The emergence and early fate decisions of stem and progenitor cells in the haematopoietic system

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Doctor of Philosophy

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“For the strength of the Pack is the Wolf,

and the strength of the Wolf is the Pack”

Joseph Rudyard Kipling

Abstract

The emergence and early fate decisions of stem and progenitor cells in the haematopoietic system

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The alternative road map describes the separation of lympho-myeloid and myeloid-megakaryocyte-erythroid (myeloid-Mk-E) lineages as the earliest haematopoietic commitment event. However, a number of aspects of this lineage restriction process remain poorly understood. Herein this work identified a lympho-myeloid restricted progenitor in the embryo, which resembles the adult LMPP, and demonstrated that lymphoid lineage restriction is initiated prior to definitive haematopoiesis, much earlier than previously appreciated. *In vivo* fate mapping showed that lympho-myeloid progenitors significantly contribute to steady state myelopoiesis in the embryo. The early thymic progenitor (ETP) as most primitive cell in the thymus was characterised and demonstrated to sustain B, T and myeloid but not Mk potentials at the single cell level. The ETP therefore largely resembles the cellular properties of lympho-myeloid progenitors in bone marrow and foetal liver, which points to these cells as candidate thymus seeding progenitors (TSP). Furthermore the existence of a putative Mk progenitor was explored within the LSKCD150^{+}CD48^{+}*Gata1*^{pos} compartment of a *Gata1* reporter mouse providing the basis for a future prospective characterisation. Finally, this work evaluated the earliest lineage restriction of von Willebrand factor (*Vwf*)-EGFP^{+} and EGFP^{-} haematopoietic stem cells (HSCs) through *in vitro* paired daughter fate mapping. Single *Vwf*$^{+}$ HSCs showed heterogeneous Mk priming and more frequently sustained Mk potential after cell division. Moreover, analysis of lineage priming between daughter cells revealed the asymmetric expression of key lineage determinants and stem cell regulators, which might be employed as reporters for future fate mapping studies.

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Commonly used abbreviations

AGM	Aorta-gonad-mesonephros
BM	Bone marrow
cDNA	Complementary DNA
CLP	Common lymphoid progenitor
CMP	Common myeloid progenitor
DN	Double negative (T cell)
DP	Double positive (T cell)
E	Erythroid
E	Embryonic day
EDTA	Ethylenediaminetetraacetic acid
EPO	Erythropoietin
ETP	Early thymic progenitor
FACS	Fluorescence activated cell sorting
FCS	Foetal calf serum
FL	Foetal liver
Flt3	Fms-like tyrosine kinase 3
Flt3L	Fms-like tyrosine kinase 3 ligand
G	Granulocyte
GATA1	GATA binding protein 1
G-CSF	Granulocyte-colony stimulating factor
GFP	Green fluorescent protein
GM-CSF	Granulocyte-monocyte-colony stimulating factor
GMP	Granulocyte-macrophage progenitor
h	human
HSC	Haematopoietic stem cell
IL	Interleukin

IL-7Rα	Interleukin 7 receptor *alpha*
Lin	Lineage
LMPP	Lymphoid-primed multipotent progenitor
LSK	$Lineage^{-}Sca\text{-}1^{+}c\text{-}Kit^{+}$
LT-HSC	Long term repopulating haematopoietic stem cell
m	murine
M	Monocytes/macrophages
M-CSF	Macrophage-colony stimulating factor
MEP	Megakaryocyte-erythroid progenitor
Mk	Megakaryocyte
MkP	Megakaryocyte progenitor
Mpl	Myeloproliferative leukaemia virus oncogene
MPP	Multipotent progenitor
NK	Natural killer
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PDC	Paired daughter cell
Rag1	Recombination-activating gene 1
SCF	Stem cell factor
ST-HSC	Short term repopulating haematopoietic stem cell
TE	Tris EDTA
TPO	Thrombopoietin
tris	Tris(hydroxymethyl)aminomethane
TSP	Thymus seeding progenitor
Vwf	Von willebrand factor homolog
YFP	Yellow fluorescent protein

1. Introduction

1.1. Haematopoiesis

The mammalian blood system consists of several different mature cellular lineages that can be categorised according to their functional roles. The lymphoid lineage includes B, T and natural killer (NK) cells, the main players of the adaptive immunity. Granulocytes (G), which can be further separated into neutrophils, eosinophils and basophils, and monocytes/macrophages (M) are cells of the innate immune system and belong to the myeloid lineage. Traditionally the megakaryocyte (Mk) lineage, which gives rise to platelets, as well as the erythroid (E) lineage are also considered as myeloid cells. However, according to more recent findings the consideration of Mk and E lineages as separate entities, that give rise to the only non-immunological blood cells, has been accepted as more appropriate.

The majority of mature blood cells is short lived and has to be replenished continuously. In the process of haematopoiesis pluripotent haematopoietic stem cells (HSCs) that sit atop a hierarchy of blood cell progenitors, through a stepwise process of cell division and differentiation, give rise to all mature blood cell lineages. Thereby multipotent progenitors successively lose the ability the generate most of the mature cell types and eventually commit to one blood cell lineage.

1.1.1. Purification of haematopoietic stem and progenitor cells

Flow cytometry and fluorescence activated cell sorting (FACS) enables the analysis as well as prospective isolation of haematopoietic stem and progenitor populations, and has

evolved as a key method in haematopoiesis research. The technique uses cell surface marker labelling via fluorochrome conjugated antibodies, staining with small molecule dyes, and more recently also the expression of fluorescent reporters, to identify phenotypically distinct cell populations. Although most surface markers and reporters are widely expressed on various haematopoietic cell types, the combination of several markers allows for the isolation and analysis of highly purified cell populations. In the flow cytometer sample cells are separated from each other by hydrodynamic focussing and subsequently pass one or several beams of laser light of different wave lengths. The cell labelling fluorochromes get thereby excited by a particular laser and emit light of a characteristic spectrum. The emitted light is separated by specific filter sets and converted into an electric signal by a photomultiplier tube. The intensity of the emitted light corresponds to the expression level of the respective surface maker, fluorescent reporter or small molecule dye staining. Additional data about size and granularity is obtained by measuring the intensity of light scattered from each cell. State of the art flow cytometry, that employs antibody conjugates with a broad variety of fluorochromes and flow cytometers with multiple lasers, allows the detection of more than ten parameters from a single cell. The method is limited by the fact that the used fluorochromes usually have rather broad and partly overlapping emission spectrums. Although this is accounted for by application of compensation between the emission channels, increasing noise limits the number of parameters that can be separated. Based on the described parameters cells can be purified using cell sorting. The hydro-dynamically focused cells pass through a nozzle immediately after their fluorescence properties have been measured. All cells get separated into single droplets while a charge is applied to cells of interest that subsequently are deflected through an electromagnetic field and deposited into a collection tube (Fig. 1.1). This allows for the purification of cells with defined fluorescence properties and therefore specific combinations of markers down to the single cell level (Shapiro, 2003).

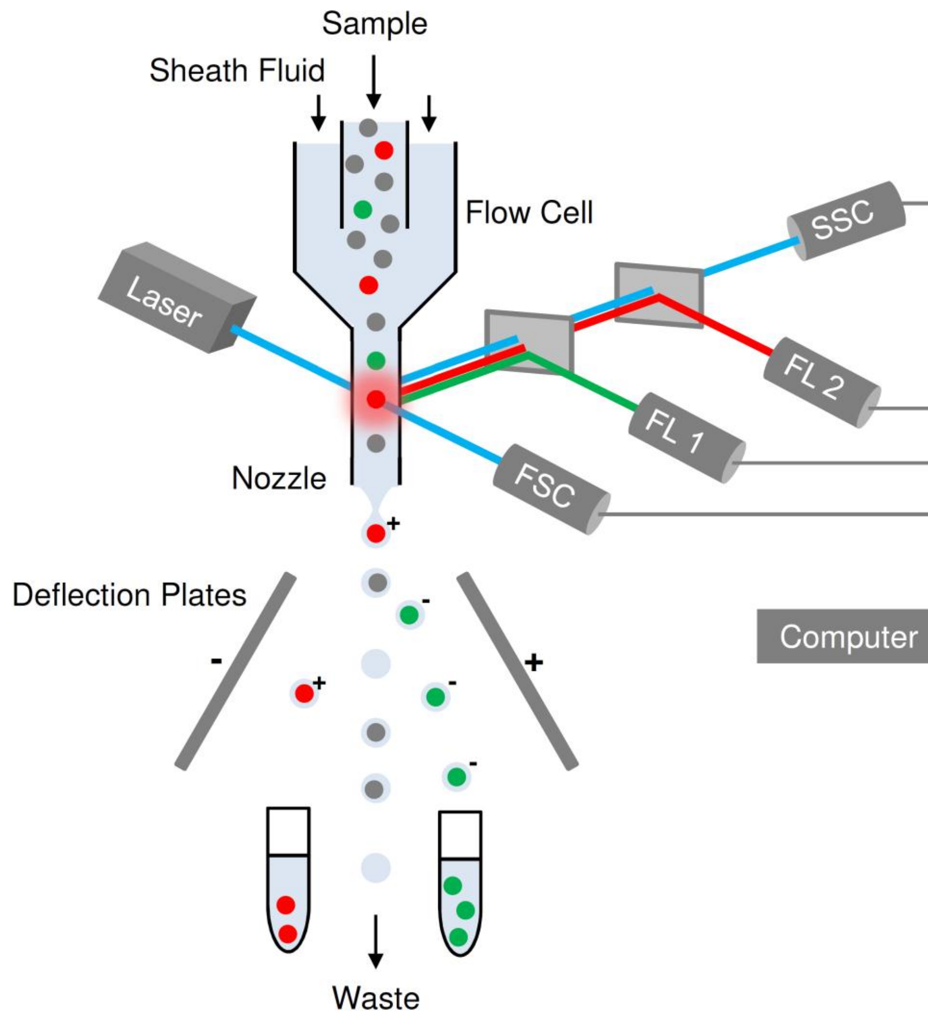


Figure 1.1: Schematic representation of flow cytometry analysis and cell sorting

In the flow cytometer cells are aspirated from the sample tube and transferred to the flow cell through the sample line. Sample cells are separated via hydrodynamic focussing after which they pass the excitation laser. Fluorescence emission is detected by photomultiplier tubes (PMT, FL 1, FL 2) after spectral separation. The side scatter (SSC) parameter indicates the granularity of a cell and forward scatter (FSC) measures the size of a cell. In a cell sorter cells pass through the nozzle where single cells are separated into individual droplets. Cells with predefined scatter or fluorescence properties are charged and deflected into collection tubes. By utilizing several excitation lasers modern flow cytometers allow the detection of more than 10 fluorescence parameters.

1.1.2. Lineage commitment in the haematopoietic system

Haematopoietic stem cells give rise to all mature blood cell lineages through a series of lineage commitment events. This process involves a hierarchy of haematopoietic progenitor populations starting from multipotent progenitors that retain the potential to differentiate into multiple lineages to unilineage restricted cells, and is regulated by cell intrinsic as well as extrinsic regulators. Over the last two decades several models of lineage commitment have been proposed. Most of them had to be revised over time and although much is known today cellular pathways governing lineage fate are still a major focus of attention in the field.

1.1.2.1. The classical model

The hallmark of the classical model of lineage commitment is the early separation of lymphoid and myeloid lineages. According to this traditional view haematopoiesis originates from a homogeneous population of long term self-renewing HSCs (LT-HSC) (Morrison and Weissman, 1994). Theses give rise to short term stem cells (ST-HSC) and multipotent progenitors (MPP), which retain all lineage potentials but lack the ability to maintain haematopoiesis over long periods of time (Morrison et al., 1997; Yang et al., 2005). All three populations are part of the lineage marker (Lin) negative, Sca-1 and c-Kit positive (LSK) population of bone marrow cells. Long and short term stem cells can be separated by the expression of CD34 (Yang et al., 2005). The subsequent division into myeloid and lymphoid lineages is based on the discovery of their common progenitors. This was in line with the historical view of an early separation of myeloid cells that constitute the innate immune system and lymphoid cells as part of adaptive immunity. The common lymphoid progenitor (CLP) defined as $Lin^{-}IL\text{-}7R^{+}Thy1^{-}Sca\text{-}1^{lo}c\text{-}Kit^{lo}$ can give rise to all lymphoid cells such as B, T and NK cells but lacks the potential to give rise to myeloid cells including Mks or erythrocytes (Kondo et al., 1997). Conversely the common myeloid progenitor (CMP) has the ability for generation of all myeloid lineages, which include granulocytes, monocytes/macrophages, Mks and erythrocytes,

but does not give rise to lymphoid cells and has been purified as the Lin$^-$IL-7R$^-$Sca-1$^-$c-Kit$^+$CD34$^+$FcγR^{lo} fraction of bone marrow cells (Akashi et al., 2000). Granulocytes and monocytes/macrophages are derived through the granulocyte/monocyte progenitor (GMP) defined as Lin$^-$IL-7R$^-$Sca-1$^-$c-Kit$^+$CD34$^-$FcγR^{hi}, whereas Mks and erythroid cells are generated via the Mk/E progenitor (MEP) that is characterised by the Lin$^-$IL-7R$^-$Sca-1$^-$c-Kit$^+$CD34$^-$FcγR^{lo} surface marker phenotype (Akashi et al., 2000). As the only cell population with that property, dendritic cells can be derived through both lymphoid as well as myeloid pathways (Fig. 1.2) (Manz et al., 2001a; Manz et al., 2001b).

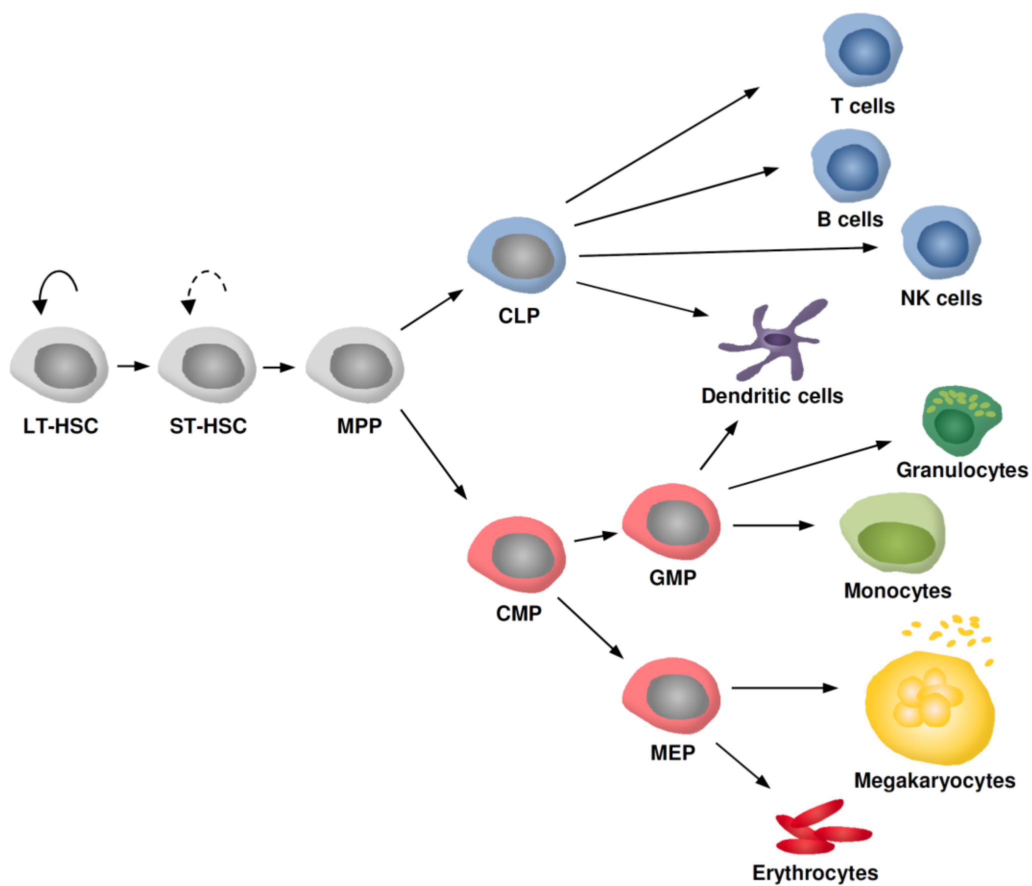


Figure 1.2: Classical model of haematopoietic lineage commitment

The classical model illustrates the strict separation of lymphoid from myeloid (including Mk/E) lineages as the first commitment event accompanied by the generation of common lymphoid progenitor (CLP) and common myeloid progenitor (CMP). Straight arrows indicate commitment events, while curved arrows represent long term (solid) or

restricted (dashed) self-renewal. Long term reconstituting haematopoietic stem cell (LT-HSC), short term reconstituting haematopoietic stem cell (ST-HSC), multipotent progenitor (MPP), granulocyte/monocyte progenitor (GMP), megakaryocyte/erythroid progenitor (MEP).

1.1.2.2. Alternative models of lineage commitment

In 1983 Ogawa and colleagues proposed the stochastic model of lineage commitment that describes the lineage choice of a multipotent cell as a completely random process. The model is based on results from *in vitro* as well as *in vivo* colony assays. After transplantation of whole bone marrow (BM) or analysis of *in vitro* colony formation haematopoietic progenitors were found to give rise to very heterogeneous colonies that contained many different combinations of mature lineages (Ogawa et al., 1983). Although in the meantime several studies have generated compelling evidence that mature lineages are derived via defined lineage commitment pathways, the concept of random lineage choices within the scope of such pathways and under the influence of extrinsic regulators still applies today (Huang et al., 2007).

