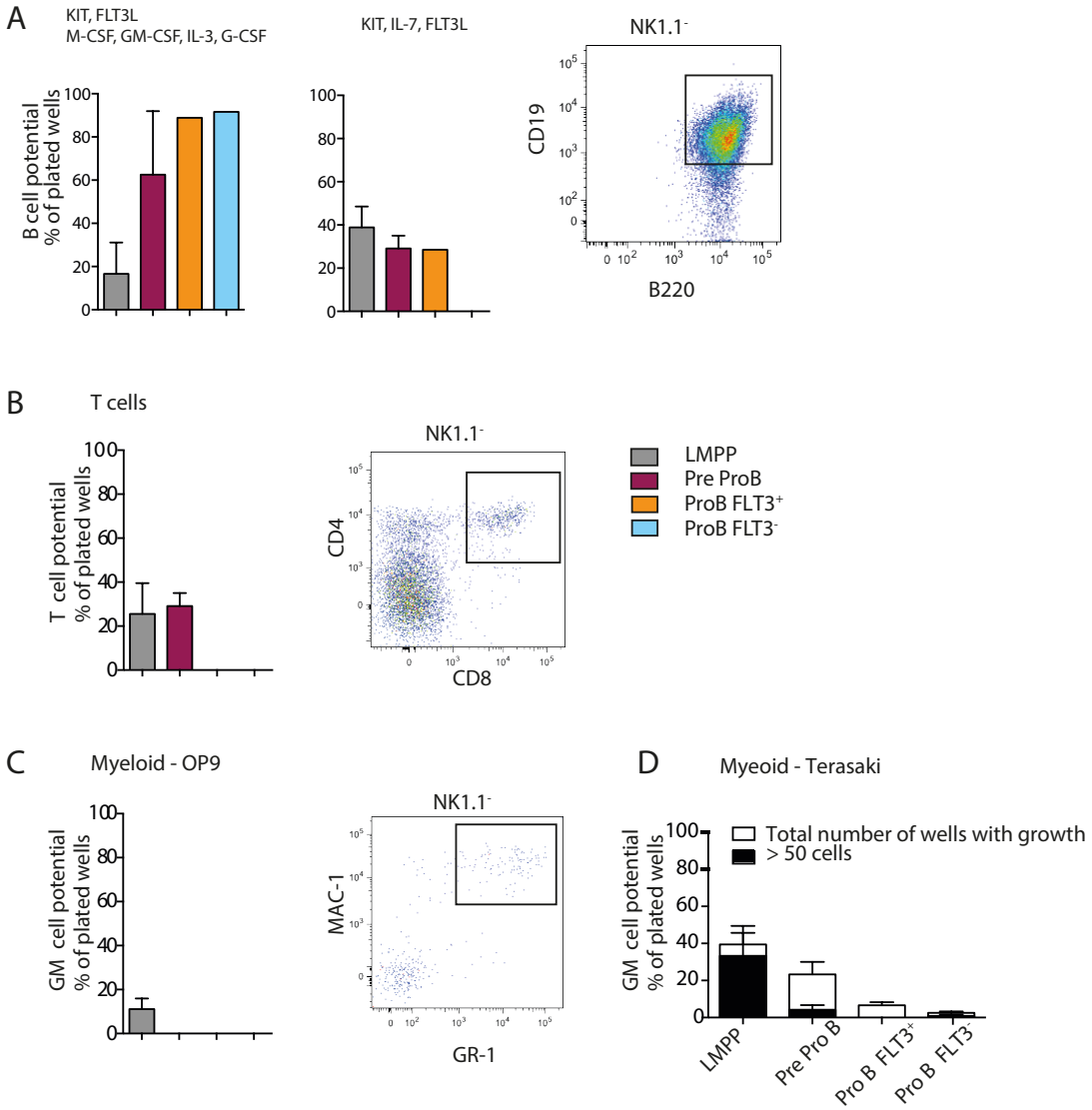


Figure 5.10: Fetal Pre Pro B cells have T cell and B cell potential, but Pro B cells are B lineage restricted

B cell potential ($NK1.1^{-}CD19^{+}B220^{+}$) was analysed by sorting cells and plating at a density of 7 cells per well (left hand graphs 9-12 wells plated, right hand graph 7-12 wells plated) in the presence of mSCF, hFL, h-Csf1, mGM-CSF, mIL-3, hGSCF (left hand graph), or mSCF, hFL and IL-7 (right hand graph). Lineages were analysed after 10-14 days by flow cytometry

B: T cell ($NK1.1^{-}THY1.2^{+}CD25^{+}$ or $NK1.1^{-}CD4^{+}CD8^{+}$) potential was analysed by sorting cells and plating at a density of 7 cells per well on OP9 DL stroma,

supplemented with mSCF and hFL and analysed 21 days after plating. Right hand panel shows representative flow cytometry plots.
C: Myeloid ($NK1.1^{-}$ $MAC1^{+}GR1^{+}$) potential was analysed by plating cells at a density of 7 cells per well on OP9 stroma supplemented with mSCF, hFL, h-Csf1, mGM-CSF, mIL-3, hGSCF (as in 'A') and lineages were scored after 10 days as myeloid if they expressed myeloid cell antigens by flow cytometry.
Right hand panel shows representative flow cytometry plot of wells scored positive for B, T and Myeloid cell potential.
D: GM lineage potential in liquid culture (right hand panel) was evaluated by sorting and plating single cells in terasaki assays. White bars represent total cloning, black bars represent clones with >50 cells.
Mean ± SEM

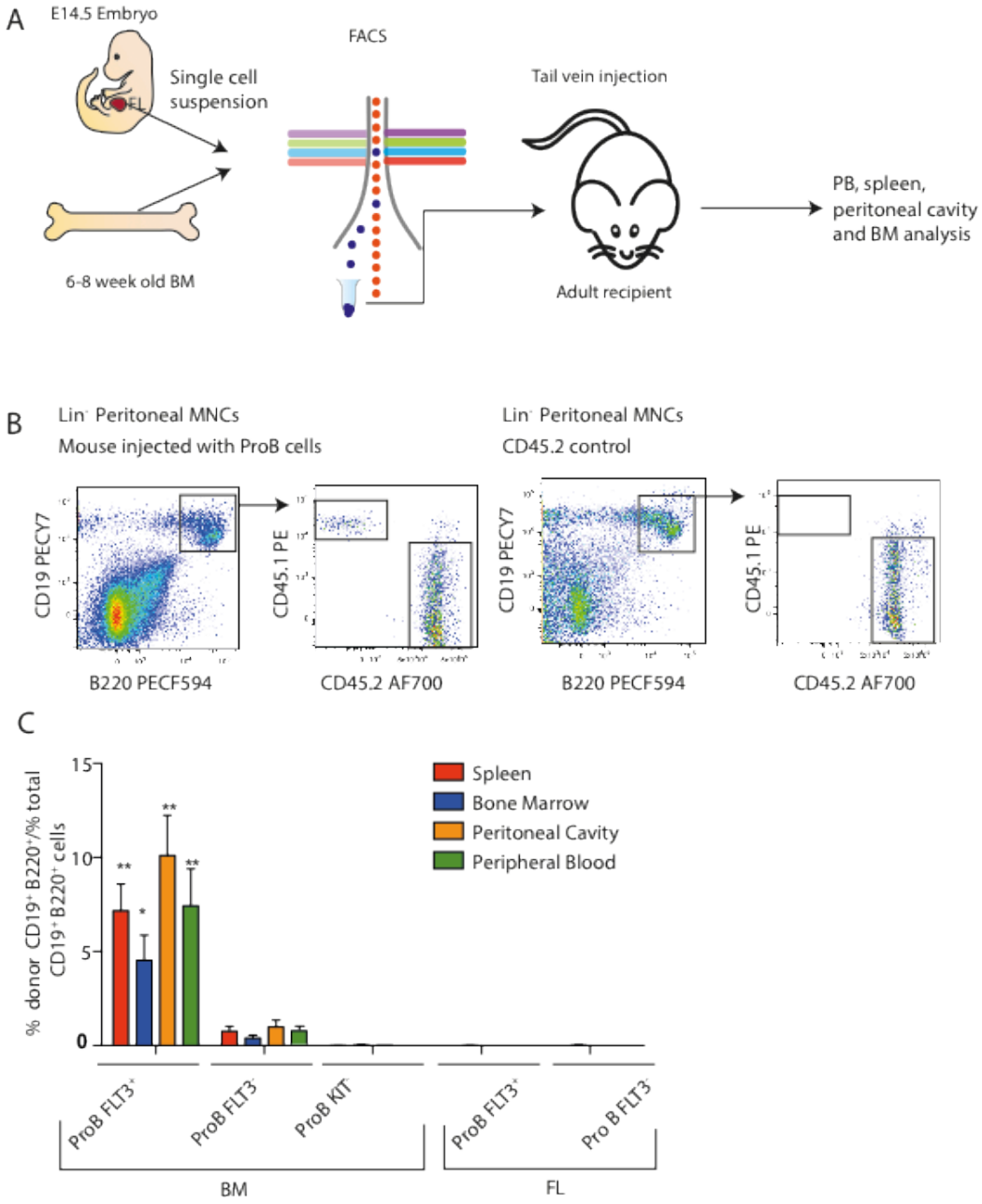


Figure 5.11: FLT3$^+$ Adult Pro B cells show high levels of engraftment in vivo

A: Schematic outline of *in vivo* transplantation experiments

B: Gating strategy for analyses of CD45.2 mice injected with 100 CD45.1 Pro B cells and 200,000 CD45.2 RAG-1 deficient support cells. Left hand panels show an experimental mouse, right hand panels show a control CD45.2 mouse not injected with any cells. Panels here show the peritoneal cavity but a similar gating strategy was used for the spleen, bone marrow and peripheral blood to calculate percentages in

C. PB analysis at 3 weeks and all other organs at 4 weeks post transplantation, % engraftment calculated as No. Donor CD19$^+$B220$^+$ cells/Total number of CD19$^+$B220$^+$ cells. 3 biological replicates, 6 mice total per population, Mean ± SD.

Subsequently we decided to investigate the potential of the B cell restricted fetal and adult Pro B cells to produce the different mature B cell lineages (B1a, B1b, MZ and Follicular B cells). There is no reliable in vitro assay for distinguishing these subsets so we therefore adopted an in vivo approach. We injected 100 B cell progenitors (>95% purity) from the FL or BM into lethally irradiated adult recipients, with immune deficient *Rag-1*$^{-/-}$ support cells, before analysing the PB after 3 weeks and the spleen, peritoneal cavity and bone marrow after 4 weeks (figure 5.11A). Within the CD19^{+}B220^{+} or CD19^{+} IgM^{+} B cell compartment, the level of chimerism was assessed by calculating the percentage of cells marked by CD45.1 and therefore derived from the donor (figure 11B). Strikingly, the Pro B FLT3^{+} adult bone marrow cells (Lin^{-}CD43^{+}B220^{+}CD19^{+}CD24^{int} AA4.1^{+} KIT^{+}IL-7Rα^{+}FLT3^{+}) demonstrated markedly higher levels of chimerism compared to an identical number of Pro B FLT3^{-} cells (Lin^{-}CD43^{+}B220^{+}CD19^{+}CD24^{int} AA4.1^{+} KIT^{+}IL-7Rα^{+}FLT3^{-}) in the spleen, bone marrow, peritoneal cavity and peripheral blood (figure 5.11C). Pro B KIT^{-} cells (Lin^{-}CD43^{+}B220^{+}CD19^{+}CD24^{int} AA4.1^{+} KIT^{-} IL-7Rα^{+}FLT3^{-}) did not show significant engraftment (figure 5.11C). Cellularity calculations revealed that the 100 transplanted Pro B FLT3^{+} cells expanded to generate 51,000 IgM^{+}CD19^{+} cells per recipient spleen and 4,000 IgM^{+}CD19^{+} per recipient tibia. Contrastingly Pro B FLT3^{-} cells produced 4,100 IgM^{+}CD19^{+} cells per recipient spleen and Pro B KIT^{-} cells produced only 180 IgM^{+}CD19^{+} cells per recipient spleens.

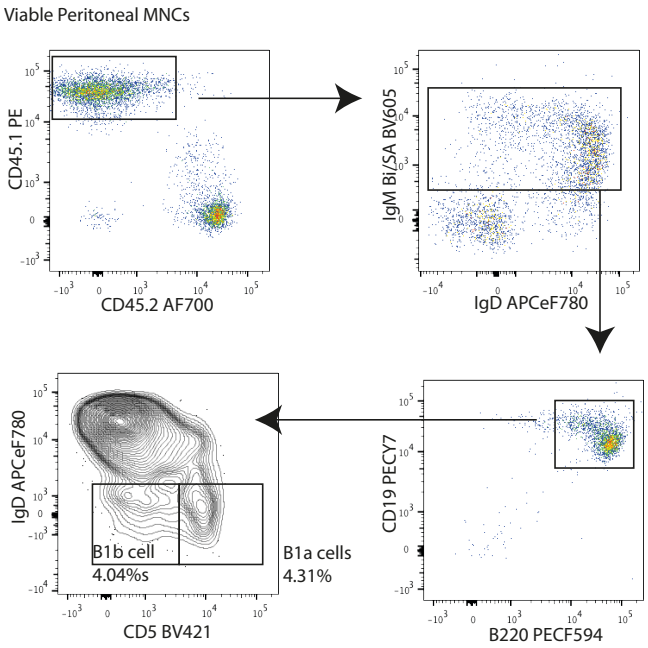


Figure 5.12: Analysis of B1 cells in the peritoneal cavity

Flow cytometry gating strategy for analysis of B1a and B1b cells in the peritoneal cavity of transplanted mice. % shown of total MNCs in relevant organ system. % are shown of total peritoneal MNCs in a wild type, non-irradiated mouse.

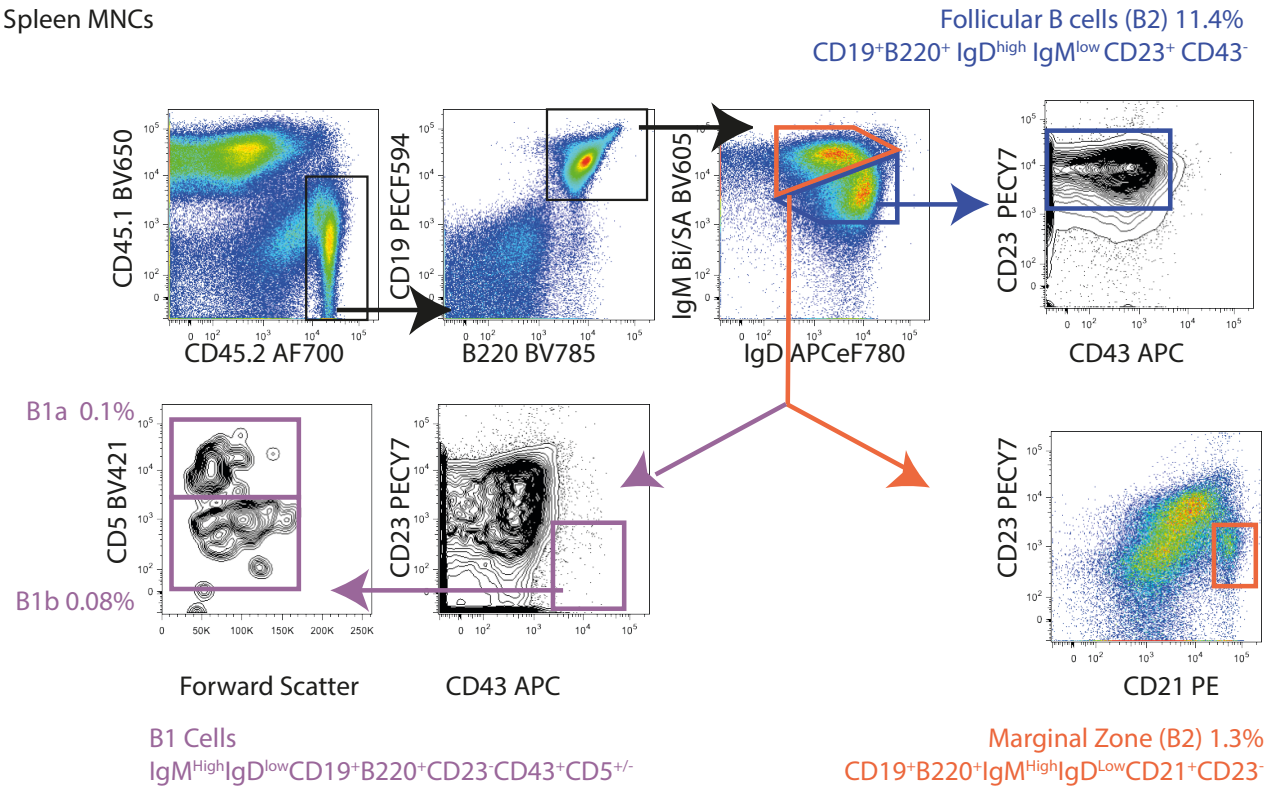


Figure 5.13: Gating strategy to assess B cell subsets in the spleen

11 colour flow cytometry for analysis of B1a, B1b, Follicular and Marginal Zone B cells in the spleen of transplanted mice. % are shown of total spleen MNCs in the non transplantation setting

A

Mouse	Spleen				Peritoneal Cavity	
	B1a	B1b	Follicular	MZ	B1a	B1b
1	x	y	y	y	x	y
2	x	y	y	y	x	y
3	x	y	y	y	x	y
4	x	y	y	y	x	y
5	x	y	y	y		
6	x	y	y	y	x	y

B

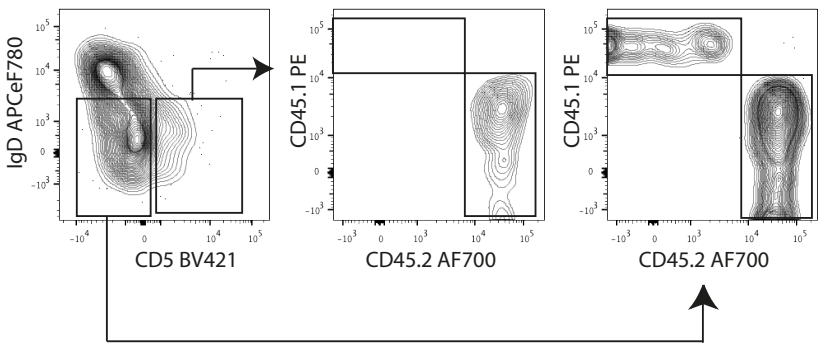


Figure 5.14: Adult Pro B cells do not generate B1a cells, but generate all other mature B cell subsets

A: Table showing the detection of different peripheral B cell subsets in the 6 mice injected with 100 Pro B FLT3$^+$ cells, 4 weeks after transplantation. Subsets defined as in figure 5.13 and figure 5.14. Greyed out: missing sample. X = not detected. Y = detected.

B: Representative flow cytometry plots of peritoneal cavity analysis of a mouse transplanted with 100 adult FLT3$^+$ Pro B cells.

Next we analysed the contribution of the engrafted donor B cells to the different mature B cell subsets by analysing the peritoneal cavity and spleen. Due to their high frequency, B1 cells can be relatively easily identified in the peritoneal cavity by expression of cell surface markers. However B1 cell identification in the spleen is more difficult, due to a decreased frequency and the down regulation of the integrin CD11b by B1 cells on leaving the coelomic cavities (Baumgarth, 2011). Additionally, CD5 expression on B1 cells is 10 fold lower than on T cells making detection more difficult. I developed a 9 colour staining panel to analyse the B1 cell compartment in the peritoneal cavity (figure 5.12) and an 11 colour

panel to identify follicular marginal zone, B1a and B1b cells in the spleen (figure 5.13) based on published studies (Copley et al., 2013; Esplin et al., 2009b; Gyory et al., 2012; Vosshenrich et al., 2004b). 'Brilliant Violet' conjugates were used to detect 'dim' markers such as CD5 and to increase resolution (Chattopadhyay et al., 2012). Due to the high levels of engraftment on injection of $FLT3^+$ Pro B cells, it was possible to analyse the mature B cell subsets in the periphery, summarised in figure 5.14A. In all 6 animals injected with 100 $FLT3^+$ Pro B cells from the adult BM, MZ, follicular and B1b cells, but not $CD5^+$ B1a cells (figure 5.14B) were detected. A summary of the markers used to identify the different subsets is shown in table 5.2. As a control, unfractionated FL and BM cells were transplanted into recipient mice to demonstrate that $CD5^+$ B1a cells could be generated and detected using this method, and that FL cells generated more B1a cells than adult BM progenitors, agreeing with previous published work (Hayakawa et al., 1985) (appendix 6).

Table 5.2: Antigens used for the identification of mature B cell subsets in the spleen and peritoneal cavity

Tissue	Population	Markers
Peritoneal Cavity	B1a	$IgM^{high}IgD^{low}CD19^+B220^+CD5^+$
Peritoneal Cavity	B1a	$IgM^{high}IgD^{low}CD19^+B220^+CD5^-$
Spleen	MZ	CD19+B220+IgMHighIgDLowCD21+CD23-
Spleen	Follicular	CD19+B220+IgDhigIgMlowCD23+CD43-
Spleen	B1a	IgM High IgD low CD19+B220+ CD23-CD43+CD5+
Spleen	B1b	IgM High IgD low CD19+B220+ CD23-CD43+CD5-

(Barber et al., 2011; Copley et al., 2013; Esplin et al., 2009a; Gyory et al., 2012; Kikuchi and Kondo, 2006; Vosshenrich et al., 2004a; Yang et al., 2007b)

In contrast to adult cells, Pro B cells derived from the E14.5 FL showed no detectable engraftment into adult recipient mice in either WT or immune

deficient (NSG) adult recipients (figure 5.11C). We attempted to increase the engraftment of fetal cells by minimizing the age difference between donor and recipient by sorting Pro B cells from the E18.5 FL, and transplanting them into 1-2 day old neonatal recipients via the anterio-facial vein (Park et al., 2008) figure 5.15). We detected low levels of mature, IgM^+ B cells from the donor (figure 5.16A) in up to 50% of recipient mice (4/8 spleens and 3/8 peritoneal cavities) compared to control mice (figure 5.16B). However, engraftment was too low to draw confident conclusions about the B cell subsets generated. Of the low number of donor $CD19^+B220^+IgM^+IgD^+$ cells in the spleen, the majority (93.5%, 4 mice analysed) lacked expression of CD5 but expressed CD21, a marker associated with MZ cells. In contrast, in the peritoneal cavity, 92.1% of donor fetal B cells expressed CD5, compared to only 36.7% of $CD19^+$ cell from the recipient (p=0.002, 3 mice, figure 5.16C), strongly indicating fetal Pro B cells can generate peritoneal B1a cells in vivo.

