On the origin of B cells. Elucidating the Role of Tyrosine Kinase Receptors in B Cell Development.

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LUND
UNIVERSITY

DOCTORAL DISSERTATION
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Abstract

B cells are essential cells that are part of the adaptive immune system with the capacity to produce antibodies against exogenous antigens. These cells are produced through a process called hematopoiesis, tightly regulated by extrinsic and intrinsic factors. Among these, tyrosine kinase receptors and their cytokines have been shown to have an important role in adult lymphopoiesis regulation. However, little is known about their involvement in the ontogeny of the blood system, and specifically, the B lineage that takes place in the embryo. In this thesis, I have aimed to deepen our understanding of the regulation and differentiation of hematopoietic cells in the embryo, both in mouse and human.

In the first paper, we have identified a new distinct B cell progenitor in mouse that is uniquely present in a narrow window of fetal liver lymphopoiesis and that possesses B/myeloid potential and gene identity. We have also shown that the tyrosine kinase receptor, CSF1R, marks and regulates this progenitor and that upon loss of CSF1R signaling, embryonic B lymphopoiesis was specifically impaired. These $CSF1R^+$ ProB progenitors are relevant for infant and childhood leukemia in which CSF1R has recently been found to be rearranged.

In the second paper, we have demonstrated that the first $CD19^+$ B cell emerging in the human embryo expresses IL7R and seems to derive from a newly identified $IL7R^+$ progenitor. Using single cell gene expression analysis, we have shown that this $IL7R^+$ progenitor shifts from a myeloid to a lympho-myeloid gene signature throughout development. Importantly, we have developed an *in vitro* modeling system that allowed us to recapitulate this transition and hierarchy using human pluripotent stem cells and used this system to study one of the most commonly found fusion genes, ETV6-RUNX1, in childhood B cell leukemia (B-ALL). Our investigation suggests that the $IL7R^+$ progenitor can be a relevant target cell for *in utero* B-ALL, initiating pre-leukemic mutation.

In the third paper, we have developed a new mouse model that allows a time or stage specific deletion of the tyrosine kinase receptor, FLT3. Previous loss of function studies, using mice deficient either for the receptor or for its ligand, FLT3L, have demonstrated that FLT3 signaling is required for lymphoid and also myeloid development. However, they have not been able to address the specific cellular stage of FLT3 requirement in the hematopoietic differentiation since the receptor was constitutively lost in the entire blood hierarchy and from the first HSC in the embryo. We showed here that FLT3 is required for adult hematopoiesis, and also after lymphoid commitment, while being dispensable after commitment to the B lineage.

Overall, the papers included in this thesis highlight the need for studying embryonic hematopoiesis specifically, since regulatory requirements can change during ontogeny. It also shows the importance of developing new tools in order to study the onset of infant and childhood blood disorders that are thought to derive from mutations occurring already *in utero*.

Key words: Hematopoiesis, B cell development, cytokine, tyrosine kinase receptors

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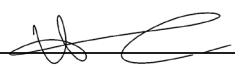
Signature  Date 2016-08-15

On the origin of B cells

Elucidating the Role of Tyrosine Kinase Receptors in B Cell Development

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To my father

*Oser ; le progrès est à ce prix.**

Victor Hugo, Les Misérables, 1862

*"To dare; that is the price of progress."

Contents

Original papers included in this thesis

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Alya Zriwil, Charlotta Böiers, Lilian Wittmann, Joanna C.A. Green, Petter S. Woll, Sten Eirik W. Jacobsen* and Ewa Sitnicka*

Blood, 2016, 128(2): 217-226.

Paper II. *A Human IPS Model implicates Embryonic B-myeloid Fate Restriction as a Developmental Susceptibility to ETV6-RUNX1*

Charlotta Böiers*, Simon E. Richardson*, **Alya Zriwil**, Emma Laycock, Virginia A. Turati, John Brown, Jason P. Wray, Dapeng Wang, Javier Herrero, Stefan Karlsson, Andrew J. H. Smith, Sten Erik W Jacobsen and Tariq Enver

Submitted manuscript

Paper III. *Stage-specific Roles of FLT3 in Adult Lympho-Myelopoiesis*

Alya Zriwil, Charlotta Böiers, Trine Ahn Kristiansen, Lilian Wittmann, Claus Nerlov, Joan Yuan, Ewa Sitnicka* and Sten Eirik W. Jacobsen*

Manuscript

* These authors contributed equally to the paper

Papers not included in this thesis

Inhibition of miR-9 de-represses HuR and DICER1 and impairs Hodgkin lymphoma tumour outgrowth in vivo.
Leucci E**, Zriwil A**, Gregersen LH, Jensen KT, Obad S, Bellan C, Leoncini L, Kauppinen S, Lund AH.
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Immunity, 2015, 43:394-407

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Drissen R, Buza-Vidas N, Woll P, Thongjuea S, GambardellaA, Giustacchini A, Mancini E, **Zriwil A.**, Lutteropp M, Grover A, Mead A, Sitnicka E, Jacobsen SE, Nerlov C.
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Cellular barcoding links B-1a B cell potential to a fetal hematopoietic stem cell state at the single-cell level.
Kristiansen TA, Jaensson Gyllenbäck E, **Zriwil A**, Björklund T, Daniel JA, Sitnicka E, Soneji S, Bryder D, Yuan J.
Immunity, 2016, 45:1-12

Abbreviations

AGM	aorta-gonad-mesonephros
ALL	acute lymphoid leukemia
AML	acute myeloid leukemia
BCR	B cell receptor complex
BM	bone marrow
CEBP	CCAAT/enhancer-binding protein
CLL	chronic lymphoid leukemia
CLP	common lymphoid progenitor
CML	chronic myeloid leukemia
CS	Carnegie stage
CSC	cancer stem cell
CSF	colony stimulating factors
CSF(1-2-3)R	colony stimulating factor (1-2-3) receptor
E	embryonic day
E2A	immunoglobulin enhancer-binding factors E12/E47
EBF1	early B cell factor 1
EHT	endothelial to hematopoietic transition
EMP	erythro-myeloid progenitor
Er	erythroid
ES	embryonic stem cell
ETP	early thymic progenitor
FL	fetal liver
FLK2	fetal liver kinase 2
FLT3	fms-like tyrosine kinase 3
FLT3L	FLT3 ligand
FOXO1	forkhead box O1
G-CSF	granulocyte colony stimulating factor
GM-CSF	granulocyte-macrophage colony stimulating factor
GMP	granulocyte monocyte progenitor
HSC	hematopoietic stem cell
Ig	immunoglobulin

IgH	immunoglobulin heavy chain
IgL	immunoglobulin light chain
IL	interleukin
IL7R	interleukin receptor 7
IPS	induced pluripotent stem cell
KITL	KIT ligand
LMPP	lymphoid primed multipotent progenitor
LSK	$Lin^{-}SCA\text{-}1^{+}KIT^{+}$
M-CSF	macrophage colony stimulating factor
MegE	megakaryocytic
MLL	mixed lineage leukemia
MPL	myeloproliferative leukemia virus oncogene
MPP	multipotent progenitor
NOD	non-obese diabetic
NSG	non-obese diabetic-severe combined immune deficiency IL2Rgnull
PAX5	paired box gene 5
PI3K	phosphatidyl-inositol 3' kinase
PSC	pluripotent stem cells
PsP	para-aortic splanchno pleura
RAG	recombination activating gene
RTKs	receptor tyrosine-kinase
Runx1	runt-related transcription factor1
SCA-1	stem cell antigen-1
SCF	stem cell factor
SCID	severe combined immune deficiency
SCL	stem cell leukemia
SDF-1	stromal cell-derived factor-1
SLAM	signaling lymphocyte activation molecule
SLC	surrogate light chain
TF	transcription factor
TKI	tyrosine kinase inhibitors
TPO	thrombopoietin
TSLP	thymic stromal-derived lymphopoietin
YS	yolk sac

Preface

It has been almost 5 years that I have been trying to understand the hematopoietic development and I believe that I have gained knowledge of various mechanisms involved in this process. When starting this PhD work, I had in mind that my work could, in the long-term, lead to the improvement of therapies used in cancer treatment, in particular, infant and children disorders. Leukemia accounts for 30% of all childhood cancers and although around 80% of the patient can be cured, the origins and causes of this disease remain widely unknown. In 1962, Irving J. Wolman, when reporting on a pair of monozygotic female infant twins with acute leukemia, suggested for the first time that the disease may have originated *in utero* [1]. This hypothesis was subsequently further investigated and is nowadays widely accepted [2]. This emphasizes the need of a deep understanding of fetal development, and more specifically, hematopoietic embryonic onset. In this thesis, I will try to convince you that the origin does matter and that understanding the embryo is of importance for understanding the origin of the disease.

1. Ontogeny of hematopoiesis

Hematopoiesis, from the Greek “haima” meaning “blood” and “poiesis” meaning “to make”, is the process of continuous blood cell production. On average, an adult produces around one trillion blood cells per day, making it one of the most efficient and regenerative systems of the human body. This production occurs in the bone marrow (BM) in adults and in the fetal liver (FL) in the embryo. A small subset of cells, the so-called hematopoietic stem cells (HSCs), has the ability to generate all of the blood cell types by following a complex and multi-step process termed differentiation [3, 4]. During this process, intermediate progenitor cells are produced and have the capability to further differentiate into a large number of mature cells. The generated cells take part in various specialized functions, such as immune response and protection (lymphocytes, macrophages and granulocytes), oxygen transport (erythrocytes) and healing through blood clotting (platelets).

Understanding the hematopoietic process requires one to gain a deep knowledge of its initiation and regulation at its earlier stage, meaning in the embryo.

Onset of the hematopoietic system

Origin of hematopoietic cells

Where do they come from? Almost 100 years ago, a close relationship between the hematopoietic system and the endothelial cells through a common mesodermal ancestor, termed hemangioblast, was suggested with the observation of blood islands in the chick yolk sac (YS) containing both cell types [5]. This concept was then further supported by the discovery in mouse of common essential genes

expressed in both lineages such as stem cell leukemia (*Scl*) and Flk-1, which when absent, resulted in embryonic lethality and failure to produce both hematopoietic cells and endothelial cells [6-9] (**Figure 1**). In addition, precursors with combined hematopoietic and endothelial potential have been found *in vitro* using Embryonic Stem cells (ES) [10, 11] and by isolating cells from the primitive streak at embryonic day 7.5 (E7.5) with similar bi-potential *in vitro* [12]. The generation of the hematopoietic cell was further described with the identification and characterization of a so-called hemogenic endothelium, consisting of a transition stage between the hemangioblast and hematopoietic cells [13, 14]. The hemogenic endothelium is distinct from other endothelial cells by the expression of, and by being fully dependent of, the runt-related transcription factor1 (RUNX1) with an absence of definitive hematopoiesis in Runx1 deficient embryos [15-18].

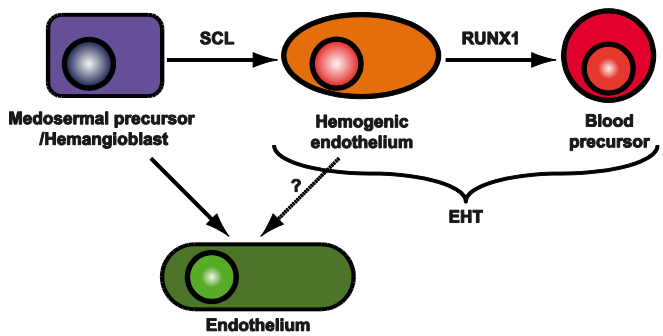


Figure 1 – Emergence of hematopoietic cells from the mesoderm through an endothelial to hematopoietic transition (EHT)

Despite all of these studies, the common precursor hypothesis with the existence of the hemangioblast has been recently challenged by *in vivo* labelling of pre-gastrulation embryos, showing that the majority of the labelled clones contain either blood or endothelial cells [19]. This demonstrates a more linear specification pathway in which mesodermal cells give rise to hemogenic endothelium committed to blood formation, rather than being generated through a bipotential precursor. Further work using single-cell resolution and labelling would help to better understand these early steps of blood emergence and the potential place of the hemangioblast in this process.

Primitive hematopoiesis

The first blood cell appears in an extra-embryonic tissue, the YS, at around E7-E7.5 [20] (**Figure 2**). These cells are runx1-independent, suggesting that they are derived directly from the mesoderm without transitioning through hemogenic endothelium precursors [17, 21]. Cells found in this blood island primarily constitute erythroblasts are distinct from adult erythroblasts in that they are large, nucleated cells that synthesize specifically embryonic globin [22, 23]. Some rare cells with macrophage potential have also been identified at this time during ontogeny [20, 24, 25]. This constitutes the ”primitive hematopoiesis”, which is the first wave of blood cells in the conceptus, appearing prior to the initiation of the circulatory system [26].

Transitory/definitive hematopoiesis prior to HSC emergence

The first transitory hematopoietic cells can already be found at E8.5 in the embryonic YS. At this time, erythro-myeloid progenitors (EMPs) are produced through the endothelial-hematopoietic-transition (EHT) via the “budding” of individual hemogenic endothelial cells rounding up and detaching from the endothelial layer [27-29]. This early wave of definitive EMPs is distinct from the definitive HSC emergence as it is suggested to derive from a different subset of hemogenic endothelium cells [24, 30]. The generation of EMP through EHT is regulated by Runx1, but, in contrast to definitive HSC emergence, is independent of the transcription factor MYB [31, 32]. EMPs migrate to the FL and contribute to the production of erythrocytes, monocytes, granulocytes and mast cells until replacement by HSC-derived cells later during fetal development [33-35]. Recently, using fate mapping mouse models, the Geissmann group suggested that EMPs are the main, if not the only, cells capable of producing long-lived tissue-resident macrophages in the adult [34] (a heterogeneous immune cell population with different functions such as clearance of cellular debris, immuno-surveillance and response to infection), which has been confirmed by other groups [36].

However, another study suggests that indeed, HSCs have the potential to produce these cells, thus the tissue-macrophages origin still remains under debate [37-39].