According to the sequential model that was first proposed by Brown and co-workers haematopoietic progenitors originate from a multipotent progenitor and go through a sequence of lineage programs with related potentials. During differentiation progenitors would first produce erythroid, then myeloid cells, B cells and eventually T cells. Between these steps the model includes bi-potential progenitors such as an erythroid-myeloid, myeloid-B and B-T progenitor (Brown et al., 1985). A revised sequential model termed the pairwise relationship model also incorporates several newly identified multipotent progenitors (Brown et al., 2007; Ceredig et al., 2009).

A related model is based on gene targeting experiments where haematopoiesis was studied after deletion of the transcription factors *cMyb*, *Sfpi1* (PU.1), *Ikaros*, *Tcfe2a*

(E2A). Originating from a stem cell or multipotent progenitor that can give rise to all lineages, erythroid/myeloid/lymphoid progenitors are derived under the influence of *cMyb*. PU.1 further restricts lineage potentials, whereupon a myeloid/lymphoid progenitor is produced. Ikaros is responsible for lymphoid lineage restriction and eventually E2A expression results in B lineage commitment (Singh, 1996). The respective downstream progenitors were absent after deletion of each of the four transcription factors, although subsequent studies showed an only limited effect of these deletions on T cell development (Georgopoulos et al., 1994; Scott et al., 1994; Spain et al., 1999; Wang et al., 1996).

1.1.2.3. The myeloid based or alternative haematopoietic roadmap

In 1997 the classical model of haematopoiesis was challenged by a study from Kawamoto and co-workers, who had analysed single lineage potentials of foetal liver (FL) progenitors in a combined lineage potential assay. This foetal thymic organ culture (FTOC) system, also called multilineage potential (MLP) assay, combines the co-culture with foetal thymus tissue, that can be used for efficient readout of T cell potential, with additional cytokines for improved detection of myeloid and B lineages (Godin et al., 1995). While this assay constantly generated combined myeloid/B as well as myeloid/T colonies the combination of B and T cell could never be derived from a single progenitor (Kawamoto et al., 1997, 1998). Additional evaluation of erythroid potential supported these results and led to the identification of a common myeloid lymphoid progenitor (CMLP) as well as common myeloid erythroid progenitor (CMEP) (Lu et al., 2002). Based on these results the authors proposed the myeloid based model of lineage commitment, where all lineages retain myeloid potential up to the bi-potent progenitor stage (Katsura and Kawamoto, 2001). This model completely lacks a common lymphoid progenitor cell, which is an integral part of the classical model. However, even though these studies provide strong evidence of the existence of several progenitors that retain myeloid potential, they fail to prospectively isolate them and are therefore not fully

conclusive. Additionally, foetal lineage commitment pathways might be different from the adult haematopoietic road map.

The hallmark of the classical model of lineage commitment is the separation of myeloid – including Mk/E – and lymphoid lineages as the first commitment step from multipotent progenitors. However, this view was challenged by studies that purified early progenitors from the LSK compartment by means of fms-like tyrosine kinase 3 (Flt3) surface expression. $Flt3^{hi}$ cells gave readily rise to myeloid, B and T cells in vitro as well as *in vivo* but lacked the ability to generate cells of the Mk/E lineages (Adolfsson et al., 2001; Adolfsson et al., 2005). This lymphoid primed multipotent progenitor (LMPP) was later also isolated using reporters for the transcription of *Sfpi1* (PU.1) or *Ikaros* as well as surface expression of vascular cell adhesion protein 1 (VCAM1) (Arinobu et al., 2007; Lai and Kondo, 2006; Yoshida et al., 2006). Controversy in the field emerged over the fact that the LMPPs retain Mk/E potential at a low frequency of about 3 %, and its ability to give low level Mk/E reconstitution *in vivo* (Adolfsson et al., 2005; Forsberg et al., 2006). This could be due to all cells retaining low potential to generate Mk and E cells. Utilizing the lack of expression for the MPL, a marker that is present on HSCs as well as the Mk lineage, and detailed studies of LMPP lineage potentials on the single cell level, it was later shown that only a small fraction of LMPPs retained MK and E potentials (Luc et al., 2008a; Mansson et al., 2007).

Complementing the LMPP as an early and highly proliferative progenitor the Akashi lab prospectively isolated a new CMP within the LSK compartment, using a fluorescence reporter for the transcription factor GATA-binding factor 1 (GATA1). In agreement with the CMP of the classical model, this progenitor also has the potential to generate myeloid, Mk as well as erythroid but no lymphoid cells (Akashi et al., 2000). Although the classical CMP is able to generate Mk/E or myeloid lineages, mixed colonies are rarely detected. Today the classical CMP is rather considered to represent a mix of MEPs and

GMPs (Rieger et al., 2009b). However, about 40 % of new CMPs gave rise to mixed myeloid/Mk/E colonies demonstrating their potential for all three lineages. Moreover, new CMPs have far higher proliferative potential *in vitro* as well as *in vivo* indicating their position high up in the haematopoietic hierarchy. (Arinobu et al., 2007). Unlike the strict dichotomy of myeloid and lymphoid lineages as first commitment step as described by the classical model, LMPP and new CMP would suggest an initial separation of Mk/E and lymphoid lineages (Fig. 1.3). In support of the myeloid model GM lineage potentials would be sustained in both pathways. Further support comes from the identification of lympho-myeloid progenitors in the thymus, where the majority of early thymic progenitors (ETPs) were shown to retain significant myeloid potential in addition to T cell potential (Bell and Bhandoola, 2008; Wada et al., 2008). Although a CLP would be compatible with an upstream LMPP, and CLPs retain residual myeloid potential in vitro, this has not been confirmed in vivo (Ehrlich et al., 2011; Kondo et al., 2000). Therefore the identification of this downstream T/M progenitor suggests the majority of T cell development to occur via a CLP independent pathway. This is in line with results from earlier studies that showed CLPs to have lower proliferative potential compared to thymic progenitors indicating CLPs are not their upstream progenitors (Allman et al., 2003).

As described in this chapter various models of haematopoietic lineage commitment have emerged over time. Although all of them describe haematopoiesis in a different way only the classical and the alternative or myeloid based model are incompatible with each other as to the nature of the first lineage commitment event – lymphoid versus myeloid or lympho-myeloid versus myeloid/Mk/E. Since the first description of the myeloid model in the embryo and the discovery of the adult LMPP several studies have generated experimental evidence in favour of the alternative or myeloid model (Adolfsson et al., 2005; Arinobu et al., 2007; Kawamoto et al., 2010; Lai and Kondo, 2006; Luc et al., 2008; Yoshida et al., 2006). Today this model is widely accepted and viewed as the

most accurate delineation of haematopoiesis. The additional models listed in this chapter are largely compatible with the alternative or myeloid based road map. The stochastical model, which describes lineage commitment as the completely random unilineage differentiation of multipotent cells, is not able to explain haematopoiesis in its entirety, for which pathways clearly exist (Ogawa et al., 1983). However, within each pathway, a single lineage decision, although influenced by multiple cell intrinsic and extrinsic signals, is most likely stochastical (Huang et al., 2007). Furthermore the myeloid model describes the sequential loss of lineage potentials, while maintaining myeloid potential to the bipotent progenitor stage. This is also indicated by the sequential model of haematopoiesis (Ceredig et al., 2009).

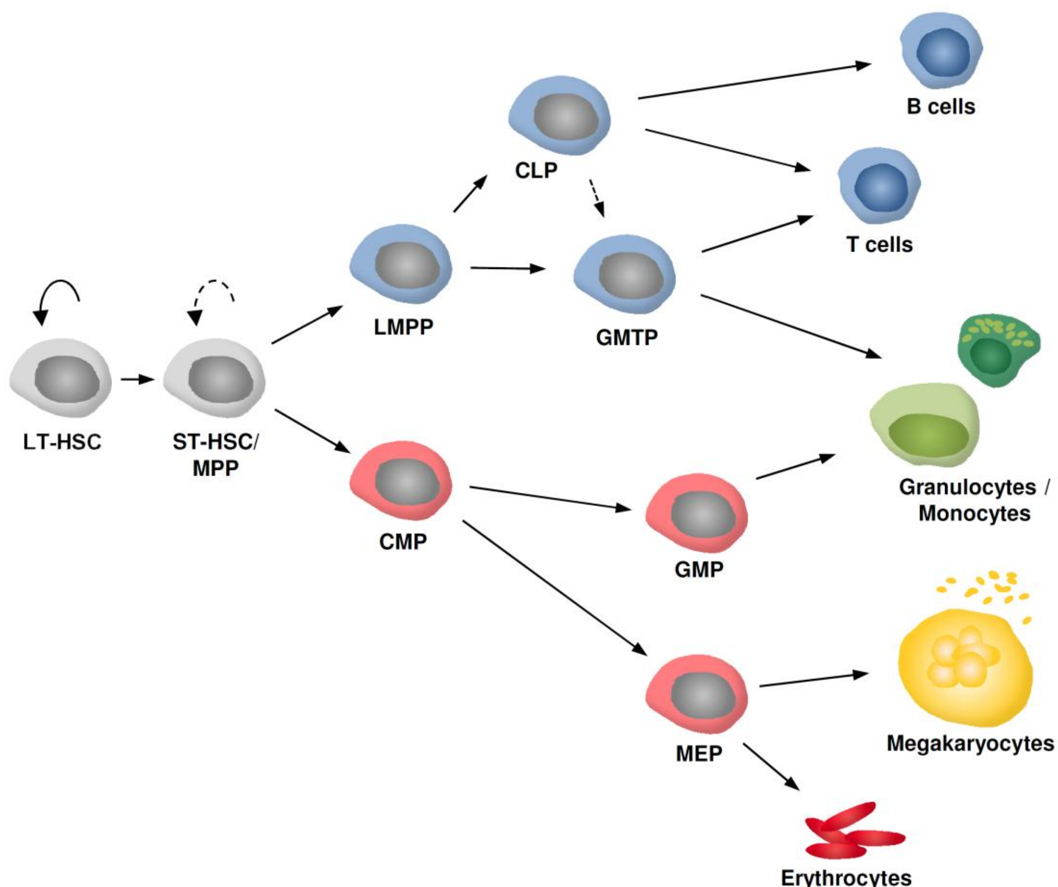


Figure 1.3: Revised model of haematopoietic lineage commitment

The revised model depicts the generation of lymphoid primed multipotent progenitors (LMPPs) with lymphoid/myeloid potentials and common myeloid progenitors (CMPs),

which retain myeloid/megakaryocyte/erythroid potentials, as earliest lineage commitment step. Straight arrows indicate commitment events, while curved arrows represent long term (solid) or restricted (dashed) self-renewal. Common lymphoid progenitors retain low lymphoid potential as indicated by dashed arrow. Long term reconstituting haematopoietic stem cell (LT-HSC), short term reconstituting haematopoietic stem cell (ST-HSC), granulocyte/monocyte progenitor (GMP), megakaryocyte/erythroid progenitor (MEP), progenitor with granulocyte/monocyte and T lymphoid potentials (GMTP).

1.2. Regulation of lineage commitment

The process of lineage commitment from multipotent HSCs to unilineage restricted progenitors and mature blood cells is highly regulated to not only ensure balanced lineage output during homeostasis, but also to respond to stress situations such as haematopoietic injury. Extrinsic regulation is mediated through cytokines that can be expressed as membrane bound forms and act via direct cell-cell contact, as well as soluble factors that are present in a local environment or produced systemically to mediate signals from the mature blood system. Intrinsically key transcription factor networks instruct a cells' lineage choice and push it towards a particular differentiation pathway.

1.2.1. Roles of cytokines

Ample evidence shows the importance of cytokines for the development of various haematopoietic lineages, and key regulatory factors for the generation of almost every mature blood cell type have been identified (Laiosa et al., 2006). However, the specific mode of action remains a highly debated topic with at least two models being discussed. According to the permissive model a cytokine promotes the growth of a cell that has already committed to a certain lineage by cell intrinsic or potentially other regulatory means. On the other hand a cell that has not yet passed the commitment step or

committed to a different lineage would not be responsive to the cytokine signal. In the case of instructive cytokine action an as yet uncommitted cell would be pushed towards a specific lineage choice (Laiosa et al., 2006). A permissive role for cytokines was suggested by studies of mouse models deficient for different myeloid cytokines. The gene knock out of granulocyte or granulocyte-macrophage colony stimulating factors (G-CSF or GM-CSF) or erythropoietin (EPO) led to a reduction of progenitors and mature cells of the respective myeloid and erythroid lineages but not to a complete loss as expected in case of an instructive role (Lieschke et al., 1994a; Stanley et al., 1994; Wu et al., 1995). The possibility of a compensatory effect mediated by functionally redundant cytokines was ruled out in double knock out studies. Mice deficient for G-CSF and GM-CSF or macrophage colony stimulating factors (M-CSF) and GM-CSF didn’t display more severe phenotypes than the single knock outs (Lieschke et al., 1994b; Seymour et al., 1997). In support of the permissive model expression of constitutively active receptors for EPO or M-CSF was compatible with normal differentiation of multipotent progenitors (McArthur et al., 1994; Pharr et al., 1994). Similarly experiments with chimeric cytokine receptors that contain intracellular and extracellular parts of different receptors indicated a role in supplying general survival signals rather than inducing lineage bias (Semerad et al., 1999; Stoffel et al., 1999). Opposing results were initially obtained from studies where ectopic expression of GM-CSF or Interleukin (IL)-2 receptors in CLPs resulted in generation of myeloid cells from these lymphoid restricted progenitors (Kondo et al., 2000). However, later detection of significant myeloid potential in wild type CLPs also suggests a potential permissive role in this case (Ehrlich et al., 2011).

Instructive modes of action have been proposed for cytokines involved in the development of the lymphoid lineage such as Flt3 ligand (Flt3L) or IL-7. In wild type mice the LMPP is primed for myeloid/lymphoid differentiation and expresses several key lymphoid as well as myeloid genes. LMPPs isolated from Flt3L deficient mice, however,

exhibit strongly reduced lymphoid lineage priming while myeloid genes remain unaffected (Sitnicka et al., 2007). Accordingly, Flt3L knockout mice have reductions in CLPs and early B cell progenitors as well as T cell progenitors including ETPs (Mackarehtschian et al., 1995; McKenna et al., 2000; Schwarz et al., 2007; Sitnicka et al., 2002). Mice deficient for IL-7 receptor signalling present with a similar phenotype characterised by the reduction of several lymphoid progenitor populations (Dias et al., 2005; Peschon et al., 1994b). Furthermore mice lacking the expression of Flt3L as well as IL-7 receptor have a more severe lymphoid phenotype characterised by the almost complete loss of all early thymic progenitor stages or mature B cells (Sitnicka et al., 2003; Sitnicka et al., 2007).

Two recent studies tried to directly address whether the two myeloid cytokines G-CSF and M-CSF act in an instructive or rather permissive manner (Enver and Jacobsen, 2009). Work performed in the Schroeder laboratory was based on the continuous microscopic observation of differentiating GMPs in the presence of either of the two cytokines. A green fluorescent protein (GFP) reporter for the late myeloid gene served as marker for unilineage differentiation to granulocyte or monocyte lineages. Analysis of their time lapse microscopy data allowed the authors to conclude that at least a fraction of GMPs were instructed to differentiate into granulocytes or macrophages by G-CSF or M-CSF, respectively (Rieger et al., 2009a). The study by Sarrazin and co-workers identified the transcription factor MafB as negative regulator of M-CSF signalling restricting the response to M-CSF in haematopoietic stem and progenitor cells. In the absence of MafB instructive M-CSF signalling results in increased myeloid lineage output (Sarrazin et al., 2009).

1.2.1.1. TPO – a key cytokine for megakaryocyte development

Thrombopoietin (TPO) is the primary physiological regulator of thrombopoiesis and its concentration in blood and bone marrow is inversely proportional to Mk and platelet

abundance. Most of systemic TPO production comes from the liver and kidney, where it is released at a constant rate into the bloodstream (Kaushansky, 1998). The regulation of systemic TPO levels occurs through an auto-regulatory feedback loop. Platelets express the TPO receptor Mpl that binds its ligand and thereby removes it from solution. Thus high platelet numbers take up large amounts of TPO resulting in low systemic TPO levels and decreased thrombopoiesis. Conversely low platelet numbers are associated with a high concentration of TPO and increased production of new platelets (Kuter and Rosenberg, 1995). TPO is the most important growth factor for progenitors committed to the Mk lineage, and in synergy with other cytokines such as stem cell factor (SCF), EPO or IL-11 it stimulates progenitor cell proliferation (Broudy et al., 1995). In culture experiments TPO has been shown to stimulate the development of large, highly polyploid Mks (Kaushansky et al., 1995). In addition to its important role in thrombopoiesis TPO is required for the maintenance of adult HSCs. TPO deficient mice have a reduction in numbers of adult phenotypic HSCs that gets more severe with age, while foetal haematopoiesis remains largely unaffected (Qian et al., 2007). In agreement transplantation experiments with adult HSC from $Tpo^{-/-}$ mice revealed a strong reduction in stem cell numbers. TPO is not only important for stem cell maintenance but also their expansion. $Tpo^{-/-}$ recipients transplanted with wild type bone marrow had reduced survival rates, lower long term multilineage peripheral blood reconstitution with strong thrombocytopenia as well as decreased stem cell numbers (Fox et al., 2002; Qian et al., 2007). While TPO is not able to stimulate HSC on its own *in vitro* it is thought to act together with SCF and IL-3 (Sitnicka et al., 1996). *In vivo* TPO deficiency leads to increased cell cycle activity in HSCs associated with reduced expression of cell cycle regulators such as *Cdkn1c* ($p57^{kip2}$) or *Cdkn2a* ($p19^{ink4a}$) as well as various *Hox* genes that are important for stem cell quiescence (Qian et al., 2007).