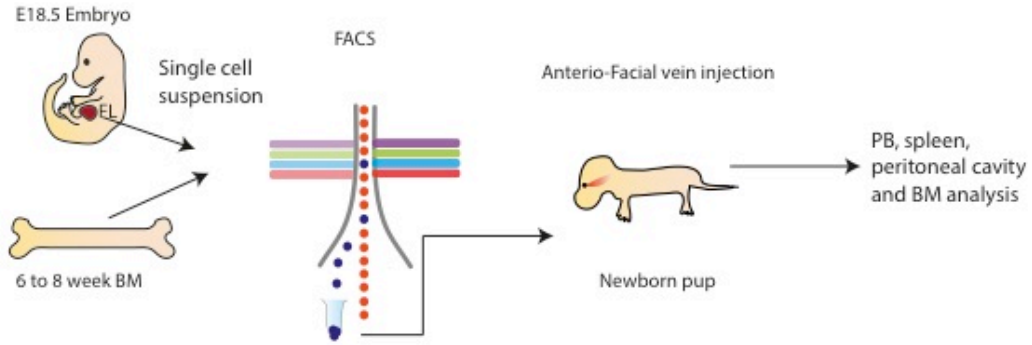


Figure 5.15: Schematic outline of transplantations into newborn recipients

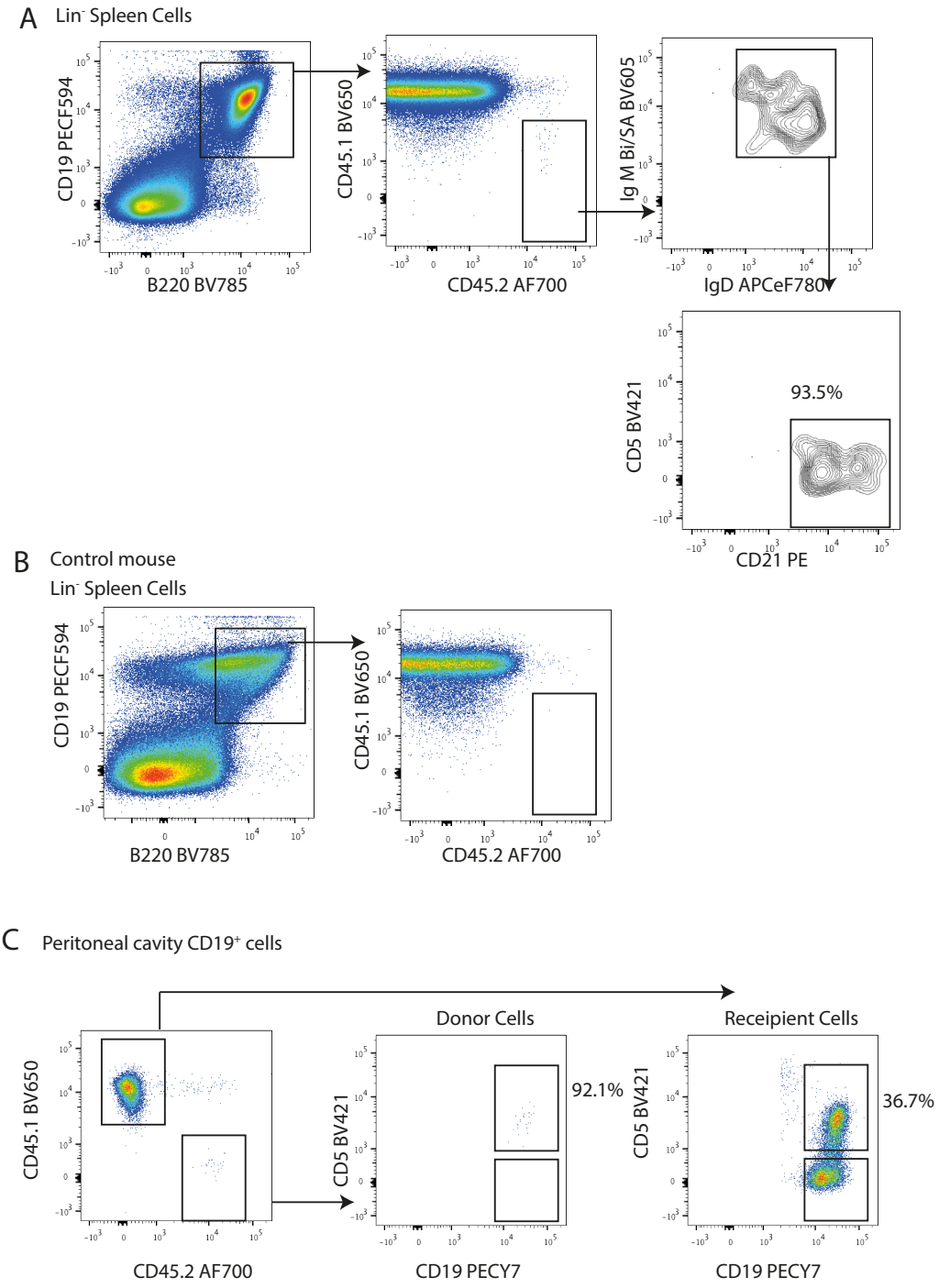


Figure 5.16: E18.5 FL cells develop into IgM$^+$ B cells in neonatal recipients

A: Analysis of spleen 8 weeks after injection of 100 E18.5 FL Pro B cells into 1 day old mouse pups. Engraftment representative of 4/9 mice injected.
B: Spleen analysis of CD45.1 mouse with no injected cells.
C: Analysis of peritoneal cavity of mice 8 weeks after injection of 100 E18.5 FL Pro B cells into 1 day old recipients. Engraftment representative of 3/9 mice analysed. Percentages are of parent population.

To gain an insight into the molecular properties of fetal and adult B cell progenitors, we sorted 100 Pre Pro B cells and 100 Pro B cells from the E14.5 FL and 6 week adult BM for global RNA sequencing, as defined in table 5.1. Matched heat maps on the aggregate differentially expressed genes from individual comparisons were generated based on both the correlation between samples and the per gene normalized counts per million values (appendix 7). The samples exhibited the expected hierarchical cluster pattern with fetal liver samples clustering together and adult BM samples clustering together, with the different B cell progenitor populations separating out within the sub groups (appendix 7).

Principal component (PC) clustering analysis was performed, including additional LMPP IL-7Rα^+ and LMPP IL-7Rα^- fetal and adult samples as an upstream progenitor comparison. PC1 appeared to separate progenitors based on maturity, and PC2 separated fetal and adult populations (figure 5.17). Interestingly FL Pre Pro B cells clustered very close to the IL-7Rα^+ LMPP. The clustering analysis showed the FLT3$^+$ Pro B cells clustered closer to the Pre Pro B than the FLT3$^-$ Pro B cells, indicating FLT3$^+$ Pro B cells are upstream in the haematopoietic hierarchy of FLT3$^-$ Pro B cells. There was a large separation between Pre Pro B cells and Pro B cells, but little separation between the FLT3$^+$ and FLT3$^-$ Pro B cell fractions, suggesting these two populations are similar at the molecular level.

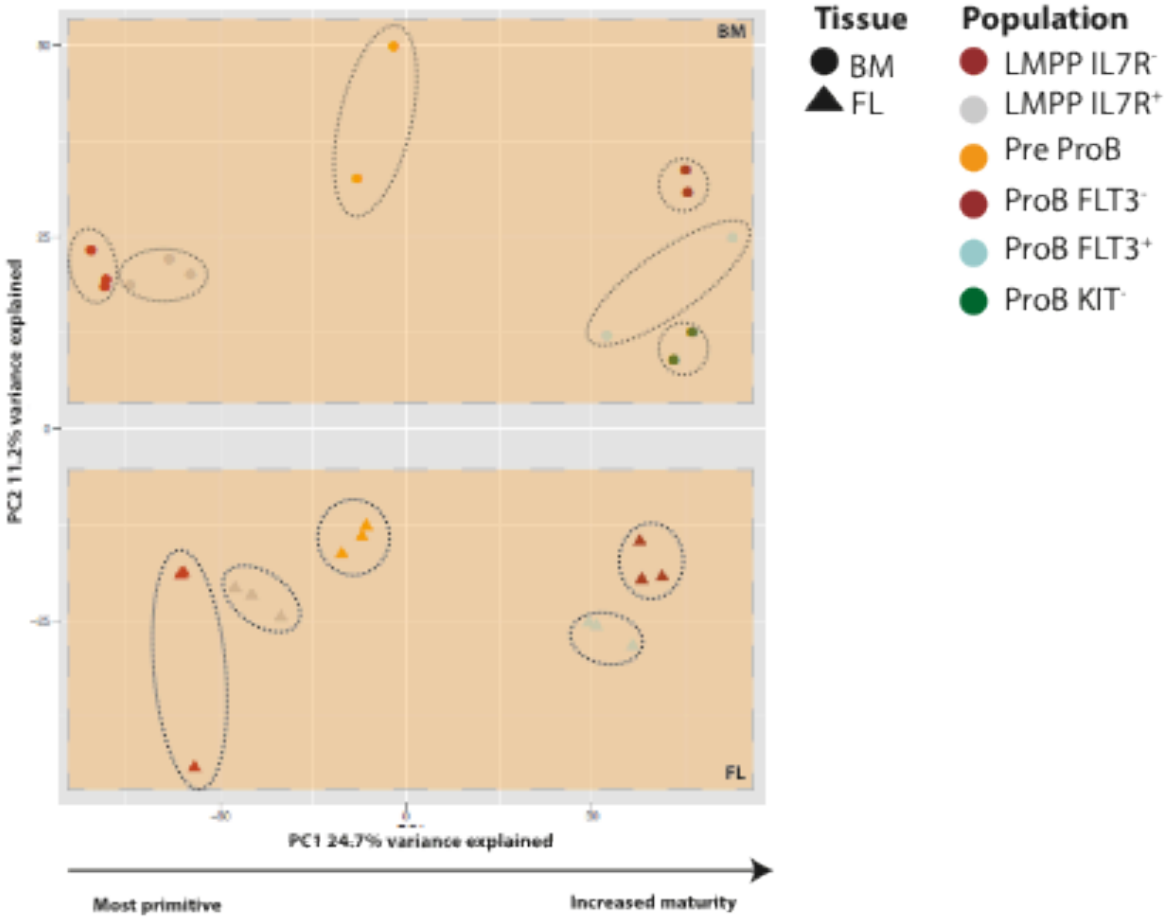


Figure 5.17: PCA analysis separates fetal and adult B cell progenitors.
Principal component analysis of fetal liver and adult bone marrow B cell progenitors. LMPPs sorted as Lin-SCA1⁺KIT⁺FLT3⁺ then IL-7Rα⁺ or IL-7Rα⁻ from the fetus and adult are also shown.

We next validated these data by verifying that B cell (*Cd19* and *Ebf1*) and lymphoid genes (*Rag1* and *Rag2*) were expressed by B cell progenitors and that there was low expression of genes associated with other lineages (*Mpl1)* (figure 5.18). Furthermore, we confirmed findings in the literature that *Dntt* and *Mylk* are not expressed in fetal B cells (Feeney, 1990; Li et al., 1993b), and that *Lin28b* and *Igf2bp* are expressed in fetal B cells, but not adult B cells (Li et al., 2015; Zhou et al., 2015) (figure 5.18). We additionally investigated expression of cytokine receptors on the B cell progenitors, which agreed with the surface expression as sorted by FACS, with FLT3 showing marked down regulation

between the Pre Pro B and Pro B cell compartment. Interestingly, fetal B cell progenitors showed a low level of gene expression of a range of genes normally associated with the myeloid lineage (5.18E).

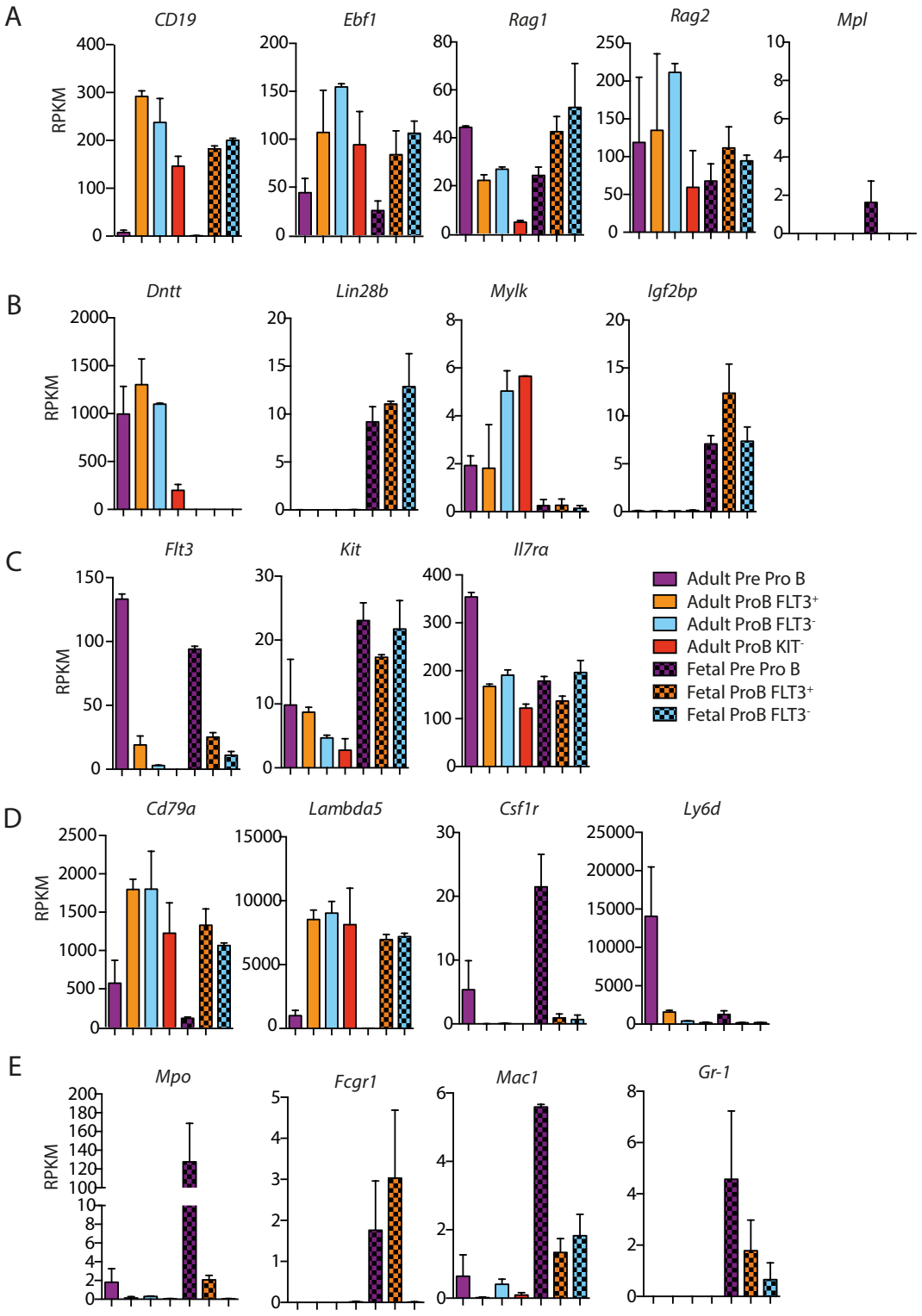
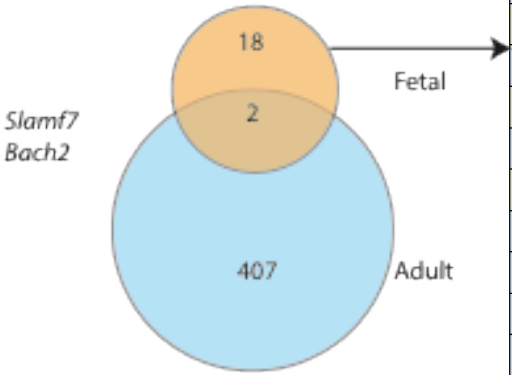


Figure 5.18: Validation of key genes from the global RNA expression analysis.

Gene Expression as determined by RNA Sequencing, shown as RPKM (reads per kilobase of exon model per million mapped reads). Mean ± SEM.

Agreeing with the data in chapter IV we noted that *CD79a* is expressed in fetal and adult Pre Pro B cells at low levels. We found that while *Lambda5* was expressed by adult Pre Pro B cells as reported by Mansson et al., (2008a), *Lambda5* is not expressed in fetal Pre Pro B cells confirming our earlier phenotypic analysis (figure 4.5B), suggesting this is not a useful maker in the fetus to identify B cell restricted progenitors in the Pre Pro B cell fraction. We also detected expression of *Csf1r* and *Ly6d* in fetal and adult Pre Pro B cells supporting the cell surface staining in chapter IV, and interestingly *Csf1r* expression was much higher in fetal Pre Pro B cells than their adult counterparts with the opposite trend seen for *Ly6d*. However as we have shown in chapter IV, the Pre Pro B cell compartment is heterogeneous, and only a small percentage of classically defined Pre Pro B cells are likely to reflect truly B lineage restricted progenitors.

The Pro B KIT^- fraction, which showed reduced functional output in vitro and in vivo compared to their KIT^+ counterparts, had down regulated lymphoid genes such as *Rag1* and *Dntt* (figure 5.18 A and B), perhaps indicating they would be incapable of further rearranging their immunoglobulin light chain loci to generate a functional BCR and therefore explaining their low functional output.



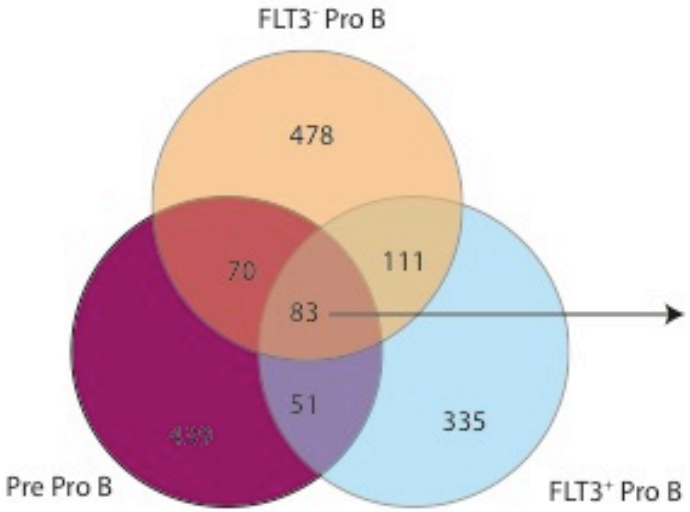
Gene	FDR
Cd34	4.08E-09
Mpo	6.70E-04
Phf11b	6.70E-04
Lef1	7.90E-04
Fcrl1	3.04E-03
Hmgn3	3.04E-03
Rgs18	3.04E-03
Slamf7	3.04E-03
Tnni1	3.04E-03
Hbb-bh1	8.01E-03
Ccr2	8.86E-03
Ermap	8.91E-03
Cd97	1.56E-02
Atp8b4	1.70E-02
Fyb	1.70E-02
Serpinb1a	1.70E-02
Spta1	2.17E-02
Bach2	3.25E-02
Cox6a2	4.36E-02
Ppt1	4.45E-02

Figure 5.19: Differentially expressed genes between fetal Pro B FLT3$^+$ and Pro B FLT3$^-$ cells

EdgeR (Robinson et al., 2010) analysis revealed 20 differentially expressed genes when comparing fetal FLT3$^+$ and FLT3$^-$ Pro B cells. Genes are listed along with adjusted p value (FDR: false discovery rate). * means DESeq2 (Love et al., 2014) analysis also suggested the gene was differentially expressed. Only two of these genes were found to be differentially expressed between adult Pro B FLT3$^+$ and FLT3$^-$ cells (*Slamf7* and *Bach2*). Blue upregulated in FLT3$^+$ Pro B, green down regulated in FLT3$^+$ Pro B.

We next compared the global gene expression profiles between FLT3$^+$ and FLT3$^-$ FL and adult BM cells. Comparative gene expression analysis using 'EdgeR' (Robinson et al., 2010) revealed only 20 genes were differentially expressed between fetal FLT3$^+$ and FLT3$^-$ Pro B cells (figure 5.19), 7 of which matched with differential analysis performing DESeq2 (Love et al., 2014). The low number of differentially expressed genes suggests the two populations are molecularly similar, whereas in the adult bone marrow 407 genes were differentially expressed when comparing FLT3$^+$ and FLT3$^-$ Pro B cells. Only two genes were found to be differentially expressed between adult and fetal FLT3$^+$ and FLT3$^-$ Pro B cells, *Slamf7* and *Bach2*. The first, *Slamf7* is up-regulated in FLT3$^-$ Pro B cells and has been shown in humans to be up-regulated on activated B cell progenitors (Lee et al., 2007). The second, *Bach2* is also up-regulated in FLT3$^-$ Pro B cells. BACH2 has been shown to be involved in somatic hyper-mutation and class switch recombination. Furthermore, BACH2 has been linked to inducing cell apoptosis in response to genotoxic stress (Swaminathan et al., 2014). Interestingly, FL FLT3$^+$ Pro B cells showed increased expression of *Cd34*, which encodes a protein that is associated with increased proliferation and reduced quiescence in stem cells (Anjos-Afonso et al., 2013) and increased expression of *Mpo*, a gene associated with myeloid and monocytic cells (Lin and Austin, 2002). *Flt3* transcripts were detected as the 85th most differentially expressed gene out of 12,638 genes when comparing FLT3$^-$ and FLT3$^+$ Pro B cells but the adjusted p value did not reach statistical significance. This may be as the criteria were set stringently, or that the level of *Flt3* transcript does not differ greatly between the two cell populations.

We next performed a comparison of genes differentially expressed between all fetal and adult B cell progenitors (Pre Pro B and Pro B). This generated a substantial list of 730 genes that demonstrated statistically significant differences in gene expression and is shown in appendix 8. To further restrict this list to genes more specific to differences in B cell regulation rather than developmental age we performed individual comparisons of equivalent B cell progenitor subsets in the BM and FL, and found of the three comparisons made (Pre Pro B, Pro B FLT3^{+} and Pro B FLT3^{-}), 83 genes were differentially expressed in all comparisons (figure 5.20). As has been seen previously many of the top 'hits' were components of the *Lin28b* pathway that are up-regulated in the fetal progenitors (*Lin28b, Hmga2*, *Igf2bp1*) (Yuan et al., 2012; Zhou et al., 2015) while *Dntt* (Feeney, 1990), which encodes TDT was down regulated. Interestingly, statistically significant genes that are down regulated in fetal B cell progenitors included *H2-K1*, *H2-D1* and *B2m*, which are either components of, or interact directly with, the major histocompatibility type I complex. The lack of MHC Class I proteins on fetal cells has been reported by others in E10.5 embryos (Kieusseian et al., 2012). Without MHC class I expression, the cells may be targeted by cytotoxic T lymphocytes (Pardoll, 2001; Watzl and Long, 2000), perhaps contributing to the low engraftment of fetal B cell progenitors in transplantation assays. Additionally, *Lef1*, which expresses a cell surface protein that binds to the T Cell Receptor facilitating T cell help, is down regulated in fetal B cell progenitors, suggesting fetal B cells conduct a T-independent B cell response, archetypal of B1 cells.



83 genes common to all comparisons

Dntt	*Ankrd33b*	*Ncl*	*Scd2*
Lef1	*Klk8*	*Ifi27l2a*	*Cox6a2*
H2-K1	*Cd97*	*Ifi47*	*Dpp4*
H2-D1	*Fstl1*	*AW112010*	*Cad*
Dlg2	*Hspe1*	*Acadl*	*S100a6*
H19	*Tpi1*	*Mif*	*Tnfsf10*
Blnk	*Hspa2*	*Fscn1*	*1190002F15Rik*
B2m	*Hmgn3*	*Ndrg1*	*Ramp2*
Mt1	*Cux1*	*Chd3*	*Cdkn1b*
Lin28b	*Ckb*	*Nhp2*	*Yes1*
1700048O20Rik	*Nr3c1*	*Tap1*	*Crip1*
Ifi44	*Nlrc5*	*Dnajc6*	*Aprt*
Aldoa	*Bach2*	*Ypel5*	*Trim34a*
Tifa	*Acsl4*	*Trim16*	*Itm2b*
Pyhin1	*Rpph1*	*Rps4y2*	*Ulbp1*
Tspan13	*Npm1*	*Gypa*	*Apobec2*
Igf2bp1	*Srgn*	*1110038B12Rik*	*Isyna1*
Hspd1	*Tcf19*	*Beta-s*	*Srm*
Cdkn2c	*Hsph1*	*Cdc42ep3*	*Tomm5*
Hmga2	*Myl10*	*Prdx6*	*Ucp2*
Lsp1	*Tapt1*	*Mt2*	

Figure 5.20: Fetal and adult gene expression comparison

Differentially expressed genes between B cell comparisons using EdgeR (Robinson et al., 2010). 83 overlapping genes expressed in order of adjusted p value.

As PAX5 is an essential TF for B cell lineage restriction and maintenance, we analysed the frequency of B cell progenitors, from dHSCs through to Pro B cells, in 6-8 week old BM of mice that did not express PAX5 by breeding *vavC*re mice with mice that had the *Pax5* allele flanked by LoxP sites (*Pax5Δ: vav*Cre$^{tg/wt}$ *Pax5*$^{tg/tg}$ (Horcher et al., 2001). As *vav* is expressed in all endothelial and haematopoietic cells, *Pax5Δ* mice lack expression of *Pax5* in the haematopoietic compartment throughout development (Georgiades et al., 2002). *Pax5Δ* animals had a marked expansion of Lin^{-}CD43^{+}B220^{+}CD19^{-}CD24^{int}AA4.1^{+} Pre Pro B cells, which all homogeneously expressed KIT, IL-7Rα and FLT3 (figure 5.21A). As expected, no CD19 expression was detected, showing the block in B cell lymphopoiesis occurs at the Pre Pro B to Pro B cell stage (figure 5.21B). *Pax5Δ* mice had a slight expansion of HSCs and LMPPs, no difference in CLPs but an approximately 10-fold increase in the frequency of the LY6D^{+} CLP, or 'BLP' (figure 5.21B). As HSCs and LMPPs do not express PAX5, this expansion is unexpected and therefore worthy of further validation.