Lymphoid potential has, for a long time, been thought to be solely derived from HSC with the identification of B/myeloid bipotent progenitors at E12 [40-42]. At a later time point, lympho-myeloid progenitors have been identified, but again without prospect identification of their origin [43, 44]. Recent studies have however challenged this idea with the identification of so-called pre-HSC prior to the definitive HSC emergence at E9.5-E10.5 in the para-aortic splanchno pleura (PsP) and in the aorta-gonad-mesonephros (AGM) [45, 46]. In addition, the first lympho-myeloid restriction has also been recently described with the characterization of an $IL7R^{+}$ lymphoid primed multipotent progenitor (LMPP) devoid of megakaryocytic-erythroid potential already at E9.5 in the YS prior to definitive HSCs [47].

Emergence of definitive HSCs

Early work by Moore and Metcalf suggested that the YS was the site of emergence of definitive hematopoiesis [48]. However, in 1975, this concept was challenged and an intra-embryonic site was suggested to be the first site of emergence of HSCs in the chick [49]. This was further investigated in the mouse system and definitive HSC capable of long-term repopulation were found in the AGM region at E10.5, while the YS was devoid of this potential [50, 51]. The E9 YS was also further investigated and shown to be able to repopulate newborn mice while other studies demonstrated that only the PsP region and not the YS had definitive HSCs [52-54]. Overall, the AGM region has been shown to be a source of definitive HSC while the contribution of the YS to adult hematopoiesis remains debated. In addition, several other embryonic sites, such as vitelline/umbilical arteries [55] and the placenta [56-58], have been suggested to contain HSC. However, the question remains regarding their capacity to generate HSC *de novo* due to blood circulation occurring at early stage in the development. Experiments using the Ncx1-deficient mouse, leading to heartbeat failure, showed *in vitro* the multilineage potential of the placenta, although this could not be verified by *in*

vivo experiments [59-61]. Finally, a recent study reported the generation of *de novo* generated HSC in the mouse head through an EHT [62]. Besides the clear evidence of newly formed HSC in the AGM at E10.5, the question of whether other HSC niches have this capacity prior to the AGM remains therefore to be established.

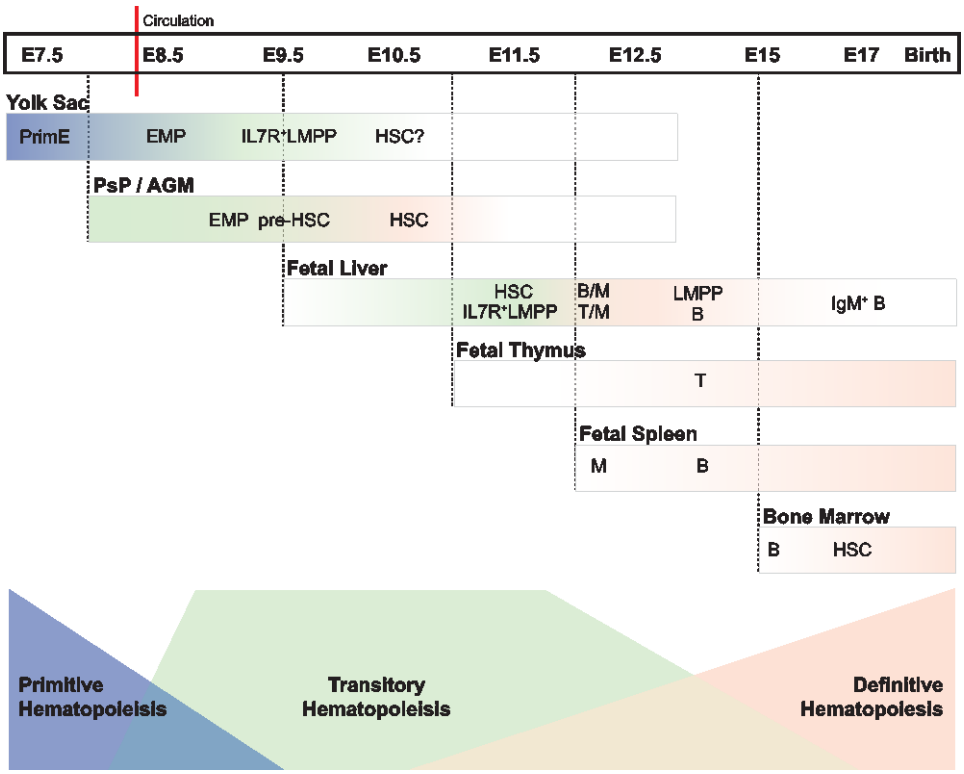


Figure 2 – Emergence of Hematopoietic Cells in extra- and intra-embryonic sites.

Hematopoietic cells emerge through three different waves: first, a "primitive hematopoiesis" (blue), followed by a "transitory hematopoiesis" (green) and finally the emergence of a "definitive hematopoiesis" (pink) that can give rise to the adult system. Of note, transitory hematopoiesis can give rise to cells that are long-lived and that remain in the adult, such as tissue-resident macrophages. For details, see main text.

PrimE, primitive erythropoiesis; EMP, Erythro-Myeloid Progenitors; LMPP, lymphoid primed multipotent progenitors; HSC, hematopoietic stem cells; B/M, bi-potent B and myeloid progenitor; T/M, bi-potent T and myeloid progenitor; B, B cell progenitor; M, myeloid progenitors or myeloid cells; T, T progenitor; IgM^+ B, mature B cells.

Building up a hematopoietic system

Fetal liver hematopoiesis

During embryonic development, the FL is the main site of hematopoiesis. It already starts to be seeded by progenitors (EMPs) through the blood circulation at E9.5 [63-65] (**Figure 2**). However, it is not until E11 that the FL is capable of long-term multilineage repopulation, demonstrating the presence of HSC [50]. The FL rapidly expands and its reconstitution capacity increase up to 38 fold between E12 and E16 when it then starts to decrease, with HSC starting to leave the FL to seed the BM [66]. The FL hematopoiesis differs largely from the adult BM hematopoietic development. FL cells are highly proliferative with better reconstitution capacity than the BM [67-69]. Moreover, specific developmentally regulated signals have been shown to distinguish FL HSC from BM HSC, such as KIT downstream signaling [70, 71]. In addition, the lineage output and differentiation of FL HSC differs from the adult BM due to distinct activated pathways [72]. This will be further discussed in Chapter 2. Why the FL is an intermediate site of hematopoiesis is still unknown, however. One explanation could be that, as has recently been reported, the FL is the safest place for rapid expansion due to its chemical secretion [73]. Unlike the BM, high levels of bile acids can be found in the FL that act as growth factors and protectors against endoplasmic reticulum stress elevation. The vasculature has also been shown to be important in creating a "niche" for the FL HSC with $Nestin^{+}$ perivascular cells driving the HSC expansion *in vivo* [74]. This is one step toward understanding the nature of the FL niche that remains to be fully elucidated.

Fetal spleen hematopoiesis

Few studies have been focused on elucidating the role of fetal spleen during embryonic development. The seeding of the fetal spleen has been shown to take place at E12 with colonization by macrophages followed by the detection of HSC at around E14 [75, 76]. The spleen keeps a hematopoietic activity until after birth,

although extra-medullary hematopoiesis can be observed under stressful conditions [77-80]. At E13, B cell progenitors start to be detected in the spleen, although the origin (FL or fetal spleen) remains unknown since HSC-derived B cells could not be observed in fetal spleen culture, nor could a long-term repopulating capacity of fetal spleen HSC [75, 81]. However, B cell differentiation could be promoted by the fetal spleen stroma, suggesting that FL already committed progenitors seed the spleen to fully differentiate [42, 81].

Bone marrow hematopoiesis

The BM is the main site of hematopoiesis in the adult and becomes the predominant hematopoietic organ at around two weeks after birth. The BM anlage starts to be colonized at E15 by B cell progenitors, and by E17.5, an active B lymphopoiesis can be observed [42]. Phenotypic HSC can be found at E17.5, being attracted by the chemokine stromal cell-derived factor-1 (SDF-1) and its receptor CXCR4 [82]. Interestingly, this chemo attraction is specific for HSC colonizing the BM, as in the SDF-1 deficient embryo, FL hematopoiesis is not impaired as compared to the BM [83]. This supports the involvement of differential mechanisms in attracting HSC to the FL and in seeding the BM.

The shift between fetal HSC to an adult, more quiescent, HSC is observed after birth, with downregulation of specific markers such as AA4.1 (a B cell marker in adult hematopoiesis) and MAC1 (a myeloid associated marker in adult hematopoiesis) [67, 84].

When finally located in the BM, HSCs are largely influenced by their environment so-called “niche”, constituted of different subsets of hematopoietic as well as non-hematopoietic cells that will regulate the self-renewal, proliferation and differentiation activity of the HSC [85]. Two main niches have been described in the BM [86, 87]. The endosteal niche is situated in the edge of the BM that contains mainly osteoblasts providing factors for HSC maintenance (for instance thrombopoietin and angiopoietin) [88-90]. The second niche is the perivascular niche, situated in the core of the BM and contains actively dividing HSCs

supported by sinusoidal endothelium, perivascular cells and neural cells [91-94]. Both niches contain perivascular nestin$^+$ Mesenchymal Stem Cells (MSCs) as a key component [86].

Hematopoiesis in human

To understand the hematopoietic development in humans, knowledge of the staging and how to evaluate the developmental age of an embryo is required. In 1942, Streeter suggested a division of the earliest stage of the development in "horizons" [95]. Later, this organization was revised by O'Rahilly and Müller (in 1987) who termed their staging "Carnegie stage" (CS), in reference to their institution [96]. They subdivided the first 8 embryonic weeks (56 days) into 23 CS. By describing not only the size, but also a thorough description of the development of structure with an accurate stage classification, they allowed researchers to make more precise statements. Most studies on the early human hematopoietic system, and also on brain development, have used this classification system as a reference. For the hematopoietic system in the human embryo, the emergence appears very similar to that found in the mouse model. In a similar way to the mouse, the first blood cells in humans emerge YS, where erythroid and myeloid progenitors are generated [97].

Human HSCs, as defined by long-term repopulating multilineage capacity, emerge in the AGM region similarly to the mouse at CS14, and can be found later at CS16 in the YS and placenta [98, 99]. Hematopoiesis then takes place in the embryonic liver, where few HSC can be found at CS17 [98, 100]. Finally, the fetal BM takes over the hematopoietic activity by first being seeded by myeloid progenitors at 10.5 weeks of gestation, followed by HSCs at around 16 weeks of embryonic gestation [101]. It is important to note that in this study, the HSC potential in the BM has not been assessed *in vivo* by transplantation and was merely defined phenotypically, using immunohistochemistry. After this last migration, the hematopoiesis is then set in the BM throughout life.

2. Lineage commitment and differentiation

After HSCs are generated in the embryo and have found their home, the differentiation process takes place. Through an asymmetric division, HSCs self-renew while producing at the same time multipotent progenitors cells that differentiate in more committed, and ultimately restricted, progenitors of unilineage potential. In this part, I will refer to "*lineage commitment*" as the step in which a cell starts to express/upregulate the signature genes of one lineage (also termed *lineage priming*), together with having its potential while still retaining low/downregulated expression of other lineage associated genes with a low potential to differentiate in those lineages. In this case, the cell is most likely to produce cells from the lineage it is committed to, but still has the potential to produce other lineages. "*Lineage restriction*" follows lineage commitment. This is the step when a cell holds only one lineage potential and gene signature and is therefore restricted to one cell type production without the ability to produce any other.

Models of hematopoiesis

Adult hematopoietic hierarchy in the mouse

Adult hematopoietic differentiation has been extensively studied (**Figure 3**). The cells at the top of the hematopoietic hierarchy, long-term HSCs (LT-HSC), have been described as the only population capable of long-term multi-lineage reconstitution with self-renewing capacities [67, 102]. Further down, short-term

HSCs (ST-HSC) exhibit a similar multi-lineage reconstitution capacity with, however, a limited self-renewal capacity [103]. Then comes the compartment of multipotent progenitors (MPP) that harbours multi-lineage potential in the short term but without any self-renewing capacity. These three populations are within the so-called LSK compartment, where cells do not express mature lineage markers (L) and are positive for the stem cell antigen-1 (SCA-1; S) and KIT receptor (K) [104].

LT-HSC have been isolated, characterized and identified by using expression of CD34 or CD48 and CD150 (also termed SLAM markers, signaling lymphocyte activation molecule) molecules [105-108]. The gold standard to evaluate the stem cell activity of a population is by performing transplantation and analyzing the contribution of this population at the single cell level to the reconstitution in all the different lineages at least 16 weeks after transplantation. Furthermore, to evaluate their self-renewal capacity, secondary transplantations of purified HSCs need to be performed [109]. Using this method, one out of 2.1 LSK $CD48^{-}CD150^{+}$ cells possesses a long-term multilineage HSC potential [107].

To subdivide the MPP compartment, similar transplantation assays have been used, showing the short-term potential of this population to reconstitute blood cells and their lack of self-renewal ability. In addition, the use of an *in vitro* differentiation system with liquid and semi-solid culture (supplemented with growth factors) has allowed a better characterization of the potential to produce myeloid, erythroid and megakaryocytic cells, overcoming the issue of their high turnover and therefore short life.

The FMS-like tyrosine kinase 3 receptor (encoded by *Flt3* gene, also called *Flk2*), FLT3, is up-regulated in a fraction of MPP. Cells expressing high levels of FLT3 lose their self-renewal capacity and their ability to reconstitute megakaryocyte and erythrocyte cells [110]. Identified as LMPP, they express lymphoid affiliated genes (*Rag1, Rag2*) while still retaining some myeloid genes (*Csf1r, Mpo, Csf2r*) [111]. The existence of the LMPP stage demonstrated that in contrast to earlier models describing a clear segregation between the lymphoid and the myeloid compartment, the commitment toward an erythroid (Er) and megakaryocytic

(MegE) fate occurs prior to a granulocyte/macrophage and lymphoid separation [112, 113].

Production of myeloid progenitor cells through differentiation into pre-granulocyte monocyte progenitors (preGMP) and GMP cells further supports the existence of an LMPP [114]. PreGMPs are committed to the myeloid lineage with low, if not any, MegE/Er potential while retaining a latent capacity to produce lymphoid cells, suggesting their placement downstream from LMPP in the hierarchy [115]. Of note, a recent study showed that the preGMP compartment can be separated into two populations according to their *Flt3* and transcription factor *Gata1* expression [116]. One population supposedly differentiated from LMPP, producing granulocytes, macrophages and neutrophils while the other is described as an erythroid-megakaryocyte primed multi-potent progenitor (EMkMPP).