1.2.2. Roles of transcription factors

Transcription factors mediate lineage commitment cell intrinsically and are part of gene expression programs that ultimately determine a cells' lineage choice. Thereby transcription factors can be induced or repressed by other transcription factors as well as cell extrinsic cues. Ultimately lineage commitment is determined by the co-expression or co-activation of a certain combination of multiple transcription factors and one transcription factor can be involved in the commitment towards multiple lineages. Although lineage commitment is regulated by transcription factor networks the manipulation of individual key determinants can be sufficient to induce commitment to a certain lineage (Laiosa et al., 2006).

While for cytokines there is experimental support for a permissive as well as lineage instructive mode of action it remains under debate as to which cytokines can really instruct lineage choice or only promote the growth of already committed cells (Laiosa et al., 2006). However, it is likely that most cytokines can act both ways. For transcription factors on the other hand there is ample evidence for an instructive mode of action. Knockout models of lineage instructive transcription factors completely lack certain lineages and overexpression has been shown to result in enhanced progenitor commitment to one lineage at the expense or even complete loss of other lineages. Most convincing evidence for a lineage instructive mode of action comes from lineage conversion experiments, where the enforced ectopic transcription factor expression in already committed cells leads to their reprogramming to a different lineage identity (Heyworth et al., 2002; Iwasaki et al., 2003; Kulessa et al., 1995; Laiosa et al., 2006).

Ikaros is widely expressed in haematopoietic progenitors and has important roles in myeloid as well as lymphoid lineage commitment. Although Ikaros deletion also affects myeloid lineages most severe reductions are observed in lymphoid lineages of adult as well as foetal haematopoiesis. Loss of Ikaros leads to absence or strong decrease of

mature B cells, NK cells, several types of T cells and dendritic cells as well as their progenitors including CLPs (Georgopoulos et al., 1994; Wang et al., 1996). Ikaros deficiency leads to the loss of Flt3 expression; however, functional LMPPs are still present (Yoshida et al., 2006). Similarly ETPs remain unaffected in Ikaros null mice (Allman et al., 2003). The investigation of specific functions of Ikaros for the above lineages is complicated by the expression of different splice variants (Klug et al., 1998).

B cell differentiation is mediated by a network of three transcription factors that act sequentially and are critical for the generation of committed B cell progenitors from CLPs. E2A is expressed at a very early stage of B cell development and initiates the up-regulation of early B cell factor (EBF) that starts to be expressed at the pro-B cell stage (Bain et al., 1994; Lin and Grosschedl, 1995). Finally the expression of PAX5 defines committed B cells associated with the expression of the B cell marker CD19 (Nutt et al., 1999).

PU.1 is a major regulator of myeloid as well as lymphoid commitment. GFP reporter studies indicate that PU.1 expression is low or absent in stem cells but up-regulated towards multipotent progenitors (Back et al., 2005; Nutt et al., 2005). In myeloid lineages the highest PU.1 expression correlates with monocyte/macrophage over granulocytic differentiation. Intermediate levels are equally required for B cell differentiation as well as the early stages of T cell development, whereas PU.1 is dispensable for more mature T cell progenitors as well as Mk and erythroid lineages (Dahl and Simon, 2003; DeKoter and Singh, 2000; Scott et al., 1994). Conditional deletion of PU.1 in adult haematopoiesis leads to a reduction in the stem and multipotent progenitor compartments (Dakic et al., 2005; Iwasaki et al., 2005). The absence of PU.1 is accompanied by the loss of CLPs and CMPs as well as downstream lineages. Since this also affects the generation of mature lymphoid and myeloid cells in long term repopulation experiments, it has not been elucidated whether PU.1 has a role in stem

cells or only in downstream progenitors. The GMP population is increased due to enhanced proliferation but unable to differentiate further, whereas Mk/E lineages are largely unaffected (Dakic et al., 2005; Iwasaki et al., 2005).

Together with PU.1 the transcription factors CCAAT-enhancer binding protein (C/EBP)α as well as C/EBPβ are important determinants of myeloid differentiation. C/EBPα is already expressed in HSCs and mice deficient for C/EBPα have increased competitive repopulation activity. In addition these mice display a block in myeloid differentiation between the CMP and GMP stages but C/EBPα is not required for terminal differentiation (Friedman, 2002; Zhang et al., 2004).Therefore C/EBPα promotes myeloid differentiation from stem cells at the expense of their self-renewal activity. C/EBPβ is most important for stress haematopoiesis where it promotes GMP formation (Hirai et al., 2006).

1.2.2.1. Transcription factors in megakaryocyte and erythroid development

GATA1 and friend of GATA1 (*Zfpm1,* FOG1) are two key regulators of megakaryocyte/erythroid lineage commitment. Both lineages widely express GATA1 in mature cells as well as progenitors. GATA1 is specifically important for the development of mature erythroid cells and GATA1 deficient embryos die around embryonic day 11 due to severe anaemia associated with an erythroid maturation block at an early pro-erythroblast stage (Fujiwara et al., 1996; Takahashi et al., 1997). Similarly adult mice with a conditional GATA1 deletion display an erythroid maturation arrest at this stage. Dissection of the bone marrow progenitor compartment using the staging developed by Pronk et al. revealed a complete absence of committed erythroid progenitors (preCFU-E and CFU-E), whereas progenitors of the GM lineage (preGM and GMP) remained unaffected (Mancini et al., 2012). Furthermore GATA1 deleted mice are thrombocytopenic but have increased numbers of Mks as well as megakaryocyte (MkP) and megakaryocyte/erythroid (preMegE) progenitors. This suggests an important role of

GATA1 in Mk maturation, whereas the increase in Mk development is likely caused by the reduction of platelets and the feedback loop mediated by TPO signalling (Gutierrez et al., 2008; Kuter and Rosenberg, 1995; Mancini et al., 2012). In Mk/E development GATA1 interacts with its co-factor FOG1 that potentially integrates developmental cues of GATA1 and GATA2 that have partially redundant functions, although GATA2 has been shown to be particularly important for Mk development (Huang et al., 2009). Mice deficient for FOG1 have reduced erythroid development, whereas Mks are completely absent (Tsang et al., 1997a; Tsang et al., 1997b). Reduced erythropoiesis as well as thrombopoiesis has also been observed after FOG1 knockdown in zebrafish. In these animals the loss of mature Mk/E cells was accompanied by an increase in myelopoiesis (Amigo et al., 2009). Elegant experiments in the mouse using FOG1 conditionally deleted bone marrow chimeras in combination with fluorescence reporters that enable the specific detection of donor derived red blood cells and platelets showed a complete loss of the two lineages. Detailed progenitor analysis also revealed an almost complete loss of Mk and E progenitors. Moreover, in agreement with the zebrafish studies GM progenitors were increased suggesting a key function of FOG1 in Mk/E versus myeloid lineage specification (Mancini et al., 2012). In addition FOG1 has been shown to have a role in the late stages of T cell development where it negatively regulates GATA3 (Zhou et al., 2001).

1.2.2.2. Antagonistic key transcription factors specify lineage fates

For several transcription factors antagonistic relationships have been identified which play a major role in determining lineage commitment decisions. The paradigm for such relationships is the interaction between GATA1 and PU.1 in lympho-myeloid versus myelo-Mk-E commitment, which according to the alternative model is the first branch point in the haematopoietic roadmap. Utilizing fluorescence reporter lines for the two factors it has been possible to identify or confirm the earliest non-multipotent progenitors, the GATA1 positive CMP as well as the LMPP that expresses high levels of

PU.1 (Arinobu et al., 2007). GATA1 and PU.1 are reciprocally regulated at the transcriptional and protein level. Direct protein-protein interaction inhibits the respective other factor. GATA1 binding blocks the interaction of PU.1 with its co-factor c-Jun and represses PU.1 dependent transcription (Nerlov et al., 2000; Zhang et al., 1999). Conversely interaction with PU.1 blocks the DNA binding of GATA1 (Zhang et al., 2000). In addition both factors are able to reinforce their own expression through an auto-regulatory loop (Okuno et al., 2005; Yu et al., 2002). Both factors have been shown to act in a lineage instructive manner and have the ability to redirect the lineage choice of already committed progenitors (Heyworth et al., 2002; Iwasaki et al., 2003; Kulessa et al., 1995; Nerlov and Graf, 1998). In *in vitro* experiments with transformed avian progenitors enforced expression of PU.1 was sufficient to reprogram MEPs into myeloblasts, and GATA1 overexpression in myeloblasts led to their conversion into MEPs (Kulessa et al., 1995; Nerlov and Graf, 1998). In two additional studies ectopic GATA1 expression was equally sufficient to change the lineage fate of restricted primary haematopoietic progenitors. CLP as well as GMP could be reprogrammed to give rise to erythroid cells and Mks (Heyworth et al., 2002; Iwasaki et al., 2003). Similarly experiments in zebrafish showed that the knockdown of GATA1 can induce the switch from erythroid to myeloid cells and the reduction of PU.1 expression induces the conversion of myeloid cells to the erythroid lineage (Galloway et al., 2005; Rhodes et al., 2005). Thus, in an antagonistic relationship of two transcription factors such as PU.1 and GATA1 the relative expression between them is particularly important and a shift in that balance can cause lineage conversion. The lineage deterministic action of PU.1 and GATA1 has also been supported by mathematical modelling approaches and similar interactions between key transcription factors might regulate binary fate decisions at other stages of haematopoiesis (Huang et al., 2007).

In addition to the PU.1-GATA1 model of lympho-myeloid versus myeloid-Mk-E bifurcation the cooperation of FOG1 and C/EBPs (here C/EBPα and C/EBPβ) has

recently been proposed to determine Mk/E versus GM fates downstream of the CMP. Whereas there is no evidence for a cross regulation by protein-protein interaction or direct transcriptional repression between FOG1 and C/EBPs, they follow reciprocal expression patterns and promote opposite lineage outputs. Conditional depletion of FOG1 leads to upregulation of C/EBP expression in preMegEs, a population otherwise restricted to Mk/E lineages, and characterised by expression of an Mk/E specific gene profile (Mancini et al., 2012; Pronk et al., 2007). Conversely, mice deficient of C/EBPα and haploinsufficient for C/EBPβ upregulate FOG1 transcription in the preGM, a population largely committed to the GM lineage (Pronk et al., 2007). These changes in gene expression are in line with altered functional output from both populations. The lack of C/EBPs results in a loss of GMPs, while phenotypic Mk progenitors as well as Mk colony potential from total bone marrow are increased. FOG1 knockout on the other hand is associated with reductions or absence of Mk/E progenitors, decreased erythroid colony forming potential *in vitro* and low platelet as well as red blood cell output *in vivo* (Mancini et al., 2012).

1.2.3. Transcriptional lineage priming

Early models of haematopoiesis assumed stem cells to be in a naïve state associated with the expression of stem cell specific genes, but the complete absence of genes specific for downstream lineages. However, up to now multiple studies have demonstrated the expression of lineage genes including transcription factors and cytokine receptors in adult HSCs as well as multipotent cells in the embryo (Delassus et al., 1999; Forsberg et al., 2005; Ye et al., 2003). Overall expression levels are usually low and genes part of multiple lineage programs are expressed simultaneously, in contrast to committed progenitors that only express one lineage program at higher levels. Similarly lineage priming occurs in progenitors. Such priming was initially reported by Enver and co-workers who detected the expression of beta-globin and myeloperoxidase in cells that retained both erythroid and granulocytic potentials (Hu et

al., 1997). However, in contrast to stem cells that are primed for all lineages, the priming of downstream progenitors is restricted to genes associated with their physiological progeny (Miyamoto et al., 2002).

1.3. Haematopoietic stem cells

1.3.1. Functional evaluation of haematopoietic stem cells

The field of haematopoietic stem cell research was first established in the early 1960s. In studies aiming to test the irradiation sensitivity of haematopoietic cells Till and McCulloch transplanted bone marrow cells and reported the formation of discrete colonies on the spleen of recipient mice (Till and McCulloch, 1961). The number of spleen colonies in these experiments appeared to be proportional to the number of cells injected. This indicated a single cell origin, and it was later confirmed by irradiation induced chromosomal marking that spleen colonies were derived from single bone marrow cells (Becker et al., 1963). Furthermore secondary transplantation of cells isolated from spleen colonies again resulted in the formation of colonies suggesting self-renewal activity and the presence of stem cells (Siminovitch et al., 1963). However, the serial colony forming capacity of these cells rapidly declined indicating that the observed spleen colonies were rather derived from bone marrow progenitor cells (Siminovitch et al., 1964). Never the less today Till's and McCulloch's work is recognised as the first evidence for the existence of HSCs (Till and McCulloch, 1961). First *in vitro* culture assays for haematopoietic cells were developed using feeder layers from mouse kidney or embryo and enabled the detection of distinct colonies with clonal origin from whole bone marrow samples (Bradley and Metcalf, 1966). Although these studies disregarded the two key definitions of HSCs today, being the ability to generate cells of all lineages in the long term as well as self-renewal, and investigated cells that would now be termed short term HSC or multipotent progenitors, they pioneered haematopoietic stem cell biology.

Despite of 50 years of research having passed since the first culture of HSCs there is to date no assay for the definitive evaluation of stem cell identity *in vitro*. Several experimental approaches, however, allow for the approximate quantification of stem cell activity in a given cell population. Long term culture on feeder layers enables the evaluation of cells with long term repopulation potential termed as long term culture initiating cells (LTC-IC) or cobblestone area-forming cells (CAFC) (van Os et al., 2008). Self-renewal activity can be approximated by long term culture with serial replating of colonies.

The gold standard experiment for the evaluation of HSC function remains the long term multilineage repopulation assay. Total bone marrow cells, purified cell populations or single cells are transplanted into conditioned recipient mice. In the majority of studies recipients are lethally irradiated. Alternatively recipients with haematopoietic defects, such as mice with a point mutation in the gene encoding c-Kit receptor, have been utilized to enhance reconstitution (Benveniste et al., 2010; Benz et al., 2012; Boggs et al., 1984; Harrison and Astle, 1991). To qualify as HSCs, transplanted cells have to be able to reconstitute all blood lineages of the recipient mouse over at least 16 weeks. However, more recent studies observed cells with intermediate term repopulation potential that give reconstitution for more than 16 weeks but decline thereafter (Benveniste et al., 2010). Therefore most recent studies have used more stringent criteria for HSCs and required the reconstitution for up to 32 weeks or even longer (Sieburg et al., 2011). The second key requirement for the HSC definition is self-renewal, the ability to maintain or even expand the HSC pool. This can be assessed by serial transplantation into secondary or even tertiary hosts (Dykstra et al., 2007).

Stem cell quality can be assessed in the competitive repopulation assay. For that purpose total bone marrow cells or a purified population are transplanted together with a defined number of competitor bone marrow cells. Evaluation of long term multilineage

peripheral blood reconstitution gives information about the ability of test cells to reconstitute the blood system or particular lineages in relation to the competitor cells. For quantitative assessment of stem cell frequencies in a given cell population the limiting dilution assay has been employed (Harrison et al., 1988; Szilvassy et al., 1990; Trevisan et al., 1996). Thereby groups of recipients are transplanted with graded dilutions of test cells with the lowest dilutions containing less than one cell on average. The probability of finding a stem cell at a given dilution follows Poisson distribution, which enables the retrospective calculation of the stem cell frequency in the original sample.

A key requirement for all reconstitution assays, in particular the competitive repopulation assay, is the ability to distinguish transplanted donor cells from competitors or residual recipient cells that remain after irradiation. Methods to achieve this have included the use of cells from mice with different haemoglobin β-chains or female versus male cells that were distinguished based to their set of chromosomes (Harrison, 1980; Szilvassy et al., 1990). Various other studies made used of different isoforms of the glucose-phosphate isomerase 1 (GPI1) that are mainly expressed by erythrocytes and can be distinguished by cellulose acetate electrophoresis (Benveniste et al., 2010; Eppig et al., 1977; Nakano et al., 1989; Trevisan and Iscove, 1995). The most commonly used system for discrimination between donor and acceptor cells in repopulation assays is based on the two isoforms of the pan-haematopoietic marker CD45 (CD45.1 and CD45.2), which can be distinguished by specific antibody staining. CD45 is expressed on all haematopoietic precursors as well as mature cells of the lymphoid, monocytic and granulocytic lineages. However, absence from mature erythrocytes and platelets impedes the analysis of these lineages (Hermiston et al., 2003). Efforts to overcome that problem have included the use of *Actb*-GFP or other reporter mice, that allow the discrimination of donor versus competitor cells based on their fluorescence reporter expression (Forsberg et al., 2006).