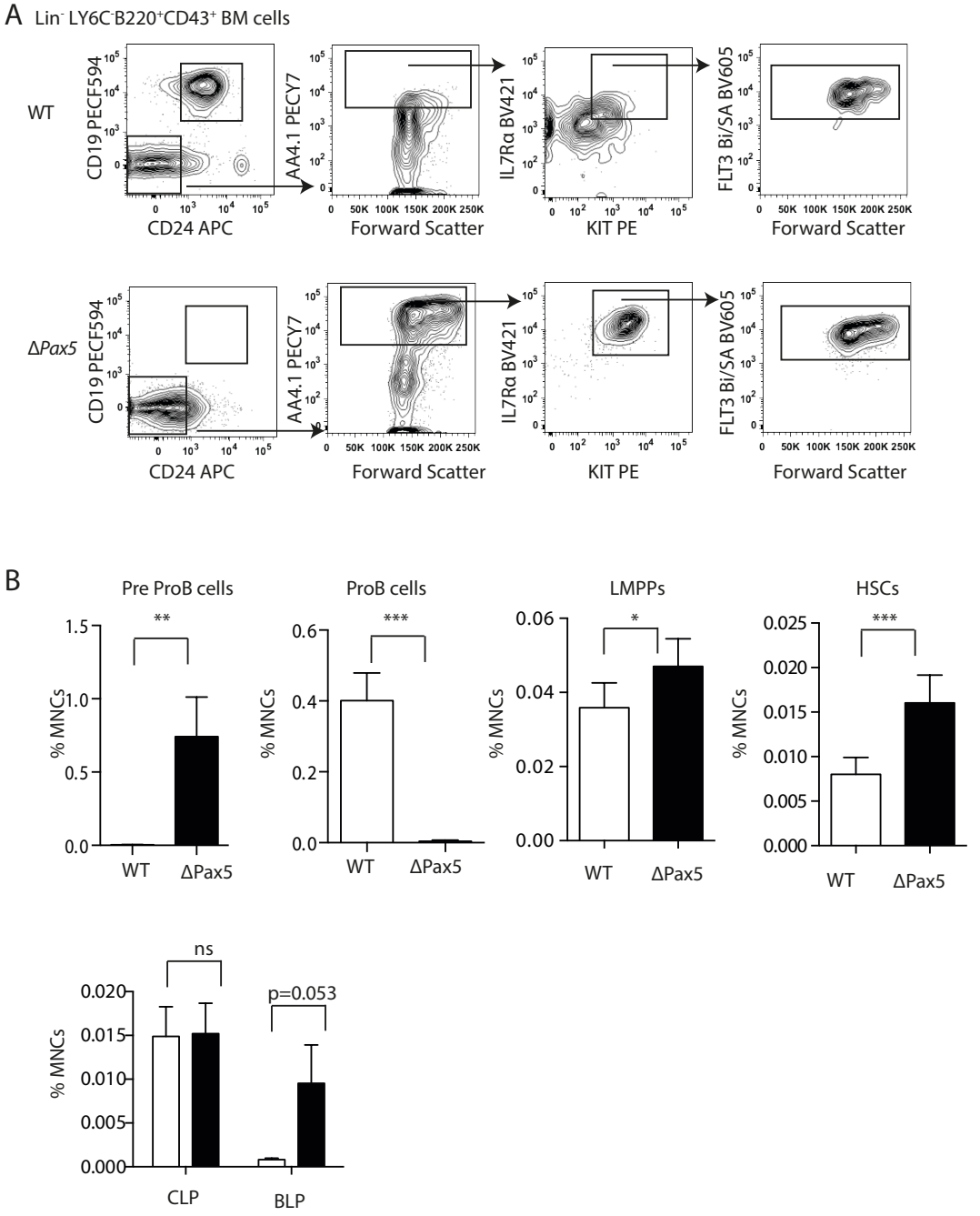


Figure 5.21: PAX5 deficient adult BM has a block at the Pre Pro B to Pro B cell stage
A: Flow cytometry analysis of Pre Pro B cells in *vavCre* $^{wt/wt}$ *Pax5* fl/fl ('WT' top panels) and *vavCre* $^{tg/wt}$ *pax5* fl/fl ('Δ*Pax5*') bone marrow (lower panels).
B: Frequency of B cell progenitors in 6-8 week in *vavCre* $^{wt/wt}$ *Pax5* fl/fl ('WT' white bars) and *vav cre* $^{tg/wt}$ *pax5* fl/fl ('Δ*Pax5*' black bars) bone marrow. Pre Pro and Pro B: 9-10 mice, LMPP and HSC 4-6 mice, CLP and BLP 6-7 mice. Mean± SEM.

It has been reported that in E14.5 FL of PAX5 deficient embryos, no B220$^+$ cells can be detected (Nutt et al., 1997), indicating B cell development is blocked prior to the heterogeneous Pre Pro B cell stage, earlier than the block at the Pro B cell

stage reported in the adult BM. However, when we investigated *Pax5Δ* progenitors in the E14.5 FL we saw a clear $Lin^{-}CD43^{+}B220^{+}CD19^{-}CD24^{-}AA4.1^{+}$ KIT^{+} IL-7Rα^{+} $FLT3^{+}$ Pre Pro B cell population (figure 5.22A). Unlike the adult bone marrow, this population was not significantly expanded (figure 5.22B). E14.5 *Pax5Δ* FLs also had an absence of $CD19^{+}$ Pro B cells, indicating a block in B cell development at the Pre Pro B to Pro B cell stage, comparable to the adult BM. In vitro culture of Pre Pro B cells demonstrated a reduction in the ability of *Pax5Δ* cells to develop into $CD19^{+}$ B cells in culture (figure 5.22C). As PAX5 is required to activate CD19 expression (Kozmik et al., 1992), it was unexpected to find that a low number of $CD19^{+}$ cells develop from *Pax5Δ* progenitors, but it is possible these cells had escaped *Pax5* deletion. These observations therefore need to be further validated. We next characterized upstream B cell progenitors from dHSCs, through LMPPs, CLPs and BLPs in the E14.5FL of *Pax5Δ* animals and found no significant difference in the size of these phenotypically defined populations (figure 5.22D). The milder phenotype associated with *Pax5* deletion in the embryo may be due to lower recombination induced by *vav* cre at early developmental time points (Ogilvy et al., 1999).

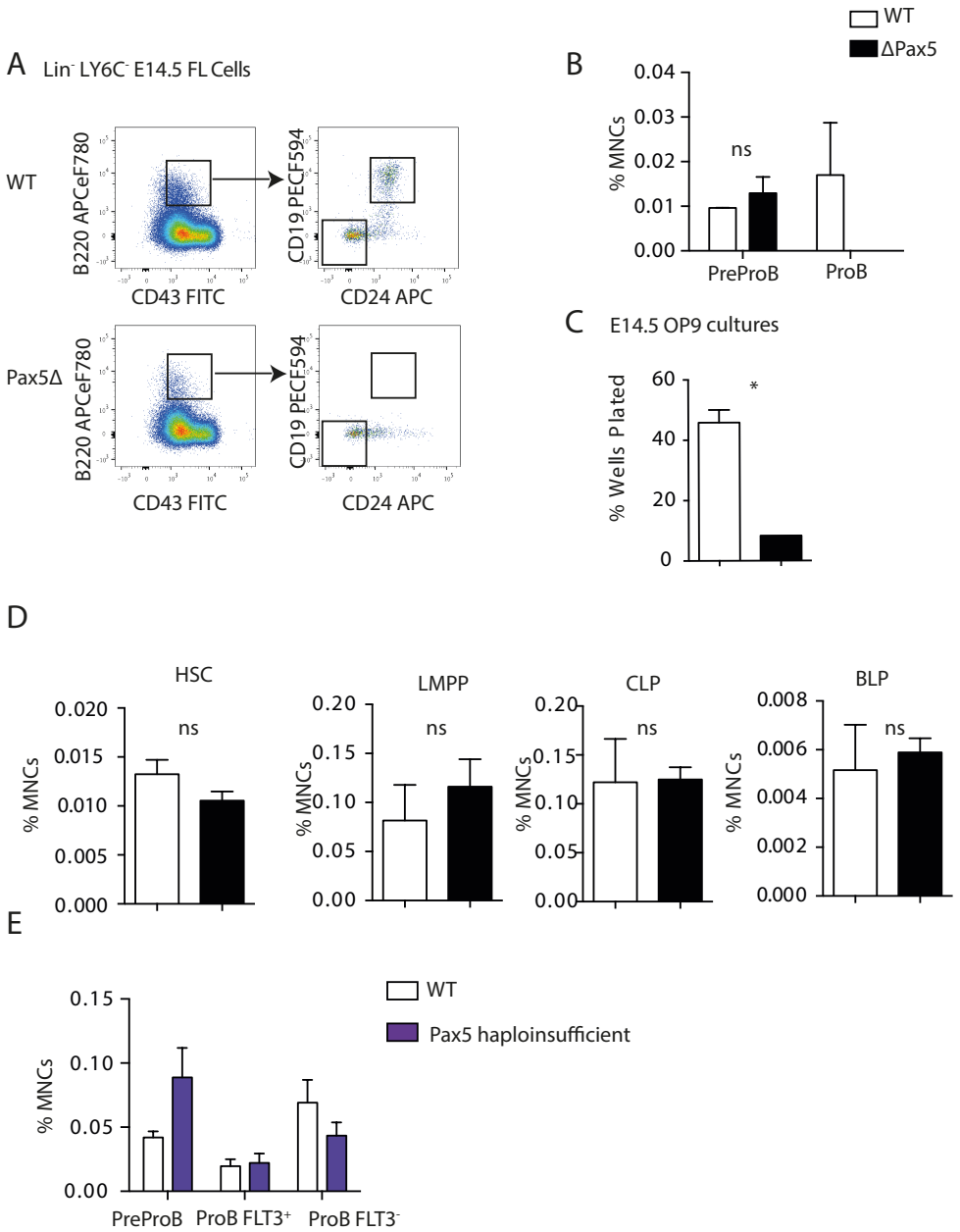


Figure 5.22: PAX5 deficient FL has a block at the Pre Pro B to Pro B cell stage

A: Flow cytometry analysis of Pre Pro B cells as defined in table 5.1 in *vav cre* $^{wt/wt}$ *pax5* fl/fl (top panels) and *vav cre* $^{tg/wt}$ *pax5* fl/fl FL (lower panels).

B: Frequency of B cell progenitors in E14.5 fetal livers from *vav cre* $^{wt/wt}$ *pax5* fl/fl 'WT' (white) and *vav cre* $^{tg/wt}$ *pax5* fl/fl FL (black '*ΔPax5'*). embryos

C: E14.5 Pre Pro B cells from *vav cre* $^{wt/wt}$ *pax5* fl/fl 'WT' and *vav cre* $^{tg/wt}$ *pax5* fl/fl FL (black - '*ΔPax5'*) were sorted and plated at single cell density onto OP9 stroma supplemented with mSCF, hFL, h-Csf1, mGM-CSF, mIL-3 and hGSCF. B220^{+}CD19^{+} B cells were analysed 10 days after plating by flow cytometry.

D: Analysis of haematopoietic progenitors from *vavCre* $^{wt/wt}$ *pax5* fl/fl 'WT' and *vav cre* $^{tg/wt}$ *pax5* fl/fl FL (black - '*ΔPax5'*) E14.5 fetal livers. Frequency of total MNCs in FL.

E: Analysis of *vavCre* $^{wt/wt}$ *pax5* fl/wt 'WT' and haplo-insufficient *vavCre* $^{tg/wt}$ *pax5* $^{fl/wt}$ E14.5 FLs (purple - '*Pax5* haploinsufficient'). One experiment, 3 embryos per group. Mean ± SEM.

Finally, as loss of one *Pax5* allele is a common secondary mutation in B Cell ALL (Mullighan et al., 2007), we investigated the effect of *Pax5* haploinsufficiency on E14.5 FL B cell progenitors. Preliminary analysis found the frequency of Pre Pro B cells and Pro B cells to be unchanged in the E14.5 *vavCre*$^{tg/wt}$ *Pax5*$^{wt/tg}$ haplo-insufficient FL*s* (figure 5.22E), indicating one *Pax5* allele is sufficient to permit B cell development.

Discussion

As fetal B cell lymphopoiesis has been poorly characterized to date, I have developed herein an advanced multi-colour flow cytometry panel to identify and characterize Pre Pro and Pro B cells in the E14.5 mouse fetal liver. We found that unlike their adult counterparts, fetal $Lin^{-}B220^{+}CD43^{+}CD19^{+}CD24^{+}$, Pro B cells homogeneously express the cytokine receptors KIT and IL-7Rα, and that 22 percent express FLT3. A Pro B $FLT3^{+}$ population ($Lin^{-}B220^{+}CD43^{+}CD19^{+}CD24^{+}KIT^{+}IL\text{-}7R\alpha^{+}FLT3^{+}$) was detectable in the adult bone marrow, albeit at a lower frequency (9% of $KIT^{+}IL\text{-}7R\alpha^{+}$ Pro B cells) with the highest frequency of $FLT3^{+}$ Pro B cells detected in bone marrow of late gestation and neonatal animals. This is compatible with previous published work that found an early Pro B cell progenitor expressing FLT3, defined as $B220^{+}CD19^{+}KIT^{lo}FLT3^{lo}\lambda5^{+}$, in the bone marrow of 1 week old mice. However, this study reported Pro B cells expressing FLT3 to be undetectable in the adult bone marrow (Ogawa et al., 2000). The reduced expression of cytokine receptors on B cell progenitors in older mice seen here and additionally shown by Ogawa et al, may be the reason why young mice deficient for KIT and FLT3 have a more severe B cell phenotype than older mice (Mackarehtschian et al., 1995).

For the first time we have shown that E14.5 fetal liver B cell progenitors have comparable lineage potentials and restrictions to their adult counterparts in vitro, with Pre Pro B cells retaining residual T cell potential, and Pro B cells being restricted to the B cell lineage. Gene expression analysis revealed Pre Pro B cells cluster adjacent to $IL\text{-}7R\alpha^{+}$ LMPP cells, highlighting how Pre Pro B cells are a poorly defined, heterogeneous population that contains progenitor cells which

are not restricted to the B cell lineage. This highlights the importance of correctly identifying the true B lineage restricted progenitors within the $CD19^-$ Pre Pro B fraction, such as the MYPs identified in chapter IV using an *Mb1*-cre fate mapping approach.

The field has largely assumed that fetal and adult B cell progenitors of equivalent phenotypic definition have comparable lineage potentials, although this has never been conclusively demonstrated until now (figure 5.4 and figure 5.10). However, the two developmental ages differ in their expression of the cytokine receptors KIT, IL-7Rα and FLT3 and global gene expression analysis highlights that at the molecular level fetal and adult B cell progenitors are markedly different. All fetal liver Pro B cells (Lin^- $B220^+CD43^+CD19^+CD24^+AA4.1^+$) expressed KIT and IL-7Rα on the cell surface, but a proportion of adult Pro B cells did not. IL-7Rα is known to be expressed on B cell progenitors until the Pre B cell stage (Fraction D) (Hardy and Hayakawa, 2001) so IL-7Rα$^-$ cells were not further characterized. We did investigate adult BM IL-7R$\alpha^+$$KIT^-$ Pro B cells, which did not express FLT3 (figure 5.2) and found that they failed to engraft in vivo (figure 5.12C) and had a reduced B cell output in vitro (figure 5.5A) compared to KIT^+ $FLT3^+$ and KIT^+ $FLT3^-$ Pro B cells, suggesting KIT^- Pro B cells do not readily develop into mature B cells at least within the constraints of the assays used herein. As the earliest analysis was performed 3 weeks after transplantation it is possible, although unlikely, that Pro B $FLT3^-$ and Pro B KIT^- populations engraft and produce short lived B cells, which are missed in this analysis.

Gene expression analysis suggested that KIT$^-$ Pro B cells have down regulated enzymes involved in somatic recombination indicating they may be incapable of generating a functional BCR by completing immunoglobulin rearrangement. Some studies have failed to obtain 'bright' signals from cell surface KIT on B cell progenitors, and concluded that certain B cell progenitors may not express KIT (Allman et al., 1999). By using bright fluorescent markers we optimized KIT detection on B cell progenitors and have shown the importance of KIT expression in identifying early B cell progenitors. This agrees with the work of others demonstrating the functional importance of KIT signalling in early B cell development (Rico-Vargas et al., 1994).

For the first time we have demonstrated that Pro B cells which express FLT3 (Lin$^-$B220$^+$CD43$^+$CD19$^+$CD24$^+$KIT$^+$IL-7Rα$^+$FLT3$^+$) show surprisingly high levels of engraftment in recipient mice, with just 100 cells proliferating to constitute as much as 10% of the B cell compartment in recipient mice (figure 5.11C). Cellularity calculations reveal 100 Pro B FLT3$^+$ cells can expand to generate 51,000 CD19$^+$IgM$^+$ cells in recipient spleens and 4,000 CD19$^+$IgM$^+$ per recipient tibia four weeks after transplantation. In contrast Pro B FLT3$^-$ and Pro B KIT$^-$ donor cells generated only 4,100 and 180 donor splenic CD19$^+$IgM$^+$ cells respectively per recipient mouse (figure 5.11C). This indicates that expression of FLT3 identifies a subset of the Pro B cell compartment that has increased potential to generate differentiated cells or perhaps that the expression of FLT3 gives the cells a functional advantage due to increased cytokine signalling stimulating growth and differentiation. Indeed compatible with this hypothesis, in vitro culture experiments have demonstrated adding IL-7, FLT3L and KIT in

combination, results in a marked increase in the growth of $B220^{+}CD19^{-}$ B cell progenitors from 1 week old mice, compared to when just two of the ligands are added (Ogawa et al., 2000).

Global RNA sequencing analysis showed that 409 genes were differentially expressed between adult Pro B $FLT3^{+}$ and $FLT3^{-}$ cells (figure 5.19), but only 20 differentially expressed genes were detected in the equivalent fetal populations (figure 5.19). This suggests that the fetal Pro B $FLT3^{+}$ and Pro B $FLT3^{-}$ cells are remarkably similar at the molecular level in the fetus, whereas in the adult the $FLT3^{+}$ Pro B cells are a more distinct population. Furthermore the global gene expression analysis highlights, that despite characterizing phenotypically identical populations, fetal and adult B cell progenitors differ greatly in terms of gene expression. In fact differences in developmental age result in more significant differences in gene expression than comparing different stages of lineage restriction from the same developmental age. 1128 genes are differentially expressed when comparing fetal and adult Pro B cells, and a further 439 genes differ between Pre Pro B cells. These differences require 3 biological replicates to become significant, a relatively low number for global RNA sequencing analysis. In fact it is likely that using this limited number of replicates, additional differentially regulated genes have been missed due to technical variability, as highlighted by the failure of our analyses to differentially detect *Flt3* when comparing fetal Pro B $FLT3^{+}$ and Pro B $FLT3^{-}$ cells. In contrast, single cell qPCR highlights how E14.5 Pro B cells can be separated into cells that express *Flt3*, and those that do not (figure 5.8). Therefore extending the global gene expression analysis by including more replicates may reveal more

differentially regulated genes. How these large molecular differences between fetal and adult B cell progenitors relate to functional differences requires further characterisation.

It has been suggested that the activity of RAG1 and RAG2, combined with the introduction of somatic mutations by AID in B cell progenitors contributes towards mutagenesis and leukaemic development (Swaminathan et al., 2015). In normal development the activity of these enzymes is segregated with RAG1 and RAG2 being active in earlier progenitors and AID in more mature B cells (Hardy and Hayakawa, 2001). However if both enzymes are active in the same cell, it has been proposed that AID mediated deamination of methylated cytosine residues at CpG islands results in the conversion of cytosine to thymine. This T:G mismatch generates a base pair 'bubble' that RAG can recognize and causes a single strand nick and subsequently introduces potentially oncogenic mutations (Swaminathan et al., 2015; Tsai et al., 2008). We found *Rag1* and *Rag2* to be expressed in Pre Pro B cells, Pro B FLT3^{+} and Pro B FLT3^{-} cells and our analysis did not reveal a marked difference in expression levels between fetal and adult progenitors (requires qPCR confirmation). Unfortunately our global gene expression analysis did not detect AID expression in any progenitor subset, perhaps suggesting it may not be correctly annotated in the reference genome. It would therefore be important to specifically investigate expression of AID by qPCR or using a published *Aicda*-GFP reporter mouse model (Swaminathan et al., 2015), and investigate whether any fetal or adult B cell subsets may harbour concurrent RAG and AID activity.

By E14.5 dHSCs have been established in the FL for 3-4 days (Dzierzak and Speck, 2008) and are therefore the most likely source of the B cell progenitors identified herein. Although it is possible that some B cell progenitors develop independently of dHSCs, such as via the immune restricted 'fetal LMPP' identified in chapter III, which have the potential to develop $CD19^+$ B cells (Boiers et al., 2013). It is likely that the Pre Pro B cells progress via the MYP population ($CD45^+B220^+CD19^-$-YFP^+KIT^+IL-$7R\alpha^+FLT3^+$) identified in chapter IV, before developing into $CD19^+$ Pro B cells that still express KIT, IL-7Rα and FLT3. We would argue that the $FLT3^+$ Pro B cell is an 'earlier ' B cell restricted progenitor than the $FLT3^-$ Pro B cell, due to the homogeneous expression of FLT3 by upstream Pre Pro B cells (figure 5.1 and 5.6) and the lack of expression of FLT3 by mature B cell progenitors. The repression of FLT3 expression by PAX5 (Holmes et al., 2006) and in vitro culture data demonstrating down regulation of FLT3 as B cells mature in culture (Ogawa et al., 2000) supports this hypothesis. This conclusion is in agreement with studies of human B cell lymphopoiesis showing FLT3 expression is down regulated as B cell progenitors mature. However, unlike in mice FLT3 expression persists until the Pre B cell stage in humans (Rappold et al., 1997).

Going forward it would be important to complement this molecular analysis by comparing the rearrangement status at the immunoglobulin heavy chain loci of the herein identified fetal and adult B cell progenitors. Such an analysis would reveal whether phenotypically defined progenitors are at comparable stages of Ig rearrangement and could contribute to studies suggesting fetal B cell progenitors preferentially use different V, D and J segments compared to their

adult counterparts (Feeney, 1990; Nutt et al., 1997). RNA sequencing confirmed previous studies that showed fetal B cells do not express *Dntt* (Feeney, 1990).

The contribution of fetal B cell progenitors to mature B cell lineages in the adult has not been definitively shown, although it has been proposed that B1 cells stem from a progenitor of fetal origin (Dorshkind and Montecino-Rodriguez, 2007a; Herzenberg and Tung, 2006; Montecino-Rodriguez and Dorshkind, 2012). Here we show that adult FLT3$^+$ Pro B cells, in addition to generating MZ and follicular B cells, can also generate B1b cells but not CD5$^+$ B1a cells, supporting previous analyses (Hardy and Hayakawa, 1991; Herzenberg, 2000; Kantor et al., 1992). However as fetal progenitors failed to engraft in conventional transplantation assays we were unable to definitively address the contribution of fetal Pro B cells towards B1a, B1b, MZ and follicular B cells in adult lymphopoiesis. Molecular data suggest that fetal B cell progenitors express lower levels of MHC class I proteins than their adult counterparts. This could target the cells for destruction by cytotoxic T cells upon transplantation (Alberts B, 2002). We obtained a low number of mature IgM$^+$ cells when transplanting E18.5 FL Pro B cells into neonatal recipients but engraftment was too low to conclude which B cell subsets had been generated. Furthermore in order to minimize the developmental age difference between the donor and recipient to maximize the chance of engraftment, we transplanted cells from late gestation E18.5 FL into newborn recipients. This approach is limited by the newborn mice only tolerating low levels of irradiation to ablate the bone marrow niche compared to adult transplantation assays, reducing the chance of donor cells engrafting. Ideally we want to assess the contribution of Pro B cells from younger

embryos to adult lymphopoiesis, as substantial molecular and functional changes may have occurred between E12.5 and E18.5. In future studies it would be valuable to investigate whether fetal B cell progenitors can generate $CD5^+$ B1a cells and investigate their contribution to the MZ compartment, as MZ cells resemble B1 cells in their innate-like response to pathogen. Intriguingly IL-7 deficient mice, which can sustain $CD19^+$ B cell progenitors in neonatal but not adult BM, demonstrate persistence of MZ cells (Carvalho et al., 2001) and molecular analysis has revealed fetal like properties of MZ B cells such as the lack of 'N' regions at rearranged immunoglobulin loci (Carey et al., 2008). Due to the limitations and problems associated with in vivo transplantation of fetal lymphoid progenitors, a fate mapping approach may be more fruitful.