The T cell differentiation pathway is also in line with the existence of a primary segregation of the Meg/Er lineage from the granulocyte/macrophages and lymphoid potential. The first population committed to the T cell lineage, the early thymic progenitors (ETPs) found in the thymus, has a similar potential to LMPPs with, in addition to T potential, B, NK and myeloid cell potential, suggesting that the LMPP might be the thymus seeding progenitor cell [117].

In the BM, subsequent differentiation of the LMPP population towards lymphopoiesis leads to the emergence of a lymphoid committed progenitor, the CLP (common lymphoid progenitor) compartment. Defined as lineage negative and low in expression for KIT and SCA-1, this population expresses the interleukin receptor 7 (IL7R) and FLT3 [118, 119]. Interestingly, CLPs can be further subdivided into LY6D positive and negative populations [120]. The $LY6D^{+}$ CLPs display a strong B cell lineage potential both *in vitro* and *in vivo*, coupled with expression of the critical B cell gene. *In vitro* data also shows that the $Rag1^{high}$ CLP (the majority of them express also LY6D molecule) has rather low, but still present, T cell potential. This demonstrates a commitment to the B cell lineage prior to the expression of the classical B cell marker, rather than a restriction [121, 122]. In Chapter 4, the B cell development will be described in further detail.

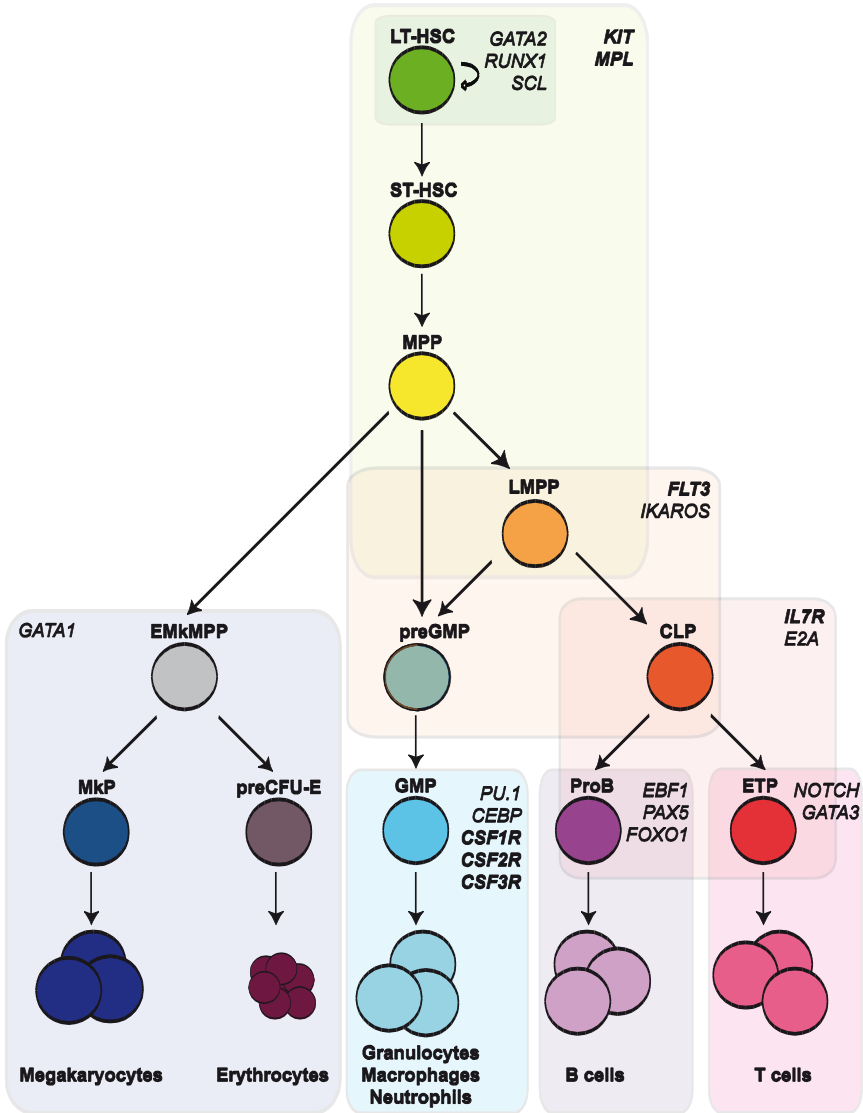


Figure 3 – Representation of the adult hematopoietic hierachy and its main regulators.

Hematopoietic stem cells give rise to the entire blood system in a stepwise process regulated by ***extrinsic*** and *intrinsic factors*.

LT-HSC, ST-HSC, long-term/short-term hematopoietic stem cells; MPP, multipotent progenitors; LMPP, lymphoid primed multipotent progenitors; EMkMPP, erythroid-megakaryocyte primed multipotent progenitors; CLP, common lymphoid progenitors; pre-GMP, GMP, granulocyte monocyte progenitors; ETP, early thymic progenitors; MkP, megakaryocyte progenitors; pre-CFU-E, pre colony forming unit erythroid.

Embryonic hematopoietic hierarchy in the mouse

Compared to the adult, the embryonic hematopoietic hierarchy is still not a fully understood process (**Figure 4**). Phenotypic characterization of HSC has shown that unlike adult HSC, they express the surface marker MAC1 and AA4.1 and are more proliferative (see Chapter 1). While LMPPs have been identified in the FL so far, no lymphoid-restricted progenitors capable of differentiating into T and B cells have been found [44]. When looking for such a restricted population, using the adult CLP phenotypic definition, the identified CLP-like cells retain macrophage potential [43]. Interestingly, and not seen in the adult, several groups reported the existence of B/myeloid bipotent progenitors in the E12 and E13 FL, suggesting a close developmental relationship between the two lineages in the embryo [40, 41, 123]. In addition, Kawamoto *et al* identified in the E12 embryo, cells with combined T and myeloid potential [40]. The prospective isolation and characterization of such bi-potent B/myeloid or T/myeloid progenitors remains, however, to be fully established with the determination of surface molecules allowing the distinction between these progenitors and their placement in the embryonic hematopoietic hierarchy (as discussed in Paper I).

Another important difference between adult and embryonic hematopoiesis is the capacity of fetal HSC to produce specifically some lymphoid cells resembling innate immune cells, which can rapidly respond to defined antigens [124].

For the T cell lineage, a specific subset of gamma-delta T cells (IL-17 producing cells; γδ-T) is found in the embryonic thymus but are not produced by adult BM cells, suggesting their exclusive fetal origin and subsequent persistence in adult mice as self-renewing, long-lived cells [125].

Similarly for the B cell lineage, long-lived B1-a cells, found in the adult peritoneal cavity, are mainly derived from fetal hematopoiesis since, unlike fetal HSC, adult HSC are not able to efficiently reconstitute this pool upon transplantation [126, 127]. The origin of these cells will be further discussed in Chapter 4.

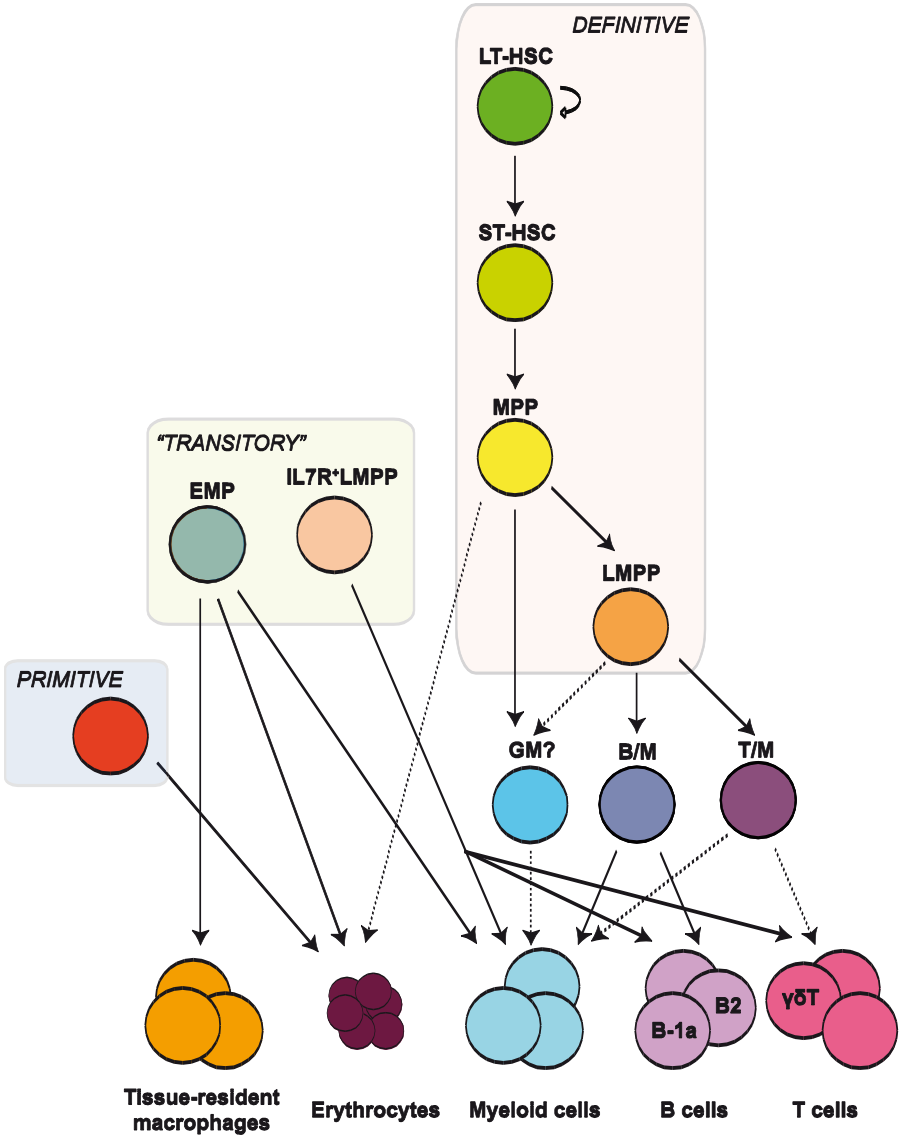


Figure 4 – Representation of the embryonic hematopoietic hierachy.

In the embryo, hematopoietic cells can differentiate from either an HSC through the so-called definitive hematopoiesis, or from earlier waves, such as the primitive and the "transitory" hematopoiesis. The lineage relationship between the different cell types is however not fully established, and the lineage output of the different waves would require further studies.

LT-HSC, ST-HSC, long-term/short-term hematopoietic stem cells; MPP, multipotent progenitors; LMPP, lymphoid primed multipotent progenitors; EMP, erythro-myeloid progenitors; B/M, bi-potent B and myeloid progenitors; T/M, bi-potent T and myeloid progenitors.

Regulation of hematopoiesis

Hematopoietic differentiation and commitment is a process tightly regulated by extrinsic (cytokines) and intrinsic (transcription factors, epigenetic regulators) factors. Using genetically modified animals and loss of function approaches, key regulators have been identified. However, the question as to whether they have a permissive (supporting the growth of already pre-committed cells) or instructive (actively initiating the commitment toward a specific cell lineage) role, or both, is still not fully resolved. In this thesis, the work has been focused on studying extrinsic regulators and their receptors. A brief overview on intrinsic factors is, however, given below.

Intrinsic factors

Transcription factors (TFs) can either have an activating or repressing role and often multiple TFs are required for commitment to a specific lineage. By binding to specific DNA responsive elements, they regulate the expression of other critical genes supported by coactivator/corepressor and chromatin-modifiers. By using a gene targeting approach, several TFs (**Figure 3**) have been shown to be important for HSC emergence and regulation, such as RUNX1 [15, 128, 129], SCL [130, 131], and GATA2 [132, 133]. Other TFs are more specifically involved in differentiation toward one lineage. For example, PAX5 (paired box gene 5) is critical for B cell lineage restriction [134, 135], IKAROS for lymphoid specification [136, 137], GATA3 through NOTCH signaling for T cell development [138] while GATA1 is essential for the erythroid lineage [139, 140] and PU.1 and CEBPa (CCAAT/enhancer-binding protein) are crucial for myeloid development [141-144]. TFs involved in lymphoid development and B cell differentiation will be further described in Chapter 4.

Lineage reprogramming, consisting of transdifferentiating cells from one lineage to another, from lymphoid to myeloid for example, further supports the importance of these TFs. Studies have shown that by introducing CEBPa and PU.1, committed

T cells could be reprogrammed into macrophages and dendritic cells although the reprogramming capacity of fully mature cells was not determined [145]. In a similar way, expression of CSF1R or CEBPa/CEBPb in early B cell progenitors or mature B cells induces a switch to the macrophage lineage by remodeling the B cell transcription network [146, 147]. Both of these studies suggest an instructive role of the involved TF.

Extrinsic factors

Cytokines are important extrinsic regulators of hematopoietic development, produced by cells from the hematopoietic niche or other tissues. They are involved in differentiation, proliferation, maintenance and commitment of hematopoietic cells.

For the maintenance of HSC, the tyrosine kinase receptor, KIT, and its ligand (KITL or stem cell factor, SCF) play a crucial role (**Box 1 and Figure 5**). KIT is expressed on HSCs, but also early multipotent progenitors [148], while SCF is produced in a membrane-bound or soluble form by several cell types, including endothelial cells [94]. Partial loss of KIT signaling leads to a decrease in HSC repopulating capacity associated with severe multilineage hematopoietic deficiencies [149-151]. At steady-state, it is also suggested that KIT is involved in the maintenance of HSC while being dispensable for their emergence [148, 152]. Another cytokine, thrombopoietin (TPO) and its receptor, MPL (myeloproliferative leukemia virus oncogene), play an important role in HSC function. Originally identified as a regulator of megakaryocyte and platelet development [153, 154], MPL is also shown to be highly expressed in HSCs [155]. Mice deficient in TPO exhibit reduced numbers of HSC and impaired repopulating functions [156, 157]. Interestingly, FL HSCs expansion does not require TPO suggesting a role of TPO in regulation of quiescent HSC, but not for highly cycling and expanding HSCs [156].

Differentiation toward lineage-committed cells is also driven by specific cytokines. Cytokines involved in lymphoid differentiation, and more specifically B cell development, will be described in more detail in Chapter 4.