1.3.2. Analysis and purification of haematopoietic stem cells

Over several decades of research HSCs have become the best characterised adult stem cell population. Current flow cytometry enables the isolation of highly purified stem cell population and various different protocols utilizing HSC specific combinations of surface markers, the ability for dye efflux or low cell cycle activity have been developed for that purpose (Benveniste et al., 2010; Challen et al., 2010; Dykstra et al., 2007; Kiel et al., 2005; Osawa et al., 1996). However, the purification of pure stem cell populations still remains a challenge. Limiting factors are the infrequent occurrence of HSCs, which has been estimated to about 0.05 % of total bone marrow cells, but also the absence of markers exclusively expressed on HSCs (Benveniste et al., 2010; Trevisan et al., 1996). The lack of means to efficiently evaluate stem cell function further complicates the situation. The gold standard assay to functionally evaluate haematopoietic stem cells remains the long term multilineage reconstitution analysis after transplantation. In addition to the long term nature of these experiments, inefficiencies in homing and engraftment could falsify obtained results and lead researcher to underestimate stem cell frequencies in a given population of cells. Evidence for efficient homing of all transplanted stem cells comes from a study that reported initial engraftment of 90 % of purified single stem cells but a declining frequency in the long term. This suggests the efficient homing of all stem cells, whereas only a fraction of them has long term reconstitution ability. (Benveniste et al., 2003). However, another study only obtained initial reconstitution from approximately one third of single purified HSC, but all of them maintained long term donor chimerism. This would be compatible with two thirds of transplanted cells failing to home (Camargo et al., 2006).

Since there is to date no marker that is expressed exclusively on stem cells and could on its own be used for HSC purification, various combinations of surface markers have been utilized. The first step of enrichment of haematopoietic stem and progenitor cells from mouse bone marrow is usually the exclusion of lineage markers, expressed on

mature haematopoietic cell populations. Standard lineage mixes, which for flow cytometry are often conjugated to a common fluorophore and measured together on only one channel, contain antibodies against markers such as CD4, CD5 and CD8 (T lineage), B220 (B lineage), Mac-1 and Gr-1 (GM lineage) and Ter119 (erythroid lineage). Using the additional markers Sca-1 and c-Kit, which are almost exclusively expressed on primitive haematopoietic cells, the $Lineage^{-}Sca\text{-}1^{+}c\text{-}Kit^{+}$ population has been defined (Ikuta and Weissman, 1992; Okada et al., 1992). The LSK population contains various multipotent progenitor populations and only enriches for HSCs but it forms the basis of several strategies for further stem cell purification (Adolfsson et al., 2005; Arinobu et al., 2007).

Flt3 is important for lymphoid development and is expressed on the LMPP (Adolfsson et al., 2005; Lyman et al., 1993; McKenna et al., 2000). The Flt3 receptor is absent on HSCs and as such has been used in HSC purification protocols in combination with the LSK phenotype (Adolfsson et al., 2001; Christensen and Weissman, 2001).

A surface marker that is commonly used for HSC enrichment together with the LSK phenotype is CD34. It is present on the majority of multipotent progenitors but absent on adult and quiescent stem cells (Nakauchi et al., 1999; Osawa et al., 1996). Its expression is, however, dependent on the cell cycle status and thus CD34 is expressed on all foetal HSC as well as adult HSC isolated from young mice. Similarly HSC do express CD34 in stress haematopoiesis such as after transplantation (Sato et al., 1999). The $LSKCD34^{-}$ HSC definition has been used for pioneering single cell transplantation studies, where multilineage donor reconstitution was obtained in more than 20 % of the recipients (Osawa et al., 1996). Together Flt3 and CD34 have been used to define a short term stem cell population ($LSKFlt3^{-}CD34^{-}$) that has the ability for rapid multilineage but not long term reconstitution (Yang et al., 2005).

The signalling lymphocytic activation molecule (SLAM) family members are expressed on mature immune cells and have roles in lymphocyte development, cell survival and adhesion, autoimmunity, humoral immunity as well as cytotoxicity (Cannons et al., 2011). Comparison of gene expression profiles of highly purified HSC and multipotent progenitors revealed three family members to be differentially expressed between these two populations, and which have been used subsequently to aid HSC purification. CD150 (SLAMF1) is present on HSC but not expressed on multipotent progenitors, whereas HSC lack surface expression CD48 and CD244, which are both present on multipotent progenitors (Kiel et al., 2005). CD150 and CD48 have been used together with the exclusion of lineage markers to isolate HSCs. Cells purified using this phenotype or c-Kit$^+$Sca-1$^+$ in addition gave long term multilineage reconstitution in 45-47 % of recipients in single cell transplantation experiments (Kiel et al., 2005). SLAM family marker expression seems to be independent from cell cycle status and has been successfully used to isolate foetal liver HSCs. Single CD150$^+$CD48$^-$Sca1$^+$Lin$^-$ foetal liver HSCs long term reconstituted 37 % of recipient mice (Kim et al., 2006). The results obtained by Kiel et al. clearly suggest that HSCs lack the expression of CD48 and no long term reconstituting HSCs could be found in the CD48$^+$ fraction (Kiel et al., 2005). However, another study identified stem cells with long term reconstitution potential in the LSKCD150$^+$CD48$^+$ bone marrow subset (Grassinger et al., 2010). The controversial results are explained by a role of CD48 for stem cell homing. CD48 function is blocked by the antibody used for cell purification by Kiel et al., whereas Grassinger et al. utilized a different non-blocking antibody clone (Grassinger et al., 2010).

Gene expression profiling also led to the identification of endothelial protein C receptor (EPCR, CD201) as a highly specific marker for HSCs (Balazs et al., 2006). EPCR has subsequently been used in various studies together with the SLAM markers CD150 and CD48 as well as CD45 to isolate highly pure populations of HSCs. These ESLAM (CD45$^+$EPCR$^+$CD48$^-$CD150$^+$) cells identify HSCs in adult bone marrow as well as foetal

liver. In single cell transplantation experiments ESLAM cells were able to reconstitute about 30 % of secondary recipients (Benz et al., 2012).

Endoglin is part of the transforming growth factor beta receptor (TGF beta) and plays important roles in angiogenesis as well as haematopoiesis. It is expressed on HSCs but absent from direct downstream progenitors, and as such has been used together with the LSK phenotype or other stem cell markers for the purification of HSC (Chen et al., 2003; Chen et al., 2002; Pronk et al., 2007).

Thy1 (CD90), for which there are two alleles in mice (Thy1.1, CD90.1 or Thy1.2, CD90.2), is present on thymocytes where it was initially identified. At low levels it is also expressed on HSCs but completely absent on downstream progenitors and as such has been used for HSC purification (Spangrude et al., 1988; Wagers and Weissman, 2006)

In addition to the cell surface markers described above various other stem cell specific properties have been used to isolate HSCs. In contrast to almost all other haematopoietic cell populations quiescent HSCs exhibit a multidrug resistance phenotype characterised by the expression of several trans membrane pumps of the ATP-binding cassette (ABC) transporter family. These transporters actively clear small molecules including fluorescent dyes from the cells cytosol. This principle is used for isolation of the side population, a small fraction of BM cells that is highly enriched for HSCs. When BM cells are stained with the DNA binding dye Hoechst 33342 and visualized at two different wavelengths, HSCs are highly enriched in the tip of a characteristic tail shaped profile that retains the lowest levels of dye (Chambers et al., 2007; Goodell et al., 1996). Similarly HSCs can be purified using the dye Rhodamine 123 that labels mitochondria (Benveniste et al., 2010; Chen et al., 2003; Phillips et al., 1992). In addition to multidrug efflux properties of a cell, Rhodamine 123 staining also depends on the mitochondrial activation state resulting in low labelling of quiescent stem

cells with low mitochondrial activity (Chaudhary and Roninson, 1991; Kim et al., 1998). The side population phenotype has also been combined with Rhodamine 123 staining for the isolation of highly purified HSCs that were able to long term reconstitute at least 25 % of recipients in single cell transplantation experiments (Dykstra et al., 2007; Dykstra et al., 2006; Uchida et al., 2003).

Other purification strategies utilize the quiescent properties of stem cells, particularly the reduced cell cycle activity compared to downstream haematopoietic progenitors. In a pulse labelling approach all haematopoietic cells are first marked *in vivo*. In the following long chase period the label will be diluted depending on the cells' proliferative behaviour. Quiescent haematopoietic stem cells with low cell cycle activity will therefore retain the highest level of labelling (Foudi et al., 2009; Nygren and Bryder, 2008; Wilson et al., 2008). A commonly used label is 5-bromodeoxyuridine (BrdU), a thymidine analogue that is incorporated into the cellular DNA during replication and can be detected using BrdU specific antibodies (Wilson et al., 2008). However, BrdU has been shown to be not very specifically retained in HSC (Kiel et al., 2007a). As the BrdU incorporation itself is dependent on cell cycle activity quiescent HSCs can also be identified by the lack of BrdU labelling after a short exposure period (Hock et al., 2004). Other approaches use the labelling with an esterified form of biotin, that binds to cell surface proteins and can be detected by antibody staining, or staining with the fluorescent dye carboxyfluorescein succinimidyl ester (CFSE) (Nygren and Bryder, 2008). CFSE couples covalently to intracellular molecules, is therefore retained for long periods and only diluted through cell division (Lyons and Parish, 1994). A transgenic mouse line that expresses a Histone2b-GFP fusion protein upon induction through doxycycline enables the ubiquitous labelling of all haematopoietic cells. Due to the long half-life of histones the GFP label is stable over time and only diluted through cell division. Work utilizing this reporter line has led to the identification of highly dormant HSCs, which have been suggested to not enter cell cycle for periods of up to 140 days. Interestingly, the stem cell population as a whole has

been shown to be highly heterogeneous in their proliferation rates (Foudi et al., 2009; Wilson et al., 2008).

1.3.3. Haematopoietic stem cell – fate and regulation

The most primitive HSCs are believed to be quiescent, a status of low metabolic activity and very infrequent cell division. In the steady state these dormant cells preserve haematopoiesis in the long term, whereas other more actively self-renewing and differentiating stem cells produce downstream multipotent progenitor populations and maintain mature blood cells (Wilson et al., 2008). Other results suggest that all stem cells constantly alternate between quiescent and active cycling states, which is supported by the fact that all stem cells are activated in response to stress such as haematopoietic injury or after transplantation (Wilson et al., 2008).

Upon cell division a stem cell has the option for three different fate decisions. Through symmetric renewal one HSC gives rise to two daughter stem cells resulting in expansion of the stem cell pool. Evidence for the existence of symmetric renewal comes from countless transplantation experiments, in particular with single cells that gave rise to numerous stem cell clones, as well as successful *in vitro* stem cell expansions (Sieburg et al., 2011; Takizawa et al., 2011). HSC differentiation results in the generation of two downstream progenitors and the loss of stem cell activity. This cell division can be symmetric where two progenitors of the same type are generated or asymmetric where the stem cell gives rise to two different progenitors. Asymmetric self-renewal leads to the generation of one HSC and one downstream progenitor and would achieve both maintenance of the stem cell pool as well as simultaneous replenishing of mature blood cells. Although this type of division has been observed for stem cells in non-vertebrates such as *Drosophila melanogaster* or *Caenorhabditis elegans*, due to technical limitations there is to date no conclusive experimental evidence for the asymmetric self-renewal of HSCs (Kipreos, 2005; Morrison and Kimble, 2006; Yamashita and Fuller, 2005).

The majority of HSCs are maintained in the bone marrow. A small number of HSCs is constantly released into the bloodstream, while others simultaneously return back to the bone marrow to maintain equilibrium (Adams et al., 2009; Wright et al., 2001). This process is non-random and influenced by circadian rhythms through the sympathetic nervous system, which regulates the expression of chemokine C-X-C motif ligand (CXCL)-12 in stromal cells as well as its receptor CXCR-4 (Lucas et al., 2008; Mendez-Ferrer et al., 2009). In response to stress such as haematopoietic injury including bone marrow ablation as well as increased cytokine signalling, for instance the treatment with G-CSF in a clinical setting, the number of HSCs released into the bloodstream can strongly increase (Suarez-Alvarez et al., 2012). HSCs find their way back to the bone marrow through the multistep process of homing, which involves chemokine/cytokine signalling, cytoskeleton rearrangements and matrix-metalloproteinase (MMP) activation. Important regulators are again CXCL-12 / CXCR-4 but amongst others also SCF, VCAM1 and CD44 (Lapidot et al., 2005).

1.3.4. Haematopoietic stem cell niches

HSCs reside in specific stem cell niches, cellular and molecular microenvironments in the bone marrow that control stem cell function and maintain HSCs over the course of a life time. This includes the regulation of quiescence, self-renewal and differentiation in homeostasis but also in response to external cues (Schofield, 1978). HSCs are primarily found in the trabecular cavities of long bones, strongly indicating that the stem cell niche is located in the same region (Lord and Hendry, 1972; Nilsson et al., 2001). Through more detailed evaluation two distinct areas were identified to harbour HSCs. Quiescent stem cells were found in the endosteal region in the immediate proximity to bone lining cells, primarily osteoblasts but also osteoclasts, CXCL-12-abundant reticular cells (CAR) and stromal fibroblasts (Ehninger and Trumpp, 2011). This endosteal niche is only sparsely vascularised and provides a hypoxic microenvironment to the residing cells. Compared to downstream progenitors HSCs have a low metabolic status and generate

the low levels of energy they require by anaerobic metabolism through a high rate of glycolysis. Low metabolic activity reduces the production of reactive oxygen species and enables the maintenance of HSCs protected from oxidative stress (Simsek et al., 2010; Suda et al., 2011). On the other hand active, cycling stem cells reside in the perivascular niche within a highly vascularised region close to the centre of the bone, where they are associated with the sinusoidal endothelium (Kiel et al., 2005). This normoxic environment supports aerobic metabolism, higher cell cycle activity and enables stem cells as well as downstream progenitors to enter the bloodstream (Wilson et al., 2007).

In the niche, HSCs are regulated through direct cell-cell contact as well as multiple locally expressed soluble factors. HSC quiescence is maintained through Angiopoietin1 (Ang-1) / Tie2 signalling. Ang-1 is expressed on osteoblasts as well as mesenchymal cells and inhibits HSC division through interaction with the tyrosine kinase Tie2 (Arai et al., 2004). A similar role has been suggested for the cell adhesion molecule N-cadherin, although its importance is still debated. In one study no evidence for N-cadherin function in HSC maintenance could be obtained. However, others reported a reduction of reconstitution ability after N-cadherin knock down as well as reduced HSC cell cycle activity when N-cadherin was overexpressed (Arai et al., 2012; Kiel et al., 2007b). Key cytokines important for stem cell maintenance include TPO with its receptor Mpl, as well as SCF with its receptor c-Kit. TPO deficient mice show a strong reduction of HSC numbers along with elevated cell cycle activity in remaining stem cells (Qian et al., 2007). Accordingly, mice deficient for the TPO signalling inhibitor LNK have an expanded stem cell compartment due to their increased self-renewal potential (Buza-Vidas et al., 2006; Seita et al., 2007). SCF does exist as a soluble form but is also expressed as membrane bound protein on the surface of various bone marrow stromal cells. Naturally occurring mutations in the c-Kit receptor or the ligand itself all lead to haematopoietic defects and reduced stem cell function (Brannan et al., 1991; Flanagan et al., 1991; Nocka et al., 1990; Thoren et al., 2008). In a recent study HSCs were

specifically lost when SCF was locally deleted from endothelial or leptin receptor-expressing perivascular stromal cells but remained unaffected after SCF deletion in haematopoietic cells or osteoblasts (Ding et al., 2012). This shows the importance of local expression of cytokines in specific cell types that are part of the stem cell niche. Pathways such as Notch or Wnt signalling that fulfil key regulatory functions at multiple stages of haematopoiesis have also been implicated in the regulation of HSCs. Several studies analysing the effect of overexpression of different members of the notch signalling pathway, such as hairy and enhancer of split 1 (*Hes1*) or Jagged1 (*Jag1*) resulted in increased stem cell self-renewal and elevated reconstitution potential in competitive transplantations (Calvi et al., 2003; Kunisato et al., 2003). Accordingly, in a separate study the overexpression of a dominant negative form of the recombining binding protein suppressor of hairless RBP-Jk led to reduced reconstitution potential (Duncan et al., 2005). However, contrasting results obtained using a RBP-Jk conditional knock out mouse model or overexpression of another dominant negative member of the Notch pathway indicated a redundant role of Notch for HSCs (Maillard et al., 2008). In contrast to Notch signalling multiple studies provide conclusive evidence for an important role of the Wnt pathway for stem cell function and maintenance. However, different gain- or loss-of-function approaches gave controversial and partly contradicting results as to what role the Wnt pathway plays. Early studies used retroviral expression of Wnt activators or inhibitors. While Wnt activation led to an increased number of HSCs with enhanced reconstitution ability, the forced expression of Wnt inhibitors resulted in reduced stem cell function (Reya et al., 2003; Willert et al., 2003). Opposing results were generated in studies that used transgenic mice with constitutively active Wnt signalling, which cause a block in differentiation, increased cell cycle activity with a transient HSC expansion but eventual exhaustion of the stem cell pool (Kirstetter et al., 2006; Scheller et al., 2006). Loss of function studies were performed using mice that either lacked a key component of the Wnt signalling cascade or constitutively expressed an inhibitor of the Wnt pathway. In both cases the loss of Wnt signalling resulted in loss of HSC self-

renewal (Fleming et al., 2008; Luis et al., 2009). The discrepancy in the results of the above studies can partly be explained by the different approaches used. Retroviral mediated expression levels likely vary from those obtained with the transgenic models, and specific levels have been shown to be particularly important for different haematopoietic cells including HSCs (Luis et al., 2011).