Investigating the in vivo potential of fetal B cell progenitors will contribute towards the controversial debate over whether B1 cells originate from distinct fetal progenitors. The identification of a unique B1 cell progenitor that could not generate B2 cells (Montecino-Rodriguez et al., 2006) supported the hypothesis that B1 and B2 cells differ in their progenitors. Our transplantation studies indicated that adult BM Pro B cells could reconstitute both B1b and B2 cells. However, unless B cells can be functionally analysed in vivo at the single cell level with high efficiency, currently beyond the limits of experimental techniques, it will be difficult to conclude whether progenitors are entirely restricted to the B1 or B2 cell lineage.

Although we can identify phenotypically comparable B cell progenitors in the fetal liver and adult bone marrow with comparable lineage potentials in vitro, we

have not proven that the cells have the same function in vivo. Indeed molecular analysis highlights that fetal and adult B cell progenitors differ greatly in their gene expression (figure 5.18, figure 5.20). Culture data suggested that fetal, but not adult B cell progenitors, show increased proliferation in response to myeloid cytokines (figure 5.4A and figure 5.10A). Furthermore, global gene expression analysis showed that fetal B cell progenitors express a low, but significant level of the myeloid lineage genes such as *Csf1r*, *Mpo, Fcgr1, Mac1* and *Gr-1* (figure 5.18E). Furthermore single cell qPCR of E14.5 FL Pro B cells suggested *Csf1r* transcripts were detectable in 8/39 cells analysed (figure 5.8). The expression of myeloid lineage genes by the Pre Pro B cell fraction is not surprising as this is known to be heterogeneous population that contains progenitors which are not B lineage restricted. However, it is noteworthy that fetal Pre Pro B cells express myeloid lineage genes to a higher level than their adult counterparts. The expression of low levels of myeloid lineage genes by $CD19^{+}$ committed Pro B cells from the fetal liver but not the adult bone marrow, suggests that fetal B cell progenitors may retain some myeloid lineage priming. This may be of relevance to paediatric and infant leukaemias that express antigens of both the B cell and myeloid lineage (Bene, 2009).

Given the difficulties with transplantation studies, to definitively address the contribution of fetal B cell progenitors to B cell lymphopoiesis in the adult, an in vivo fate mapping approach is needed. Fetal Pro B cells could be labelled during a specific embryonic time window by pulse labelling to temporarily activate expression using a *Rag1* or *Cd19* inducible cre mouse line. Subsequently labelled progenitors could be tracked to determine to which mature B cell subsets they

contribute in the adult, without the need for cytotoxic irradiation. This analysis would address the question whether fetal Pro B cells are capable of generating $CD5^+$ B1a cells, supporting the hypothesis that these cells have a fetal progenitor.

Herein we show that in the absence of PAX5, fetal and adult B cell lymphopoiesis are blocked at phenotypically comparable stages, although we have not yet compared the populations at the molecular level nor confirmed that their rearrangement status and deletion efficiency were equivalent. It has not been verified whether the same regulatory networks apply in the fetus, which is known to respond differently to cytokines such as IL-7 that mediate transcriptional networks (Carvalho et al., 2001). Perhaps counter-intuitively, it has been suggested that PAX5 deficient fetal livers demonstrate a block in B cell development at the Pre Pro B stage due to failure to detect any $B220^+$ cells (Nutt et al., 1997). This is in contrast to the adult bone marrow where $B220^+$ Pre Pro B cells are detectable and the block in B cell lymphopoiesis occurs later, prior to the $CD19^+$ Pro B cells stage (Nutt et al., 1997). The flow cytometry analysis used in this study separated cells based only on three markers with a resulting limited resolution. As B220 expression is now known to be found on progenitors of the T, NK, DC and Myeloid lineage and the Pre Pro B cell compartment is so heterogeneous, it seems unusual that these cells would not develop in the absence of PAX5 (Huntington et al., 2007; Kouro et al., 2002; Rumfelt et al., 2006a, b). As these data have not been confirmed by further publications, it is possible that the scarcity of $B220^+$ cells in the fetal liver may have resulted in their failure to be detected by Nutt et al. Interestingly the 'blocked' Pre Pro B cells unanimously express high levels of FLT3, supporting the observation that

PAX5 represses FLT3 expression (Holmes et al., 2006). It would be interesting to examine whether MYP cells, as characterized in chapter 4, are present in the absence of PAX5 in the fetal liver and bone marrow, as we would hypothesize that MYPs sit between the heterogeneous $CD19^-$ Pre Pro B cell compartment and the $CD19^+$ Pro B cell compartment in B cell development.

In the adult bone marrow of PAX5 deficient animals there is a marked expansion of Pre Pro B cells, and the LYD^+ CLPs, or 'BLPs', functionally corroborating the suggestion that BLPs are involved in B cell development (Inlay et al., 2009a). The corresponding Pre Pro B cells and BLPs in the E14.5 FL on PAX5 deficient embryos do not show any marked expansion, perhaps simply reflecting the early developmental time point and lack of progenitors.

The analysis of *Pax5* deficient haematopoiesis performed herein relied on the pan haematopoietic *vavCre* to delete the *Pax5* alleles. The block in B cell progenitors is compatible with the literature (Nutt et al., 1998; Thevenin et al., 1998), but we should also analyse the B cell progenitors in *vavCre* only mice (with wild type *Pax5*) to control for possible cre mediated toxicity influencing the phenotype. There are examples in the literature of dramatic phenotypes associated with cre expression that have subsequently been discovered in *cre* only mice, with no floxed target allele. This is most likely due to the cre enzymes causing DNA breakages at cryptic loci resulting in widespread genome damage and cell death (Schmidt-Supprian and Rajewsky, 2007).

Having begun to phenotypically, functionally and molecularly characterize fetal B cell progenitors in the wild type setting, we next wanted to investigate how

genetic lesions involved in childhood leukaemias influenced fetal B cell development. It was noticeable that the highest frequency of Pro B cell progenitors was found in late embryogenesis and early neonatal animals (figure 5.10), a potentially key time window for the occurrence of pre-leukaemic events in young children that present with TEL-AML1^{+} B cell ALL (Greaves et al., 2003). We therefore proceeded to investigate the effect of the TEL-AML1 oncoprotein on fetal B cell lymphopoiesis (chapter VI).

Chapter VI: Investigating the impact of the TEL-AML1 oncoprotein on fetal lymphopoiesis

INVESTIGATING THE IMPACT OF THE TEL-AML1 ONCOPROTEIN ON FETAL LYMPHOPOIESIS

Introduction

The initiating genetic event in childhood leukaemias is often a genomic translocation, which alone is insufficient for overt disease (Greaves and Wiemels, 2003). 25% of children with B cell ALL are characterized by the TEL-AML1 (TA) oncoprotein, the latter caused by a fusion of the genes *Etv6* and *Runx1*. The initial genetic lesion occurs in utero (Wiemels et al., 1999) with subsequent accumulation of secondary mutations refined by Darwinian natural selection resulting in the evolution of sub-clonal populations and overt disease (Nowell, 1976).

In the normal setting *Etv6* and *Runx1* encode TFs that are essential for the emergence of dHSCs and the establishment of haematopoiesis with mice lacking these genes dying at E10.5 and E12.5 respectively (further discussed in chapter I, (Okuda et al., 1996; Wang et al., 1997). Despite attempts to model TEL-AML1 ALL using cellular, viral (Tsuzuki et al., 2004) and mouse model approaches (Schindler et al., 2009; van der Weyden et al., 2011), how the TEL-AML1 fusion subverts B cell lymphopoiesis is still poorly understood. Epidemiological studies indicate that the evolution of pre-cancerous sub-clones is driven by delayed exposure to infection, followed by over-active aberrant responses to pathogens during childhood (Gilham et al., 2005; Greaves, 2006; Greaves and Buffler, 2009; Greaves and Maley, 2012; Urayama et al., 2010). The early pathogenesis of TEL-AML1 leukaemias have been linked to stalled B cell development due to either

genetic lesions or altered cytokine responses to infections. It has been suggested that if both RAG1/2 and AID enzymes, whose expression is normally strictly segregated between earlier and mature B cell progenitors respectively (Hardy and Hayakawa, 2001), are active in the same cell then the generation of a base pair mismatch by AID can be recognized by the RAG enzymes causing a potentially oncogenic translocation (Papaemmanuil et al., 2014; Swaminathan et al., 2015).

Retroviral studies indicate that TEL-AML1 can induce a block in B cell lymphopoiesis, as transduction of TEL-AML1 into cells followed by transplantation into murine recipients, results in a deficiency of B cells after the Pro B cell stage and reduced expression of IL-7R. However viral studies are difficult to standardize between labs and results can be obscured by off target effects. Expression of TEL-AML1 in the early haematopoietic compartment of zebrafish supported the viral studies by showing TEL-AML1 induces an early B cell differentiation block (Sabaawy et al., 2006).

Mice have to date been poor models for human B cell ALL as they do not normally develop B cell ALL, either spontaneously or after genetic modification or mutagen treatment (Hauer et al., 2014). However, rather than abandon mouse models it is more likely that the approaches taken to model leukaemia have been insufficient to recapitulate a highly complex disease. For example, unlike in the human where a mutation occurs in a single cell that then 'evolves', mouse genetic models artificially introduce a genetic lesion in multiple heterogeneous cell types, resulting in a non-physiological model. Murine haematological

malignancies are often T cell or myeloid acute leukaemias or lymphomas (Hauer et al., 2014). The first attempt to model TEL-AML1 B cell ALL in the mouse expressed the oncoprotein under the control of the *IgH* promoter. The animals did not develop any haematological disorders (Andreasson et al., 2001). More recently a conditional TEL-AML1 knock-in demonstrated that TEL-AML1 mice had an expansion of adult HSCs when the expression of the oncoprotein was induced in all haematopoietic compartments (Schindler et al., 2009). The authors showed increased longevity of uncharacterized fetal liver progenitors in a serial re-plating assay in the presence of IL-7, KIT and FLT3L. However the model failed to generate B-ALL and, in the presence of ENU, T cell malignancies prevailed (Schindler et al., 2009). A third mouse model involved the generation of a constitutive TEL-AML1 knock in followed by introducing the *Sleeping beauty* transposase ('TA' mice), that on crossing to mice carrying the *T2Onc* transposon array, allowed in vivo random mutagenesis and the generation of secondary mutations (van der Weyden et al., 2011). This is the only model to have thus far generated a B cell ALL, but at a low frequency (13/90) with other haematological malignancies dominating (AML 34/90 and T-ALL 21/90). Furthermore, the B cell ALLs that presented were unusual as the blast cells were characterized as $B220^{+}CD19^{-}CD24^{+}IL\text{-}7R\alpha^{-}$, unexpectedly not expressing CD19 and IL-7Rα. Therefore in the absence of molecular data it remains unclear whether the disease generated was B cell lineage leukaemia.

None of these mouse models attempted to include a targeted secondary mutation such as loss of the second *Tel* allele or *Pax5* haploinsufficiency, the two most common secondary mutations in TEL-AML1 B cell ALLs (Mullighan, 2012).

Recently, the synergy of two mutations has been used to reproduce a more faithful model of B cell ALL in the mouse. Combining a constitutively active form of *Stat5b*, a mutation associated with a poor prognosis in humans (Heltemes-Harris et al., 2011), with haploinsufficiency of either of the key B cell transcription factors *Pax5* or *Ebf1*, rapidly induced $CD19^{+}IgM^{-}$ B cell progenitor ALL in mice with 100% penetrance (Heltemes-Harris et al., 2011). This indicates models of B cell ALL may be more successful if multiple mutations are included.

Achieving a realistic model of B cell ALL would be a useful tool and help elucidate how the TEL-AML1 oncoprotein influences B cell lymphopoiesis and collaborates with RAG and AID driven somatic rearrangements and mutations. Before attempting to model disease, it is first important to try to understand the effect of a pre-leukaemic hit on fetal B cell development. Most studies of TEL-AML1 have focused on the adult bone marrow. However as the TEL-AML1 translocation occurs in utero and disease affects young children, we decided to take a new approach and first characterize how TEL-AML1 influences B cell lymphopoiesis in the fetus using the staging developed in previous chapters.

Results

In order to investigate the effect of TEL-AML1 on fetal B cell lymphopoiesis, we obtained a constitutive TEL-AML1 knock in mouse model (TA: *Etv6*$^{Runx1/wt}$, (van der Weyden et al., 2011), which is the only murine model that has demonstrated any incidence of B cell ALL, albeit at a low frequency with a questionable B cell identity. Exons 1-6 of human *Runx1* had been inserted into the mouse *Etv6* locus (figure 6.1) with a *Sleeping beauty* transposase, the latter gene being redundant in this study. This results in the TEL-AML1 oncoprotein being expressed under the same promoters as in human patients.

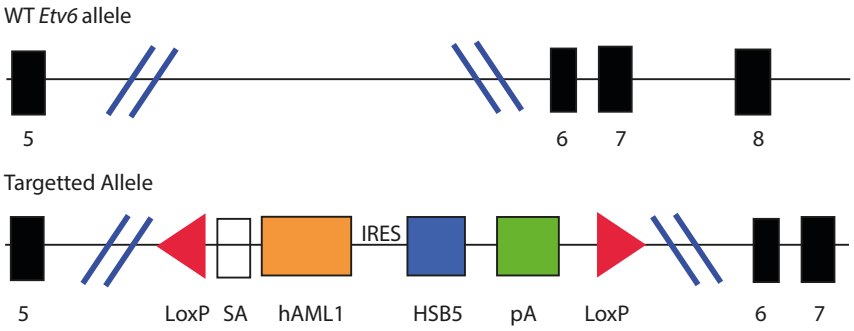


Figure 6.1: A Constitutive TEL-AML1 knock in at the Tel locus
Structure of the TEL-AML1 (*Etv6-Runx1)* constitutive knock in mouse model (van der Weyden et al., 2011). The mouse *Etv6* locus has been targeted to introduce a splice acceptor (SA), exons 1-6 of human RUNX1, an internal ribosomal entry site (IRES), and a hyper-active variant of the *Sleeping Beauty* transposase (HSB5). The construct is flanked by *Lox66* and *Lox71* sites for potential *Cre* mediated inactivation of the TEL-AML1 fusion gene. Upper panel depicts WT *Tel* allele and lower panel depicts targeted allele.

As the original studies on this mouse model focused on introduction of mutations using the *Sleeping beauty* transposase, the haematopoietic progenitors had not been characterized in the adult BM or FL. We therefore first analysed the adult BM and found TEL-AML1 mice had an expanded HSC compartment, corroborating the findings of an independent TEL-AML1 mouse model (Schindler et al., 2009) figure 6.2A). We found no significant difference in LMPPs (figure

6.2A) but a reduction in Lin$^-$ CLPs (figure 6.2B). Notably there were no changes in frequency of Pre Pro B cells, Pro B FLT3$^+$, Pro B FLT3$^-$ or Pro B KIT$^-$ cells (figure 6.2C-F) in the adult BM. We also used a crude 4 colour B cell staining to reproduce the B cell analysis used by others (Schindler et al., 2009) and in agreement with published work again saw no change in the frequency of B cell progenitors in the adult BM (figure 6.2G).

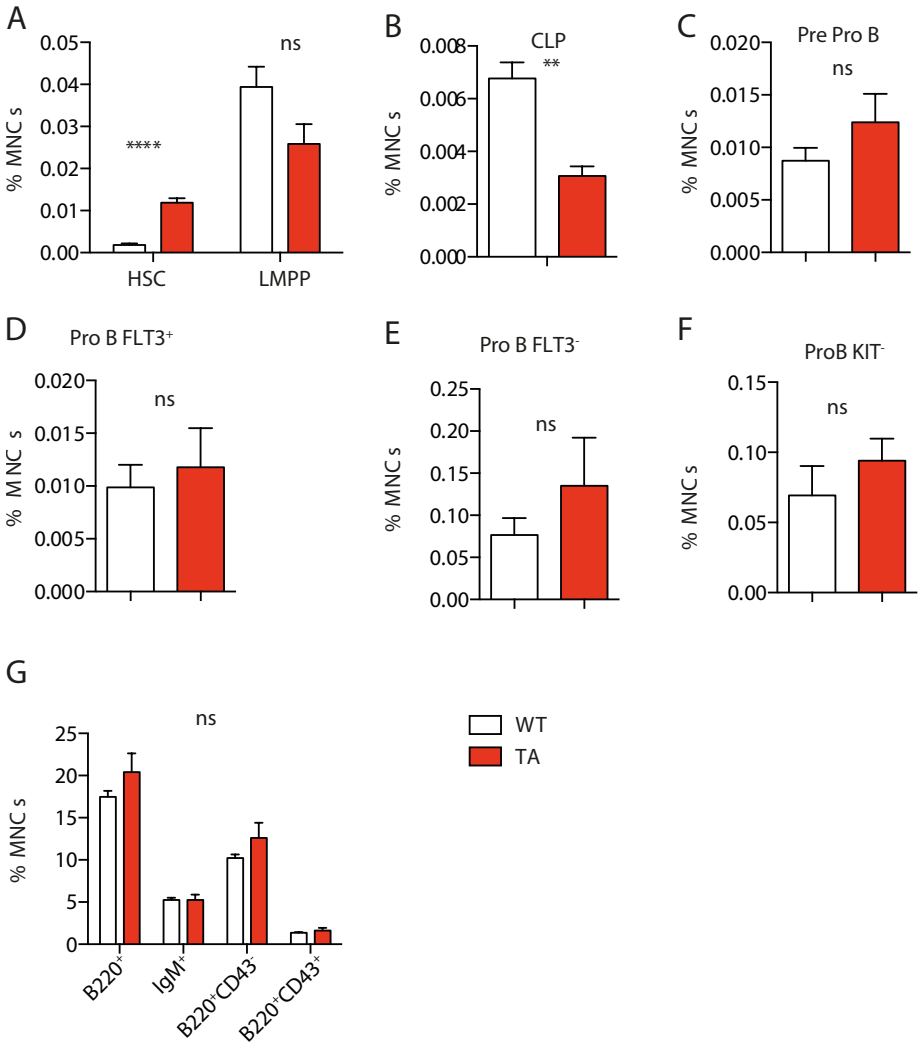


Figure 6.2: HSCs are expanded in adult BM of TEL-AML1 mice

Analysis of haematopoietic progenitors in bone marrow of 6-8 week old TEL-AML1$^+$ mice. A: HSCs and LMPPs, B: CLPs, C: Pre Pro B cells, D: Pro B FLT3$^+$ E: Pro B FLT3$^-$, F: Pro B KIT$^-$, G: B cell progenitors analysed on surface expression of IgM, B220 and CD43 as in (Schindler et al., 2009), with the populations counted as annotated in the figure. 6-7 animals per group, except CLP (B) with 3 animals per group. Mean ± SEM.

We next characterized haematopoietic compartments in the E14.5 FL of TEL-AML1 embryos and littermate controls. Due to the constitutive nature of the mouse model, the translocation is expressed in all cells from conception. At E14.5 TEL-AML1 embryos were viable and found at the expected allelic frequency (39/66 embryos, 59%), whereas by birth only 26.3% of mice (42/160) carried the TEL-AML1 translocation indicating a lethality late in embryogenesis, confirming reports by others (van der Weyden et al., 2011).

FLs from TEL-AML1 embryos showed a slight, but significant reduction in cell number (WT: 32.2±2.4x10^6 cells/FL, n=29, TA: 25.6±1.72 x10^6 cells, n=33, p=0.0267) (figure 6.3A). There was no detectable difference in the sizes of the Pre Pro B (figure 6.3B) or Pro B FLT3$^-$ (figure 6.3C) cell compartments between WT and TA$^+$ livers but unlike the adult BM (figure 6.2D) we found the Pro B FLT3$^+$ compartment was doubled in size in E14.5 TA$^+$ FLs (0.006% of MNCs in WT FL, 0.012% of MNCs in TEL-AML1 FL, p=0.015, figure 6.3D). However, despite the increased frequency in vivo of Pro B FLT3$^+$ cells from TA$^+$ FL, we could not detect any increased growth in culture of single E14.5 Pro B FLT3$^+$ cells from TA$^+$ mice compared to WT littermate controls (figure 6.3F). Using crude 4-colour B cell staging to reproduce the results of others (Schindler et al., 2009), this subtle expansion in Pro B cells is missed (figure 6.3E) and B cell progenitors appear equivalent between WT and TEL-AML1 mice, emphasizing the importance of inclusion of cytokine receptors in B cell progenitor analyses.

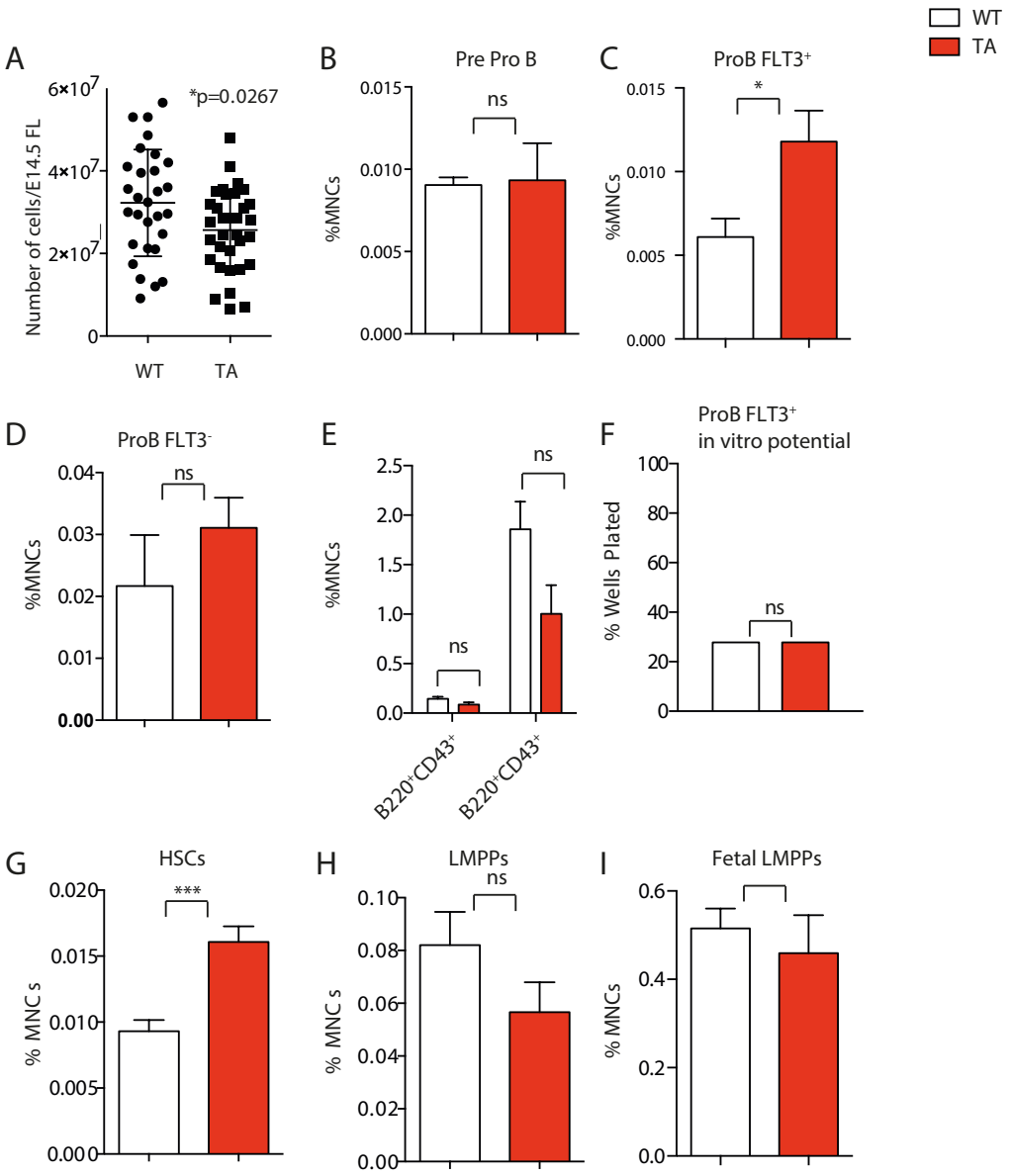


Figure 6.3: Pro B FLT3⁺ cells are expanded in E14.5 TEL-AML1 Fetal Livers

A: Cell counts of E14.5 WT or TEL-AML1⁺ fetal livers. Mean ± SD

Frequency of Pre Pro B cells (B), Pro B FLT3⁺ (C) and Pro B FLT3⁻ (D) in the E14.5 FL. Pre Pro B 5-12 embryos per group, Pro B 14-15 embryos per group, five independent experiments. Mean ± SEM.