For the myeloid lineage, the colony stimulating factors (CSFs), macrophage CSF (M-CSF or CSF1), granulocyte-macrophage CSF (GM-CSF or CSF2) and granulocyte CSF (G-CSF or CSF3) were first identified as *in vitro* hematopoietic growth factors, acting through interaction with their respective receptors, CSF1R (**Box1 and Figure 5**), CSF2R and CSF3R (colony stimulating factor 1/2/3 Receptor) [158-161]. Studies on mice deficient in these cytokines have demonstrated that at steady-state, M-CSF is essential for the maintenance of macrophage lineage populations and bone modeling, while GM-CSF is needed for the maturation of macrophages and G-CSF is important in neutrophil generation [162]. In addition, CSF1R and its ligand are highly expressed in the embryo proper and in the placenta, suggesting an important role of this cytokine during fetal development [163, 164]. Importantly, early lympho-myeloid progenitor cells in the mouse embryo, possessing combined B, T, NK and myeloid-lineage potential, but not megakaryocyte-erythroid lineage potential, were found to co-express CSF1R and IL7R in the embryo, suggesting that CSF1R may not exclusively play a role in myelopoiesis but also in lympho-myelopoiesis (as discussed in Paper I) [47, 165].

Box 1 - Class III Tyrosine Kinase Receptors

Class III tyrosine kinase receptors (RTKs) play an important role in the regulation of hematopoiesis **[166]**. This family of receptors includes KIT (CD117) **[167]**, FLT3 (CD135) **[168]** and CSF1R (CD115) **[169]**. They represent specific receptors for KITL, FLT3L (FLT3 ligand) and CSF1 respectively.

Class III RTKs have a homology in their structure and are characterized by five immunoglobulin-like domains in the extracellular matrix ligand binding region, a single transmembrane domain, a juxtamembrane domain and two intracellular kinase domains that are divided by a kinase insert domain **[167]**. When the ligand binds to RTKs, it induces a receptor dimerization that activates an intrinsic tyrosine kinase activity, leading to a transphosphorylation of specific tyrosine kinase residue **[170]**. After the receptor's phosphorylation, several docking proteins are recruited and induce a downstream signaling cascade that involves phosphorylation and activation of multiple cytoplasmic molecules. The phosphatidyl-inositol 3' kinase (PI3K), the Grb2 and the MAP kinase pathways are activated by both FLT3 **[171]** and CSF1R **[172]**.

As a results of those pathways, KIT and FLT3 **[173]** have a key role in cell survival, proliferation and differentiation of hematopoietic progenitors, while CSF1R is crucial for the growth and differentiation of the monocyte and macrophage lineage **[174]**.

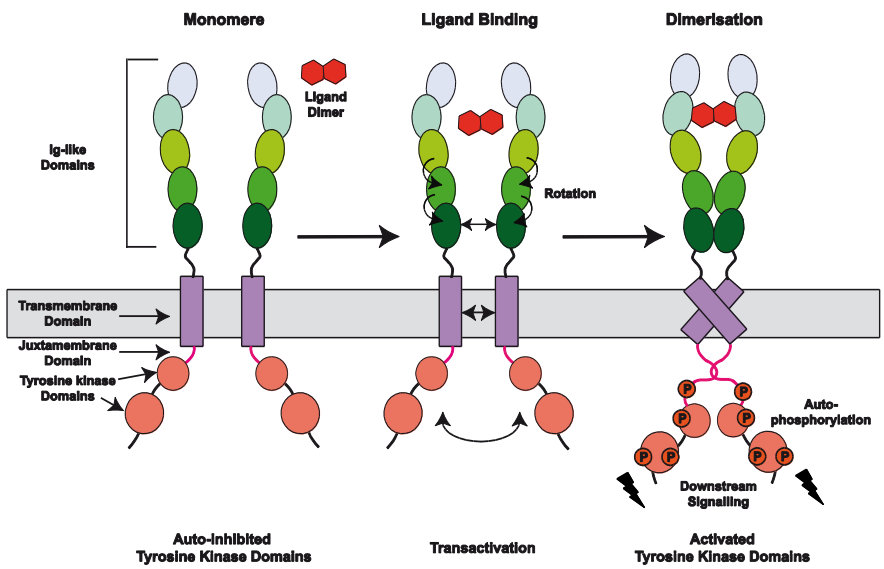


Figure 5 – Schematic representation of RTKs class III and their mechanism of action

3. Modeling and studying hematopoiesis

In this thesis, the research has been conducted using mouse models (Papers I and III) and both human ES/IPS cells and primary human fetal samples (Paper II). In this section, I will discuss the relevance of these models, the limitations and the ethical considerations that we, as researchers, try to address prior to and while performing our investigations.

The mouse as a model

Use of mice to model hematopoiesis

In 1937, Life Science magazine published an article on the "war against a new enemy: cancer." In this article, between pictures of X-Ray machines and tumor sections, we are informed of the mouse as a model with "similar physiological structures" to humans, and that "*Its life cycle is man's life cycle in miniature*" [175]. By providing genetically homogenous models, being inbred at a reduced cost making experiments easier to repeat, mice have been widely used as hematopoietic models *in vivo*. In 2007, the Nobel Prize for physiology and medicine was awarded to Mario R. Capecchi, Martin Evans, and Oliver Smithies for their generation of the first knock-out model in 1989, by introducing specific gene modifications in mice using ES cells.

Following this, phenotypic characterization with the analysis of cell population frequencies of inbred mouse strains, combined with loss of function and genetic modifications, have revealed the molecular and cellular mechanisms of a wide

range of biological process and disorders with inherited or acquired mutations found in human diseases. In addition, the development of conditional and inducible knockouts using the Cre/LoxP system and Flp/Frt, both derived from other organisms, allows a temporal, tissue or cell specific control of the ablation or induction of the expression of the gene of interest [176-180]. Another important aspect of mouse models is their ability to support regeneration of the entire hematopoietic system from a single cell, allowing an assessment of gene function in transplantation settings [106]. In addition, the different isoforms of the pan-hematopoietic marker, CD45, enables the distinction between recipient and donor cells.

Development of leukemic mouse models, such as the acute myeloid leukemia (AML) models, has also been extensively used to study the disease onset and initiating populations [181, 182]. In addition, introduction of immune deficient NOD-SCID IL2Rg^{null} (non-obese diabetic-severe combined immune deficiency, NSG) mice has led to an important improvement of *in vivo* xeno-transplantation assays to study human hematopoiesis [183, 184]. These mice lack mature T cells, B cells and NK cells, making them a suitable host when performing xeno-transplantation with oncogenic human primary or transduced cells, for example.

Furthermore, humanized mouse models are being developed to express human genes, encoding essential cytokines of the hematopoietic development, aiming to recapitulate human hematopoiesis and its immune system *in vivo* [185-187].

Limitations of mice models

Besides the improvement and development of models to understand hematopoiesis, the use of mouse models still retains some limitations. Difference in the transformation capacity of mouse cells compared to human, differential cytokine requirements (for example FLT3L and SCF for HSC development), as well as genetic differences, can complicate the use and interpretation of data generated through mouse model characterization [188, 189].

While they allow the development of both human B and T cells, humanized mouse models such as NSG are limited in their capacity to generate a cellular and humoral response after immunization or infection, preventing the generation of a fully mature immunological system. For example, class-switching appears to be inefficient, sustaining a pool of immature B cells, which is suggested to be due to the lack of formation of organized lymphoid structure in the periphery required for their maturation [190]. In addition, humanized mouse models are compromised in their capacity to generate circulating human platelets, erythrocytes and myeloid cells [187].

Moreover, transplantation of human $CD34^+$ shows a low long-term maintenance of human engraftment, but genetic modifications of the mouse niche or implementation of human niche cells could potentially overcome this issue [191, 192].

For a leukemic model, the mouse system is strongly biased toward the development of AML in contrast to human [193]. For example, MLL-AF9 fusion (mixed lineage leukemia-AF9), which can progress to either acute lymphoid or myeloid leukemia in patients, only develops into myeloid leukemia when developed in the mouse system. Improved genetic manipulation and transduction of primary human cells transplanted into humanized mice could lead to a better recapitulation of the human disease [194-196].

In-vitro models (hES/hIPS system)

Human Embryonic and Induced Stem Cells for modeling

While the use of mouse models provides a first line of research, the findings still need to be validated in the human setting. However, access to primary human fetal tissue can often be a challenge, therefore, establishing cell lines mimicking the development of human tissues is of great interest. Human ES cells (hES) are pluripotent stem cells derived from the inner cell mass of a blastocyst (**Figure 6**).

Human ES have the capacity to differentiate into any cell type of the body and also to self-renew. The first human ES cell lines were derived in 1998 [197] and are nowadays widely used, both for comparative research (comparing adult, embryonic and tissue-specific stem cells) and also for *in vitro* testing, to assess the genotoxic and other properties of chemical compounds (EUROSTEMCELL and CARCINOGENOMICS European Union financed projects). These cells can be grown in 3D and differentiate into specialized tissues and cells [198].

In 2007, reprogramming of human somatic cells to generate induced pluripotent cells (hIPS) was demonstrated by the 2012 Nobel Prize Laureate Shinya Yamanaka [199]. Having the same properties as hES to differentiate into all cell types, they have the potential to be a great alternative for tissue regeneration and also for modeling human development and disease [200].

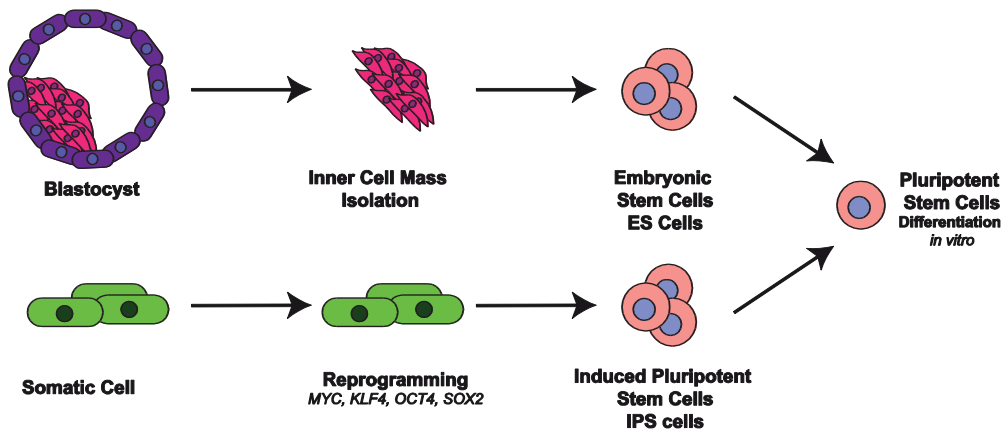


Figure 6 – Sources of Pluripotent Stem Cells

Pluripotent Stem Cells can be obtained from the inner cell mass in the blastocyst or by re-programming somatic cells. They can give rise to the three germ layers (endoderm, ectoderm, mesoderm) but not to extra-embryonic tissue such as the placenta.

In addition, new genome-editing technologies such as CRISPR/Cas9 combined with pluripotent stem cells (ES or IPS) should allow the study of gene functions, disease modeling and gene therapy [201].

Limitations of *in vitro* modeling

Besides the ethical consideration in the use of hES or hIPS cells, as discussed in the next paragraph, some biological limitations still need to be considered. For both hES and hIPS cells, their undifferentiated state gives them the potential to form all three germ layers and therefore to form teratomas [202]. Elimination of undifferentiated cells is then a requirement if the use of these systems is going to be pursued further on, for example, in cell-based therapies [203].

For hIPS, reprogramming strategies are often associated with the use of potentially dangerous genome integrating viruses to deliver reprogramming factor genes that can increase the risk of tumour formation [204]. In addition, to which extent hIPS are similar to hES and to which extent their differentiation gives rise to functional fetal or adult systems (for example fetal globin versus adult) remains to be fully established (see Paper II) [205, 206].

Ethical considerations

Whether research is performed on animal models or using human cells, the benefits and risks need to be properly and thoroughly thought through and evaluated.

> « *Science sans conscience n'est que ruine de l'âme* »*, Rabelais, 1542

The "Three Rs"

Performing research on animals confronts one with a moral dilemma: it is wrong to harm animals, but it is also wrong to neglect human health. When performing research using animals, a set of guidelines and principles allows the ethical evaluation of such use, trying to evaluate the harm versus the benefits. The three R rules consisting of Replacement, Reduction and Refinement, described in 1959,

*"Knowledge without conscience is but the ruin of the soul"

serves as a first guide. Replacement strategies, such as the one described above (*in vitro* system), are being used. However, the need of *in vivo* evaluation of the resulting research can sometime not be avoided, as for example in the case of HSCs studies when engraftment and long-term reconstitution can only be tested *in vivo*. When using animals, researchers try to maximize the data generated in order to reduce the overall animal number involved by planning carefully and designing strategically their experiments. In addition, by providing good animal husbandries and experimental procedures that minimize the pain and distress, researchers try to refine their work, enhancing the welfare of the animals used.

Patient and donors consent and the use of human material

When using patient or volunteer donor samples, an ethical application evaluated by an independent committee is first written and assessed. Only planned, well-organized and well-risk assessed researches are allowed to be pursued and need to follow high ethical standards. In 1964, the World Medical Association published and adopted a set of ethical principles for medical research involving human subjects or materials in a 2000 word document termed the Declaration of Helsinki. Practically, in basic research, for all tissues collection (cord blood, BM and fetal samples in this thesis), voluntary donors or their legal guardians have to be informed of the aims, methods and potentials risks of the study, together with being informed of the right to refuse to participate or to withdraw consent at any time. Only after understanding this information, the subject can freely give informed consent. In this context, informed-consent is key for any type of medical research. The Declaration of Helsinki is however a "living text" since it has already been modified seven times, and some paragraphs still remain under debate on their application. For example, in paragraph 36 of the latest updated text, it is stated that "negative and inconclusive as well as positive results should be published or otherwise made publicly available". The question is how can this be achieved in practical terms? Publishing positive results which may change medical research is often perceived as more interesting than negative results. But can bias in research be considered as something related to an ethical issue? Maybe by only

giving one part of the picture we do not clearly assess which treatment/discovery could be the best or more interesting to follow. Also, the Declaration of Helsinki is addressed primarily to clinicians, but shouldn't scientists be considered also? Besides this questioning, in a general perspective, human tissues should only be used after a thoughtful assessment of the benefits and risks with also a commitment of the researchers to be fully transparent with their use of such material.

A human in a dish?