1.3.5. Heterogeneity within the haematopoietic stem cell population

For a long time the most primitive haematopoietic stem cells were thought to be a homogeneous population sitting atop a hierarchy of progenitor cells, where every HSC has the ability to give rise to all mature haematopoietic lineages with equal probability. According to this model the heterogeneity of the blood system could be achieved by cell intrinsic stochastic variation, the regulation through external cues such as the niche environment or cytokines, or most likely a combination of both. However, in recent years several studies provided evidence that the most primitive stem cell compartment in itself is heterogeneous. Individual HSCs show distinct reconstitution patterns and are biased to give rise to particular lineages (Benz et al., 2012; Challen et al., 2010; Dykstra et al., 2007; Morita et al., 2010; Sieburg et al., 2006). In a recent study the transplantation of individual HSCs via a limiting dilution approach revealed 16 different reconstitution patterns. Cells increased, decreased or maintained the percentage of reconstitution over a seven month period (Sieburg et al., 2006). Successive serial transplantation showed that the life span of HSCs is intrinsically limited, ranging from 10 to almost 60 months. In these experiments daughter HSCs within a clone behave synchronously and eventually exhaust at the same time (Sieburg et al., 2011). Cells with the longest reconstitution potentials have also been shown to have a myeloid lineage bias. Often these HSCs, that were classified as α and β cells, reside in the $CD150^+$ fraction or the side population tip and give only low reconstitution in primary recipients, but achieve high reconstitution after secondary transplantation (Challen et al., 2010; Dykstra et al., 2007; Morita et al., 2010). On the other hand lymphoid biased HSCs, which are also termed γ and δ cells,

most efficiently reconstitute lymphoid lineages. These cells show high initial reconstitution which starts to decline after a 3-4 months period (Dykstra et al., 2007). Such intermediate term reconstituting cells were also purified based on the expression of CD49b (Benveniste et al., 2010). Taken together these results suggest a hierarchy within the stem cell compartment with the most dormant myeloid biased long term HSC on top of lymphoid biased HSC with limited life span (Challen et al., 2010; Morita et al., 2010). Recent evidence establishes the existence of such hierarchy already in foetal liver haematopoiesis (Benz et al., 2012). Interestingly, the balance between myeloid and lymphoid biased stem cells changes with age. In old mice the majority of HSCs show a myeloid bias potentially reflecting the constant need for replacement of short lived myeloid cells. The repertoire of lymphoid cells on the other hand has long been established at that point (Beerman et al., 2010).

1.3.6. HSC and megakaryocyte commitment

Various findings are compatible with close relationship between HSCs and Mks with the possibility of a direct hierarchical link in between. The first lineage restriction from stem cell towards LMPPs involves the loss of Mk as well as erythroid potentials (Adolfsson et al., 2005). The simultaneous generation of a progenitor restricted to these two lineages would be plausible, but to date there is no direct experimental evidence for the existence of such a progenitor. However, a large fraction of the recently identified primitive CMP population produced pure Mk colonies *in vitro*, which points towards an Mk progenitor in this population (Arinobu et al., 2007). HSC and Mks share various cell surface markers as well as transcription factors. Both express Mpl, the receptor for TPO and a key cytokine required for Mk differentiation. Mpl is also important for HSCs with Mpl deficient HSCs displaying a long term repopulation defect (Alexander et al., 1996; Gurney et al., 1994; Kimura et al., 1998). Likewise the knockout of TPO itself reduces HSC numbers and *in vivo* reconstitution potential (Qian et al., 2007). CD150 and CD41 are commonly used to identify MkPs in the LSK bone marrow fraction (Pronk et al., 2007). CD150 is

also a key marker for stem cells and has been applied as part of the SLAM definition (Kiel et al., 2005). CD41 which is further up-regulated during Mk differentiation and highly expressed on mature platelets is present on adult HSCs as well as the first definitive HSCs during embryonic development (Mikkola et al., 2003). Various other surface markers including c-Kit, CD105, Tie2, CD31 and KDR are shared between Mks and HSCs. In addition both cell types express many transcription factors in common. *Runx1* plays an essential role during the emergence of the first definitive HSC from the Aorta-gonad-mesonephros (AGM) region, and *Runx1* has been implicated in the regulation of adult HSCs (Ichikawa et al., 2008; North et al., 2002; Okuda et al., 1996). During Mk development Runx1 plays an important role in the later stages of Mk maturation and conditionally Runx1 deleted mice develop thrombocytopenia (Ichikawa et al., 2004). GATA2 has a role in HSC regulation and is also involved in megakaryopoiesis, where it has overlapping roles with GATA1. The *Hox*-related gene *Meis1* is highly expressed in HSC, AGM mesenchyme and aortic endothelium. In addition *Meis1* deficient embryos are anaemic with complete absence of the Mk lineage demonstrating the importance of this transcription factor for Mk development (Azcoitia et al., 2005; Hisa et al., 2004). Taken together HSC and the Mk lineage are closely related, and although supporting experimental evidence has yet to be obtained, several lines of data are compatible with a direct HSC-Mk differentiation pathway.

1.4. Mapping the fate of haematopoietic stem cells

In the process of haematopoiesis all mature cell lineages of the mammalian blood system develop from a pool of stem cells via hierarchically organised progenitor cells. Over the last decades progenitor populations were prospectively purified using cell surface antigens and characterised on the functional as well as molecular level. Subsequently multiple progenitor populations were placed in relation to each other according to their functional lineage potentials and lineage specific gene expression

properties, which led to the postulation of different models of lineage commitment (Ceredig et al., 2009; Kawamoto et al., 2010; Luc et al., 2008b) (A detailed review of haematopoietic lineage commitment models is given in section 1.1.2.). Within these hierarchies, however, the detailed cellular pathways and the direct relationships between stem as well as progenitor populations are largely unknown and likely involve a sequence of progressive cell fate decisions. Detailed tracing of individual fates would ideally require the continuous observation at the single cell level and *in vivo*, while constantly evaluating cell identities using a set of surface markers or transcriptional reporters. Although, due to technical limitations, to date this is not fully possible for the haematopoietic system several studies have attempted to investigate single cell fates *in vitro* and *in vivo* (Rieger and Schroeder, 2008).

1.4.1. Mapping of stem cell fates in different model organisms

Pioneering work in stem and progenitor cell tracking has been done in model organisms like *Drosophila melanogaster* and *C. elegans*, where it has been possible to continuously follow cell development from the stem to the mature cell at the single cell level. The adult *C. elegans* has a defined cell number of less than 1000 cells. Through serial electron microscopy or more recently time lapse imaging it has been possible to trace the development of the entire adult organism and to define the lineage history of each single cell (Bao et al., 2006; Kipreos, 2005; Sulston et al., 1983; White et al., 1986). Detailed insights in stem cell behaviour and their interaction and regulation through the niche also come from the observation of germline and somatic stem cells in the *Drosophila* testis. Germline stem cells reside in contact to their niche at the apical end of the testis. Upon asymmetric stem cell division one daughter cell remains in contact with the niche, whereas the second daughter cell is displaced away from the niche and differentiates. This gonial blast undergoes a defined number of cell divisions and maturation steps before mature sperm cells are generated. Due to the relatively simple spatial organisation and the limited number of cells involved, it has been possible to elucidate

this process in great detail and to characterise all participating cells (de Cuevas and Matunis, 2011; Yamashita et al., 2010). In the more complex murine haematopoietic system such detailed analysis is prevented 1) by technical limitations in observing the bone marrow *in situ*, 2) the more sophisticated spatial organisation of the stem cell niche, 3) the higher number of cells as well as 4) the continuing challenge for prospective identification of haematopoietic cells, in particular HSCs. However, the work with *C. elegans* or *Drosophila* has also helped to understand haematopoiesis, particularly stem cells, which are thought to divide asymmetrically during homeostasis, although this is yet to be shown (Knoblich, 2008; Morrison and Kimble, 2006).

1.4.2. Mapping the fate of murine haematopoietic stem cells *in vivo*

Two recent studies attempted to live image HSCs in the bone marrow environment using confocal microscopy in combination with two photon excitation technique. According to their imaging protocol termed *ex vivo* imaging stem cells (EVISC) Xie et al. transplanted phenotypic stem cells defined as LSKFlt3$^-$ from an *Actb*-GFP reporter mouse. 4 hours after transplantation they harvested the femur of recipient mice and imaged a cross section of the trabecular bone. In irradiated but not in non-irradiated hosts GFP$^+$ stem cells homed close to the endosteal region, which has stem cell niche function, and underwent cell division (Xie et al., 2009). In the second study Lo Celso *et al.* imaged dye labelled and transplanted haematopoietic stem or progenitor cells in the calvarium bone marrow of live mice. Also in this study close homing of stem cells to the endosteum in irradiated hosts was observed. The most primitive cells defined as negative for the surface marker CD34 were located closest to the bone surface. For some engrafted cells it has been possible to track the first cell division within the first two days after transplantation (Lo Celso et al., 2009). These studies achieved for the first time the live observation of HSCs in their bone marrow microenvironment. However, both experimental systems have several drawbacks. HSCs are initially only purified by phenotypic markers, the time span for observation is limited to a few days, and most

importantly cell identities cannot be traced .The latter could be addressed by the utilisation of reporter lines, however, this would also only give an indication about the identity of the observed cells. Therefore it has not been possible to make definitive conclusions about stem cell fates in these studies.

1.4.3. *In vitro* fate mapping by time lapse microscopy

The development of haematopoietic cells can be traced *in vitro* via time lapse imaging. A major challenge of the technique remains the provision of an environment for intermediate to long term cell culture while achieving good imaging quality. If both needs are met single or low numbers of purified haematopoietic cells can be followed over a few days and surface marker as well as fluorescence reporter expression can be recorded. For long term imaging or tracking of larger cell numbers specific software packages for analysis of data have been developed. Wu et al. attempted to investigate the division characteristics of HSCs in a stroma co-culture system. Haematopoietic cells were isolated from a transgenic Notch-GFP reporter line based on the LSK phenotype. GFP^+ cells from these mice are enriched in the HSC fraction and GFP is down regulated upon differentiation (Duncan et al., 2005). Based on the GFP reporter expression Wu et al. observed symmetric cell division that could be either commitment (GFP down regulation) or self-renewal (maintenance of GFP expression) or asymmetric cell division associated with down regulation of GFP in only one daughter cell. The frequencies of each type of cell division was dependent on the stroma used for co-culture and was altered by ectopic expression of leukaemia associated fusion proteins. Further asymmetric GFP expression in daughter cells correlated with asymmetric distribution of Numb a negative regulator of Notch signalling (Wu et al., 2007). Numb has been most extensively studied in *Drosophila*, where it is important for asymmetric cell division during development, particularly in the nervous system. However, it is also asymmetrically segregated during neurogenesis as well as T cell division during the

adaptive immune response (Cayouette and Raff, 2002; Chang et al., 2007; Zhong et al., 1996).

Long term time lapse imaging experiments usually require the continuous tracking of a large number of cells, even if the culture is initiated with only a single but highly proliferative haematopoietic cell. The co-culture on a layer of stroma cells is often desired to provide specific environmental cues for maintenance of cell identity, or induction of differentiation along a particular pathway, which further complicates the tracking of cultured haematopoietic cells. To achieve continuous tracking of cells of interest at single cell resolution the Schroeder laboratory has developed a cell tracking software. This tool allows the retrospective analysis of long term time lapse movies, tracking of single or multiple colonies and evaluation of experimental parameters such as cell morphology or fluorescence readout. Transcriptional reporters or even in culture antibody staining has been applied (Rieger and Schroeder, 2008). To date this method has been used for two different studies. In the first study the authors attempted to continuously image the generation of haematopoietic cells from the hemogenic endothelium. During embryonic development endothelial cells have been suggested as precursors of the first definitive HSCs. During multiple days of observation murine mesodermal cells derived from embryonic stem cells gave first rise to cells with endothelial morphology and VE-cadherin expression. Later they started to detach and initiated expression of CD41 and the pan haematopoietic marker CD45 (Eilken et al., 2009). The second study investigated the effect of cytokines on the lineage choice of haematopoietic cells for which an instructive or permissive mode of action has been discussed. M-CSF or G-CSF drive GMPs to differentiate into either the macrophage or granulocyte lineage, respectively. In presence of one of the two cytokines GMPs proliferated and differentiated until final unilineage commitment without significant cell death being observed. Thus, these results suggest the instructive action of both cytokines that drive all cells towards a particular lineage rather than supporting cell

survival and growth of only intrinsically committed cells as expected in a permissive mode of action (Rieger et al., 2009a).

1.4.4. *In vitro* paired daughter fate mapping

An additional method for tracking of haematopoietic cells *in vitro* is paired daughter fate mapping. Unlike time lapse microscopy, that enables observation of multiple divisions or differentiation steps, paired daughter fate mapping evaluates a cells fate over one single cell division. In comparison to time lapse microscopy where cells are only phenotypically identified, paired daughter fate mapping enables the phenotypic but also functional analysis *in vitro* and *in vivo* of traced cells as well as their molecular characterisation (Ema et al., 2000; Takano et al., 2004). The method was developed and first performed by Suda and colleagues in 1983, who analysed cells isolated from the spleen after HSC mobilisation. Initial colonies from cultures in semisolid medium were separated by micromanipulation and lineage potentials of single cells evaluated by May/Grünwald-Giemsa staining after a secondary culture period. Single cells gave rise to colonies that contained various mature lineages and allowed the retrospective characterisation of the initiating cells (Suda et al., 1983). Follow up experiments included additional micromanipulation steps, which allowed for the analysis of paired progenitors of single cells and the underlying cell division (Suda et al., 1984b). Pedigrees of initiating single cells were derived by repeated micromanipulation and analysis of granddaughter cells (Suda et al., 1984a). While the above studies selectively analyse cells with high proliferative potential that had the ability to form colonies *in vitro*, their cell identities remain unknown. Paired daughter fate mapping of prospectively isolated LSKCD34$^-$ phenotypic stem cells was performed by the Nakauchi laboratory. FACS sorted single cells were cultured in presence of various combinations of cytokines. After the first cell division daughter cells were micromanipulated and cultured in presence of SCF, IL-3, TPO and EPO. Resulting colonies were evaluated for the presence of neutrophils, monocytes, megakaryocytes and erythrocytes, with cells giving rise to all 4 cell types

being defined as stem cells. For assurance that the initiating cell was in fact a multipotent cell, only cells that gave rise to all 4 cell types in the combination of both daughter colonies were analysed. The authors observed symmetric cell divisions with both daughter cells giving rise to all cell types and asymmetric divisions, where only one daughter cell remained a stem cell with frequencies depending on the cytokines present. Depending on the cytokine combination 20 – 50 % of cells divided asymmetrically and symmetric stem cell renewal was best promoted by SCF + TPO. In the event of asymmetric cell division the colonies derived from cells that had lost one or more lineage potentials contained more than 10 different combinations of mature lineages (Ema et al., 2000; Takano et al., 2004). Due to the nature of the assay such heterogeneity could be the result of inefficient detection of lineage potentials rather than real lineage commitment. Thus, although the results of these studies suggest the existence of asymmetric stem cell divisions *in vitro* this remains to be conclusively proven.

The analysis of paired progenitors has also provided important information about the role of several genes in HSC self-renewal and differentiation. Using the experimental setting described above Nakauchi and colleagues performed gain and loss of function studies of the polycomb gene product BMI1 that has been implicated in stem cell maintenance, as well as the adaptor protein *LNK*, a negative regulator of TPO signalling. Deletion of BMI1 resulted in accelerated differentiation and a profound self-renewal defect. Conversely, ectopic expression of BMI1 led to an increase in stem cell self-renewal underlining its importance for this process (Iwama et al., 2004). The effect of LNK deletion on HSCs is TPO dependent and LNK deficient stem cells show advanced self-renewal potential, which was confirmed by investigation of the cells long term repopulation activity (Seita et al., 2007). Sarrazin et al. recently reported the monocytic transcription factor MafB as negative regulator of M-CSF signalling. Absence of MafB resulted in enhanced sensitivity to M-CSF and increased expression of the transcription factor PU.1 in HSCs. Using the paired daughter cell assay together with a GFP reporter for PU.1 the authors

revealed an increased asymmetric upregulation of PU.1 in stem cell derived paired daughters. However, due to the lack of functional analysis it remains elusive whether a MafB deficiency causes asymmetric cell division (Sarrazin et al., 2009).

1.5. Haematopoiesis in the embryo

1.5.1. Primitive haematopoiesis

Embryonic haematopoiesis occurs in two distinct waves, the primitive wave primarily characterised by the emergence of red blood cells, which initially express foetal haemoglobin, and the definitive wave that in large parts resembles adult haematopoiesis and is derived from definitive HSCs (Fig. 1.4) (Dzierzak and Speck, 2008). The first primitive blood cells emerge in the mouse embryo at day 7-7.5 after conception (E7-7.5) and are thought to be derived from a mesodermal precursor. This haemangioblast can give rise to haematopoietic as well as endothelial cells and was first described in the chick embryo almost a century ago (Murray, 1932; Sabin, 1920). Functional evidence for the existence of the haemangioblast came from *in vitro* studies with embryonic stem cells where so called blast colony-forming cells could give rise to haematopoietic as well as endothelial precursors (Choi et al., 1998; Fehling et al., 2003). In early stage mouse conceptuses haemangioblasts could be identified in the posterior region of the primitive streak expressing the mesoderm marker brachyury as well as the vascular endothelial growth factor receptor Flk1 (Huber et al., 2004). From there they migrate to the yolk sac (YS) where they generate progenitors of haematopoietic as well as endothelial cells, give rise to primitive erythrocytes as well as parts of the YS vasculature, and contribute to the formation of the embryonic blood islands (Ferkowicz and Yoder, 2005; Ueno and Weissman, 2006).