E: FL B cell progenitors analysed only on B220 and CD43 expression as in (Schindler et al., 2009). 8-9 embryos per group, 2 independent experiments. F: Single E14.5 Pro B FLT3⁺ cells were plated on OP9 stroma with mSCF, IL-7 and FLT3L and clones evaluated for B cell potential by flow cytometry 14 days after plating, 18 wells plated from a pool of 3 TEL-AML1 FL and 8 WT FLs.

G and H: Frequency of HSCs and LMPPs in E14.5 FL cells. 12-14 FLs per group. Mean ± SEM.

I: Frequency of Lin-KIT⁺IL-7Rα⁺FLT3⁺ 'fetal LMPPs' in the E11.5 FL, 4 mice per group. Mean ± SEM.

As in the adult bone marrow, we found an expansion of HSCs in E14.5 FL of TEL-AML1 embryos (figure 6.3G) but no significant difference in LMPPs (figure 6.3H) or E11.5 Lin-KIT$^+$IL-7Rα$^+$FLT3$^+$ 'fetal LMPPs' (characterized in chapter III, figure 6.3I).

Using the same approach as in chapter V (figure 5.12A) we transplanted 100 E14.5 FL Pro B cells per mouse that were either FLT3$^+$ or FLT3$^-$, into lethally irradiated adult mouse recipients. Unfortunately, as seen with WT cells (figure 5.12C) the FL cells from either the TA$^+$ or WT FL failed to engraft into adult recipients in standard competitive transplantation assays (figure 6.4).

E14.5 FL Pro B cells were sorted from TEL-AML1 mice and littermate controls for global RNA sequencing analysis in order to understand the molecular effect of the TEL-AML1 oncogene. Unfortunately, principal component analysis of the data sets revealed a large variation between samples and lack of clustering between FLT3$^+$ and FLT3$^-$ Pro B cells (appendix 9), unlike the molecular analysis of B cell progenitors from C57 BL/6 animals reported in chapter V. This variation is most likely due to the mixed genetic background of the TEL-AML1 mouse strain, and therefore this analysis should be repeated when the strain has been backcrossed to C57BL/6 animals to reduce the genetic variation that might mask subtle molecular effects of the TEL-AML1 oncogene.

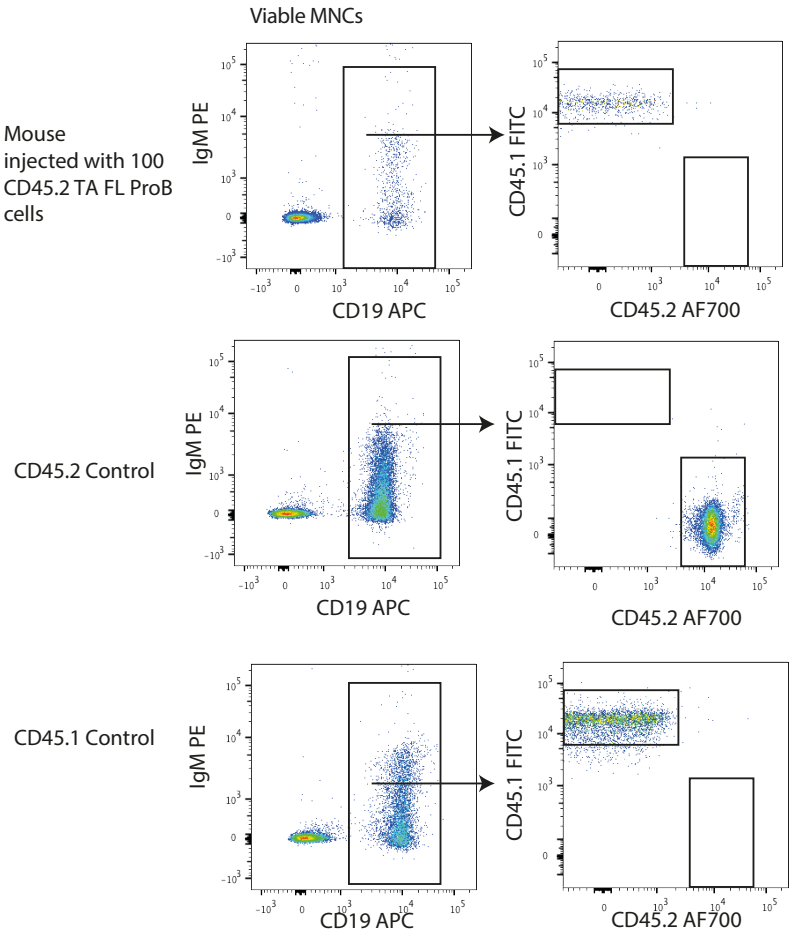


Figure 6.4: TEL-AML1 Pro B cells, like WT counterparts, fail to engraft into adult recipients in vivo

Peripheral blood analysis 3 weeks after injection of 100 Pro B cells from E14.5 TEL-AML1^{+} FLs and littermate controls into lethally irradiated CD45.1 adult recipients. Representative of 8 mice, 4 injected with TEL-AML1^{+} cells and 4 injected with WT Pro B cells. Top panel is representative of an experimental mouse. Bottom two panels show CD45.2 and CD45.1 control mice.

Discussion

We have characterized fetal B cell progenitors to a high resolution in a mouse model that expresses TEL-AML1, an oncoprotein associated with 25% of childhood BCP ALLs, focusing on fetal lymphopoiesis to probe the developmental age relevant to human disease. We identified a small but potentially biologically important expansion of Pro B FLT3$^+$ cells in the E14.5 FL, a phenotype that is not sustained in the adult BM.

The expansion of HSCs in the adult BM and FL of mice expressing TEL-AML1 seen herein and by others (Schindler et al., 2009) has been suggested to result in a self-renewing pool of cells that is available for accumulation of subsequent genetic lesions. The molecular mechanism for this HSC expansion has not been elucidated (Schindler et al., 2009). Furthermore no B cell progenitor phenotype has been reported to date in TEL-AML1 mouse models. The authors reported that E14.5 FL cells show increased serial re-plating in vitro in the presence of KIT, IL-7 and FLT3L, cytokine receptors for which are expressed by early B cell progenitors, a phenotype not seen with IL-7 alone (Schindler et al., 2009). Additionally Schindler et al showed in the presence of IL-7, that E14.5 TEL-AML1 progenitor cells demonstrate approximately a 3-fold increase in the number of colonies produced in vitro. Both these observations and our finding that FLT3$^+$ Pro B cells are expanded in E14.5 fetal livers in the presence of TEL-AML1 are compatible with TEL-AML1 exerting a subtle effect on fetal B cell lymphopoiesis.

TA does not direct a transforming hit and disease presents with on average six secondary mutations (Mullighan et al., 2007). Given that transcripts for TEL-

AML1 can be detected at 100 fold higher incidence in neonates compared to the rate of disease (Mori et al., 2002a), it seems plausible that TEL-AML1 is a 'mild' oncogene rather than directly driving leukaemic development. Instead it may act to increase susceptibility to the acquisition of secondary mutations.

Aberrant activity of the lymphoid enzymes RAG and AID have been proposed to drive secondary mutation accumulation (Papaemmanuil et al., 2014; Swaminathan et al., 2015). The RNA sequencing and single cell gene expression data presented in chapter V confirm that *Rag1* and *Rag2* are expressed in E14.5 FL Pro B cells, although unfortunately the data did not reveal whether AID was expressed in Pre Pro B or Pro B cells. Additionally TEL-AML1 leukaemias are associated with high levels of FLT3 expression in blast cells (Chillon et al., 2012). Both these findings are compatible with the Pro B $FLT3^+$ cells providing a reservoir for the acquisition of secondary mutations during disease development. The increased engraftment of BM $FLT3^+$ Pro B cell progenitors in vivo (figure 5.12C) is compatible with the notion that these B cell progenitors may persist and accumulate the multiple mutations needed for overt disease (Mullighan et al., 2007).

Studying only the TEL-AML1 translocation alone, while not a model for overt leukaemia, will give useful insights into how pre-leukaemic mutations increase the risk of disease development. Here we show firstly that the Pro B $FLT3^+$ cells are phenotypically expanded in the presence of TEL-AML1 in the E14.5 FL (figure 6.3) but not the adult bone marrow (figure 6.4). The fact that this expansion is not seen in the adult BM, and that a greater proportion of adult BM

Pro B cells are FLT3$^-$, may provide insight into why, despite its high prevalence in childhood B Cell Progenitor (BCP) ALL, TEL-AML1 is rarely associated with adult ALL (2-3% of cases in adult but 25% of cases in children) (Aguiar et al., 1996).

A limitation of these studies is the failure to obtain engraftment of fetal B cell progenitors to assess their in vivo characteristics, a problem which is shared by the field (Delassus and Cumano, 1996; Godin et al., 1995). Transplantation assays are designed for adult progenitor cells and to evaluate self-renewing long-lived HSCs, not downstream progenitors. It would be highly informative to understand the behaviour of TEL-AML1 fetal Pro B FLT3$^+$ cells in vivo, and investigate whether their increased frequency in the FL correlates with an altered longevity and contribution to mature B cell lineages. Going forward, an inducible fate mapping approach whereby fetal progenitors are labelled with an inducible *Mb1*-cre (Hobeika et al., 2015), in the presence or absence of TEL-AML1, could provide a useful insight into the effect of TEL-AML1 on B cell progenitors in vivo. The constitutive mouse model used here would not be suitable for this analysis as the TEL-AML1 construct is flanked by *LoxP* sites. Using CRISPR technology (Wang et al., 2013; Yang et al., 2013), the lab is developing a new conditional TEL-AML1 knock in that can be used to address this question. We could investigate how long fetal Pro B cells persist in the presence of TEL-AML1 and whether or not they show altered responses to infection (Gilham et al., 2005; Greaves, 2006; Greaves and Buffler, 2009; Greaves and Maley, 2012; Urayama et al., 2010).

Going forward, given the difficulty in obtaining functional data from fetal B cell progenitors in vivo, it will be critical to molecularly characterize the expanded E14.5 FL Pro B FLT3^{+} cells in order to gain an understanding of the functional effect of the TEL-AML1 translocation on fetal B cell lymphopoiesis. Specifically whether expression of *Rag1*, *Rag2* and *Aicda* is altered in the presence of the TEL-AML1 oncogene.

We have attempted for the first time to investigate the effect of a pre-leukaemic hit, TEL-AML1, on fetal B cell lymphopoiesis. A further step could be to investigate the collaboration of multiple mutations on fetal and postnatal B cell lymphopoiesis. We could use lentiviral knock down to mimic *Pax5* haploinsufficiency, a common secondary hit in leukaemia. In chapter V we reported no B cell phenotype in a mouse model that lacked one *Pax5* allele in the haematopoietic compartment, but the combined effect of TEL-AML1 and *Pax5* haploinsufficiency could be investigated. The restricted expression of *Pax5* to the B cell lineage should minimize the chance of haematological malignancies in other lineages, which have been prevalent in previous mouse models (Schindler et al., 2009; van der Weyden et al., 2011). Furthermore a viral approach will allow us to control the developmental time window in which the secondary mutation is introduced, to better reproduce the physiological setting. It has been recently shown for myeloproliferative neoplasms that the order in which mutations are accumulated may be key to disease development, highlighting the need for temporal control over the introduction of genetic lesions to successfully model disease (Klampfl et al., 2013; Nangalia et al., 2013). Viral approaches have limitations and any results would have to be verified to ensure phenotypes are

not due to shRNA off target effects, renowned for causing experimental artefacts by binding to unrelated partially complementary gene transcripts, thereby influencing translation or inducing an interferon response (Jackson, 2004).

The characterization of fetal B cell lymphopoiesis in this thesis could be useful in modelling the biology of other translocations associated with paediatric and infant B cell leukaemias in the mouse. MLL translocations are found in more than 70% of infant leukaemias, but are less frequent in leukaemias of older children and adults (Krivtsov and Armstrong, 2007). Leukaemias characterized by the MLL-AF9 translocation are associated with 33% of paediatric AMLs and 18% of paediatric ALLs, whereas adult MLL-AF9 leukaemias are predominantly myeloid with only 2% of cases presenting as ALL (Krivtsov and Armstrong, 2007). Additionally, the high penetrance of the MLL translocations, with only on average 1.3 additional mutations in infant leukaemias (Andersson et al., 2015; Mullighan et al., 2007), means insights may be gleaned without the need to introduce secondary mutations.

Interestingly, many MLL-AF9 blast cells express both lymphoid and myeloid markers, and it has been observed that MLL-AF9 ALLs can relapse as AMLs after therapy, whereas AMLs relapsing as ALLs are extremely rare (Dorantes-Acosta and Pelayo, 2012). We could therefore hypothesize that to drive paediatric ALL, MLL-AF9 can target a unique fetal B cell progenitor that retains residual myeloid potential but not T cell potential. Despite support from numerous in vitro studies for the existence of a B/Myeloid restricted progenitor (Cumano et al., 1992; Katsura and Kawamoto, 2001; Kawamoto et al., 2000b), it has eluded phenotypic

characterization. In the future it would be interesting, both virally and using mouse models, to model the effect of introducing the MLL-AF9 translocation into fetal B cell progenitors to gain an insight into potential cells of origin of MLL-AF9 lymphoid leukaemias. By specifically targeting B cell progenitors, we can reduce the chance that animals will succumb to myeloid leukaemias before lymphoid leukaemias have the chance to develop.

Chapter VII: Discussion and Conclusions

DISCUSSION AND CONCLUSIONS

Fetal Lympho-Myelopoiesis

By identifying a fetal progenitor with lympho-myeloid but no MkE potential that emerges in the E9.5 YS (chapter III), we provide evidence for the 'alternative' haematopoietic road map, whereby lymphoid progenitors retain myelomonocytic capacity but lose MkE potential in the early embryo, prior to the emergence of dHSCs at E10.5-E11 (Boiers et al., 2013). These progenitors have a comparable lineage restriction to lympho-myeloid progenitors that emerge after the establishment of dHSCs in the E14.5 FL (Mansson et al., 2007) and adult BM (Adolfsson et al., 2005). Furthermore, as childhood and paediatric leukaemias often co-express both lymphoid and myeloid antigens (Bene, 2009), and the initial events leading to disease occur in utero (Greaves and Wiemels, 2003), the E9.5 'fetal LMPP' may be a relevant cell in disease ontogeny.

Whether the MYP cells found in the E12.5 FL (chapter IV) develop from the E9.5 YS fetal LMPP cells or dHSCs that seed the FL at E10.5-11, is unknown. Future experiments using an inducible *Rag1*-cre to specifically fate map cells that express *Rag*-1 between E9.5 and E10.5 would evaluate to what degree YS fetal LMPPs contribute towards the E12.5 MYPs and also whether they make a significant contribution to adult lympho-myelopoiesis, such as the B1 cell and myeloid lineages. As 1% of fetal myeloid cells had detectable YFP expression in *Mb1*-cre $^{tg/wt}$ Rosa26 YFP$^{eYFP/wt}$ animals, fate mapping would help address

whether this is genuine labelling of a fetal progenitor with combined B cell and myeloid cell potential, or alternatively due to 'leakage' of the cre line.

It has been proposed that two waves of B cell development may exist in the embryo, a pre dHSC wave that gives rise to B1 cells, and a post dHSC wave that results in B1 and B2 cells (Montecino-Rodriguez and Dorshkind, 2012). The identification of a lympho-myeloid progenitor reported in this thesis prior to dHSC emergence identifies a progenitor compatible with the pre-dHSC wave of B cell development.

B cell lymphopoiesis in the embryo: the importance of KIT, IL-7R and FLT3 signalling

The fetal LMPP (chapter III), the E12.5 *Mb1* expressing CD19^{-} B cell restricted progenitor (chapter IV), and the CD19^{+} FLT3^{+} Pro B cell (chapter V) characterized in this thesis all share the expression of the cytokine receptors KIT, IL-7Rα and FLT3, highlighting the importance of the cognate lymphoid cytokines in fetal B cell development. We demonstrate that the rare fraction of adult Pro B cells that express FLT3 show an increased ability to generate mature B cells in vivo relative to their FLT3^{-} counterparts, and that E14.5 FLT3^{+} Pro B cells are expanded in FLs that express TEL-AML1. We demonstrate that adult Pro B cells that did not express KIT had much lower propensity to develop into B cells in vitro and in vivo, and had begun to down regulate lymphoid genes involved in somatic recombination suggesting they will not develop a functional B cell receptor required for B cell maturation.

In vitro cultures have long indicated a synergistic effect of KIT, IL-7R and FLT3 signalling on B cell progenitor growth (Lyman and Jacobsen, 1998; Veiby et al., 1996). Additionally mouse models show relatively mild effects on B cell lymphopoiesis in the absence of signalling from one of the aforementioned cytokine receptors, whereas the combined loss of IL-7 and FLT3L results in a complete absence of B1 and B2 cells (Jensen et al., 2008) . Furthermore analysis of a murine TEL-AML1 model indicates FL progenitors demonstrate increased serial re-plating in the presence of the oncogene and all three cytokines, but not IL-7 alone (Schindler et al., 2009). Going forward, work to understand how these receptors elicit their function at the molecular level could lead to important insights and possible therapeutic targets for lymphoid malignancies. FLT3 inhibitors are a promising new therapy in treating AML (Wander et al., 2014) and similar therapies targeting cytokine receptors may also be fruitful in treating ALL (Small, 2008).

The heterogeneous Pre Pro B cell compartment

We and others (Rumfelt et al., 2006a) have demonstrated that the classically defined $Lin^{-}B220^{+}CD43^{+}CD19^{-}CD24^{-}AA4.1^{+}KIT^{+}IL\text{-}7R^{+}$ Pre Pro B cell compartment is heterogeneous and contains non-B lineage progenitors. Furthermore, the global gene expression analysis of B cell progenitors in chapter V showed that classical Pre Pro B cells cluster very closely to upstream IL-7Rα^{+} LMPP cells which retain lymphoid and myeloid cell potential, indicating that at the molecular level Pre Pro B cells and LMPPs are similar. The use of a fate mapping approach based on *Mb1*-cre (chapter IV) identified a sub-fraction of Pre Pro B cells that appear to be entirely B cell lineage restricted and express the

cytokine receptors cKIT, IL-7Rα and FLT3. At E13.5 11.1% of $B220^{+}CD19^{-}$ cells express YFP in *Mb1*-$cre^{tg/+}$ $R26R^{eYFP/+}$ animals (MYPs), suggesting these may be the true B lineage restricted Pre Pro B cells. Furthermore, MYPs appear to represent the earliest B cell lineage progenitor that emerges in the mouse embryo, found in the E12.5 FL one day before cell surface CD19 detection in the E13.5 FL. Whether MYPs include all the B lineage restricted progenitors within the heterogeneous 'Pre Pro B' cell compartment is unknown, but the data indicate that most studies analysing 'Pre Pro B cells' have largely been analysing heterogeneous progenitors, with the true B lineage restricted Pre Pro B cells in a minority. It would be interesting to characterize MYPs using global gene expression analysis to investigate whether they cluster closer to more mature $CD19^{+}$ B cell progenitors at the molecular level, and to thoroughly assess whether they express genes associated with any other lineages. Furthermore, for the MYP to become established it will be important to identify cell surface markers that allow it to be identified in WT mice, without the need for the reporter mice. Finally, it would be interesting to investigate whether the MYP is a relevant cell population for the development of paediatric and childhood leukaemias, and if it is altered in frequency, function and molecularly in the presence of genetic lesions such as TEL-AML1.

B1 cells

The mature blood cell lineages produced during fetal and adult haematopoiesis have different functions. Key characteristics of fetal haematopoiesis include the production of fetal macrophages (Schulz et al., 2012), γδ T cells (Mold et al., 2010), erythrocytes expressing fetal haemoglobin (Brotherton et al., 1979) and,

of relevance to this thesis, the generation of B1 cells (Hayakawa et al., 1985). It is known that fetal B lymphopoiesis generates B cell subsets that function without T cell help as part of the innate immune system, secreting natural IgM and undergoing minimal class switch recombination. These B1 cells have an altered VDJ recombination repertoire and are abundant in the peritoneal cavity (Montecino-Rodriguez and Dorshkind, 2012). Whether B1 cells emerge from a unique fetal progenitor ('layered immune system model'), or are driven into the B1 lineage based on the antigen specificity of the BCR and the developmental age at which they are produced ('selection hypothesis') has been historically controversial (Dorshkind and Montecino-Rodriguez, 2007b).

It has been shown herein and by others, that B1b cells can be generated by adult B cell progenitors in vivo (Hardy and Hayakawa, 1991; Herzenberg, 2000; Kantor et al., 1992), but to date studies have failed to show that adult progenitors can generate B1a cells, indicating B1a cells may have a fetal restricted progenitor.

Studies into the origins of B1 cells have been hindered by the lack of any definitive in vitro assays, coupled with poor definitions in vivo that vary between anatomical locations due to down regulation of cell surface markers such as MAC-1 outside of the coelomic cavities (Baumgarth, 2011). Furthermore, few differences in molecular properties have been characterized between B1 and B2 cells, other than differential preferences for V, D and J segments during somatic recombination. A putative fetal $B220^{low}CD19^{+}$ progenitor that could generate B1 cells but not B2 cells has been proposed (Montecino-Rodriguez et al., 2006) and

multiple studies have now been published based on this progenitor (Esplin et al., 2009a; Ghosn et al., 2011; Tung et al., 2006; Yoshimoto et al., 2011), supporting the 'selection hypothesis' that proposes the two lineages have different ontogeny. However this $B220^{low}CD19^{+}$ progenitor is not fully accepted by the field due to limited in vivo data, making it unconvincing that the 'B1' specified progenitor entirely lacks the ability to generate B2 cells.

Going forward the use of inducible fate mapping to investigate whether the fetal LMPP and early fetal B cell progenitors contribute to the B1a as well as other compartments in the adult, would help elucidate whether progenitors that are entirely B1 cell restricted exist and may help understand if B1 cells provide a relevant cellular context for the development of paediatric and childhood leukaemias.

Switch between adult and fetal lymphopoiesis

The distinction between fetal and adult B cell lymphopoiesis is perhaps highlighted by the temporal bimodal frequencies of leukaemias. Incidence peaks twice, once in 5 year olds then again in the elderly. Genetic lesions are often exclusively associated with either paediatric or adult leukaemias, for example TEL-AML1 is found in paediatric leukaemias but BCR-ABL is detected predominantly in adult leukaemias. This suggests that the cellular context or microenvironment interplay closely with the oncogene in driving disease development (Babovic and Eaves, 2014).

It remains to be shown when precisely the transition between 'fetal' and 'adult' B cell progenitors occurs. Late in embryogenesis haematopoiesis migrates from the fetal liver to the adult bone marrow, and the first B cell progenitors are detected in the fetal bone marrow at E15.5 (Delassus and Cumano, 1996). It would be interesting to sort and characterize the earliest B cell progenitors in fetal bone marrow and ascertain whether at the molecular level they resemble fetal liver or adult BM B cell progenitors. It is unclear whether the large number of genes that we find to be differentially regulated between fetal and adult B cell progenitors is due to differences in developmental age or anatomic location or both.

Whether fetal B cell progenitors gradually transition to adult B cell progenitors, or exist as entirely separate waves of B cell development, developing from either dHSCs in the FL or from progenitors prior to dHSCs such as the fetal LMPP is unknown. Supporters of the specified B1 progenitor (Herzenberg and Tung, 2006; Montecino-Rodriguez et al., 2006) argue that separate B1 cell progenitors exist in the FL and are not generated 'de novo' in the adult BM.

Recently, a molecular mechanism that controls fetal B cell lymphopoiesis has been elucidated by the discovery that the *Lin28b-Let7* micro RNA axis, which mediates the properties of fetal dHSCs (Copley et al., 2013; Yuan et al., 2012), is also active in fetal but not adult Pro B cell progenitors, a finding confirmed by our RNA sequencing data (Li et al., 2015).

The relationship between the B cell and myeloid lineages: a fetal B/M progenitor, bi-phenotypic leukaemias and lineage switching in acute leukaemia

The functional similarities between B and T lymphocytes, highlighted by molecular similarities in their use of VDJ rearrangement enzymes, suggested that these two lineages diverge from a common progenitor late in haematopoietic development. This was supported by the identification of a common lymphoid progenitor (Kondo et al., 1997).