Work performed using ES cells brings up an important and necessary society debate. To obtain ES cells, the early embryo (4-5 days post-fertilization) has to be destroyed. This means destroying a potential human being for research purposes, even though those embryos obtained were meant to be destroyed. But, yet again, a moral dilemma arises: ES cells could lead to the discovery of new therapies that have the potential to reduce the suffering of many people, but don't we have a duty to respect human life? Choosing here would mean addressing an important question: does the embryo have a status as a person? Also, what happens after we obtain the cells? In 1984, the Warnock report (United Kingdom) recommended that "*no live human embryo derived from in vitro fertilisation, whether frozen or unfrozen, may be kept alive, if not transferred to a woman, beyond fourteen days after fertilisation, nor may it be used as a research subject beyond fourteen days after fertilisation*". This report setting the limit at 14 days post fertilization for *in vitro* research on an intact human embryo was drawn because of the formation of the primitive streak representing the earliest point at which an embryo is fully individualized.

In the use of ES cell lines, the 14-day rule does not apply: indeed, separation of the inner cell mass from the trophectoderm implies the loss of cells with the potential to form extra-embryonic tissues. However, recent studies bring up again the question of how long and what type of research we can perform with these cells. Several groups reported from *in vitro* cultures of ES cells (mouse and human) a

recapitulation of some aspects of the spatial organization of the embryo during and after gastrulation [207, 208]. A human in a dish? This raises new ethical questions that need to be addressed.

In this thesis, some of the work was performed using ES or IPS cells without the potential of regenerating *in vitro* a full organism. Indeed the comparison that we are making in Paper II between primary tissues, ES and IPS cells has the objective to allow the modeling of hematopoiesis without requiring the use of primary or embryonic derived tissues. In our study, we also use the genome-editing technology CRISPR/Cas9. While we are using it here to model a disease, another group has used it for genome editing in "non-viable" human embryos [209]. Not only does this raise ethical questions on how ethical modifications of germline cells are, but it can also bring up the damages that such controversial studies can have on scientific research [210, 211]. Overall, questioning ourselves, where do we stand while being transparent with our work, is, in my mind, a primary and important aspect of our work.

4. B cell development

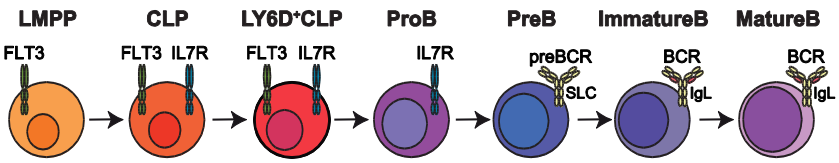
B cell is a lineage first discovered through its function and what it produces: antibodies. In 1890, Emil von Behring, the winner of the first Nobel Prize in Physiology and Medicine, together with Shibasaburo Kitasato discovered the implication of "circulating anti-toxins" in the immune response against diphtheria. Later, Paul Ehlrich suggested that cells producing these anti-toxins had specific receptors that bind to the toxic substance. It then took 50 years to get an insight on such specificity and diversity with the two hypotheses made by Niels K. Jerne, the "natural selection", and by Frank Macfarlane Burnet, the "clonal selection" [212, 213]. The second hypothesis was then further demonstrated by showing that one cell always produces one antibody [214].

Yet, the producing cells and their origin remained unknown. In a Nature paper, Max Cooper, using irradiated chickens, showed that with no bursa, they were not able to produce antibodies when injected with bovine serum albumin [215]. The equivalent organs in mammals were subsequently found as the FL, and as the adult, BM [216, 217]. Since then, B cells have been considered as a fully distinct lineage of the adaptive immune cells.

Towards a B cell

Adult B cell development

The early steps of B cell development take place in the BM in the adult (**Figure 7**). The Ly6D$^+$ CLPs (described in Chapter 2) further differentiate in the first CD19$^+$ progenitor cells, the ProB cells, while also undergoing a process called VDJ



B220	-	-	+/-	+	+	+	+
CD19	-	-	-	+	+	+	+
CD43	+	+	+	+	-	-	-
CD93	+	+	+	+	+	+	-
IgM	-	-	-	-	-	+	+
IgD	-	-	-	-	-	-	+
KIT	+	+	+	+	-	-	-
FLT3	+	+	+	-	-	-	-
IL7R	-	+	+	+	+/-	-	-
IgH	Germline	Germline	V-D↔J	V↔DJ	VDJ	VDJ	VDJ
IgD	Germline	Germline	Germline	Germline	V↔L	VL	VL

Figure 7 – B cell development in the adult mouse

Expression of the surface markers at different stages of B cell differentiation and recombination status.

LMPP, lympho primed multipotent progenitors; CLP, common lymphoid progenitors; IgH, immunoglobulin heavy chain; IgL, immunoglobulin light chain; SLC, surrogate light chain.

recombination [218, 219]. This initial recombination consists of a rearrangement of the diversity (D) and joining (J) regions of the gene encoding the immunoglobulin heavy chains (IgH), followed by a rearrangement of the variable (V) to the DJ. This process is mainly operated by the enzymes RAG1 and RAG2 (recombination activating gene) and has been shown to be initiated already at earlier stages in the development [220-223]. The B cell development classification developed by Hardy's lab has another fraction of cells, the so-called PreproB cells, thought to derive from CLPs and to further differentiate in ProB cells. This cell population expresses similar surface markers to CLPs with the addition of low surface expression of B220 [224]. However, single cell gene expression analysis

reveals a rather heterogeneous population with only few cells expressing the RAG genes and with, in addition to B cell potential, a strong T and myeloid potential (unpublished observations).

ProB cells then differentiate into PreB cells that lose the expression of CD43 and that have fully completed the VDJ rearrangement. At this stage, the pre-B cell receptor complex (preBCR) is expressed with the newly rearranged IgH, a surrogate light chain (lamda5 and VpreB) and the Ig-associated protein CD79a and CD79b (encoded by *Mb1* and *B29* genes) [219, 225]. Signaling through the preBCR terminates the recombination through down-regulation of *Rag1* and *Rag2* expression and triggers a high proliferation of the cells [226, 227]. Later signals, again through the preBCR, re-establish a quiescent state, allowing a recombination of the V and light (L) regions of the light chain (IgL) gene [228, 229].

After completing the second rearrangement, the cells then become immature B with the expression of a complete BCR (with IgM isotype) [219]. Immature B cells develop further passing through a negative selection where autoreactive cells are either depleted or undergo receptor editing [230]. Finally, mature B cells expressing both IgM and IgD leave the BM for the spleen and secondary lymphoid organs, where they further develop to become fully mature and functional parts of the adaptive immune system [231].

Embryonic B cell development

While extensive studies have allowed the characterization of B cell development in the adult, little is known about the staging of this lineage in the embryo. In fact, whether the embryonic B cell development follows the same differentiation path as in the adult is unclear. The presence of progenitors with B cell potential is already detected at E8.5 in the YS and PsP, although ability to generate other lineages has not been assessed [232, 233]. Whether both sites can generate *de novo* B cell progenitors is still under debate [53, 234, 235]. Importantly, these previous studies only show that a potential can be detected and it is not until E13.5 that PreB cells are found, while IgM^+ cells are detected at E17 in the FL [165, 236].

Applying the adult B cell staging in the embryo led to the identification of a $CD19^{+}B220^{-/low}$ cell population in the FL that is also sustained at low level in the adult BM. These cells display a restricted potential to the fetal B1 B cell lineage and cannot produce adult B2 B cells [237, 238]. The B1 B cells are characterized as $IgM^{high}IgD^{low}MAC1^{+}$cells and are found mainly in the peritoneal and pleural cavities. They can be further subdivided using CD5 expression ($CD5^{+}$, B1-a and $CD5^{-}$, B1-b). The B1 B cells have a less diverse repertoire compared to B2 cells and secrete spontaneously "natural" antibodies as a first line of defense, and can respond quickly in a T-cell independent manner against specific pathogens (such as *Streptococcus pneumoniae*) [239].

Of note, the origin of B1-a has been under debate for many years and several models have been suggested. Evidence for B1-a lymphoid potential prior to HSC emergence in the YS and PsP suggests that a distinct progenitor gives rise to B1-a lineage [240-243]. However, a recent study using single cell barcoding has shown that indeed, single fetal HSC are able to generate B1-a cell and that this potential is lost during ontogeny [244]. This indicates that the B1-a cell lineage is fully part of the fetal HSC differentiation potential, though it does not exclude the existence of restricted progenitors prior to HSC emergence.

In humans, the existence of such B-1 cells is still not fully defined. One piece of evidence is the identification of a human cell population that is functionally similar to mouse B-1 cells in the cord blood and peripheral blood [245]. The origin of these cells remains however to be determined.

As already discussed in Chapter 1, embryonic B cell development also differs from the adult, through the existence of B/myeloid bipotent progenitors (see Chapter 1). Again, the origin and regulation of such cells is unknown but the potentially close developmental connection between the B and myeloid lineages is of high relevance for understanding infant and childhood leukemias displaying bi-phenotypic (B-myeloid) cells [246].

Regulation of B cell development

For the development of B cells, some major TF and cytokines are involved, and through a complex network, they orchestrate the B lineage fate.

Intrinsic regulators

The specification and commitment to the B cell lineage is dependent mainly on four important TFs (**Figure 8**). E2A, (immunoglobulin enhancer-binding factors E12/E47) encoded by the gene *Tcfe2a*, is critical prior to the B cell commitment, since in its absence, B cell development is blocked at early progenitor stages (LY6D$^-$ CLPs) [120]. E2A is also essential for the expression of *Foxo1,* (forkhead box O1), encoding for another critical early B cell regulator, FOXO1 [247]. This protein is required at several stages during differentiation, and is critical for the modulation of the expression of *Rag1* and *Rag2*, and for the cell surface expression of IL7R [248, 249].

Another critical factor of the early B cell commitment is the TF EBF1 (early B cell factor 1) [250, 251]. When lacking either FOXO1 or EBF1, B cell development is blocked at the LY6D$^+$ CLP stage, and gene expression analysis of the deficient cells shows strong similarity, suggesting a cross-regulation between the two TFs [247, 252, 253].

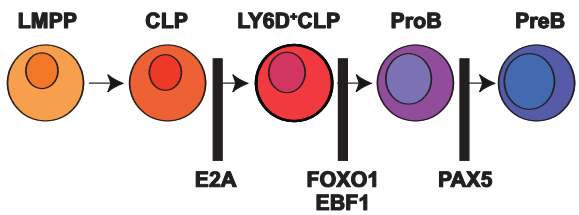


Figure 8 – Stage-specific B cell development block in mice deficient for essential B cell transcription factors

LMPP, lymphoid primed multipotent progenitors; CLP, common lymphoid progenitors; E2A, immunoglobulin enhancer-binding factors E12/E47; FOXO1, forkhead box 01; EBF1; early B cell factor 1; PAX5, paired box protein 5.

Once all are activated, these three proteins induce the expression of PAX5 [254]. PAX5 is essential to maintain the B cell identity and acts as an important suppressor of non-B lineage fates [255, 256]. *Pax5*-deficient mice display a complete block of B cell differentiation at the early ProB stage [134, 135]. Interestingly, *Pax5* deficiency in the embryo induces a block prior to the ProB stage, suggesting a requirement already for B cell specification in the embryo while being required only for B cell development progression in the adult [257].

Extrinsic regulators

Two cytokines and their receptors have been shown to be key for B cell commitment and differentiation: FLT3 and IL7R [258]. IL7R belongs to the family of hematopoietin receptors and dimerizes with the common gamma chain upon IL7 ligand binding [259, 260]. IL7R is expressed on the earliest lymphoid progenitors and its expression remains until the PreB stage [119, 218]. IL7 is produced by BM stromal cells [261]. This cytokine is required for survival and differentiation of B cells and also for up-regulation of EBF1 [253, 262-264]. The absence of IL7R signaling induces a striking deficiency in lymphoid development both for B cells and T cells (**Figure 9**) [265]. However, B1 B cells and marginal zone B cells seem to be generated independently of IL7 signaling, suggesting a differential requirement of adult B2 cells compared to fetal derived populations [266].

Of note, *Il7r*$^{-/-}$ mice display a more severe phenotype compare to *Il7*$^{-/-}$ [267]. The thymic stromal lymphopoitein receptor (TSLPR) has been shown to dimerize with IL7R upon TSLP binding and could potentially explain this difference with an IL7-independent lymphopoiesis [268, 269]. In the embryo, TSLP together with FLT3L has been suggested to mediate fetal B lymphopoiesis, but this has been questioned in other studies [270-272]. It is therefore not clear what role TSLP has in IL7 independent B lymphopoiesis, and whether other cytokine pathways may be involved.

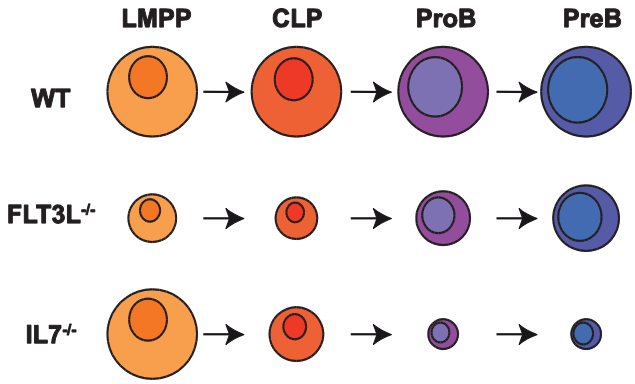


Figure 9 – Reduced cell populations in adult mice deficient in key B cell cytokines

LMPP, lymphoid primed multipotent progenitors; CLP, common lymphoid progenitors; FLT3L, flt3-ligand; IL7, interleukin 7

FLT3 is a type III receptor tyrosine kinase (see **Box 1 and Figure 5** for description of the receptor). Two groups independently cloned FLT3 in 1991 from mouse tissues [273, 274]. FLT3 and FLT3L have a key role in lymphopoiesis in mice [173, 275-277]. Within mouse hematopoietic hierarchy, FLT3 is expressed earlier than IL7R, and found already on LMPPs, CLP and on ProB cells, but not thereafter [111, 118, 278]

Constitutive loss of FLT3 or its ligand induces a decrease in B and T cell progenitors as well as upstream precursor cells in the hematopoietic hierarchy, such as LMPPs and CLPs (**Figure 9**) [279-282]. In the more mature stage, functional B cells are present at near normal levels in the periphery at steady state while being strongly reduced following transplantation or myelo-ablation [283]. Combined signaling through IL7R and FLT3 selectively promotes B cell commitment and differentiation [284]. In line with this synergistic interaction, mice double deficient in FLT3L and IL7R almost entirely lack mature B cells and B cell progenitors, both in fetal and adult hematopoiesis [282].