During primitive haematopoiesis the vast majority of cells formed are primitive erythrocytes, and erythroid as well as macrophage progenitors are the first cells

detected in the embryo. Primitive erythroid progenitors can be found in the YS from E7 on but are only maintained for two days after which they get replaced by erythroid cells that express the adult isoforms of haemoglobin (Palis et al., 1999). Furthermore this wave has been shown to produce the first Mk progenitors that have been isolated as early as E7.5 from the YS and form Mk colonies *in vitro* when cultured in the presence of SCF, IL-3, IL-6, EPO and TPO (Xu et al., 2001).

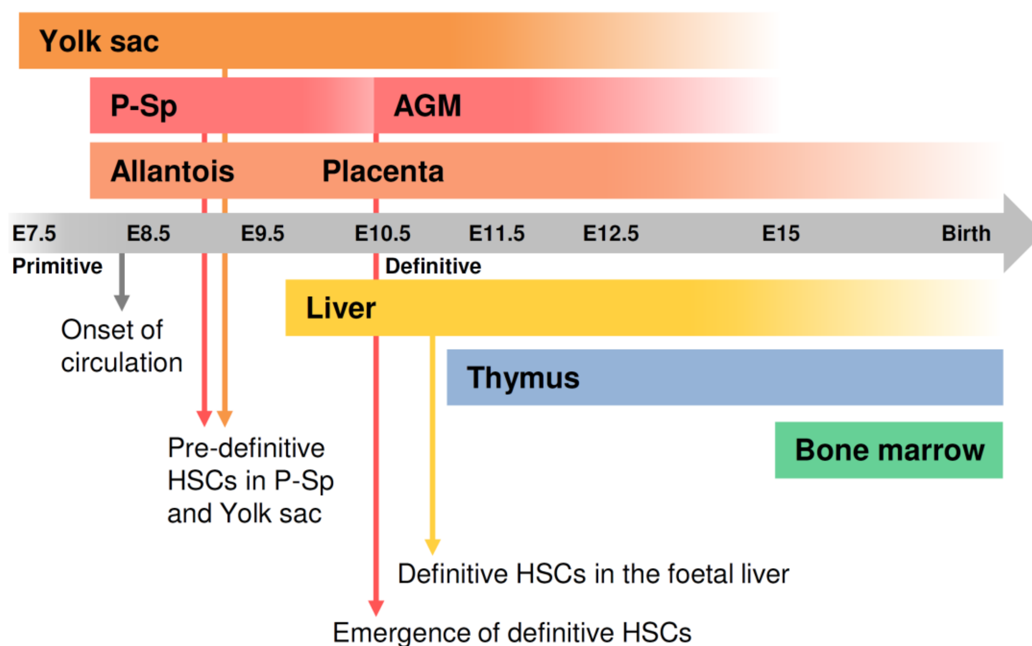


Figure 1.4: Timeline of emergence and development of haematopoietic activity in the mouse embryo

First primitive haematopoietic cells emerge around day E7 in the yolk sac. Definitive haematopoiesis is initiated at E10.5 when first definitive HSCs emerge in the AGM. Bars represent the presence of haematopoietic activity in the respective embryonic tissues. Arrows indicate the times when pre-definitive or definitive HSCs can first be found in different tissues. Embryonic day (E), para-aortic splanchnopleura (P-Sp), aorta-gonad-mesonephros (AGM). Adapted from Dzierzak and Speck, 2008.

1.5.2. Emergence of HSCs and definitive haematopoiesis

Definitive HSCs are defined by their ability to give long term reconstitution of irradiated adult hosts. In the embryo these cells first emerge at E10.5 (after the 35 somite stage) when they can be isolated from a structure consisting of the aorta, gonads and mesonephros (Medvinsky and Dzierzak, 1996). Slightly later, at E11 (or 42 somite stage) definitive HSCs can also be found in the foetal liver (Muller et al., 1994; Sanchez et al., 1996). This very stringent definition of definitive stem cells led to the conclusion that no stem cells are present at earlier stages of development. However, earlier HSCs might simply require the specific microenvironment present in the embryo and therefore could be unable to reconstitute adult hosts. In fact there are now several lines of evidence suggesting the existence of immature or pre-HSC that despite lacking the potential to reconstitute adult wild type mice have stem cell properties. Such multipotent haematopoietic progenitors have first been identified at E9 in the para-aortic splanchnopleura (P-Sp) that develops into the AGM at later stages. These cells can give rise to multiple lineages *in vitro* and are phenotypically characterised by the expression of c-Kit, CD41 and a combination of CD31, CD34 and AA4.1, whereas they lack the pan-haematopoietic marker CD45. When isolated at E10 from the P-Sp/AGM region they are able to long term reconstitute sub-lethally irradiated mice deficient of *Rag2* and the *common gamma chain* (*Rag2cγ*$^{-/-}$) (Bertrand et al., 2005; Cumano et al., 1996; Cumano et al., 2001; Godin et al., 1995; Godin et al., 1999). Recent studies show that immature HSCs can give rise to definitive HSCs *in vitro*. After culture for 7 days on OP9 stroma in the presence of TPO or in P-Sp organ culture immature HSC upregulated the expression of the definitive stem cell markers CD150 and Sca-1 and gained the ability to reconstitute lethally irradiated wild type recipients. Thus, immature HSCs might also give rise to definitive HSCs during embryonic development (Kieusseian et al., 2012).

Early on also the YS was proposed to be the first site of definitive haematopoiesis in addition to or instead of the P-Sp/AGM (Moore and Metcalf, 1970). However, tissue

grafting experiments with avian embryos showed that cells from the definitive haematopoietic wave rather originate from the embryonic body that includes P-Sp/AGM and extra-embryonic allantois that later develops into the placenta (Dieterlen-Lievre, 1975; Turpen et al., 1981). More recently further evidence supporting the YS as site of origin of HSCs in the mouse came from several studies performed by Yoder and colleagues. By using neonatal rather than adult recipients they were able to reveal long term reconstitution potential in $CD34^{+}$ yolk sac cells as early as E9 (Yoder et al., 1997). However, since circulation is established and the heart starts to beat already at E8.25, it was still unclear whether these cells were generated in the yolk sac or transferred from other tissues (Ji et al., 2003). To circumvent that problem *Ncx1*$^{-/-}$ embryos were analysed that do not develop a beating heart (Koushik et al., 2001). Although haematopoietic progenitors could be detected in the yolk sac but not in the embryo proper as early as E8.5, no reconstitution experiments were performed, and therefore no definitive data about stem cell activity obtained. Furthermore despite the lack of a beating heart progenitors could still have transferred between tissues by diffusion (Lux et al., 2008).

In addition to P-Sp/AGM and YS haematopoietic cells have been detected in the allantois as well as the placenta before the onset of circulation. These are mainly definitive erythroid as well as myeloid cells; although after pre-culture multipotent clonogenic progenitors could be detected in semisolid medium. The expression of c-Kit as well as CD41 also indicates the presence of haematopoietic cells at that early point (Corbel et al., 2007; Zeigler et al., 2006). Definitive stem cells with long term reconstitution potential in the placenta were only observed at embryonic day 11 (Gekas et al., 2005; Ottersbach and Dzierzak, 2005). While these results demonstrate general haematopoietic potential in the allantois/placenta there is no evidence for de novo generation of HSCs in these tissues.

1.5.3. The hemogenic endothelium as origin of definitive HSCs

Several studies of murine as well as avian haematopoiesis demonstrate a close relationship of endothelial and first haematopoietic cells, and point towards the hemogenic endothelium as origin of definitive HSCs (Jaffredo et al., 1998; Nishikawa et al., 1998). The first long term repopulating stem cells can be found in the AGM at embryonic day 10.5. At the same time clusters of haematopoietic cells emerge in close proximity to the ventral endothelium of the dorsal aorta, the vitelline and umbilical arteries (Jaffredo et al., 2005). Metabolic lineage tracing and retroviral labelling confirmed these clusters to be derived from endothelial cells (Jaffredo et al., 1998). AGM stem cells express a set of markers including CD45, Sca-1, c-Kit, CD34, Runx1 and GATA2 that with exception of CD45 are all also present on the aortic endothelium (de Bruijn et al., 2002; North et al., 2002; Sanchez et al., 1996). Furthermore the majority of HSCs in the AGM express VE-cadherin a surface protein otherwise considered as a marker for endothelial cells (North et al., 2002).

Other studies also provide some evidence for HSC generation from mesenchymal cells underlying the endothelium in the ventral area of the dorsal aorta as well as the so called subaortic patches. Cells from both regions that do not express CD45 have been shown to harbour some reconstitution potential in immune deficient recipients (Bertrand et al., 2005). However, $CD45^+$ cells located in the haematopoietic clusters and aortic endothelium are able to reconstitute wild type recipients and are far more potent (de Bruijn et al., 2002; North et al., 2002).

The transcription factor Runx1 has been identified to play a key role in the development of definitive haematopoiesis. Although primitive erythrocytes are not affected by Runx1 deletion, definitive progenitors including definitive stem cells fail to develop (Okuda et al., 1996). Experiments with a *lacZ* reporter system for Runx1 show the initiation of its expression before the emergence of haematopoietic clusters in the dorsal aorta, vitelline

and umbilical arteries (North et al., 1999). A recent study utilizing conditional Runx1 deletion shows that Runx1 is specifically required for the transition from hemogenic endothelium to HSCs, but is dispensable for stem cell maintenance or immediate downstream progenitors (Chen et al., 2009).

Recent attempts to directly observe the generation of HSCs from endothelial cells using time lapse imaging have provided further support for the hemogenic endothelium. In zebrafish embryos haematopoietic cells emerge by egress and transformation of single endothelial cells from the aortic wall without cell division (Kissa and Herbomel, 2010). Subsequently these cells give rise to the complete adult haematopoietic system (Bertrand et al., 2010). In vitro continuous single cell tracing of endothelial cells on stroma co-culture showed the direct generation of haematopoietic cells also in the mouse system (Eilken et al., 2009). This was confirmed by imaging studies of the live mouse embryo. Ventral aortic hemogenic endothelial cells gave directly rise to phenotypic stem cells defined by the expression of Sca-1, c-Kit and CD41 (Boisset et al., 2010). Although these studies allow the direct visualisation of the emergence of haematopoietic cells they are limited by the only phenotypic identification of HSCs. Long term reconstitution experiments would be required to unambiguously confirm stem cell identity.

1.5.4. Haematopoiesis in the foetal liver

The first haematopoietic cells appear in the foetal liver around E9 (Houssaint, 1981). These primarily erythroid and myeloid progenitors are not de novo generated in the foetal liver but rather arrive through blood circulation from multiple sites including AGM and yolk sac (Delassus and Cumano, 1996; Kumaravelu et al., 2002). The first definitive HSC migrate to the foetal liver around E11 from the AGM where they emerge slightly earlier (Muller et al., 1994). However, quantification of HSC numbers during foetal liver seeding suggest that in addition to the AGM also yolk sac and placenta contribute to the

foetal liver stem cell pool. These tissues might not be the origin of HSCs but could serve as sites for per-HSC maturation or expansion of definitive HSCs (Kumaravelu et al., 2002). During the following days of embryonic development foetal liver haematopoiesis massively expands with stem cell numbers increasing 30-40 fold between E12 and E16 (Ema and Nakauchi, 2000). Definitive stem cells isolated from the foetal liver differ from their adult counterparts in several ways. They are characterised by their high cell cycle activity, in contrast to quiescent adult HSCs, and express the surface markers CD4, CD34, Mac-1 and AA4.1. Their higher capacity to proliferate also makes them more efficient in repopulation of lethally irradiated recipient mice (Bowie et al., 2007; Jordan et al., 1990; Morrison et al., 1995). At E15 haematopoiesis eventually moves to the bone marrow and foetal liver haematopoiesis declines again (Dzierzak and Speck, 2008).

The results from several studies suggest foetal liver haematopoiesis to be slightly different from the classical model describing its adult counterpart. CLP and CMP are the key progenitors supporting this model of haematopoiesis in the adult. However, attempts to purify corresponding populations from the foetal liver have not been successful. Phenotypic CLPs isolated from E14.5 foetal liver possess besides B and T cell potential also the ability to generate macrophages, potentially due to lower expression of the myeloid-suppressing transcription factor PAX5 (Mebius et al., 2001). Similarly phenotypic CMPs retain residual B cell potential in addition to myeloid/Mk/E potentials, which stands in conflict with the lympho-myeloid bifurcation of the classical model (Traver et al., 2001). Based on the characterisation of foetal liver progenitors in their *in vitro* foetal thymus organ culture system, Katsura and colleagues first proposed the myeloid model of haematopoiesis in the embryo. In their hands foetal liver progenitors always retained GM potential until unilineage differentiation and cells with exclusive B/T restriction were never observed (Kawamoto et al., 1997; Lu et al., 2002). Since more recent findings are also supporting a myeloid based model for adult haematopoiesis, the

pathways of lineage commitment in the two systems might not be as different as the initial results suggested (Kawamoto, 2006; Kawamoto et al., 2010; Luc et al., 2008b).

1.5.5. The emergence of embryonic lymphopoiesis

Erythroid, Mk and myeloid lineages already appear at very early stages of haematopoietic development before the emergence of definitive HSCs and respective progenitors have been identified. Experimental results for the lymphoid lineage are different and lymphoid restricted progenitors have not been identified until E12. Cells isolated at that or later stages harbour, in agreement with the myeloid model, either B, T and myeloid potential or have been shown to be bipotent for B and macrophage potentials (Cumano et al., 1992; Lacaud et al., 1998; Mebius et al., 2001). Thus, these results suggest that lymphoid commitment in the embryo might only emerge downstream of definitive HSCs. However, lymphoid potential has been identified at stages before the emergence of definitive stem cells as early as E9. Immature or pre-HSCs isolated from P-Sp/AGM or yolk sac are multipotent and have the ability to give rise to lymphoid lineages (Godin et al., 1995; Yoder et al., 1997). Furthermore low level expression of the early lymphoid genes encoding for RAG1 and IL-7Rα has been detected at embryonic day 10.5. Cells that expressed a GFP reporter for RAG1 could be detected by flow cytometry, however, they were not isolated or characterised (Kawamoto et al., 2000; Yokota et al., 2006). In a very recent study Yoder and colleagues were able to identify a T lymphoid progenitor in the E9.5 yolk sac. The cells produced T cells when cultured on OP9DL1 stroma and pre-cultured cells can generate mature T cells after transplantation in vivo. Thus, these experiments indicate the existence of lymphoid restricted cells already prior to definitive haematopoiesis (Yoshimoto et al., 2012).

1.6. T cell development

While most haematopoietic cell lineages fully develop in the bone marrow large parts of thymopoiesis take place in the thymus that provides the specific microenvironment required for T cell generation. The thymus itself does not harbour any long term self-renewal potential and thymopoiesis is therefore dependent on regular replenishment by stem cell derived progenitors from the bone marrow (Donskoy and Goldschneider, 1992; Scollay et al., 1986). In the thymus these cells undergo T lineage restriction and T cell receptor rearrangements accompanied by positive and negative selection before they eventually enter the blood stream as naïve T cells (Fig. 1.5). Although later stages of T cell development have been elucidated, thymus seeding and the identity of the thymus seeding progenitor as well as the early T lineage restriction process are yet to be completely understood (von Boehmer et al., 2003).

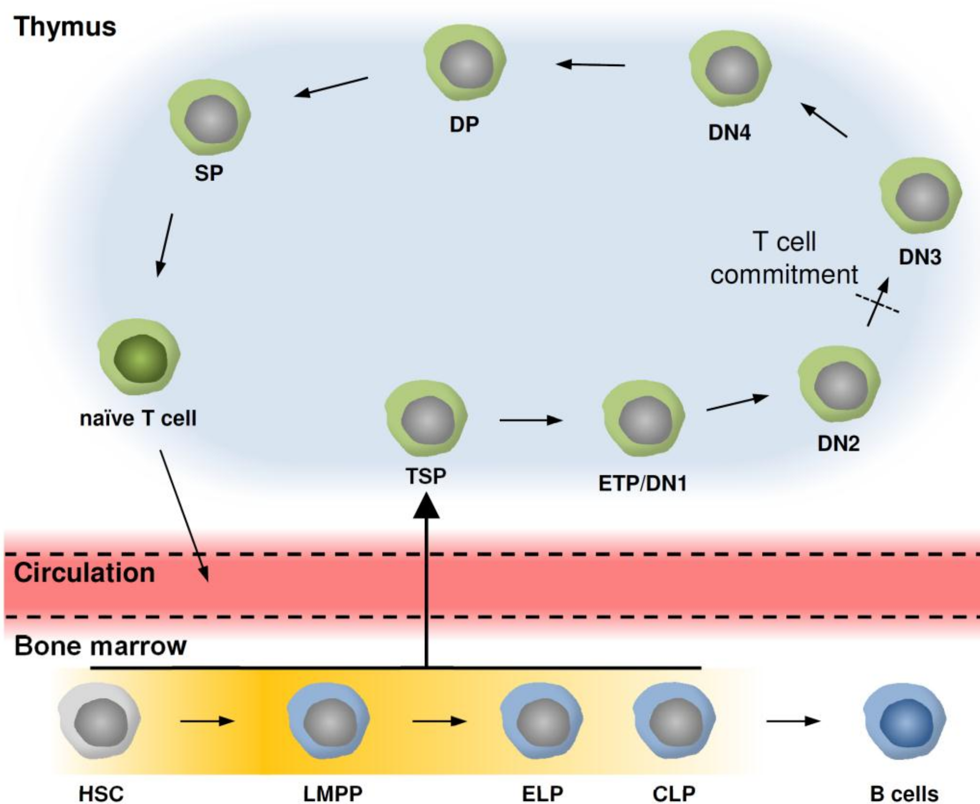


Figure 1.5: Schematic representation of T cell development

Candidate thymus seeding progenitors (TSPs, yellow bar) transfer from the bone marrow via circulation to the thymus. TSP derived early thymic progenitors (ETPs) represent the most primitive cell in the thymus. T cell development progresses via double negative (DN, $CD4^{-}CD8^{-}$) populations, DN1 (overlapping with the ETP, $CD44^{+}CD25^{-}$), DN2 ($CD44^{+}CD25^{+}$), DN3 (first T committed stage, $CD44^{-}CD25^{+}$), DN4 ($CD44^{-}CD25^{-}$) and the double positive (DP, $CD4^{+}CD8^{+}$) population. Eventually single positive (SP, $CD4^{+}$ or $CD8^{+}$) naïve T cells leave the thymus into blood circulation. Haematopoietic stem cell (HSC), lymphoid-primed multipotent progenitor (LMPP, $LSKFlt3^{hi}$), early lymphoid progenitor (ELP, $Lin^{-}CD27^{+}cKit^{hi}Sca1^{hi}$*Rag1*-$GFP^{+}$), common lymphoid progenitor (CLP, $Lin^{-}IL\text{-}7R\alpha^{+}c\text{-}Kit^{lo}Sca\text{-}1^{lo}$). Adapted from Bhandoola *et al.*, 2007.