Several features of the B and myeloid cell lineages are surprisingly similar, compatible with divergence occurring as a late step in lineage restriction. Functionally, both B and some myeloid cells bind antigen and perform endocytosis followed by presentation of antigen on the cell surface to T cells via MHC proteins. At the molecular level, the transcription factor PU.1 is involved in the specification of the B, T, myeloid and erythroid lineages (Back et al., 2004; McKercher et al., 1996; Scott et al., 1994). Interestingly, low levels of PU.1 induce B cell differentiation and high levels of PU.1 protein induce myeloid cell differentiation (DeKoter and Singh, 2000; Mak et al., 2011). Transdifferentiation of committed pre B cell lines (Bussmann et al., 2009) and primary $CD19^{+}$ splenic B cells (Xie et al., 2004) into macrophages can be readily induced by C/EBPα, with penetrance reaching 100% in the most efficient transdifferentiation system thus far described. Interestingly the cell line studies (Bussmann et al., 2009) used cells of fetal origin, as the authors found fetal B cells to more efficiently convert to macrophages than their adult counterparts.

We observed that early fetal B cell restricted MYP progenitors showed higher expression of myeloid lineage genes such as *Csf1r* than equivalent populations from the adult or older embryos. Global gene expression analysis performed in chapter V suggested that fetal Pre Pro B and Pro B cell progenitors expressed low levels of myeloid genes such as *Fcgr1, Mac1, Gr-1, Mpo* and *Csf1r*, not seen in the phenotypically identical adult populations. A small number of fetal $CD19^{-}B220^{+}$ cells co-expressed *Mb1*-YFP and the myeloid antigen CSF1R. We found that when culturing E14.5 fetal B cell progenitors on OP9 stroma, more cells developed into B cell clones when the cytokines CSF, GM-CSF, G-CSF and IL-3, which traditionally stimulate myeloid cell growth (Metcalf, 2008), were included in the culture media. Additionally we observed that *Mb1*-cre fate mapping labelled 1% of fetal myeloid cells, but only 0.20% of adult myeloid cells and 0.27% of $CD4^{+}CD8^{+}$ adult T cell progenitors, compatible with, but not proving, more myeloid lineage priming in fetal B cells compared to their adult counterparts. Taken together, these data indicate that fetal B cell progenitors have a more active myeloid lineage programme than their adult counterparts.

Previous in vitro cultures of E12.5 fetal liver progenitors detected bi-potent progenitors but failed to find any evidence of B/T progenitors (Katsura and Kawamoto, 2001). Furthermore this study was limited by failing to prospectively isolate any relevant populations and therefore reliant on one in vitro assay to efficiently permit the development of multiple lineages. In vitro studies have long reported the presence of fetal B/Macrophage progenitors in the E12.5 FL (Cumano et al., 1992) and more recently in the adult BM (Montecino-Rodriguez et al., 2001). However, a follow up study reported that the fetal E12.5

B/Macrophage progenitor also had T cell potential in different assays, so is more likely to be multipotent (Delassus and Cumano, 1996), highlighting how data interpretations should consider the limitations of in vitro assays.

In the disease setting, the close relationship between the B and myeloid lineages is clear. Leukaemias can be bi-phenotypic where blasts co-express cell surface antigens of B and myeloid cells, but rarely the B and T cell lineage. Furthermore, lineage switching between relapse and diagnosis in patients often occurs between B cell ALL and AML, but not usually between the B and T cell ALLs (Dorantes-Acosta and Pelayo, 2012). Studies of healthy and malignant human myeloid cells have demonstrated detectable expression of immunoglobulins, highlighting the relationship between the lineages in humans (Huang et al., 2014). We did not identify a fetal progenitor that is B/M restricted during these studies; however this was not the main focus of our assays. Within the bulk Pre Pro B fraction we found cells to have B and T cell potential in vitro, reflective of the heterogeneity of the Pre Pro B cell compartment.

Problems of understanding fetal cells in vivo: Fate mapping

A major limitation of these studies is the failure to definitively investigate the in vivo potential of rare fetal progenitors, a problem shared by the field (Durkin et al., 2008; Yoder and Hiatt, 1997). We attempted numerous strategies to evaluate in vivo potential, including using *Rag-1* deficient support cells, NSG immune deficient recipients, and newborn recipients in which the microenvironment is closer to the fetal niche. However current transplantation assays are designed for either adult stem cells, which have both an increased self-renewal and output

compared to fetal lymphoid progenitors. The gene expression analysis herein revealed that fetal B cell progenitors, sorted from the E14.5 FL, express lower levels of MHC class I proteins than their adult counterparts. This suggests that upon transplantation into the adult BM, fetal B cells are targeted by cytotoxic T cells (Alberts B, 2002), explaining their failure to engraft. Studies have managed to get early embryo cells to engraft into adult recipients but by first transplanting YS cells into an intermediate niche in the neonatal bone marrow. This approach demonstrated that fetal progenitors can in principle persist and contribute towards adult haematopoiesis (Schmitt et al., 2014).

Studies that managed to obtain successful engraftment of fetal lymphoid progenitors used very large numbers of cells. In addition populations were not so strictly defined by the multiple cell surface markers used in this study and so were likely impure (Li et al., 2015). Secondly studies used recipient mice that lacked both *Rag-1* and *Il-2rγ* chain. The advantage of these animals over NSG mice is that, in addition to lacking B and T lymphocytes, they tolerate the high levels of irradiation needed to ablate the BM niche giving donor cells a competitive advantage (Pearson et al., 2008). Studies have also manipulated fetal cells to express anti-apoptotic proteins such as BCL-2 which may result in non-physiological cell outputs (Nechanitzky et al., 2013).

A strategy that would avoid the caveats above associated with in vivo transplantation of fetal progenitors would be to use inducible fate mapping. As two of the populations studied herein were identified based on reporter expression (*Rag-1* for the fetal LMPP in chapter III and *Mb1* for the MYP B cell

restricted progenitor in chapter IV) we concurrently validated a tool for inducible fate mapping. Inducible fate mapping is obviously reliant on obtaining the appropriate genetic tools and these were unfortunately not available in time for this study. Recently an inducible *Mb1*-cre has become available (Hobeika et al., 2015) and the lab is currently attempting to generate an inducible *Rag1*-cre using CRISPR technology. Using these tools fetal progenitors could be labelled in a specific time window and their contribution towards adult lympho-myelopoiesis traced without artificial irradiation or engraftment related challenges. By breeding mouse models containing leukaemic translocations, the effect of oncogenes on the development of these progenitors could be established. In vivo fate mapping could be used to address the contribution of early fetal progenitors to the B1a compartment in the adult, addressing the controversial area of whether B1a cells develop from a fetal specific B1 progenitor.

One advantage of studying B cell lymphopoiesis is the availability of faithful cre lines, such as the *Mb1*-cre used and validated in Chapter IV. However, *cre* mediated fate mapping is not without limitations. The approach is reliant on the faithfulness of the promoters used to drive *cre* expression. Poor promoters will result in 'leaky' *cre* expression or multi-lineage transcriptional priming in multi-potent progenitors (Hu et al., 1997). Furthermore *cre* mediated toxicity due to the enzymes targeting cryptic loci and causing wide spread genome damage could result in cell death. This is however less of an issue for cre mediated fate mapping and more relevant when *cre* expression is used to delete a gene, as

widespread genotoxic stress due to the cre expression can be mistaken for a phenotype of the deleted gene (Schmidt-Supprian and Rajewsky, 2007).

TEL-AML1 and Disease

Understanding the evolution of TEL-AML1 leukaemia and the cell type in which the initial events occur will be useful in designing treatment therapies. Key questions include in which cell does the initial translocation occur, in what cell(s) do subsequent mutations accumulate, and finally which cell drives and sustains disease and what are the associated molecular mechanisms.

It is known that the TEL-AML1 translocation occurs in utero (Wiemels et al., 1999) in an early fetal B cell or an upstream progenitor. Genetic mouse and zebrafish models of TEL-AML1 suggest that the fusion protein must be expressed early in haematopoietic development to reveal a block in B cell lymphopoiesis. Expression of TEL-AML1 in lymphoid-restricted progenitors does not reveal a phenotype (Sabaawy et al., 2006; Schindler et al., 2009). However, this does not mean that subsequent 'hits' are also accumulated in the same progenitor, and it is possible that the cre lines used to induce expression in later progenitors may have failed to target the earliest B cell progenitors such as MYPs. It has recently been proposed that the TEL-AML1 translocation causes stalling in B cell development when the enzymes RAG1, RAG2 and AID, which are normally segregated in their expression (Hardy and Hayakawa, 2001), are both active (Papaemmanuil et al., 2014; Swaminathan et al., 2015). The partial stalling in lymphopoiesis provides an opportunity for these enzymes to aberrantly introduce the secondary mutations that drive leukaemic development

(Papaemmanuil et al., 2014; Swaminathan et al., 2015). *Rag1* transcripts are detectable as early as the LMPP, at higher levels in the fetus than the adult (Boiers et al., 2013). Therefore the LMPP is a possible progenitor in which secondary mutations can accumulate. However, whether the RAG1 protein is translated and is functionally active at this stage, is unknown.

Nevertheless, the $FLT3^+$ Pro B cell progenitor, identified and characterized herein, has features compatible with a fetal B cell progenitor that is stalled by expression of TEL-AML1, and therefore a candidate cell for the accumulation of secondary mutations. Firstly, $FLT3^+$ Pro B cells are more prevalent in the FL and neonate than the adult BM, a feature that may be linked to the association of TEL-AML1 with childhood but not adult leukaemias. Secondly, $FLT3^+$ Pro B cells express RAG1 and RAG2, the enzymes proposed to drive accumulation of secondary mutations. Unfortunately our global gene expression data did not reveal whether AID was expressed in $FLT3^+$ Pro B cells, and furthermore it would be interesting to investigate if the expression of RAG1, RAG2 and AID is altered in the presence of the TEL-AML1 protein. Thirdly $FLT3^+$ Pro B cells are increased in frequency in E14.5 TEL-AML1 FLs, suggesting perhaps that development may be partially stalled at this time point. In light of finding that the earliest $CD19^+$ Pro B cell is expanded in the presence of the TEL-AML1 translocation, it would be important to investigate whether the $CD19^-$ B cell restricted MYP, which also expresses $FLT3^+$ and emerges just prior to the $FLT3^+$ Pro B cells, has altered frequency, function and gene expression in the presence of the TEL-AML1 oncoprotein.

Loss of a PAX5 allele is one of the most common secondary mutations in TEL-AML1 leukaemias (Mullighan et al., 2007) and as PAX5 is a B lineage specific gene, we can assume that *Pax5* haploinsufficiency only exerts a functional phenotype in B cell progenitors. Therefore I favour the proposal that, despite TEL-AML1 initially occurring in an upstream multi-potent progenitor as supported by genetic models, disease evolves after the accumulation of secondary mutations in an early B cell progenitor that expresses RAG and PAX5, such as the Pro B FLT3$^+$ cell identified herein.

Partly due to the subtle nature of its function, we know very little about how TEL-AML1 subverts and potentially stalls fetal B cell development. Studies have given initial insights and suggested that the oncogene has a myriad of functions such as increasing expression and therefore signalling from the erythropoietin receptor (Torrano et al., 2011), increasing the number of reactive oxygen species promoting mutation accumulation (Kantner et al., 2013), attenuating mitotic check points (Krapf et al., 2010) and altering the response to TGFβ signalling (Ford et al., 2009). However, much remains unclear and further studies are required to understand the mechanism by which TEL-AML1 establishes pre-leukaemic clones.

In continuing investigations, the TEL-AML1 mouse line is being backcrossed to C57 Bl/6 to minimize genetic variation and permit whole genome expression analysis of E14.5 FL Pro B cells in order to gain an insight into altered molecular pathways in the presence of the TEL-AML1 lesion.

Modelling Disease in Mouse

In this thesis we have used the mouse to characterize fetal B cell lymphopoiesis in the WT and leukaemic setting. The use of the mouse to study haematopoiesis has historically provided major breakthroughs in the field, from understanding lineage restriction to the development of the cancer stem cell hypothesis (Bonnet and Dick, 1997). The availability of tissues and the ability to introduce mutations in a spatio-temporal controlled fashion are major advantages of the mouse over human studies. However, mouse models are not without limitations. Importantly, mice do not readily develop B cell ALLs, either spontaneously or with the aid of genetic or random mutagenesis, limiting disease modelling (Hauer et al., 2014). Mouse genetic models attempt to introduce a relevant mutation into a specific progenitor cell type. However, unlike in the human physiological where the fusion protein initially develops in a single cell (Greaves et al., 2003), most mouse models artificially target many cells at various stages of lineage commitment, generating a non-physiological environment for disease evolution.

Few B cell leukaemias have been faithfully modelled in the mouse, but we would propose that this is most likely reflective of the complex nature of the disease, rather than the insufficiency of the model organism. Models should take into account targeting the genetic lesion to the correct cell type, at the correct stage of development, and the accumulation of secondary mutations in an appropriate order. It therefore seems that rather than the mouse not being a useful tool, the approaches used to model B cell ALL have not reflected the complex aetiology of leukaemia.

Relevance of studies to the human

To what degree early human B cells that express FLT3 resemble mouse populations is not clear. Phenotypically comparable subsets exist but it remains to be shown whether they represent equivalent populations and whether the cells are candidates for the accumulation of pre-leukaemic events. Recently studies have begun to find human lymphoid progenitors with equivalent lineage potentials to populations characterized in the mouse. A progenitor that had lymphoid and monocytic, but not erythroid potential has been characterized in human bone marrow, supporting the lympho-myeloid pathway of haematopoietic development (Doulatov et al., 2010; Goardon et al., 2011; Kohn et al., 2012), although in contrast to the mouse LMPP, this progenitor marked by L-Selectin and CD10 (Kohn et al., 2012) and or CD45RA (Doulatov et al., 2010; Goardon et al., 2011) expression, does not have granulocyte potential (Kohn et al., 2012). Granulocytes are short lived and it is possible that the assays used by Kohn et al. did not investigate the correct kinetic time points to reveal granulocytic potential. Analysis of $CD34^{+}$ AML patient samples identified a separate $CD10^{-}$ LMPP-like cell with lymphoid and myeloid, including granulocytic potential, that approximates to the murine LMPP and functions as a leukaemic stem cell-like population to drive disease (Goardon et al., 2011). Studies in this lab have looked to identify early B cell restricted progenitors in human cord blood samples and early data suggest that a majority of fetal Pro B cells, in contrast to adult Pro B cells, expresses the cytokine receptors *KIT* and *IL-7Rα,* and approximately 20% of human fetal Pro B cells express *FLT3* (Charlotta Böiers, unpublished observations). Therefore some degree of caution must be

taken when applying data from the mouse to gain useful insights into human diseases.

Despite the potential discovery of functionally analogous lymphoid progenitors in the human, uncertainties still remain about the relevance of murine B cell lymphopoiesis to humans. Firstly, patients with Severe Combined Immunodeficiency Disease (SCID), due to a mutation in IL-7R pathway, have greatly diminished T cell numbers, but a relatively normal B cell compartment (Puel and Leonard, 2000). However mice with disrupted IL-7 signalling show total loss of $CD19^+$ progenitor cells in the adult BM (Carvalho et al., 2001; Miller et al., 2002b). The BM B cell progenitors in SCID patients have not been analysed in detail and it is possible that similar to what is seen in the mouse, long-lived mature B cells generated during fetal haematopoiesis persist, but BM B cell progenitors are absent or severely reduced. Secondly, attempts to identify B1 cells in humans have been controversial. Investigations have been limited as B1 cells are largely functionally defined and are poorly defined in vitro, which is the major approach used to characterize human haematopoietic progenitors. Recently one study claimed to identify a B1 cell population in human cord blood (Griffin et al., 2011b) but this has been challenged by groups suggesting that the gating strategy was flawed and the authors have unwittingly analysed MZ cells, memory B cells and T cells (Descatoire et al., 2011; Griffin et al., 2011a; Perez-Andres et al., 2011).

Conclusions: Insights into fetal B cell lymphopoiesis

The earliest immune restricted progenitor with combined B cell, T and myeloid cell potential, emerges in the vascular plexus of E9.5 YS, prior to the emergence of dHSCS.

One day prior to the detection of cell surface CD19 in the E13.5 FL, a B cell restricted progenitor emerges in the FL, characterized as $CD45^{+}B220^{+}CD19^{-}$*Mb1*-$YFP^{+}KIT^{+}$IL-$7R\alpha^{+}FLT3^{+}$ (MYPs).

The MYP defines a sub fraction of the heterogeneous Pre Pro B cell compartment that is truly B cell lineage restricted.

A novel Pro B cell fraction that expresses FLT3 is prevalent in late embryogenesis (Pro B $FLT3^{+}$). Pro B $FLT3^{+}$ cells from the adult bone marrow demonstrate an increased capacity to generate mature B cells in vivo compared to Pro B $FLT3^{-}$ cells.

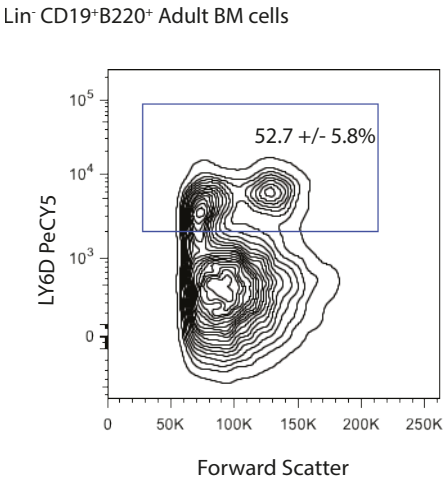
Pro B $FLT3^{+}$ cells that express the TEL-AML1 oncoprotein are expanded in frequency in E14.5 FLs. *Rag1*, which is thought to drive the accumulation of secondary mutations in TEL-AML1 leukaemia, is expressed by Pro B $FLT3^{+}$ cells indicating that these cells may be a relevant cell in leukaemia development.

Appendix

Appendix

Appendix 1

Percentage of adult BM Lin$^-$CD19$^+$B220$^+$ cells that express LY6D on the cell surface. 7 week old adult BM. Average of 5 mice. Mean ± SEM

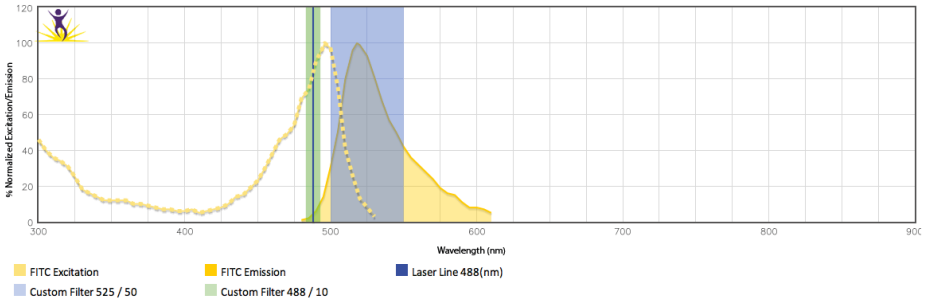


Appendix 2

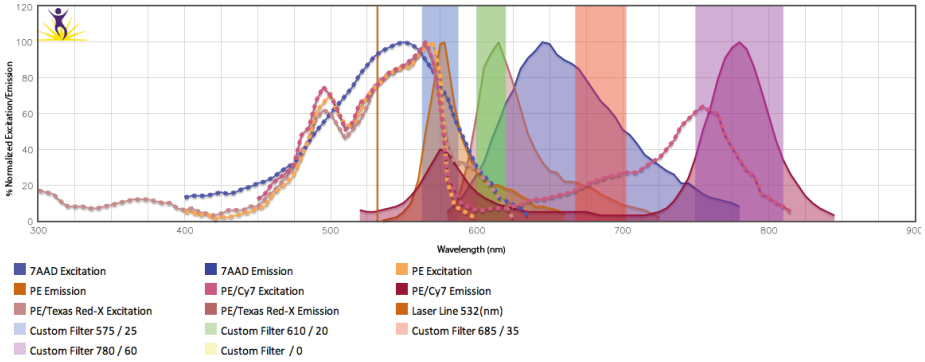
Spectral panels for each of red, green, blue and violet laser for the 10 colour pre pro B cell panel. Made using BioLegend spectral analysis tool.

Appendix: 10 colour panel spectral overlap

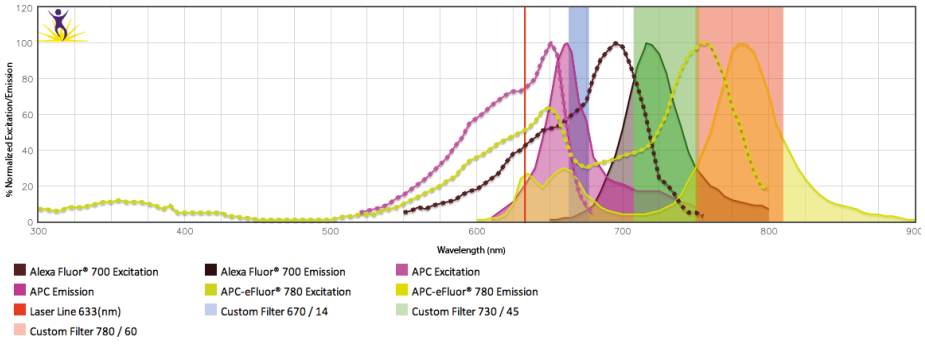
Blue Laser 488nm



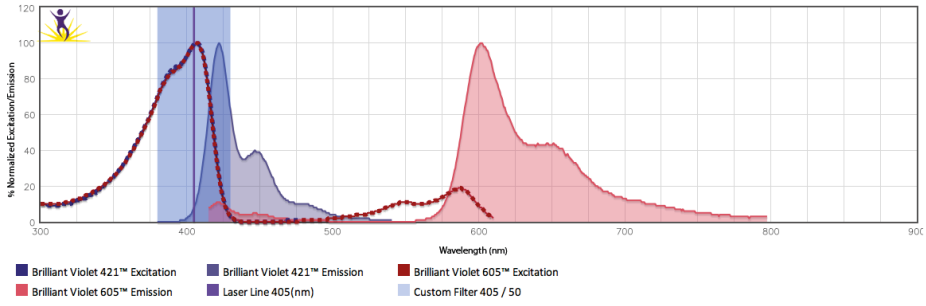
Green Laser 532nm



Red Laser 638nm



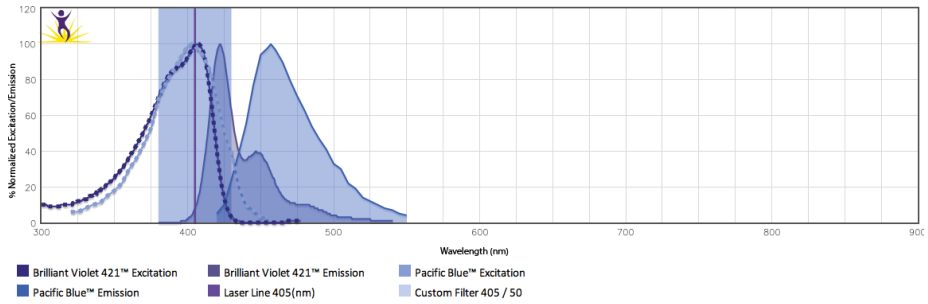
Violet Laser 405nm



Appendix 3

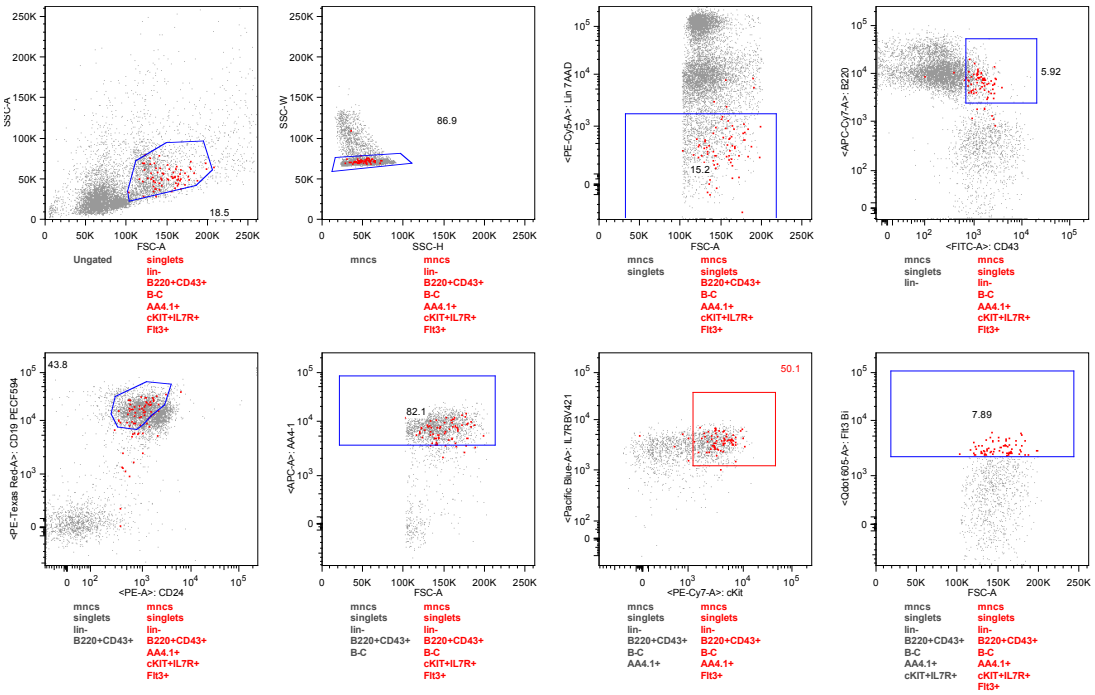
Comparison of the emission spectra of Brilliant Violet 421 and Pacific Blue. Made using BioLegend spectral analysis tool.