5. Stem cells and Leukemia

As mentioned at the beginning of this thesis, understanding hematopoiesis in the embryo is highly relevant for understanding the origin and onset of infant and childhood blood disorders. Although leukemia occurs most often in the older adult, it is the most common form of childhood cancer (30%). Acute lymphoblastic leukemia (ALL) accounts for approximately 75% of all childhood leukemias. In contrast, the most common types of leukemia in adults are AML and chronic lymphoid leukemia (CLL). Here, I will give a brief overview on infant and childhood leukemia, and more specifically on the biology of ALL.

The complexity of cancer

Cancer cells are highly heterogeneous and it has been proposed that among all these cells, only a few act as tumour-initiating cells or "cancer stem cell" (CSCs). These CSCs can sustain the disease and have the same ability as normal stem cells to self-renew (**Figure 10**).

The first demonstration of the existence of CSCs was made in 1997 in John Dick's laboratory using sample from AML patient [285]. Upon transplantation into immuno-deficient mice, only the most primitive fraction of cells was able to engraft long-term and to reproduce the human disease, suggesting a hierarchical organization of the cancer. This discovery has been followed by identification of other CSCs in blood cancer and also in solid tumours [286-288].

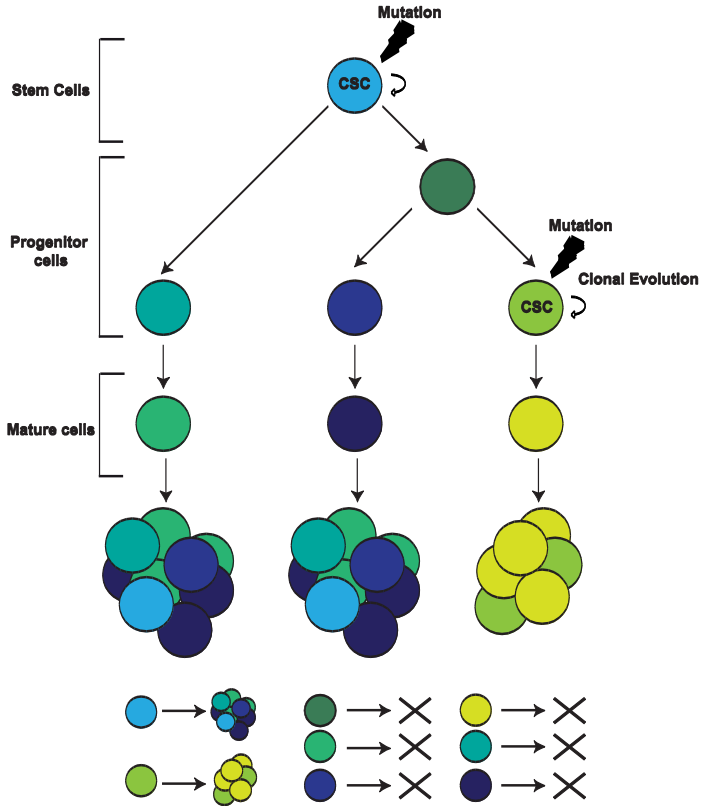


Figure 10 – Clonal evolution of the Cancer Stem Cell (CSC) model

In the CSC model of tumour generation, only a few tumour cells have the ability for self-renewal and can give rise to more committed progenitors with limited proliferative potential and tumour re-generation potential. New somatic mutations in progenitor cells can generate a clonal evolution with generation of new initiating tumour cells that increase the heterogenitiy of the tumor.

The concept of CSC is however still debated since multiple studies suggest that these cells are not necessarily rare or phenotypically distinct [289, 290]. In addition, the generation of sub-clones harbouring secondary mutations conferring a resistance to drugs in CML (chronic myeloid leukemia) treatment provides the evidence for clonal evolution in cancer and for the leukemogenesis contribution of more differentiated cells (**Figure 10**) [291]. Both conceptions of cancer organization could however be combined with new genetic approaches, allowing a mapping of the disease evolution with the identification of common ancestors [288, 292].

Pediatric Leukemias

Pediatric leukemia, when occurring before 12 months of age, is termed infant leukemia, while after this and before reaching adulthood, is called childhood leukemia. Identifying the common ancestor or cell of origin in child leukemia appears to be a real challenge. Indeed, it is now widely accepted that childhood and infant leukemias are mainly the result of initiating chromosome translocations arising before birth, during *in utero* haematopoiesis [2, 293]. Considering the difference between adult and embryonic hematopoiesis (see Chapter 2), identification of such target cells requires first a better understanding of the embryonic blood development. Moreover, not only may the cellular founders of infant and childhood disease differ, but also the mutations observed. More than 200 genes have been found to be involved in translocations in childhood leukaemia, but many of these have only been reported sporadically, and few genes appears to be very frequently mutated.

Infant leukemia

Rearrangement of the MLL gene is found in up to 80% of the infant ALL (less than 12 months old), and is characterized by immature B cells lacking CD10 expression and expressing myeloid surface markers [294, 295]. This unique B/myeloid feature of MLL (majority rearranged as MLL-AF4) was suggested to be the result of oncogenic events occurring in bi-potent progenitors which are active specifically during fetal life [296]. However, prospective identification and characterization of such cells in the human fetus remain technically challenging. Recently, as another distinction between infant, childhood and adult ALL, a study demonstrated that MLL gene fusion alone could lead to an aggressive leukemia in infants, while in older children, a more complex mutational landscape with additional somatic mutations was required for disease development [297, 298].

Childhood leukemia

In childhood leukemia, TEL-AML1 (also known as ETV6-RUNX1) is the most common fusion gene observed, accounting for around 20% of childhood ALL cases [299]. Monozygotic twins concordant for ALL were found to share the same fusion gene break point, suggesting that a common pre-leukemic clone able to generate a pre-leukemic state was generated *in utero* and shared between the twins [300, 301]. Indeed, the TEL-AML1 fusion gene can already be detected at birth, prior to the development of leukemia, however, only a few cases will develop into leukemia, suggesting that secondary genetic mutations are required for the disease onset [302]. Supporting this, studies of TEL-AML1 have allowed the identification of several other genetic alterations involving the B cell TFs, *Pax5* and *Ebf1* [303]. In addition, analysis of IgH rearrangement revealed that the first targeted cells may be early ProB cells not fully-restricted, since similar DJ rearrangement was observed in twin pairs [304]. Once again, further functional studies are needed to pinpoint the cell of origin and would require the development of a relevant model to study the disease emergence.

Tyrosine kinase receptors in leukemia

Identification of mutation or overexpression of TK at the surface of cancer cells has been widely described and has led to the development of drugs inhibiting their activity. Since the description of the first inhibiting compound of the epidermal growth factor receptor in 1988, numerous tyrosine kinase inhibitors (TKI) have been generated and proven to be efficient as anti-tumorigenic such as Imatinib against BCR-ABL fusion protein in CML [305, 306].

FLT3 in leukemia

FLT3 represents a potential target, given the fact that its overexpression is reported in the majority of leukemia cases and considering the high rate of FLT3 mutations in human leukemia. It is found aberrantly overexpressed in PreB ALL and in AML

primary samples [307-309]. In addition, activating mutations of FLT3, leading to a constitutive activation in almost 30% of AML cases, led to the development of several inhibitors for the treatment of these patients, showing some promising initial results [310, 311].

Further, gene-expression profiling of MLL patient samples, compared to PreB ALL and AML, demonstrated a unique signature of MLL rearranged leukemia with a consistent over-expression of FLT3 [312]. FLT3 mutations associated with an over-expression are also common in hyperdiploid ALL, while not being frequently detected in TEL-AML1 childhood B-ALL [313]. FTL3 targeting could therefore be a potential therapeutic approach in pediatric leukemias.

However, the acquired resistance against TKI appears to be the new challenge: most of the response is transient and followed by progressive disease reappearance [304]. Different mechanisms could explain this resistance to FLT3 inhibitors, for example, the pharmaco-kinetic barriers and the fact that mutated FTL3 in the kinase domain has a different conformation and therefore cannot be targeted by classical inhibitors. Other pathways might also be involved in compensatory mechanisms. It is therefore important to monitor FLT3 inhibition and to combine FLT3 TKI with other treatments (such as chemotherapy, targeting other pathways, monoclonal antibodies, etc.).

CSF1R in leukemia

CSF1R has been reported to be mutated (point-mutations) in human AML cell line [314]. More recently, a study indicated that PU.1 mediates an up regulation of *CSF1R*, leading to an increase of leukemia stem cell potential induced by the fusion protein MOZ-TIF [315]. Interestingly, ablation of *CSF1R* in those leukemic stem cells resulted in a suppression of the AML induction. In lymphoid disorders, several cases have been reported in which the myeloid *CSF1R* is rearranged in childhood PreB B ALL [316-318]. In addition, *in vitro* studies using human leukemic PreB ALL cells have demonstrated sensitivity to CSF1R-specific inhibitors. This supports that CSF1R might be a relevant therapeutic target [318].

6. Present investigations

While the development of the hematopoietic system in adults has been largely investigated, little is known about the fetal hematopoietic hierarchy. In particular, the cytokine requirement for the lympho-myeloid development is not well established. In addition, suitable models for studying this development in humans remain challenging to generate.

In this thesis, I have addressed the cellular and molecular differences between adult and embryonic hematopoiesis, within the different stages of the hematopoietic hierarchy. I also studied the initiation of lymphopoiesis in human primary fetal tissue in comparison to *in vitro* models. The main aims of this thesis were as follows:

Paper I

To investigate whether the tyrosine kinase receptor CSF1R has a previously unknown role in B lymphopoiesis.

Paper II (Manuscript)

To investigate the human lympho-myeloid development in the early embryo and how to model it *in vitro* to study the onset of childhood leukemia.

Paper III (Manuscript)

To investigate the stage role specifics of FLT3, trying to underpin the cellular stage requiring its regulation.

7. Summary of results and discussion

Paper I

Macrophage colony-stimulating factor receptor marks and regulates a fetal myeloid-primed B-cell progenitor in mouse.

Background

Recently CSF1R mutations have been found in cases of B-ALL [317, 318]. However, whereas its requirement for myeloid development has been established, so far, no clear role of this receptor or its ligand have been reported in B lymphoid development [161]. Leukemic mutations in childhood disorders are proven to occur already *in utero*, suggesting that the target cell for leukemia initiating cells may be a fetal restricted progenitor cell [2]. A specific embryonic population with combined B/myeloid potential has been previously found in the mouse embryo but its prospective isolation and characterization has been lacking [40, 41].

In this study, we aimed to further investigate the relationship between the B and myeloid lineage by analysing the first B cell progenitors emerging in the embryo and characterizing their potential regulation by CSF1R.

CSF1R is expressed by the earliest B progenitor cells in the mouse embryo

We first investigated whether CSF1R is expressed on B cell progenitors at different time points during embryonic development. We found that at E13.5, 20% of the FL ProB cells expressed CSF1R at the surface, and that this percentage decreased to 4.1% at E14.5 while being totally absent in adult BM ProB cells. More differentiated B cell progenitor populations, PreB and immature B, were found not to express CSF1R, neither in embryonic nor adult hematopoiesis. We validated the E13.5 ProB surface expression by performing single cell gene

expression analysis and could detect single cells co-expressing both B lineage specific genes (*Mb1, Ebf1, Pax5*) together with *Csf1r*. Thus, the hypothesis that CSF1R may play a role in lymphoid development was further narrowed down to a specific role in the embryo, at the earliest stage of B cell development.

CSF1R marks a fetal bipotent B myeloid-primed progenitor cell

We next investigated whether the $CSF1R^+$ ProB cells were distinct from the $CSF1R^-$ ProB by comparing their gene expression profiles. We found that while both populations (as well as the adult ProB) expressed key lymphoid and B cell genes (*Rag1, Rag2, Ebf1, Pax5, Mb1*), only the $CSF1R^+$ cells expressed myeloid genes (*Mpo, Csf3r, Fcgr3, Cebpa, Cebpb*). In addition, when cultured *in vitro* using the OP9 system, we observed B and myeloid clones generated from $CSF1R^+$ ProB, while adult ProB and E13.5 $CSF1R^-$ ProB gave rise only to B cells. Together, these results suggest that CSF1R marks a developmentally restricted myeloid-primed B cell progenitor.

Loss of CSF1R impairs fetal B lymphopoiesis

To address the requirement of CSF1R signaling for B lymphopoiesis, we next investigated B cell compartment in embryos lacking *Csf1r*. We observed a reduction of ProB cells already at E13.5, and in ProB cells, as well as downstream populations, at E14.5 and E17.5. Importantly, upstream populations, HSC, LMPP and CLP, were not affected by the loss of CSF1R, suggesting a specific requirement of CSF1R for the B cell lineage. In addition, transplantation assays demonstrated that, in agreement with CSF1R not being expressed on adult ProB cells or subsequent B cell precursors, CSF1R signaling was dispensable for adult B cell progenitors regeneration.

Complementary role of CSF1R and FLT3 for the emergence of B cells

Our gene expression analysis showed a high level of co-expression of *Csf1r* and *Flt3*. We then assessed the impact of the loss of both receptor pathways on fetal B cells and observed a more severe impairment in double deficient embryos compared to single mutants. Thus, CSF1R and FLT3 signaling have a critical and complementary role in fetal B lymphopoiesis.

Final remarks and future directions

In this study, we identified a distinct B cell progenitor subset uniquely present in a narrow window of FL lymphopoiesis that expresses and is regulated by CSF1R (**Figure 11**). This finding, showing for the first time the importance of a myeloid cytokine for lymphoid development only in the fetus, highlights the need for studying specifically embryonic hematopoiesis, since regulatory requirements can change during ontogeny.