1.6.1. Early thymic progenitors and adult thymopoiesis

Early progenitor populations in the thymus are characterised by the lack or low level expression of the T cell co-receptors CD4 and CD8. This double negative (DN) subset of thymocytes has been further subdivided by the surface expression of CD25 and CD44 into 4 developmental stages. The DN1 population expresses CD44 and is negative for CD25. The following stages include the sequential up-regulation of CD25 (DN2), down-regulation of CD44 (DN3) and eventually the down-regulation of both markers (DN4) (Godfrey et al., 1993). The change in surface marker expression goes along with progressive maturation and significant expansion of the cells. At DN2 T cell receptor rearrangement is initiated with β, γ as well as δ but not yet α chains and continues through DN3, which is also the stage of definite T lineage commitment (Fehling and vonBoehmer, 1997; ZunigaPflucker and Lenardo, 1996). Proceeding from DN4 thymocytes start the expression of CD4 and CD8. At this double positive (DP) stage the cells express functional T cell receptors and undergo positive selection, which also determines the fate to become an either $CD4^{+}$ or $CD8^{+}$ T cell, followed by negative

selection (Starr et al., 2003). After about 3 weeks thymocytes leave the thymus as naïve T cells into the bloodstream.

The most primitive population of double negative thymocytes (DN1) has been further subdivided based on the expression of c-Kit and the heat stable antigen (HSA) CD24 into 5 subsets termed as DN1a to DN1e, which can all produce T cells but differ in their kinetics of differentiation and proliferative potential (Porritt et al., 2004). The c-Kit positive ETPs, which overlap with the DN1a and DN1b subsets are regarded as the most primitive cells isolated from the thymus, and as such are thought to be directly derived from thymus seeding progenitors (Allman et al., 2003; Porritt et al., 2004; Sambandam et al., 2005). Further purification of the ETP was achieved using Flt3 receptor expression that is present on approximately 10 % of ETPs but completely absent from DN2 cells as well as all downstream progenitors. $Flt3^+$ ETPs are up to 20-fold more proliferative when injected intrathymically and show slower differentiation kinetics in *in vitro* cultures compared to their $Flt3^{lo}$ counterparts (Sambandam et al., 2005). In a separate attempt ETPs were subdivided by CC chemokine receptor 9 (*Ccr9*) reporter labelling. CCR9 plays a role in thymus seeding and is therefore likely expressed on the most primitive ETPs (Zlotoff et al., 2010). Indeed the 20 % of ETPs that expressed high levels of *Ccr9* were most proliferative and gave slower rise to downstream progenitors (Benz and Bleul, 2005).

In addition to strong T cell potential thymocytes have been shown to produce B cells, NK cells, dendritic cells as well as myeloid cells. Mice with defective T cell development caused by the absence of Notch signalling have strongly increased numbers of B cells in the thymus (Radtke et al., 1999; Wilson et al., 2001). The origin of these cells, however, remains controversial since they could be derived from thymic progenitors that commit to the B rather than the T lineage, or B cells might just colonise the empty thymus (Feyerabend et al., 2009). This is supported by the fact that B cells express the

chemokine receptors CCR7 and CCR9 that are involved in attracting cells to the thymus (Bowman et al., 2000; Zlotoff et al., 2010). Similarly, besides their ability to generate T cells ETPs also possess the potential for development of NK cells, dendritic cells and retain some B and myeloid potential (Lu et al., 2005; Matsuzaki et al., 1993). Recent studies showed that the B cell potential is limited to ETPs that express either Flt3 or *Ccr9*. When evaluated *in vitro* 3 % of single *Ccr9*$^+$ ETPs gave rise to colonies containing cells from B as well as T lineage, confirming that they are not just B cell progenitors (Benz and Bleul, 2005). Using a limiting dilution approach B cell potential could be detected *in vivo* at an estimated frequency of about 1 in 500 cells (Sambandam et al., 2005). As the frequencies of B cell potential in both studies are very low and others failed to detect B cell potential at all, this remains a subject of debate (Balciunaite et al., 2005b). The above studies analysed ETPs that were isolated from adult mice at the age of 3 weeks or older. Thymic B cell potential has been shown to be age dependent with highest frequencies at birth and declining thereafter (Ceredig et al., 2007; Montecino-Rodriguez et al., 1996). However, due to the lack of combined lineage potentials from single cells it is unclear whether this reflects higher B cell potential in primitive thymocytes, increased frequencies of such cells or simply the presence of B cell progenitors.

As indicated above, ETPs have been shown to harbour significant myeloid potential. This is not confined to the most primitive *Ccr9*$^+$ ETP fraction but can be found in all Lin$^-$ CD25$^-$c-Kit$^+$ ETPs (Benz and Bleul, 2005). A more detailed analysis of ETPs using an *in vitro* culture system for combined readout of T and myeloid potentials revealed that more than 80 % of single ETPs can give rise to both lineages (Bell and Bhandoola, 2008). This was confirmed by studies of the most primitive double negative subpopulation (DN1) that largely overlaps with ETPs. Single DN1 cells retained significant myeloid potential and gave predominantly rise to macrophages but only low frequencies of granulocytes (Wada et al., 2008). Although at lower frequencies, the myeloid potential is

also maintained in downstream lymphoid progenitors. 13 % of DN2 cells were found to give rise to combined T and myeloid colonies but exclusively generated macrophages and no granulocytes (Bell and Bhandoola, 2008; Wada et al., 2008). The likely overlapping populations of ETPs and DN1 cells that possess combined T and myeloid potentials probably also contain the $Flt3^+$ or $Ccr9^+$ subsets with B lymphoid potential. However, since the frequency of B potential is very low, even within the $Flt3^+$ or $Ccr9^+$ subsets, it was not detected in the total ETP or DN1.

The erythroid and Mk potentials of thymocytes have not been studied in much detail and both potentials were not investigated in the recent ETP studies described above (Bell and Bhandoola, 2008; Benz and Bleul, 2005; Sambandam et al., 2005; Wada et al., 2008). One early study addressed the question by analysing *in vivo* and *in vitro* erythroid potential of primitive $CD4^{lo}$ T cell progenitors. Upon intravenous transplantation these cells did not give rise to any erythroid colonies on the spleen and neither were they able to form erythroid colonies *in vitro*. However, in contrast to several recent studies described above the examined progenitors also failed to generate myeloid cells (Wu et al., 1991). Thus, it remains unclear whether there is in fact no erythroid potential in the thymus or the used assays simply failed to detect it. The evaluation of Mk/E potential in the thymus has particularly important implications for the nature of the as yet unknown thymus seeding progenitor, and presence of both potentials would point to a multipotent or stem cell like cell seeding the thymus.

1.6.2. Thymus seeding progenitors

The thymus does not contain a permanent self-renewing stem cell population and thymopoiesis is maintained by the import of bone marrow derived progenitors through circulation (Donskoy and Goldschneider, 1992). Although several attempts have been made to identify the particular progenitor that is responsible for thymus seeding, and multiple candidates have been proposed, the thymus seeding progenitor (TSP) is yet to

be identified (Bhandoola et al., 2007). All TSP candidates fulfil the important requirements for thymus seeding: significant proliferative potential, the ability to give rise to T cells and presence in the blood while transferring from bone marrow to thymus. However, since progenitors might only be present in the blood for a short period of time and at limited numbers the actual TSP might not have been detected yet. Candidate thymus seeding cells include HSCs, multipotent progenitors, LMPPs and early lymphoid progenitors (ELP, $Lin^{-}CD27^{+}c\text{-}Kit^{hi}Sca\text{-}1^{hi}$*Rag1*-$GFP^{+}$), CLPs and the CLP-2 as well as circulating T cell progenitors (Balciunaite et al., 2005a; Gounari et al., 2002; Igarashi et al., 2002; Martin et al., 2003; Perry et al., 2006; Schwarz and Bhandoola, 2004; Umland et al., 2007; Wright et al., 2001). While ongoing efforts towards identification of the TSP are being made it remains possible that the thymus is seeded by multiple different progenitors from the bone marrow (Bhandoola et al., 2007).

According to the classical model of haematopoiesis the CLP gives rise to unilineage committed T and B cell progenitors and as such would be a candidate for seeding the thymus (Kondo et al., 1997). CLPs were shown to retain significant myeloid potential compatible with myeloid potential of downstream ETPs (Ehrlich et al., 2011). However, in intrathymic transplantations ETPs were more potent than CLPs in giving rise to T cells making the CLP unlikely to be the precursor of ETPs. This is supported when comparing the surface marker phenotypes of both populations. ETPs do express high levels of the early bone marrow progenitor markers c-Kit and Sca-1, which are only present at low levels on CLPs. In mice deficient for the transcription factor Ikaros CLPs were absent from the bone marrow, whereas ETPs could be detected at normal levels suggesting that ETPs develop through a CLP independent pathway (Allman et al., 2003).

More primitive candidate thymus seeding progenitors including HSCs are more potent and therefore potential precursors for ETPs. However, despite the fact that HSCs produce T cell efficiently in *ex vivo* cultures or after intrathymic injections, they fail to

home to the thymus after intravenous injection into non-irradiated recipients. LMPPs on the other hand readily generated double negative as well as double positive T cell progenitors after intravenous transplantation (Schwarz et al., 2007). The chemokine receptors CCR7 and CCR9 that are expressed on LMPPs but not on HSCs have been shown to be important for thymus seeding. Mice deficient for both receptors, that are thought to have at least partly redundant functions, have severe reductions in ETPs and cells deficient for both receptors completely fail to home to the thymus in a competitive setting (Lai and Kondo, 2007; Zlotoff et al., 2010). Furthermore the upregulation of CCR9 has been demonstrated to be dependent on Flt3 signalling, since Flt3 ligand deficient bone marrow progenitors fail to express the CCR9 protein or transcript (Schwarz et al., 2007). Collectively these results point towards a thymus seeding progenitor such as the LMPP that expresses Flt3 as well as CCR7 and CCR9, and in accordance with its proliferative and lineage potentials can function as a precursor of the ETP (Adolfsson et al., 2005; Lai and Kondo, 2006, 2007; Schwarz et al., 2007; Zlotoff et al., 2010). The exact identity of such progenitor, however, remains to be determined.

1.6.3. Regulation of T cell development

The development of unilineage committed T cell progenitors and naïve T cells from multipotent early thymic progenitors is highly regulated stage specific process, that requires the interplay of multiple extrinsic and intrinsic regulators (Rothenberg et al., 2008). Notch signalling in T cell development has been extensively studied and Notch plays a key role in T lineage specification (Radtke et al., 2004). Other important extrinsic regulators include cytokines such as SCF and IL-7, but also Wnt signalling that is specifically required for the later stages of T cell development (Kang and Der, 2004; Massa et al., 2006; Staal and Clevers, 2003). T cell specification is regulated by multiple transcription factors, several of which have important functions in other differentiation programs such as PU.1, Ikaros, E2A, Runx1 and many more. Others including GATA3,

HES1, TCF1 and BCL11B are much more specific for the T cell lineage (Rothenberg, 2012).

1.6.3.1. Notch signalling in T cell specification

The Notch signalling pathway is highly conserved between species, essential for early development and deletions of Notch receptors in mice lead to embryonic lethality (Swiatek et al., 1994). In vertebrates there are 4 Notch receptors (Notch1–4) that bind 5 different ligands (Delta-like (DLL)1, -3, 4 and Jag1 and -2) (Bray, 2006). In the thymus different Notch ligands are expressed in thymus epithelial cells (TEC) as well as the vasculature, with DLL4 expression in TECs being the key determinant for T cell specification (Hozumi et al., 2008; Koch et al., 2008; Thompson and Zuniga-Pflucker, 2011). The important role of Notch in thymopoiesis has been supported by multiple loss and gain of function studies. Conditional deletion of the Notch1 receptor, DLL4 or RBP-Jk, a transcription factor and integral part of canonical Notch signalling, results in the block of T cell differentiation at the DN1 stage. At the same time B cells are accumulating in the thymus (Han et al., 2002; Hozumi et al., 2008; Koch et al., 2008; Radtke et al., 1999; Wilson et al., 2001). Whether this is the result of an altered lineage choice of ETPs or B cells entering from the periphery is a matter of controversy (Feyerabend et al., 2009). When the Notch1 intracellular domain was overexpressed in bone marrow progenitors B cell development was blocked and double positive T cells could be detected in the bone marrow (Pui et al., 1999). These results suggest that Notch signalling is both required and sufficient to instruct T cell commitment in the thymus. In further support Notch ligands have also been shown to promote T cell differentiation of various haematopoietic progenitors *in vitro* (Schmitt and Zuniga-Pflucker, 2002). Although Notch signalling is the key driver of thymopoiesis, once T cell progenitors are past the DN3 stage and are fully committed to the T cell lineage Notch signalling is no longer required (Wolfer et al., 2001).

1.6.3.2. Transcriptional regulation in interplay with Notch

GATA3 is a T lineage specific transcription factor and one of the earliest downstream targets of Notch signalling with important roles from the ETP stage to mature T cell activation (Hendriks et al., 1999; Ting et al., 1996). Studies with mice bearing different GATA3 alterations demonstrated that GATA3 is redundant for LMPPs or CLPs, but ETPs are strongly reduced in its absence (Hosoya et al., 2009). GATA3 regulation is not very well understood, but gain and loss of function studies suggest that T cell development requires a specific dose of GATA3. While at normal levels it promotes T cell specification together with Notch, GATA3 overexpression results in a block at DN2 and drives T cell precursors into the mast cell lineage (Taghon et al., 2007).

The transcription factor HES1 is a direct target of Notch signalling and expressed from ETPs to the DN4 stage (Tan et al., 2005). Since the deletion of HES1 results in embryonic lethality its role in T cell development was recently evaluated in mice using a conditional knockout approach. Similar to Notch ligand and receptor deletions these mice had severe reductions in all T cell progenitor populations including ETPs. However, this did not lead to accumulation of B cells in the thymus as seen for Notch suggesting that the suppression of B cell potential through Notch might be mediated by a HES1 independent pathway. Furthermore HES1 appears to be specifically important for the earliest ETP/DN1 populations but is dispensable for later stages of T cell development (Wendorff et al., 2010).

An additional T lineage transcription factor and target gene of Notch signalling is the transcription factor BCL11B. Unlike HES1 BCL11B is only upregulated when cells progress from ETPs to the DN2 stage (David-Fung et al., 2009). In accordance BCL11B deficient mice show a differentiation block at the DN2/DN3 transition but not at earlier stages. These mice have normal DN2 cellularity, however, when cultured *in vitro* *Bcl11b*$^{-/-}$ DN2 cells continuously divide for more than 4 weeks without further

differentiation. Gene expression analysis revealed that knockout DN2 cells failed to repress several stem and progenitor genes and upregulated NK genes. In contrast to their wild type counterparts *Bcl11b*$^{-/-}$ can generate NK cells and even myeloid cells when cultured in myeloid promoting conditions (Ikawa et al., 2010; Li et al., 2010). These results suggest that BCL11B maintains T cell development and contributes to T cell specification by downregulation of other lineage programs.

Notch signalling is the major driver of T cell commitment and differentiation in the thymus that feeds into a network of multiple downstream transcription factors. GATA3, HES1 and BCL11B are three of those regulators involved in promotion of T cell development at different stages but many more are required during thymopoiesis.