Appendix
BV421 vs PB



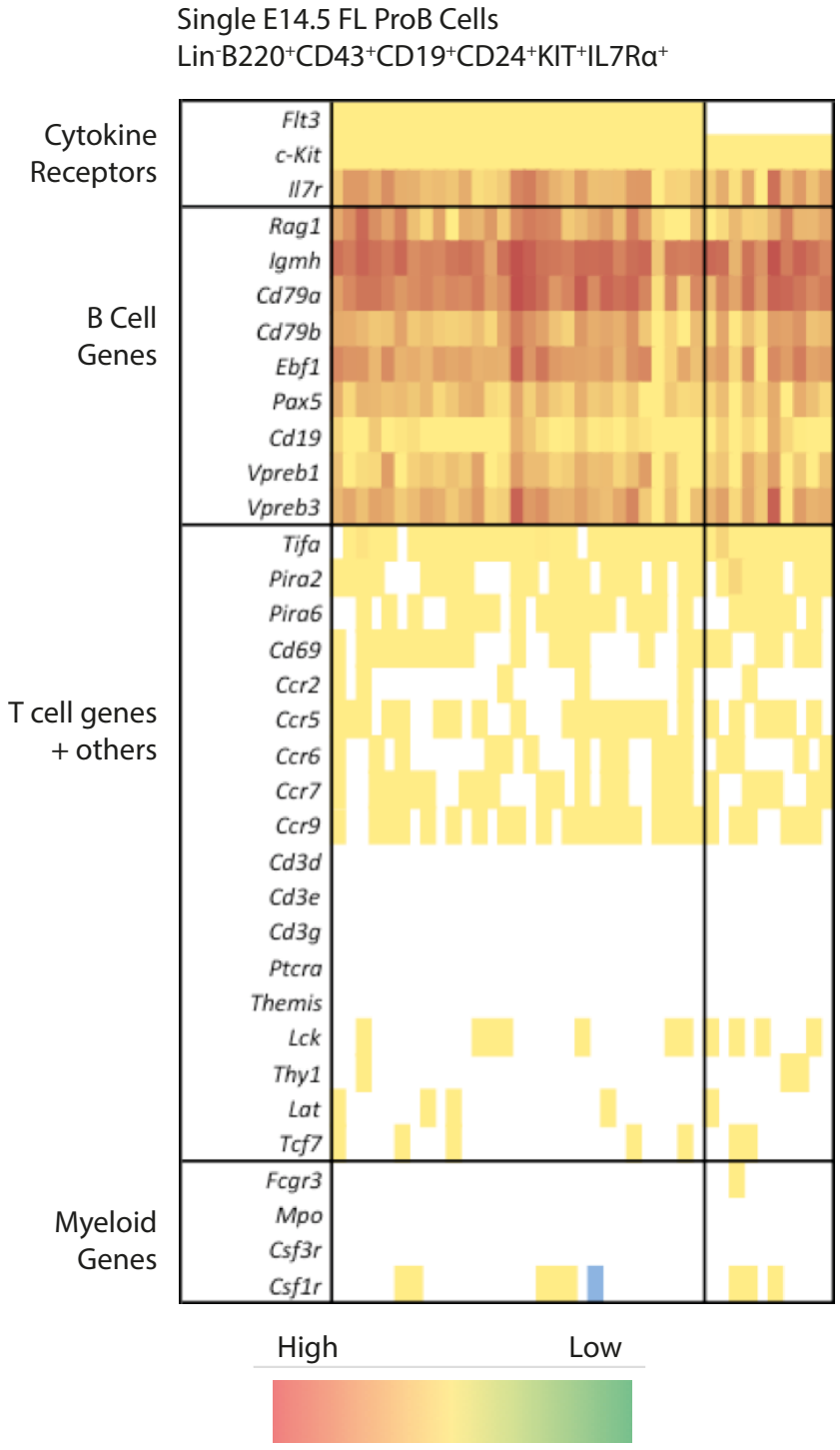
Appendix 4

Back gating analysis of BM FLT3+ Pro B cells showing unanimous expression of IL-7R and cKIT



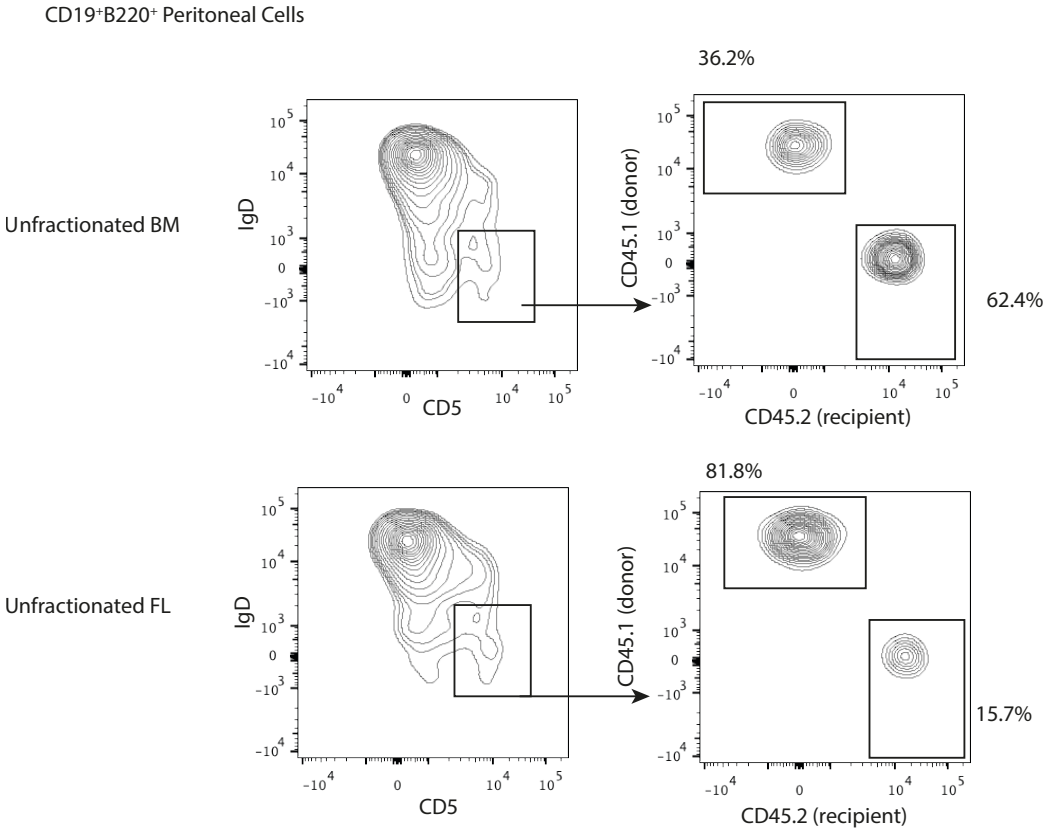
Appendix 5

Quantitative gene expression analysis of single Lin$^-$CD43$^+$B220$^+$CD19$^-$ CD24$^-$ AA4.1$^+$cKIT$^+$IL-7Rα$^+$ Pro B cells from the E14.5 FL. Expression of genes are shown as Δ Ct values, each row represents a single cell (39 cells).



Appendix 6

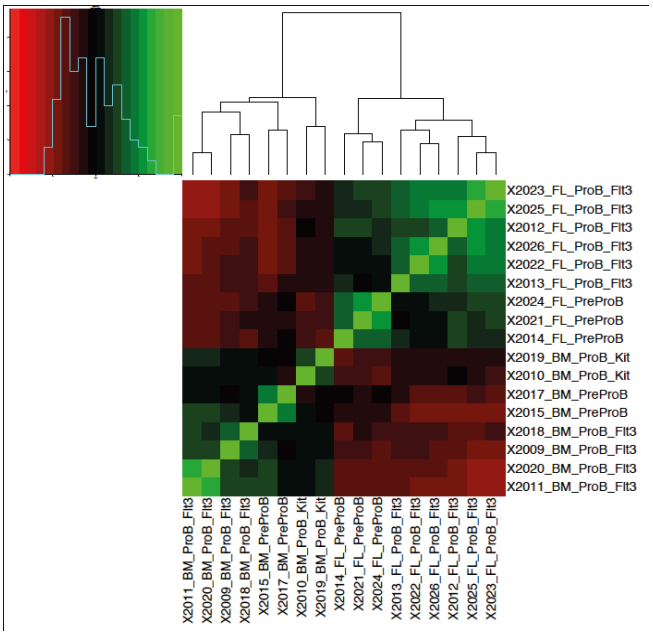
As a control to demonstrate detection of $CD5^+$ B1a cells, 200,000 unfractionated FL or BM cells were transplanted into lethally irradiated recipient mice and the peritoneal cavity analysed after 4 weeks. Percentages are shown of the total $CD19^+B220^+CD5^+IgD^{low}$ cells. One mouse analysed per group.



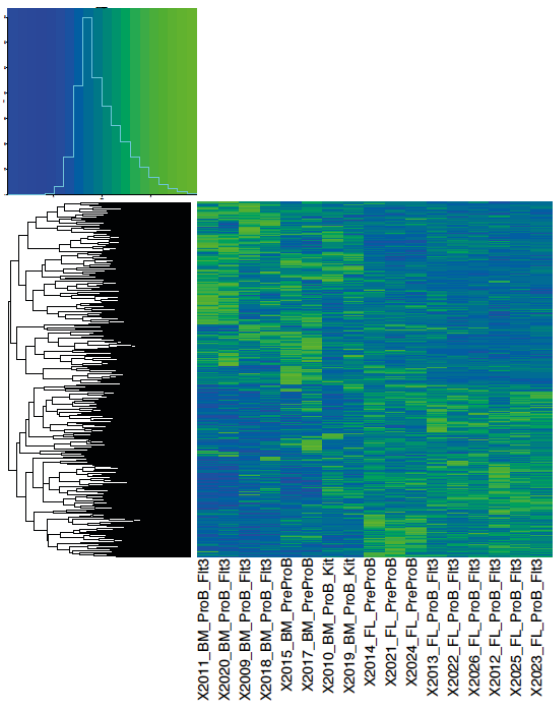
Appendix 7

Matched heat maps on the aggregate differentially expressed genes from individual comparisons between B cell populations (fetal and adult, Pre Pro B cells, FLT3^{+} Pro B cells, FLT3^{-} Pro B cells and KIT^{-} Pro B cells – same populations as defined in figure 5.17). The top plot is a heat map of correlation between samples. The bottom heat map displays, per gene normalised, counts per million values. The columns, representing individual samples, are in identical order in both plots and are arranged according to the hierarchical clustering of the top plot.

A



B



Appendix 8

Global gene expression comparison of fetal and adult B cell progenitors using DiSeq2 – genes whose adjusted P value reaches statistical significance.

Gene	Log Fold Change	P value	Gene	Log Fold Change	P value
Dntt	-10.03210884	5.74E-146	*Lrif1*	-1.019486263	0.008362678
Lin28b	7.628415676	8.32E-93	*Cdkn1b*	-1.21617853	0.008362678
Igf2bp1	5.86637432	8.61E-71	*Emd*	-1.029158491	0.008433075
Fstl1	6.332504361	7.06E-52	*Cep57l1*	-0.935153352	0.008492603
H19	6.757137657	7.85E-47	*Cnot2*	-0.587963336	0.008492603
H2-K1	-3.493501311	2.49E-42	*Mrpl38*	1.016259517	0.008492603
B2m	-2.326949066	1.30E-40	*Foxn2*	-0.77830545	0.008492603
H2-D1	-3.025531366	1.23E-36	*Ptplad1*	0.912448353	0.008492603
Ifi44	-5.679620499	2.77E-28	*Whsc1*	-0.723230526	0.008649445
Tspan13	-1.762759316	6.28E-18	*Tmppe*	-1.163002052	0.009155594
Ckb	4.524128642	5.69E-17	*Sema4b*	1.019268166	0.009283968
Yes1	4.307622227	4.77E-16	*Ankrd37*	1.882128296	0.009283968
Klk8	2.320000448	4.60E-15	*Herpud1*	-0.978754022	0.009283968
Fscn1	4.25756543	2.54E-14	*Uba1*	0.622469532	0.009283968
H2-T10	-4.205566529	2.72E-14	*Cdc42bpb*	1.875683406	0.009420769
Pyhin1	-2.363069287	4.90E-14	*Cstf2*	-0.7558201	0.009832651
Chd3	-4.078730925	1.78E-13	*Nfia*	-1.371543612	0.009912748
Nes	3.964702358	7.15E-13	*Ezr*	-0.771463738	0.010105308
Rps4y2	3.161561017	1.90E-12	*Anks1*	-0.876966186	0.010221602
Myo1b	3.877518609	6.05E-12	*Hspa4*	0.792322684	0.010334686
Srgn	-1.598194732	6.05E-12	*Kifc1*	1.445314929	0.010334686
Ndrg1	-3.966801417	9.40E-12	*Rhag*	1.843547621	0.010334686
Gpc3	3.597183395	1.09E-11	*Pias1*	-0.70693624	0.010334686
Dpp4	-2.353125235	1.57E-11	*Eif4g1*	0.963548142	0.010348959
Ulbp1	-3.03168547	1.61E-11	*Snai3*	1.847277699	0.010348959
Fjx1	3.810787033	2.59E-11	*Ly6e*	-0.876713421	0.010384426
Acp6	-2.428365288	1.09E-10	*Ulk2*	-1.224018921	0.010449788
Trim16	-3.497723303	5.24E-10	*9530077C05Rik*	-1.759456892	0.010491981
AW112010	-3.481307558	3.80E-09	*Trim30a*	-1.323303791	0.010590935
Nr3c1	-2.507541884	4.68E-09	*Tspyl1*	-0.85693907	0.010798987
Tifa	-2.014070944	4.68E-09	*Slc44a2*	-1.028912138	0.010919746
Dlk1	3.405920718	5.61E-09	*Mt2*	1.847196512	0.010975081
Myl10	-3.110662292	7.67E-09	*Scp2*	-0.70141817	0.011027356
Snn	-1.871541944	8.60E-09	*Zfp59*	1.786088647	0.0110626
Mboat1	3.379474674	1.11E-08	*Gnb4*	-1.499387356	0.011177303
H2-Ob	-2.448187018	1.31E-08	*Frmd6*	-1.652318009	0.011508473
Psmb9	-1.726799647	1.80E-08	*Morn2*	-0.928773733	0.01152598

Cdkn2c	-2.370718272	2.41E-08	*Wdr43*	0.733129684	0.011841064
Hspa2	-2.208056169	3.43E-08	*Hjurp*	-1.147073591	0.011883569
Tnfsf10	-3.015995617	3.43E-08	*Dstn*	0.955293968	0.01210947
Acsl4	-1.355246131	3.56E-08	*Lrpprc*	0.931168658	0.012248384
Bmpr1a	3.215211455	4.57E-08	*Mylk*	-1.843322707	0.012314151
Spry2	-1.523839886	5.78E-08	*Abce1*	0.66710671	0.012359923
Mcts2	3.065909483	5.98E-08	*Dnahc8*	-1.731925355	0.012579686
Ifi27l2a	-2.476209482	6.63E-08	*Cdc25b*	-1.391772369	0.012645418
Tcf19	-1.623051649	6.63E-08	*Asb13*	-1.769549471	0.012651191
Ankrd33b	-3.244390221	7.17E-08	*Cnn1*	-1.823690739	0.012685514
Cdc42ep3	-1.783693857	1.04E-07	*Aatf*	1.280059898	0.013048222
AB124611	-2.399877364	1.04E-07	*Hspa5*	0.780620571	0.013170984
Plaur	3.225363832	1.36E-07	*Arhgap15*	-0.705259325	0.013170984
Cpne2	-2.848486363	1.84E-07	*Smarce1*	0.546916059	0.013172952
Tnni2	2.942279754	2.03E-07	*Alpk1*	-1.8236903	0.013172952
Ifi47	-2.093623188	2.82E-07	*Capg*	1.158698399	0.013172952
2610305D13Rik	3.108878354	3.14E-07	*Dek*	-0.701292257	0.013196727
Acadl	-1.822893636	3.52E-07	*Ermap*	1.795023095	0.013196727
Tram1	-0.951721845	3.93E-07	*Zfp592*	-0.799379538	0.013196727
Cdkn2d	-1.602685828	4.05E-07	*Gadd45gip1*	1.244733182	0.013196727
Adat3	3.039396549	4.27E-07	*Lta*	1.822845585	0.013257088
Ndn	3.11242332	4.51E-07	*Zc3h6*	-1.728926939	0.013257088
Tmem107	-1.224400043	4.58E-07	*Itpr2*	-1.706938904	0.013273336
Zfp593	1.52209863	4.68E-07	*Gm5088*	1.368156481	0.013286913
Mier1	-1.027366288	4.94E-07	*Ankrd13a*	-0.889657236	0.013498857
Rasa4	-2.348585211	5.75E-07	*9130206I24Rik*	-1.822946629	0.013559494
Hipk1	-1.157086769	6.97E-07	*Trim28*	0.637739129	0.013560926
Scd2	1.913269932	8.18E-07	*Pdcd4*	-0.9491001	0.01375457
Fgd5	3.005866136	9.23E-07	*Pola1*	-0.662703073	0.013916581
AI597468	-1.525279479	1.18E-06	*Khdrbs3*	-1.685489644	0.014077014
2700089E24Rik	-0.990880009	1.34E-06	*Dnase2a*	-1.359239793	0.014077014
Tmem134	-1.00629138	1.39E-06	*Eif3g*	0.679148938	0.014077014
Ypel5	-1.443928228	1.46E-06	*Gng12*	-1.301595156	0.014113491
Hmga2	2.970924426	1.63E-06	*Pla2g16*	-1.773730081	0.014188158
1190002F15Rik	-1.515480695	1.81E-06	*Ezh2*	-0.631523653	0.014188158
Crip1	-1.334442193	1.90E-06	*Snora68*	0.831147435	0.014188158
Etnk1	-1.121297445	2.06E-06	*Rrp9*	1.509002503	0.014188158
Rtkn	2.964254411	2.11E-06	*3110052M02Rik*	-1.509904921	0.014531553
Igf2bp3	2.354674451	2.41E-06	*Slc25a16*	-1.385711131	0.01457272
Marcksl1	1.134208872	2.47E-06	*Ankmy2*	-0.806986698	0.01458247
Snord52	1.32884618	2.77E-06	*Ccnd3*	-1.061806939	0.01458247
Xaf1	-1.820799808	2.81E-06	*Pdia3*	0.604847709	0.01458247
Rell1	-2.045472434	2.81E-06	*Csf1*	1.781680137	0.01458247
4931429I11Rik	2.914881483	3.43E-06	*Spata6*	-1.470389243	0.01458247
Lsp1	1.922020269	3.51E-06	*Anp32a*	0.722070097	0.01458247

Endod1	-2.109935594	3.57E-06	*Spn*	-1.040453204	0.014602951
Ppp3cc	-1.866554118	3.84E-06	*Snrpa*	0.743183509	0.014801166
Insl6	-2.861011405	5.54E-06	*Bbs2*	1.803062938	0.014801166
Sipa1l1	-1.922011517	5.92E-06	*Atp4a*	1.798039601	0.014879887
Tshz1	-1.336722227	6.08E-06	*Tfdp2*	-0.757153083	0.015029598
Tmem179b	-0.880647654	6.37E-06	*Bbx*	-1.107889569	0.015063799
H2afv	-1.017635889	7.32E-06	*Mir17hg*	0.927391877	0.015156142
Slain1	-2.459329523	9.60E-06	*Cdkn3*	-1.071976571	0.01516627
Ptk7	2.73562671	9.60E-06	*Cdca4*	-0.625887805	0.0153177
Klhl6	-1.421611992	1.10E-05	*Slc43a3*	-0.749885208	0.0153177
Cd97	-2.252288038	1.30E-05	*Slc25a40*	-1.57259889	0.015352349
Morn4	2.768984612	1.41E-05	*Clk4*	-0.831106854	0.015419287
H2-Q4	-2.741575837	1.79E-05	*Mzt1*	-0.702574288	0.01549208
Epg5	-1.825222401	1.88E-05	*St6gal1*	-1.28229769	0.01549208
Trmt1	1.141947027	1.91E-05	*Pisd-ps1*	-1.228663744	0.015610228
Calcrl	-1.497976267	1.92E-05	*Trim12a*	-1.344531517	0.015739244
Tmem131	-1.333968933	2.56E-05	*Adrb2*	1.783273459	0.016070255
Cux1	-1.292057642	2.68E-05	*Mki67ip*	0.780840591	0.016404277
Pitrm1	0.879817222	2.70E-05	*Tmpo*	-0.660129369	0.016404277
Mpp1	-1.145174931	3.33E-05	*Tspan8*	1.742166901	0.016404277
Dlg2	-2.425444796	3.48E-05	*Hvcn1*	-1.757918902	0.016404277
Arxes2	2.679312813	3.52E-05	*Lonrf3*	-1.722420531	0.016404277
Blk	-1.96734569	3.64E-05	*Msra*	-1.023253948	0.016668553
C1galt1c1	-1.215871577	3.64E-05	*Tnfrsf18*	-1.744321869	0.016704073
Kcna3	-1.883092434	3.71E-05	*2010315B03Rik*	-1.401824288	0.016704073
Ucp2	-1.257027352	3.72E-05	*Glrx5*	0.776005814	0.01708113
Hilpda	-1.404357324	5.05E-05	*S100a13*	-0.715871663	0.01708113
Stac2	2.544579724	5.09E-05	*AI504432*	-1.274863139	0.017330077
Rrp1b	1.450469105	5.23E-05	*Atp8a1*	-1.04492458	0.017330077
Epb4.1l4b	-1.76016817	5.28E-05	*Reep3*	-1.000345733	0.017450084
Klra1	-2.621257101	5.46E-05	*Rnf5*	0.555742374	0.017450084
Cenpl	-1.051756138	5.79E-05	*Rbm42*	0.965881746	0.017450084
2210404O07Rik	-1.631376075	5.98E-05	*Zfp560*	-0.687286028	0.017450084
Fam129c	-1.684760396	6.37E-05	*Samm50*	1.002852639	0.017726489
Osbpl8	-1.433334407	6.65E-05	*Polr1a*	0.984185257	0.017726489
Chd7	1.838604	6.66E-05	*Marcks*	-0.944611875	0.017731478
Tap1	-2.007674376	7.22E-05	*Cklf*	-0.721639545	0.017777193
Dnajc6	-2.583747972	7.43E-05	*Gngt2*	-0.961399576	0.017844234
Pag1	-1.531432212	7.88E-05	*Ppp1r13l*	1.733658257	0.017909896
Zeb1	-1.301904503	8.45E-05	*Ddx18*	0.852773096	0.018087592
Tapt1	-1.33668149	9.43E-05	*Cep95*	-1.005909975	0.018087592
Aldh18a1	1.275336511	1.00E-04	*Elac1*	1.759042875	0.018087592
4930523C07Rik	-1.254448061	0.000103883	*Chchd4*	1.06867941	0.018250181
Gstt2	-1.524119776	0.000108374	*Selrc1*	0.992571521	0.018404256
Crispld2	2.5454831	0.000109246	*Ppat*	0.762990494	0.018404256