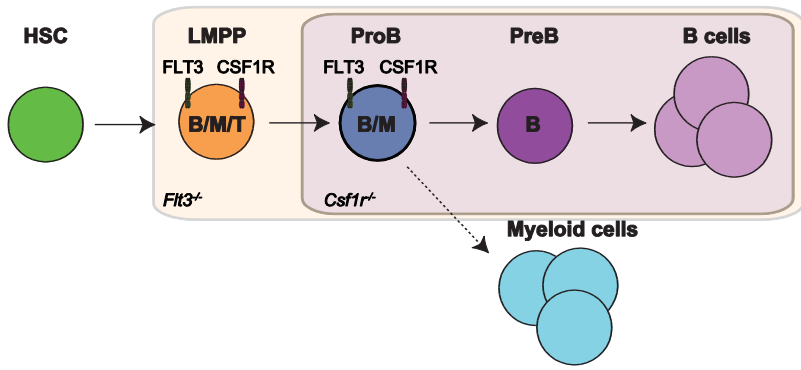


Figure 11 – Suggested hierachy for the emergence of B progenitor cells in the embryo

Expression of CSF1R and FLT3 in early B cell progenitors and reduced cell populations in embryo deficient for their signaling (shaded area).

Establishing the identity of the earliest fetal B cell progenitors, and that these, unlike their adult counterparts sustain myeloid lineage gene expression and potential, is highly relevant for understanding the biological origin of pre-leukemic and leukemic lesions in infant and childhood PreB ALL that can lead also to bi-phenotypic leukemias [246].

Additional studies of the mechanisms behind the CSF1R expression would be needed to understand the potential role of the cells identified in this study in blood disorders, and to assess their leukemic potential. Identifying the human counterpart of this population would be a first step, as well as comparison of

CSF1R expression in the adult BM, cord blood and also FL. The challenging access to human fetal tissue might limit further studies, but the development of a new *in vitro* model of early human hematopoiesis could help such work.

Paper II

A Human IPS Model implicates Embryonic B-myeloid Fate Restriction as a Developmental Susceptibility to ETV6-RUNX1.

Background

Childhood and adult ALL are clinically and biologically distinct blood disorders. The spectrum of mutations is very different with for example 20% of childhood ALL expressing the fusion gene ETV6-RUNX1, while this fusion gene is only found in less than 3% of the adult cases. Besides different mutations observed, different initiating cells may further explain the difference between the adult and childhood forms of disease. Indeed, the ETV6-RUNX1 mutation can already be detected at birth, suggesting that the initiating events occur already *in utero* in embryonic specific populations [2]. Several studies in mouse have pointed out the lineage hierarchy differences in the embryo compared to the adult (for example the identification of B/myeloid progenitors and the expression of the lymphoid gene *Rag1* already in lympho-myeloid progenitors); it is therefore important to better characterize the embryonic hematopoiesis in order to understand the disease and its onset [40, 41, 47].

In this study, we aimed to characterize the earliest stages of B cell development in primary human FL and to investigate whether IPS cells can recapitulate this early lymphopoiesis. We developed *in vitro* fetal hematopoietic modeling tools using hES and hIPS cells comparable to primary samples in order to study the impact of ETV6-RUNX1 in embryonic hematopoiesis.

The earliest B progenitor cells express IL7R

Using multicolor flow cytometry, we analyzed the emergence of $CD19^+$ cells in the early human embryo. We observed that the earliest $CD19^+$ cells, (ProB cells also expressing CD34) could be detected at CS17, while PreB cells were found later, at CS20. The majority of ProB cells (60 to 88%) at CS17 expressed IL7R, while less than 20% of cells in the CB or adult BM counterpart were $IL7R^+$.

Identification of a novel lympho-myeloid progenitor

We next attempted to assess the upstream progenitor cells giving rise to these $CD19^+IL7R^+$ cells. We identified an $IL7R^+$ progenitor population whose frequency decreased throughout developmental age, with up to 10-fold reduction in adult BM compared to CS17. These FL progenitor cells showed a strong B cell output *in vitro*, together with production of macrophages but not erythroid cells, suggesting a previous restriction to a lympho-myeloid fate. In support of this, DJ rearrangement could also be detected in this population, demonstrating a commitment to the lymphoid lineage. We further characterized the $IL7R^+$ progenitors by performing a single cell gene expression assay. We found that the overall gene program changed depending on the ontogenic stage: at CS17, the majority of the cells had a myeloid primed program, while at CS20, lymphoid and B cell signatures together with myeloid were detected. Interestingly, we found at CS20 a transitory population displaying a B/myeloid signature. At birth, in the CB and in the adult BM, the majority of the $IL7R^+$ cells presented a lympho-myeloid signature without a sign of commitment to the B cell lineage. Importantly, and in line with Paper I, principal component analysis of the overall gene expression of the $IL7R^+$ progenitors at different time points showed that CSF1R contributed the most to the myeloid signature of the CS17 $IL7R^+$ progenitor.

Our results suggest a different cellular hierarchy of the early embryonic hematopoiesis compared to that of newborn and adult, with the existence of a specific early embryonic cell population likely to underpin the differences of oncogene targeting cell population in leukemia.

Modeling early human fetal lympho-myelopoiesis *in vitro* using hPSCs

As discussed in Chapter 3, modeling *in vitro* human embryonic hematopoiesis could lead to the development of useful tools to study disease onset. We differentiated *in vitro* human ES cells and human IPS cells (human pluripotent stem cells, hPSC) using an OP9/MS5 stroma system, and could observe the emergence of an $IL7R^+$ progenitor already at day 10, prior to the detection of ProB cells which occurs at day 31. This $IL7R^+$ progenitor had a similar B cell and

macrophage potential, as observed in primary tissues. Interestingly, when comparing single cell gene expression, IL7R$^+$ progenitors at day 10 had a strong myeloid signature similar to the fetal CS17 IL7R progenitor, while at day 31, IL7R$^+$ progenitors exhibited the same expression program as the CS20 progenitor, with a lympho-myeloid and B cell priming. ProB cells were clustering with CS20 FL ProB cells and were distinct from the adult BM counterpart. Thus, the *in vitro* generation of IL7R$^+$ progenitors and ProB cells, using hES and hIPS, resembles the FL hematopoietic counterpart populations functionally and transcriptionally, with a transition from myeloid primed to lympho-myeloid primed progenitor cells.

Modeling ETV6-RUNX1 mutation *in vitro* using hIPS

We then took advantage of our *in vitro* modeling of early human hematopoiesis to study the impact of the ETV6-RUNX1 mutation on hematopoiesis. Using a CRISPR/Cas9 strategy produced by the Enver's laboratory, we generated hIPS expressing ETV6-RUNX, together with a Venus-reporter. ETV6-RUNX1 hIPS clones generated a low number of CD19$^+$ ProB and PreB cells, while the IL7R$^+$ progenitor was detected at normal or near normal frequency compared to control, suggesting a block in B cell differentiation at the IL7R$^+$ progenitor stage. Global gene expression analysis (RNAseq) further supported this by showing that the few remaining CD19$^+$ ProB cells from ETV6-RUNX1 hIPS clustered with IL7R$^+$ cells rather than with primary CS20, and day 31 hPSCs derived ProB cells. Looking at single cell gene expression, we observed that IL7R$^+$ progenitors from ETV6-RUNX1 hIPS displayed a similar myeloid gene signature as observed in CS17 or day 10 hPSCs, while ProB cells generated from ETV6-RUNX1 hIPS had an enhanced co-expression of B and myeloid genes. Importantly, by removing the RUNX1 cassette, we could restore the production of ProB and PreB cells with a similar gene expression signature to ProB cells from control.

Final remarks and future directions

In this study, we have demonstrated that the first CD19$^+$ B cell emerging in the human embryo expresses IL7R and seems to derive from an IL7R$^+$ progenitor. This progenitor shifts from a myeloid to a lympho-myeloid gene signature

throughout development and we could recapitulate this transition and hierarchy *in vitro* using hPSCs. In addition, genome-engineered hPSCs expressing ETV6-RUNX1 displayed a partial block in B cell differentiation at the $IL7R^+$ progenitor cell level and the remaining ProB cells displayed aberrant co-expression of B and myeloid genes, resembling the earliest $IL7R^+$ progenitor. This supports that our identified $IL7R^+$ progenitor could represent a potential target cell for *in utero* ALL initiating pre-leukemic mutation.

The origin of the $IL7R^+$ cells cannot be fully determined and it could be that the $IL7R^+$ cells observed at CS20 are derived from those at CS17, or that these two populations are developmentally distinct. In addition, HSC emergence in human is already found at CS14, in the AGM region and therefore whether this progenitor is derived from definitive or transient hematopoiesis remains unknown [97]. One suggestion of a distinct development is the identification of a similar population in the E9.5 mouse YS, also $IL7R^+$ prior, to the emergence of HSCs, although this needs to be further demonstrated and would require the access to very early human embryo [47].

Paper III

Stage-specific roles of FLT3 in adult lympho-myelopoiesis

Background

The tyrosine kinase receptor, FLT3, is an important regulator of hematopoiesis that is widely expressed throughout the hematopoietic hierarchy. It is found at the surface of LMPP, CLP and also more lineage committed cells, such as ProB cells and ETPs, although at lower levels than upstream cells [110, 111, 117, 278]. Loss of function studies, using mice deficient either for the receptor or for its ligand, FLT3L, have demonstrated that FLT3 signaling is required for lymphoid and also myeloid development, with reduction already in early multipotent progenitors (LMPP, CLP), B, T and myeloid progenitor cells [275, 277, 279, 280, 282]. Therefore, these studies have not been able to address the specific cellular stage of FLT3 requirement in the hematopoietic differentiation, since the receptor was constitutively lost in the entire blood hierarchy and from the first blood cell in the embryo.

In this study, we aimed to broaden our understanding of the role of FLT3 by establishing at which specific stage it is required. For this purpose, we generated a conditional FLT3 deficient mouse model and induced FLT3 deletion in different lineages, and at different times, using different Cre expressing mice.

Generation of a FLT3 conditional mouse model

We first generated a mouse carrying loxP sites into the flanking intron of exon 15 of the *Flt3* gene, coding for a protein kinase ATP domain ($Flt3^{fl/fl}$). Upon the cross to a Cre-expressing mouse, exon 15 is deleted, leading to a non-functional protein. To validate our model, we crossed $Flt3^{fl/fl}$ with *Vav-cre* expressing mice, inducing a deletion in the entire hematopoietic tree. We observed similar reductions in early lympho-myeloid, B and T progenitors as previously reported in constitutive *Flt3*-deficient mice, together with the loss of FLT3 cell surface expression. Thus, FLT3 signaling is abrogated using our newly generated mouse model.

FLT3 is specifically required for adult hematopoiesis

We then pursued our study by assessing whether FLT3 is required specifically for adult hematopoiesis, or if the phenotype observed in adults was a consequence of embryonic requirement. For that purpose, we crossed *Flt3*$^{fl/fl}$ with the *Mx1-cre* mouse, allowing us to specifically induce the deletion in the adult upon interferon signaling induction with pIpC injection. We observed a reduction in early multipotent progenitors and T cell progenitors, but no changes in B progenitor cells. However, validation of the deletion at the DNA level showed that the majority of the remaining B progenitors were inefficiently deleted. We therefore generated chimeras in a competitive transplantation setting and observed here a drastic impairment in B progenitor reconstitution, suggesting that inefficient deletion at the steady-state may have allowed maintenance of the B cell pool. Overall, we propose that FLT3 is specifically required for adult hematopoiesis.

FLT3 is required after lymphoid commitment but not after adult B lineage restriction

We next crossed *Flt3*$^{fl/fl}$ with *Rag1-cre* mice in order to address whether FLT3 signaling is needed after lymphoid lineage restriction. Previous work, using transplantation of MPPs from Flt3-deficient mice, showed a defect in B cell reconstitution but no effect on the T cell lineage [276]. Our model allowed an assessment of this in steady-state conditions and we observed that both T and B progenitor cells were reduced, suggesting therefore that indeed FLT3 is required after lymphoid commitment.

Although previous work has established a critical role of FLT3 for the generation and maintenance of the B cell lineage, the cellular stages of its requirement remain unknown. We therefore crossed our new model with *Mb1-cre* mice, allowing a deletion specifically at the earliest stages of B cell commitment, and observed no reduction in the B cell compartment in adult mice. Although preliminary, these results suggest a dispensable role of FLT3 for adult B cell development.

Final remarks and future directions

Taken together, our data highlight the importance of the developmental context and cellular stage to define the precise role of hematopoietic regulators. Here, we show that FLT3 is required for adult hematopoiesis and also after lymphoid commitment, while being dispensable after B commitment.

Additional work would be needed in order to assess the deletion efficiency in the B cell compartment. In fact, when performing this study, we faced several challenges with the use of the different Cre mouse models. For instance, the inefficient deletion of *Flt3* in B cell progenitors of the *Mx1-cre x Flt3*$^{fl/fl}$ mice was unexpected. In addition, a recent study showing uncontrolled recombination in hematopoietic cells from *Mx1-cre* mice when transplanted raises the question of whether or not cells transplanted with *Mx1-cre x Flt3*$^{fl/fl}$ were already deleted after transplantation and prior to pIpC injection [319].

Further work using this mouse model could also be performed to address the role of FLT3 at different stages during ontogeny. We have recently shown that the earliest B cell progenitors found at E13.5 expressed high-levels of FLT3 compared to adult B progenitors, which show no expression [320]. Studying embryos lacking FLT3 function specifically in the B cell lineage (*Mb1-cre*) would be of great interest to better characterize hematopoietic regulation in the embryo.

Concluding remarks and future perspectives

Understanding better the embryonic hematopoietic development has the potential to optimize therapies used in the treatment of infant and childhood blood disorders. The overall aim of this thesis has been to study and characterize better this early hematopoiesis, mostly focusing on the different signaling involved in the B cell development process. In both Paper I and Paper II, we have characterized new cell populations that specifically exist in the embryo in mouse or in human.

For the first cell population in Paper I, we have identified a new progenitor, harbouring B and myeloid gene signatures and potential. We have also demonstrated its regulation by CSF1R and FLT3. However, we have not been able to demonstrate the functional existence at the single cell level of this bipotent progenitor cell. Indeed, this cell population is detected at a very low frequency and its myeloid potential remains low. Also, many questions remain to address the relevance of this finding for understanding the human development. First, does it exist? The window of detection of the $CSF1R^{+}$ ProB is narrow and we would need access to human embryonic material equivalent to E13.5 in the mouse development, meaning at CS18 (48 days of development). And then, would this population, by being targeted by oncogenic events, lead to the development of a similar disease as observed in bi-phenotypic infant or childhood leukemias, for example? We would need first to assess its capacity of transformation by oncogene in the mouse, but then, if a disease develops, how similar to the human disease characteristics would it be? Indeed, in Chapter 3, I have discussed the limitations when using animal models for disease recapitulation and the way that the mouse model is often biased in the development of disease towards a myeloid fate.

In Paper II, we have identified a population of progenitors in the embryo and have been able to recapitulate our finding in primary tissue *in vitro*, using hPSCs. We have also demonstrated that we could recapitulate a human pre-leukemic B-ALL *in vitro* using our system. While the gene expression at the single cell level clearly shows the priming of this cell towards the B and myeloid lineage, we have not been able to assess its combined B and myeloid potential using *in vitro* cultures. Also, while our *in vitro* model allows us to characterize the early effect of oncogenic events in early human hematopoiesis, we would need to specifically target different populations to clearly identify which one can act as a cell of origin for the disease. DJ rearrangement observed in twin studies supports that the first event may happen in an already lymphoid committed, but not yet restricted, progenitor cell. In addition, we have faced one of the strongest limitations in the field of hPSCs: going *in vivo*. Even when using FL primary cells, we were not able to get engraftment from specific populations. Do embryonic cells need another environment? Perhaps *in utero* transplantation would be one way to go, albeit technically very challenging. Our *in vitro* system brings, however, a useful tool to study the disease onset. In fact, we studied here the onset of leukemia after introducing an ETV6-RUNX1 mutation. It is important to remember that leukemia with this fusion gene requires secondary oncogenic events in order to develop. It would be interesting to study the beginning of infant leukemia with MLL fusion protein, where fewer mutations than non-MLL have recently been shown to be required.

In general, in Paper I and Paper II, we identified cells that could be the potential target cells of early mutation, leading to a pre-leukemic state. And after? Leukemia is a stepwise process with accumulation of mutation during a pre-leukemic phase that can ultimately lead to the disease development. We could analyse the introduction of secondary mutations to understand the pattern of oncogenic event acquisition and clonal evolution. Targeted therapies have been developed to eliminate mostly leukemic cells with, however, the persistence of pre-leukemic cells in remission, which can lead to a relapse of the disease. Eradication of both leukemic and pre-leukemic cells could result in long-term

remission and therefore, further investigation and characterization of these potential pre-leukemic cells is an important current therapeutic challenge.

In Paper III, we characterized a new mouse model, to study the role of FLT3. Indeed, most of the phenotypic characterization of mouse models has been performed using constitutive knock-out models. It has then been challenging to answer two simple questions: when and where is a gene needed? Transplantation can partially answer this, however, how much does the stressful aspect of it allow us to make a conclusion at the steady-state? In our study, we have been able to show that FLT3 is required after lymphoid commitment, but not after B cell restriction. This can answer, partially, the question as to "where" FLT3 is required. Unfortunately, regarding "when" it is needed, we have faced a strong technical issue with the inefficient deletion in the B cell lineage, using the *Mx1-cre* model. Why B cells seem more protected than, for instance, T cells from this deletion is something that remains to be determined.

Populärvetenskaplig sammanfattning

Blodcellsbildning (hematopoes) är en stegvis process där s.k. hematopoetiska stamceller, genom en rad mellansteg, ger upphov till blodsystemets alla olika celler (bl.a. T-celler, B-celler, myeloida celler, och röda blodceller). Blodstamcellerna har förmåga att producera alla typer av mogna blodceller, men ju längre ner i hierarkin utvecklingen går, desto mer begränsade blir cellerna vad gäller vilka celltyper de kan producera. Under den embryonala utvecklingen sker blodcellsproduktionen i levern, men förflyttats sedan till benmärgen där den kvarstår livet ut. Processen är tätt reglerad av både inre (inifrån cellen själv) och yttre (från omgivningen runtom cellen) faktorer. Några exempel på sådana viktiga faktorer är tyrosinkinasreceptorerna och de ämnen (ligands) som binder till dem. Receptorerna är protein som sitter på ytan av vissa blodceller. Genom att binda upp ligander från omgivningen kan de signalera till cellen vad den ska göra.

Många studier har visat att hematopoes ser annorlunda ut under embryonaltiden jämfört med under vuxentiden, bl.a. angående vissa blodcellspopulationer som endast förekommer i embryot. Man har också visat att det i sådana embryospecifika celler kan uppstå mutationer som leder till blodcellscancer (leukemi) hos nyfödda och barn. Leukemi är den vanligast diagnostiserade barncancern och står för ca 30 % av alla barncancerfall. Ökad kunskap om embryonal hematopoes skulle kunna bidra till bättre förståelse för barnleukemier och utvecklande av förbättrade behandlingsalternativ.

B-celler är en blodcellstyp som spelar en viktig roll inom immunsystemet. De bekämpar främmande ämnen i kroppen, bl.a. genom att frisätta antikroppar som binder till den invaderande substansen och startar en immunreaktion. I de ”mellanstegsceller” (progenitorer) som bildas under utvecklingen från blodstamceller till mogna B-celler sker vanligtvis de mutationer som leder till en

viss typ av barnleukemi som kallas B-ALL (*B-cell acute lymphoid leukemia*). Min avhandling sammanfattar mitt doktorandarbete där jag har fokuserat särskilt på just B-cellsutvecklingen i embryot hos både möss och människor, i hopp om att det kan bidra till ökad förståelse för barncancerutveckling.

I den första studien identifierade vi en ny progenitorcellspopulation i den embryonala B-cellsutvecklingen hos möss. Cellpopulationen existerade endast under en kort tid i levern under utvecklingen i fostret men kunde inte hittas hos vuxna möss. Cellerna kunde ge upphov till både B-celler och till myeloida celler. Då vissa leukemier påverkar både B-celler och myeloida celler, samt att dessa leukemier är vanligare bland barn än hos vuxna, är det extra intressant att studera denna cellpopulations eventuella koppling till uppkomst av barnleukemier. Vi upptäckte också att tyrosinkinasreceptorn CSF1R fanns på ytan av dessa celler, och att denna receptor hade en viktig roll under B-cellsutvecklingen. Nyligen har man även hittat mutationer i CSF1R-genen vid barnleukemier, vilket ökar intresset för receptorn och celltypen. Om receptorn skulle visa sig vara inblandad i uppkomsten av leukemierna skulle man kanske kunna utveckla terapier där man kopplar mediciner direkt till ämnen som kan binda receptorn. På så sätt kan mer effektiva och bättre riktade behandlingar utvecklas.

I den andra studien fokuserade vi på mänskliga embryon och de första B-celler som uppkommer i dem. Vi kunde visa att dessa celler har en annan receptor på sin yta, nämligen interleukin 7-receptorn (IL-7R). Under denna studie arbetade vi fram en metod som tillät oss att undersöka tidig B-cellsutveckling utanför embryot i ett cellodlingssystem. Eftersom det ofta är svårt med tillgängligheten av mänskliga embryon skulle vårt nya odlingssystem kunna vara väldigt behjälpligt vid studerandet av embryonal hematopoes och barncancerutveckling. Genom att sedan använda detta system kunde vi visa på en möjlig koppling mellan de tidiga IL-7R-uttryckande B-cellerna och uppkomst av barnleukemin B-ALL.

I den tredje och sista studien i denna avhandling gick vi återigen tillbaka till musen. Där utvecklade vi en ny musmodell som tillät oss att undersöka en tredje receptor – FLT3. FLT3-genen är förändrad på olika sätt vid de flesta leukemier. I vår nya musmodell förvinner receptorn efter födseln eller bara i en viss typ av

blodceller (till exempel endast i B-celler men inte i myeloida celler). Tidigare studier då receptorn varit borta helt och hållet (redan under embryonaltiden) har visat att FLT3 krävs för utveckling av både B- och T-celler, samt myeloida celler. Man har dock inte lyckats visa i vilket steg i hierarkin från stamceller till mogna celler som receptorn krävs. Med vår nya musmodell kunde vi visa att receptorn var nödvändig lite högre uppe i utvecklingen, i celler som kunde bilda både B- och T-celler, men inte i celler längre ner som bara kunde bilda B-celler.

Sammanfattningsvis belyser studierna i denna avhandling vad som sker under embryonal hematopoes hos möss och människor. Sådan kunskap är viktig för utveckling av nya metoder och strategier för både undersökning av uppkomsten och för behandling av blodsjukdomar hos barn.

Résumé Scientifique

Le développement des cellules du sang (cellules hématopoïétiques) est un processus par étapes durant lequel des cellules, dites cellules souches hématopoïétiques, donnent naissance aux différentes cellules du système sanguin (cellules T, cellules B, cellules myéloïdes et les globules rouges). Ce mécanisme est appelé « différenciation cellulaire ». Ces cellules souches sanguines se différencient en cellules intermédiaires, appelées cellules progénitrices, et en cellules matures. A mesure qu'elles se différencient, elles perdent leur capacité à produire différents types de cellules. Au cours du développement embryonnaire, la production du sang a lieu dans le foie. Elle se déplace ensuite vers la moelle osseuse chez l'enfant et l'adulte. Le processus de production de cellules sanguines est étroitement régulé par des facteurs intrinsèques (par la cellule elle-même) et par des facteurs extrinsèques (par l'environnement et les cellules environnantes). Les récepteurs à activité tyrosine kinase sont un exemple de facteurs extrinsèques importants. Ils sont fixés à la surface de certaines cellules sanguines et peuvent se lier à des petites molécules de l'environnement: les cytokines. Cette liaison active le récepteur qui déclenche alors série de modifications en cascade dans la cellule. Cette activation du récepteur conduit à différents effets biologiques tels que la différenciation ou la division cellulaire.

De précédentes études ont mis en évidence les différences entre l'hématopoïèse embryonnaire et l'hématopoïèse adulte, avec par exemple l'existence de populations cellulaires sanguines présentes uniquement dans l'embryon. Il a également été démontré que la majorité des mutations pouvant mener au développement de cancers du sang (leucémies) chez l'enfant a lieu pendant le développement embryonnaire. Les leucémies sont le type de cancer le plus fréquemment diagnostiqué chez l'enfant et le nourrisson avec près de 30 % des

cas. Une meilleure connaissance de l'hématopoïèse embryonnaire pourrait potentiellement conduire à une meilleure compréhension des leucémies infantiles et à l'établissement de thérapies plus efficaces et spécifiques.

Les cellules B sont des cellules importantes du système immunitaire adaptatif, capables de combattre les organismes étrangers en secrétant des anticorps par exemple. Les anticorps se lient aux substances étrangères et enclenchent une réaction immunitaire. Ces cellules B sont produites à partir des cellules souches hématopoïétiques suivant une série d'étapes intermédiaires. Les cellules souches se différencient en cellules progénitrices de B qui se différencient elles-mêmes, par la suite, en cellules B matures. Ces populations intermédiaires sont souvent la cible de mutations pouvant conduire à un certain type de leucémie infantile, appelé leucémie aiguë lymphoïde de type B (LAL-B).

Cette thèse résume mon travail sur le développement des cellules B, en particulier durant l'embryogenèse chez la souris et chez l'homme.

Dans la première étude, nous avons identifié durant le développement des cellules B de souris un nouveau type de population de cellules progénitrices. Cette population de cellules est présente uniquement durant une période restreinte du développement du foie fœtal, et est absente de la moelle osseuse des souris adultes. Ces cellules ont la capacité de produire à la fois des cellules B et des cellules myéloïdes. Sachant que certaines leucémies présentent des caractéristiques à la fois des cellules B et des cellules myéloïdes, et que ce type de leucémie est plus fréquent chez l'enfant que chez l'adulte, les cellules que nous avons identifiées sont particulièrement intéressantes pour l'étude du développement des leucémies infantiles. En outre, nous avons découvert que le récepteur à activité tyrosine kinase appelé CSF1R est détectable à la surface de ces cellules et qu'il régule le développement des cellules B uniquement chez l'embryon. Récemment, des mutations du gène CSF1R ont été observées chez des cas de LAL-B. Si l'activité du récepteur est impliquée dans le développement leucémique, l'utilisation de petites molécules inhibitrices de cette activité pourrait constituer une nouvelle voie thérapeutique.

Dans la deuxième étude, nous nous sommes focalisés sur l'embryogenèse humaine et sur les premières cellules B produites. Nous avons montré que ces cellules B de l'embryon ont un autre récepteur à leur surface, à savoir le récepteur de l'interleukine 7, contrairement aux cellules B adultes. Nous avons, par la suite, développé une nouvelle méthode *in vitro* nous permettant d'étudier le développement embryonnaire des cellules B, en utilisant un système de culture cellulaire. L'accès aux embryons humains étant souvent compliqué d'un point de vue éthique et pratique, notre nouvelle méthode de culture cellulaire pourrait faciliter grandement les études de l'hématopoïèse embryonnaire et des cancers de l'enfant. Au moyen de ce nouveau système, nous avons pu mettre en évidence un blocage de la différenciation de ces cellules B exprimant le récepteur de l'interleukine 7 lors du développement de leucémie infantile LAL-B.

Dans la troisième et dernière étude de cette thèse, nous avons repris le travail sur la souris et avons développé un nouveau modèle murin, nous permettant d'étudier une troisième molécule de surface : le récepteur à activité tyrosine kinase FLT3. Le gène codant pour le récepteur FLT3 est muté dans la plupart des leucémies, adultes et infantiles. Notre nouveau modèle de souris permet la délétion de FLT3 spécifiquement dans un type de cellules donné (par exemple uniquement dans les cellules B et non dans les cellules myéloïdes) ou à un instant précis de la vie. Des études antérieures à la nôtre ont montré que FLT3 était essentiel à la génération des cellules B, T et myéloïdes. Cependant, le récepteur étant supprimé dans tous les types de cellules et dès la période embryonnaire dans ces études, il est impossible de savoir si la réduction des cellules B, T et myéloïdes est due à un impact sur une cellule progénitrice en amont ou si ce type de cellules requiert FLT3 pour se développer. En utilisant notre nouveau modèle murin, nous avons pu montrer que le récepteur était nécessaire au début du développement des cellules lymphoïdes (B et T), pour l'émergence d'une cellule progénitrice capable de produire des cellules B et T, mais pas après la différenciation en cellules B plus matures.

Dans leur ensemble, les études incluses dans la présente thèse apportent une contribution à la compréhension de l'hématopoïèse embryonnaire chez l'humain et

la souris. Ce savoir est important pour le développement de nouvelles méthodes et stratégies tant pour la recherche que pour le traitement des pathologies de cellules sanguines chez l'enfant.

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