4933439C10Rik	-1.367388286	0.000113456	*Zfp825*	-1.249587642	0.018549915
Cad	1.196578309	0.000115513	*Ttpal*	-0.940173098	0.018549915
Evc2	2.537277334	0.000115513	*Uvssa*	-1.020881177	0.018549915
Tspan32	-1.255072861	0.000117476	*Usp4*	0.499416391	0.018549915
Nlrc5	-2.527030721	0.00012497	*Adnp2*	-1.286547445	0.018583757
Dap	-1.389873384	0.000160151	*Cdan1*	-0.890058363	0.018923915
Beta-s	2.482456532	0.000186526	*Cggbp1*	-0.691085038	0.019111179
Lgr5	-2.454031846	0.000207221	*Ruvbl2*	0.706984837	0.019276667
Sparc	2.468032928	0.000210506	*C330007P06Rik*	-0.838215467	0.019282284
Zfp367	-1.250768372	0.000211227	*Ubtd2*	1.614534925	0.019284237
Nkain1	2.467465401	0.000211227	*Cenpk*	-0.776943004	0.019633502
Purb	-1.063363012	0.000223815	*Wdr77*	0.923915502	0.019633502
Slc30a4	-2.052552559	0.000228465	*Sdcbp*	-0.659958982	0.01966762
Uimc1	-0.869111333	0.000235299	*Got1*	1.42288607	0.019692107
Osbpl9	-1.278846436	0.000235299	*Utp11l*	0.693373484	0.01975494
Neo1	2.451244697	0.000235299	*Sh3bgrl3*	-0.418528034	0.019797232
Lgals9	-1.474832184	0.000241394	*2310008H09Rik*	0.921063135	0.020143271
Zfp36	-1.431502034	0.000241394	*Spcs1*	-0.495165544	0.020333695
Rpl27a	0.854848658	0.000241394	*Rpl39l*	1.734572146	0.020462635
Lhpp	-1.916262981	0.000241394	*Tbc1d2*	1.712377475	0.0205326
Cntln	-2.055514305	0.000271141	*Ids*	-1.543524063	0.020605368
Cpt1a	-2.270515008	0.000285663	*Sh3pxd2b*	1.719386011	0.021197757
Dnmt3b	1.622823095	0.000294224	*Nucb1*	-0.66696919	0.021643064
Ankrd12	-1.494275312	0.000307343	*Btrc*	-1.119032535	0.021739952
Pck2	-1.244015434	0.000317967	*D930015E06Rik*	-0.591119722	0.021923906
Gm6525	-2.321685729	0.000320984	*Map1lc3b*	-0.668624474	0.022032787
Btg1	-1.161066257	0.000359792	*Slc25a13*	0.927849815	0.02205361
B3gnt5	-2.17876386	0.000360663	*Ninj1*	-1.440711365	0.022157624
Polr1e	1.547557682	0.00038889	*Rnf144a*	-1.247743709	0.022394675
Zfp111	-2.237496384	0.00038911	*9430076G02Rik*	1.681775656	0.022394675
Bex2	2.38278179	0.000391466	*Enoph1*	0.894776844	0.022464247
Kdm5b	1.459999334	0.000401022	*Whsc1l1*	-0.743556103	0.022464247
Aff3	-1.282007335	0.000405843	*Fam196a*	1.680869432	0.023417104
Cd52	-0.889255715	0.000424022	*Snhg6*	0.805748596	0.023514066
Lynx1	-2.356845774	0.000424933	*Gar1*	0.951738891	0.023514066
Arhgap28	2.371131948	0.000424933	*Fbxo48*	-1.53628825	0.023685397
Slc12a6	-0.943683642	0.000424933	*Pde4d*	-1.149405925	0.023704101
Fabp5	1.177164347	0.000424933	*Igf2bp2*	1.603821455	0.023977575
Cdh17	2.362766138	0.000424933	*Oasl1*	-1.684602958	0.023977575
Mxd3	-1.53014691	0.000435944	*Gprasp2*	1.70346964	0.023977575
Pdzd8	-0.689060313	0.000439088	*Impdh1*	-1.068551934	0.023999279
Smim1	2.361025221	0.000439088	*Galnt10*	1.631066502	0.024008538
Zfp385a	2.114954705	0.000452685	*Epb4.9*	1.679210226	0.024008538
Ric3	2.356426412	0.000468456	*Carhsp1*	-0.938087342	0.024008538
Thy1	-2.315760235	0.000485592	*Msh5*	-1.042652825	0.024008538

Shisa5	-1.72503183	0.000519026	*Elmo3*	1.706870146	0.024008538
Phyh	-1.257650902	0.000519528	*Fli1*	-0.651991989	0.024008538
Mbnl3	-1.080030389	0.000527911	*Ltv1*	0.871527542	0.024035249
Otud1	-1.870283644	0.000543607	*Morf4l2*	0.489520887	0.02420593
Egfl7	1.277719243	0.000543607	*Gypa*	1.65309139	0.024341147
Ube2l6	-2.014481459	0.000543607	*Gspt2*	1.701102749	0.024398319
Fam63b	-1.611598896	0.000543607	*Mtx2*	1.121498984	0.024573108
Nfil3	2.316763376	0.000613134	*Snx10*	-1.196704529	0.024573108
Pqlc3	-1.787352284	0.000628077	*Rheb*	-0.598368645	0.025113003
Selk	-0.801337463	0.000628924	*Phf3*	-0.70107244	0.025184294
Asf1b	-1.127769556	0.000661516	*Eftud2*	0.752495893	0.025301506
Ccdc82	-1.366619006	0.000668048	*Eif3a*	0.510506979	0.025305369
Hsph1	1.097823783	0.000681028	*Zfp212*	1.562158925	0.025654301
Mdfic	-2.299389248	0.000710351	*Ddb1*	0.651177287	0.025725703
Git2	-1.229164965	0.000713617	*Ngfrap1*	-0.617238412	0.025785947
Gpr12	-2.275293034	0.00074468	*Trim34a*	-1.335996852	0.026018502
1110032A03Rik	-1.470875787	0.000771795	*Zc3h7a*	-0.710884217	0.026297206
Eps15	-0.825846222	0.000783177	*Gtpbp2*	-1.345838171	0.026727662
Slc4a1	2.276851098	0.000795644	*Itm2b*	-0.774310854	0.027300774
Nat10	1.72241693	0.000843651	*Ugcg*	0.837945147	0.0277969
Ccdc135	-1.35987026	0.000870094	*Tmem126a*	0.733685408	0.028097208
Tgtp2	-2.261537387	0.000875391	*Papolg*	-0.869479381	0.02822193
Tapbp	-1.019836976	0.000875391	*Rbmx*	-0.612258262	0.02822193
Skil	-1.282722288	0.000881438	*Ikzf5*	-0.952773379	0.028255524
Entpd5	-1.413213961	0.000930198	*Ino80c*	-0.714622602	0.028506589
H2-M3	-1.919281653	0.000930198	*Tbc1d12*	1.667194025	0.028506589
Prkd3	-1.00513683	0.000930198	*Rbl1*	-0.826987503	0.028529254
5430417L22Rik	-1.214624881	0.000930198	*Gsk3b*	-0.484442014	0.028982109
Sapcd2	-0.889036893	0.000933137	*Farsa*	0.713399063	0.028982109
Idh2	0.956640387	0.000933825	*Mospd3*	-1.019200963	0.029071229
Litaf	-1.032552918	0.00096613	*Mndal*	-0.828028314	0.029298359
Bach2	-1.495694674	0.000994987	*Cenpm*	-0.566217328	0.029298359
Cib1	0.855475165	0.000997866	*Sorcs2*	1.398916535	0.029435445
Kit	1.462912571	0.000999515	*Lats2*	-1.351366151	0.029482334
Kat2b	-1.453163217	0.00100635	*Klf10*	-0.968451471	0.029482334
Snx30	-1.299900793	0.001028137	*Dyrk1a*	-0.568466673	0.029482334
Ankrd44	-1.252096327	0.001048201	*Trappc6b*	-0.847795276	0.029535087
Arid3a	1.92033045	0.00106932	*Cenpq*	-0.740995743	0.029535087
Cul7	2.050085393	0.001159819	*Gm11602*	-1.275690569	0.029561136
Blnk	-1.64741059	0.001196168	*Fgfbp3*	1.663063106	0.029561136
Ralgps2	1.578877406	0.001208726	*E230008N13Rik*	1.659316544	0.02956316
Jup	1.946747047	0.001208726	*Echdc2*	1.629749765	0.02956316
Ing1	-0.952555593	0.001208726	*Robo3*	-1.401140758	0.02956316
Pus7l	1.834426802	0.001311986	*Rab43*	-1.27639681	0.029684085
Map3k1	-0.92595476	0.001318464	*Aldoa*	1.048930231	0.029703449

Cd53	-0.889779577	0.001375942	*H2-T23*	-1.09880185	0.029973432
Bola1	0.753765515	0.00146086	*Ecscr*	1.606699591	0.030064945
Rrp15	1.251935342	0.001494564	*Klhdc5*	-0.968095508	0.030064945
Sorbs3	-2.191673562	0.001494564	*Cep57*	-0.573554323	0.0305577
Cdip1	-1.001544196	0.001494564	*Dse*	1.599668975	0.030696724
Dennd5b	-2.053727588	0.001508243	*Mbd3*	0.975788465	0.030696724
Lmtk2	-1.280702832	0.001525931	*Thop1*	1.295719151	0.030696724
E2f7	-1.412244436	0.001613267	*Bop1*	1.061627706	0.030696724
Casp4	-2.174630001	0.001641215	*Hivep3*	-1.633266129	0.030696724
Larp1b	-1.909779464	0.001646496	*Tmem9b*	-1.112127505	0.030696724
Fundc2	-0.736253058	0.001670491	*Art1*	1.625827445	0.030903969
Ccnj	1.240898871	0.001733656	*Polr1c*	0.723045121	0.030988665
Pcgf5	-1.282271607	0.001743006	*Cct6a*	0.921815834	0.031075718
Rb1	-1.436661671	0.001743118	*Atic*	0.782476243	0.031273348
Ap2a2	0.922248522	0.001778975	*Dtl*	-0.559756503	0.031273348
Rpph1	1.997117127	0.001788057	*Midn*	0.943555909	0.031273348
2410004N09Rik	1.269759541	0.001803972	*Anxa1*	-1.236242317	0.031332721
Bex4	2.017752881	0.001803972	*Nop2*	1.057872219	0.031634116
Zfp423	2.156113048	0.00182233	*Ipo5*	0.765519329	0.031737781
Usp44	2.143931927	0.001908697	*Nme4*	0.813150109	0.031737781
Rhd	2.140044306	0.001910583	*Gm5506*	1.269267009	0.031737781
1110038B12Rik	1.01071868	0.001920128	*Rela*	-0.767308255	0.031848022
Eprs	0.79340224	0.001948621	*Dusp6*	-1.054900535	0.032157984
Nop14	1.305384583	0.001948907	*Lrrc25*	1.592246079	0.032181447
Sigirr	-1.266173098	0.001963261	*Ncoa2*	-0.943221183	0.032196924
Selm	-1.816301391	0.001990681	*Lat*	-1.613547228	0.032196924
Gcat	1.865282972	0.002030325	*Siglecg*	-1.099314738	0.032450437
Btbd19	2.018565042	0.002054617	*Ftx*	-1.126595114	0.032450437
Abhd11	1.087089511	0.002095061	*Vamp8*	-0.520023798	0.032515854
Dtx3l	-1.15078466	0.002116937	*Cyfip2*	-0.531122675	0.032853539
Hspa8	0.789075408	0.002133809	*Kbtbd3*	-0.805354546	0.032853539
Mcu	-1.247809481	0.002195176	*Serf1*	0.711697597	0.033399801
Neil3	-1.162639119	0.002219558	*Ebi3*	1.6328982	0.03402481
Eif4a1	0.851923718	0.00229198	*Cd48*	-0.749356882	0.034065437
Adk	1.017642769	0.002295595	*Lenep*	1.608353052	0.034477125
Rapgef2	-1.182658216	0.002295595	*Snhg1*	0.606396469	0.03455468
Pds5b	-0.773173253	0.002295595	*Zfp959*	-0.991772657	0.034617364
Rae1	0.91885143	0.002316636	*D7Ertd715e*	1.573308651	0.034617364
Rnf150	2.119028033	0.002316636	*Msl3*	-0.783569629	0.034617364
Mbnl1	-0.760259528	0.002321971	*Neurl1b*	-1.58398926	0.035811456
Zfp39	-1.251888777	0.002359686	*Kpnb1*	0.578168306	0.03596707
Smim14	-1.027558833	0.002359686	*Gsdmd*	-1.214395833	0.03596707
Mrpl3	0.754132511	0.002476639	*Zbtb20*	-1.601039014	0.03596707
Mar-08	2.096927301	0.00258078	*Zbp1*	-1.531000108	0.036009912
Cyld	-1.043695263	0.002705691	*Zmat1*	-1.58232948	0.03605126

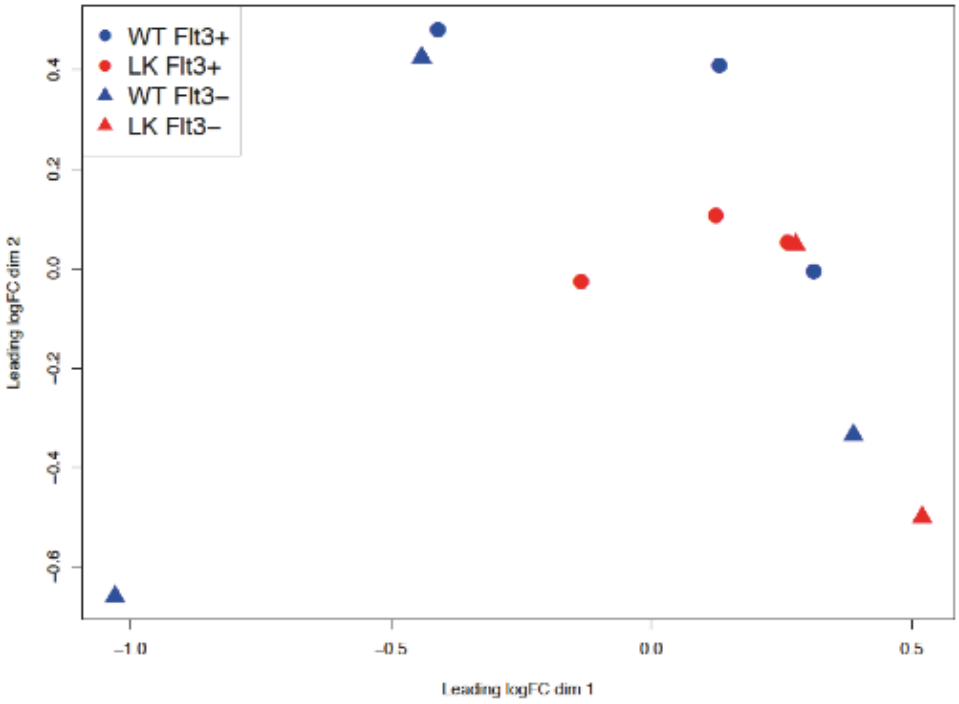
Pfkp	-1.304875468	0.002709635	*Pdcd11*	0.992364972	0.036057682
Map3k8	-1.835444529	0.00276101	*Bhlhe41*	1.4329046	0.036120357
Evc	2.087402782	0.002779876	*Mapk1ip1*	-1.074064799	0.036144248
Gm129	2.082169356	0.002788721	*Trappc5*	0.917461165	0.036144248
Scoc	-1.054658557	0.002790592	*Mrps26*	0.845987664	0.036149176
Utrn	-1.440161644	0.002803004	*Myb*	-0.609396699	0.03627058
Zcchc3	1.406673098	0.002803004	*Shmt2*	0.865599701	0.03627058
Atp2a3	-1.078269656	0.002817248	*Nudt22*	-1.284968566	0.03627058
Paip2	-0.816561329	0.002817248	*Ninl*	1.609678265	0.03627058
Zfp563	-1.928548856	0.002832108	*Zfp62*	-0.833804186	0.036681086
Rchy1	-0.785416935	0.002832108	*Phc1*	-1.113026459	0.036792519
Utp20	1.25124218	0.002869961	*Ncapd3*	-0.781511048	0.036900704
Mtmr12	-1.405917525	0.003267056	*1700016K19Rik*	1.559469465	0.036901675
Ncl	0.902906954	0.003287711	*Usp8*	-0.643213964	0.036924222
Garem	2.061498773	0.003287711	*Ncoa1*	-1.403208872	0.03731032
Hsbp1	-0.608062468	0.003287711	*Mrps6*	0.707924693	0.03731032
Pml	-1.180767235	0.003287711	*Noc3l*	1.082980761	0.03731032
Zfp386	-0.96523499	0.003413085	*Cmpk1*	-0.523211542	0.03731032
Trp53inp1	-1.407722133	0.003469625	*Csrp1*	-1.176268706	0.037405364
Gemin5	1.314774298	0.003507541	*Wls*	-1.221016858	0.037457082
Maf	-1.784425717	0.003526924	*Ap1ar*	-1.107530995	0.037620608
Lst1	1.998469219	0.003537332	*Grhpr*	1.12954568	0.037620608
Hsp90aa1	0.573154989	0.00356904	*Wdr46*	0.997355362	0.037834281
Wdr70	1.523785984	0.003782666	*Wdr36*	0.683863067	0.037906037
Pcbp4	2.043648866	0.003782666	*Casp1*	-1.607763668	0.037906037
Hcfc1r1	-0.782080245	0.003829567	*4930579G24Rik*	-0.825009692	0.037958957
Mfng	-1.149344505	0.003885601	*Rps2*	0.665722664	0.038268629
H2-Ab1	-1.575118682	0.003885601	*Elovl5*	-0.74526837	0.038280927
Ppp1r16b	-1.544853558	0.003905783	*Sfpi1*	1.274829276	0.038439929
Spats2	2.027895705	0.003920046	*Exoc8*	-0.673200913	0.038439929
Cyba	0.712220382	0.003987776	*Manf*	0.586309343	0.038439929
Ginm1	-1.269537215	0.003998713	*Etfdh*	-1.025438463	0.038772875
Pdlim1	-1.161687305	0.00413532	*Yaf2*	-1.157982619	0.038940519
9330175E14Rik	-2.017708974	0.004252478	*Bet1*	-0.66428941	0.038940519
Hmgn3	-1.447485095	0.004363509	*Hbq1b*	1.540766128	0.038968335
Tceal8	1.002661263	0.004388107	*Acsf2*	-1.413318957	0.039007569
Itgam	1.967323808	0.004478673	*Aldh7a1*	1.314357345	0.039216762
Aprt	1.085448499	0.004478673	*Emp1*	-0.942719784	0.039487084
Pitpnc1	-0.950518845	0.00451533	*Srpk3*	-1.5756491	0.039487084
Pink1	-1.391931975	0.00451975	*Med7*	-0.805896144	0.039982173
Sh3rf1	-1.161101187	0.00451975	*Cd84*	-0.761845004	0.04013192
Cbfb	-0.86219741	0.00451975	*BC005764*	1.592635845	0.040437748
Sms	1.184221984	0.00451975	*Zbtb39*	1.333722892	0.040437748
Mybbp1a	1.306791698	0.004559372	*Mthfd1*	0.84406958	0.040766707
Syt11	2.00644118	0.004572259	*B3gnt7*	1.555362103	0.041086142

Nars	0.952803496	0.004597853	*Bri3bp*	0.702681436	0.041400103
Hspd1	1.151038265	0.004620335	*Mtmr2*	-0.553623172	0.041453596
Glrx	-1.017949704	0.004678226	*Ttll12*	0.928176055	0.041505564
Mfap2	1.979357858	0.004920914	*Nolc1*	0.788641342	0.041505564
Prmt7	1.250354087	0.00492174	*Mafg*	1.541298331	0.042162507
Hmbs	0.934899688	0.005044409	*Pkm*	0.775316479	0.042845113
Lyrm5	-1.272231857	0.005325687	*Birc2*	-1.091338523	0.04300758
Crot	-1.053328019	0.005363002	*Zdhhc9*	1.398100341	0.04300758
Appl1	-0.766927962	0.005532626	*Klhl9*	-0.514151342	0.043071294
Rdm1	-1.097494934	0.005543886	*Ankle2*	-0.651924434	0.043144923
Pilra	1.958354706	0.005559044	*Scly*	-1.196084447	0.043274971
Cnp	-0.942992107	0.005623277	*Fbxw5*	-0.819474833	0.043274971
Rrp12	1.384196227	0.005680469	*Vcp*	0.605202465	0.043274971
Rbm15	-0.698933708	0.005737479	*Tmem80*	-1.081089573	0.043518438
Rsph9	-1.428604377	0.005743427	*Gid4*	-0.678780616	0.043686023
Ank2	1.959990355	0.005849592	*Wdr45b*	0.609318517	0.043686023
Atp6v1d	-0.734983113	0.00592298	*Atf6b*	1.01840318	0.043686023
Pdcd10	-0.800557553	0.00592298	*Rbm4b*	-0.978445267	0.043686023
Armc3	-1.283575873	0.005938019	*Sc5d*	-0.922839377	0.043686023
Ifit2	-1.802846898	0.006031627	*Ddx3x*	0.499305426	0.043686023
Mprip	-1.110789367	0.006038332	*Ly6a*	-1.463552058	0.043712996
Fgf13	-1.287041409	0.006038332	*Cisd2*	-0.585435406	0.043727434
Trmt1l	-1.123915932	0.00623399	*Plgrkt*	-0.786025983	0.044077676
Ypel3	-1.320470288	0.006259726	*Mrto4*	0.779074397	0.044123947
Tpi1	0.844318696	0.006278943	*Gtpbp4*	0.655367893	0.044224742
Dguok	-1.043988113	0.006280601	*Fam126b*	-1.137509214	0.044820019
Rabl6	1.231362368	0.006282083	*Bet1l*	-0.912014309	0.044820019
Cybasc3	-0.92579957	0.006335853	*Enpp6*	-1.568332208	0.044820019
Gja1	1.924877687	0.006348965	*Myo19*	1.396830361	0.044992957
Dazap2	-0.682896543	0.006348965	*Rad21*	-0.642937925	0.044992957
Msantd3	-1.95756805	0.006348965	*Ccdc86*	0.968145662	0.044992957
Isg20	-1.666922805	0.006348965	*Sh3yl1*	1.514564828	0.045250367
Cul4b	0.824444049	0.006355383	*Tln2*	1.529556157	0.045263863
2900026A02Rik	-0.941587796	0.006375799	*Mier2*	1.274079805	0.045303997
Stag2	-0.716850708	0.006464302	*Vezf1*	-0.7881452	0.045340681
Sh3bgrl	-0.667333367	0.00672755	*Sf3b2*	0.394342054	0.045455747
Vars	1.060389012	0.006778725	*Mlh1*	0.806132654	0.045871441
Nfyb	-0.909327806	0.006843896	*Srm*	0.888620046	0.045972654
N6amt2	1.598980944	0.006843896	*Sp4*	-1.20609886	0.046446395
Kbtbd7	-1.457291386	0.00688873	*Srp14*	-0.527613506	0.046601252
Rfc1	-0.729749241	0.006938158	*Herc6*	-1.241238281	0.046601252
Utp6	0.765924657	0.007137411	*Hspa9*	0.675890657	0.046675012
Ppapdc2	-1.850139317	0.007163664	*Cd163*	-1.484002393	0.046777836
4833420G17Rik	-0.647013651	0.007384767	*Hmgn1*	0.613407077	0.046816448
Ube2a	-0.616252006	0.007415423	*Pglyrp2*	1.473149385	0.046819273

Rnasek	-0.633431687	0.007492013	*8430410A17Rik*	-1.247338662	0.047644656
Rock1	-0.738379508	0.007492013	*H3f3b*	-0.433325958	0.047652109
Lig4	-1.780675779	0.007492013	*Rsu1*	-0.806860334	0.047682062
Psmb8	-0.86798466	0.007528651	*Asb1*	-1.492271023	0.04797214
Ikbkap	1.023006412	0.007529847	*AA467197*	-1.505806317	0.048065217
Lrrc32	1.901832349	0.00759268	*Krt10*	0.815172969	0.048177219
Sirpa	1.822423035	0.007955326	*Lats1*	0.970900031	0.048267055
Gata3	1.904120809	0.008030802	*Cdk19*	-0.744961312	0.048629423
Hip1r	-1.155410622	0.00804808	*Mex3d*	1.469686244	0.049272674
0610030E20Rik	-1.003023235	0.00804808	*Samd9l*	-0.99346822	0.049598341
Mll1	-0.853207648	0.00804808	*Nufip2*	-0.510574351	0.049690579
Ptgs1	1.89722564	0.008101634	*Nle1*	1.264748673	0.049788199
Znrd1	-0.730551239	0.008142964			

Appendix 9

Principal component analysis of E14.5 FL Pro B cells (Lin$^-$ B220$^+$CD43$^+$CD19$^+$CD24$^+$AA4.1$^+$KIT$^+$IL-7Rα$^+$ then FLT3$^+$ or FLT3$^-$) sorted from mice expressing TEL-AML1 ('LK' - leukaemic) or littermate controls ('WT'). The large variation, probably due to the mixed genetic background, meant analysis was not taken further.



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