1.6.4. Embryonic thymopoiesis

The embryonic thymus development starts at around E10.5 when the thymus anlage is derived from endodermal cells in the third pharyngeal pouch (Blackburn and Manley, 2004). Although the thymus is not vascularised until around E12.5 colonisation is already initiated at E11 (Mori et al., 2010). Thymus seeding progenitors exit circulation and, attracted through CCR7 and CCR9 signalling, migrate through the mesenchymal tissue to the thymus anlage (Liu et al., 2006; Liu et al., 2005). The first mature T cells emerge from the embryonic thymus at E14 and express the γδ-T cell receptor, whereas significant numbers of αβ-T cells cannot be detected before E19 (Snodgrass et al., 1985a; Snodgrass et al., 1985b). Although attempts have been made to isolate and characterise the earliest cells in the thymus or the seeding progenitors, due to methodological constrains and the infrequency of these cells, it has been difficult to prove their identity. It was suggested that early progenitors with DN1 phenotype isolated from E12 thymi were able to generate T, B and myeloid cells (Hattori et al., 1996). Another study that detected B, T and myeloid potential in thymi of E12 embryos suggested that the embryonic thymus might at least partly be seeded by HSCs (Peault

et al., 1994). Single cell analysis, however, revealed that most cells were T lineage committed and some cells gave rise to B or myeloid cells, whereas no multipotent or stem cells that could produce more than one lineage were detected (Kawamoto et al., 1998). Through later analysis it was shown that the thymus seeding cells with high T cell potential also retain NK as well as dendritic cell potential (Masuda et al., 2005).

1.7. Aims of the present work

The alternative roadmap describes the bifurcation of lympho-myeloid and myeloid-Mk-E lineages as first haematopoietic commitment event. However, several aspects of this lineage restriction process remain poorly understood. Therefore the studies described in this thesis aimed to further investigate the early lympho-myeloid pathway during embryonic development (Chapter 2) as well as adult thymopoiesis (Chapter 3), to explore early megakaryopoiesis as part of the myeloid-Mk-E pathway (Chapter 4) and to evaluate the initiation of this lineage specification in HSCs (Chapter 5).

2. Lympho-myeloid progenitors emerge prior to definitive haematopoietic stem cells

2.1. Introduction

The classical haematopoietic road map describes the bifurcation of myeloid/Mk/E and lymphoid pathways as the earliest lineage commitment event and has been supported by the isolation of common myeloid as well as common lymphoid progenitors (CMP, CLP) (Akashi et al., 2000; Kondo et al., 1997). Experimental evidence challenging this classical model was first obtained from studies of embryonic haematopoiesis that reported the detection of B/myeloid and T/myeloid restricted progenitors but never observed B/T or lymphoid restricted cells. This suggested the existence of a common lympho/myeloid progenitor rather than the CLP, and led to the proposal of the myeloid based model of lineage commitment. According to this model all progenitors maintain myeloid lineage potential up to the bipotent stage and the earliest lineage commitment event results in the generation of a CMP as well as a common myeloid/lymphoid progenitor (CMLP) (Kawamoto et al., 1998; Kawamoto et al., 2010; Lu et al., 2002). This lympho-myeloid progenitor has now been prospectively isolated in adult haematopoiesis and been characterised at the single cell level (Adolfsson et al., 2005). These LMPPs give rise to lymphoid as well as myeloid cells after transplantation *in vivo* and *in vitro* culture but have lost the potential to generate Mk/E lineages. Although, additional purification and detailed molecular characterisation confirmed the lympho-myeloid identity of the LMPP (Luc et al., 2008a; Mansson et al., 2007), several other reports claim it to possess significant *in vivo* Mk/E potential (Boyer et al., 2011; Forsberg et al., 2006). Therefore the existence of a lympho-myeloid pathway instead of or in addition to

the CLP dependent purely lymphoid pathway of the classical model remains disputed. Moreover, the classical as well as alternative haematopoietic road maps are largely based on the lineage potentials of haematopoietic progenitors that have been characterised *in vitro* or in non-physiological transplantation settings with irradiated recipients *in vivo*. The thereby detected lineage potentials might not resemble the physiological fate choice of the respective progenitor during homeostasis. The physiological relevance of the lympho-myeloid pathway was recently challenged by *in vivo* fate mapping studies using an *Il7ra* reporter line. While the majority of thymic T cells were labelled and had therefore developed through an $Il7ra^+$ lymphoid pathway, thymic myeloid cells remained largely unlabelled indicating their development through an *Il7ra* independent route (Schlenner et al., 2010; Schlenner and Rodewald, 2010). Thus, although these results are compatible with a lympho-myeloid pathway that branches before the initiation of *Il7ra* expression, its physiological relevance has yet to be established.

Foetal haematopoiesis emerges in two distinct waves. The primitive wave that comprises mainly erythroid cells, but also myeloid and Mk lineages emerges from the yolk sac at around embryonic day 7 (Medvinsky et al., 2011; Palis et al., 1999). Definitive stem cells have first been isolated from the AGM region at day 10.5 (Medvinsky and Dzierzak, 1996). Other studies point to the yolk sac as site of generation for definitive HSCs (Yoder et al., 1997). However, since circulation has already been established at this point of development the site of HSC emergence has not yet been conclusively established and definitive stem cells might even be generated at multiple sites (Delassus and Cumano, 1996; Kumaravelu et al., 2002). The foetal liver, which does not contribute to de novo HSC production, is colonized at day 11 and becomes the main site of embryonic haematopoiesis until it eventually moves to the bone marrow (Ema and Nakauchi, 2000; Muller et al., 1994). While myeloid, Mk and erythroid cells appear already early during development, lymphoid progenitors are thought to be derived from

definitive stem cells. The first progenitors with lymphoid potential have been isolated around embryonic day 12 and, in agreement with the myeloid model, possessed the combination of lymphoid and myeloid potentials (Cumano et al., 1992; Lacaud et al., 1998; Mebius et al., 2001). While in the studies by Mebius et al. progenitors were only investigated at the population level, Cumano, Lacaud and colleagues reported single cell derived colonies with lymphoid and macrophage content. However, lymphoid potential has been detected in the embryo at much earlier stages prior to the emergence of definitive stem cells. Several lines of data also suggest the existence of immature or pre-HSC, that are not able to reconstitute irradiated adult recipients, but possess all including lymphoid lineage potentials (Bertrand et al., 2005; Cumano et al., 1996; Cumano et al., 2001; Godin et al., 1995; Kieusseian et al., 2012). Further evidence for the early emergence of lymphoid potential comes from gene expression analysis that detected the low level expression of the key lymphoid regulators *Il7ra* and *Rag1* already at the time of definitive HSC emergence (Kawamoto et al., 2000; Mombaerts et al., 1992; Peschon et al., 1994a; Yokota et al., 2006). Although this could be the result of multilineage gene priming in multipotent cells, in adult haematopoiesis priming for these two genes does not initiate in HSCs but rather in the first lympho-myeloid restricted progenitors (Adolfsson et al., 2005; Hu et al., 1997; Ng et al., 2009).

In this study a lympho-myeloid progenitor is identified in the foetal liver at day E11.5 and traced back to the E10.5 foetal liver as well as E9.5 yolk sac prior to the emergence of first definitive HSCs. Moreover, using *Rag1*-Cre fate mapping this study demonstrates that embryonic lympho-myeloid progenitors contribute significantly to steady state myelopoiesis in the embryo.

2.2. Results

2.2.1. IL-7Rα and Flt3 expressing E11.5 foetal liver progenitors possess lympho-myeloid but not Mk/E lineage potentials

The upregulation of Flt3 receptor expression marks the earliest lympho-myeloid restriction in the adult bone marrow (Adolfsson et al., 2005). On the other hand IL-7Rα is expressed on lymphoid restricted adult as well as embryonic progenitors and has been detected, although at low levels, during early haematopoiesis in the embryo (Kawamoto et al., 2000; Peschon et al., 1994a). High expression of Flt3 receptor and IL-7Rα were detected in the Lin$^-$CD45$^+$c-Kit$^+$ fraction of E11.5 foetal liver and Flt3$^+$IL-7Rα$^+$ progenitors represented 0.35 % of total foetal liver mononuclear cells (Fig. 2.1A). Analysis of FACS purified Lin$^-$CD45$^+$c-Kit$^+$Flt3$^+$IL-7Rα$^+$ foetal liver cells in single cell culture assays revealed significant granulocyte/monocyte potential, while Mk or erythroid potentials were not detected (Fig. 2.1B). This was confirmed at the clonal level through cultures in semisolid medium, an assay known to efficiently detect Mk or erythroid lineage potentials *in vitro* (Fig. 2.1C). The erythroid potential was further evaluated on erythroid promoting OP9 co-cultures. Whereas significant erythroid potential was detected in the heterogeneous population of Lin$^-$CD45$^+$c-Kit$^+$Flt3$^-$IL-7Rα$^-$ foetal liver progenitors, the Flt3$^+$IL-7Rα$^+$ fraction was largely unable to produce erythroid colonies (Fig. 2.1D). In addition to their myeloid potential Lin$^-$CD45$^+$c-Kit$^+$Flt3$^+$IL-7Rα$^+$ foetal liver progenitors sustained strong lymphoid potential. In line with the important regulatory function of Flt3 and IL-7 signalling for the development of lymphoid lineages around 40 % of these cells generated B or T lymphoid cells on OP9 or OP9DL1 stroma co-cultures *in vitro* (Fig. 2.1E,F) (Peschon et al., 1994a; Sitnicka et al., 2003; Sitnicka et al., 2002; Sitnicka et al., 2007).

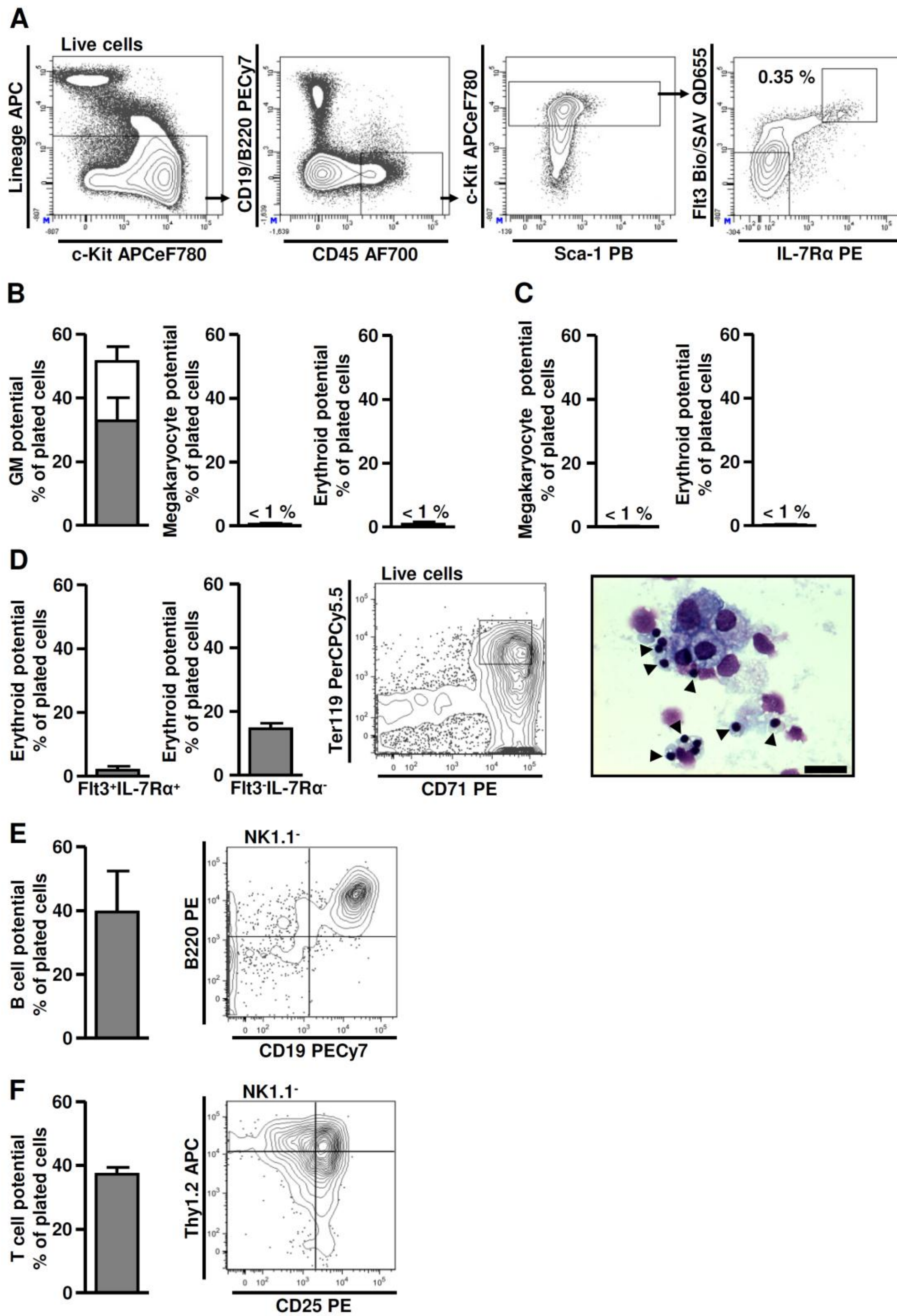


Figure 2.1: Early foetal liver progenitors that express Flt3 and IL-7Rα have lympho-myeloid restricted lineage potentials

(A) FACS purification of Lin$^-$CD45$^+$c-Kit$^+$Flt3$^+$IL-7Rα$^+$ E11.5 FL progenitors. Mean percentage of total FL cells, n=3 experiments.

(B) GM, Mk and erythroid lineage potentials in liquid culture of single Lin$^-$CD45$^+$c-Kit$^+$Flt3$^+$IL-7Rα$^+$ E11.5 FL progenitors in 60 well terasaki plates. Total colonies (white bars), colonies covering ≥ 10 % of well (grey bars). Mean percentages ± SEM, 450 – 480 cells per assay, n=4 experiments.

(C) Mk and erythroid potentials of Lin$^-$CD45$^+$c-Kit$^+$Flt3$^+$IL-7Rα$^+$ E11.5 FL progenitors in semisolid medium. Mean percentages ± SEM, 600 cells analysed per assay, n=3 experiments.

(D) Erythroid potential of Lin$^-$CD45$^+$c-Kit$^+$Flt3$^+$IL-7Rα$^+$ E11.5 FL progenitors in OP9 stromal co-cultures under conditions promoting erythroid development compared to a Lin$^-$CD45$^+$c-Kit$^+$Flt3$^-$IL-7Rα$^-$ heterogeneous control population (positive clones were Ter119$^+$CD71$^+$ by FACS and/or displayed erythroid morphology after May-Grunwald Giemsa stain). Representative FACS profile (middle panel) and morphology (right panel, examples of erythroid cells indicated by arrows, scale bar 25 μm) of a clone positive for erythroid cells. Mean percentages ± SEM, 2 cells/well, total 150 cells analysed, n=3 experiments.

(E) B cell potential of Lin$^-$CD45$^+$c-Kit$^+$Flt3$^+$IL-7Rα$^+$ E11.5 FL progenitors evaluated by OP9 stromal co-culture. Representative FACS plot of a NK1.1$^-$B220$^+$CD19$^+$ B cell clone. Mean percentages ± SEM, > 70 cells analysed, n=2 experiments.

(F) T cell potential of Lin$^-$CD45$^+$c-Kit$^+$Flt3$^+$IL-7Rα$^+$ E11.5 FL progenitors evaluated by OP9DL1 stromal co-culture. Representative FACS plot of a NK1.1$^-$Thy1.2$^+$CD25$^+$ T cell clone. Mean percentages ± SEM, > 70 cells analysed, n=2 experiments.

Single lineage potential analysis clearly established that the Lin$^-$CD45$^+$c-Kit$^+$Flt3$^+$IL-7Rα$^+$ population of foetal liver progenitors is restricted to the lympho-myeloid lineage. However, whether these cells possess the combination of B, T and myeloid potentials remained elusive. This was evaluated by gene expression analysis of key lymphoid, myeloid and Mk/E genes. As expected the expression of *Flt3* as well as *Il7ra* transcripts in Flt3$^+$IL-7Rα$^+$ progenitors largely resembled their surface marker phenotype. The

majority of cells also expressed additional lymphoid genes (*Rag1*, *sIgH*) as well as myeloid genes (*Csf3r*, *Mpo*), whereas genes of the Mk and erythroid lineages (*Vwf*, *Epor*, *Gata1*) were expressed very infrequently (Fig. 2.2A). Further analysis revealed that more than 80 % of $Lin^-CD45^+c\text{-}Kit^+Flt3^+IL\text{-}7R\alpha^+$ cells expressed a combined lympho-myeloid signature, whereas the expression of Mk/E genes was confined to a small fraction of cells with multipotent characteristics (Fig. 2.2B). In order to establish the combined lympho-myeloid potential at the functional level, $Lin^-CD45^+c\text{-}Kit^+Flt3^+IL\text{-}7R\alpha^+$ were analysed using the OP9/OP9DL1 switch assay as described previously (Fig. 2.2C) (Mansson et al., 2007). Single cell derived clones were analysed by flow cytometry for surface marker expression of CD19 (B cells), Thy1.2/CD25 (T cells) and Mac-1/Gr-1 (myeloid cells). While the output for all lineages was reduced compared to the single potential assays, out of the single $Flt3^+IL\text{-}7R\alpha^+$ progenitors that gave rise to any lineage positive haematopoietic clones more than 40 % generated the combination of B, T and myeloid cells (Fig. 2.2D, E). Thus, lympho-myeloid restricted progenitors, resembling the adult LMPP, are already present in the foetal liver at E11.5 short after colonisation by the first definitive stem cells.

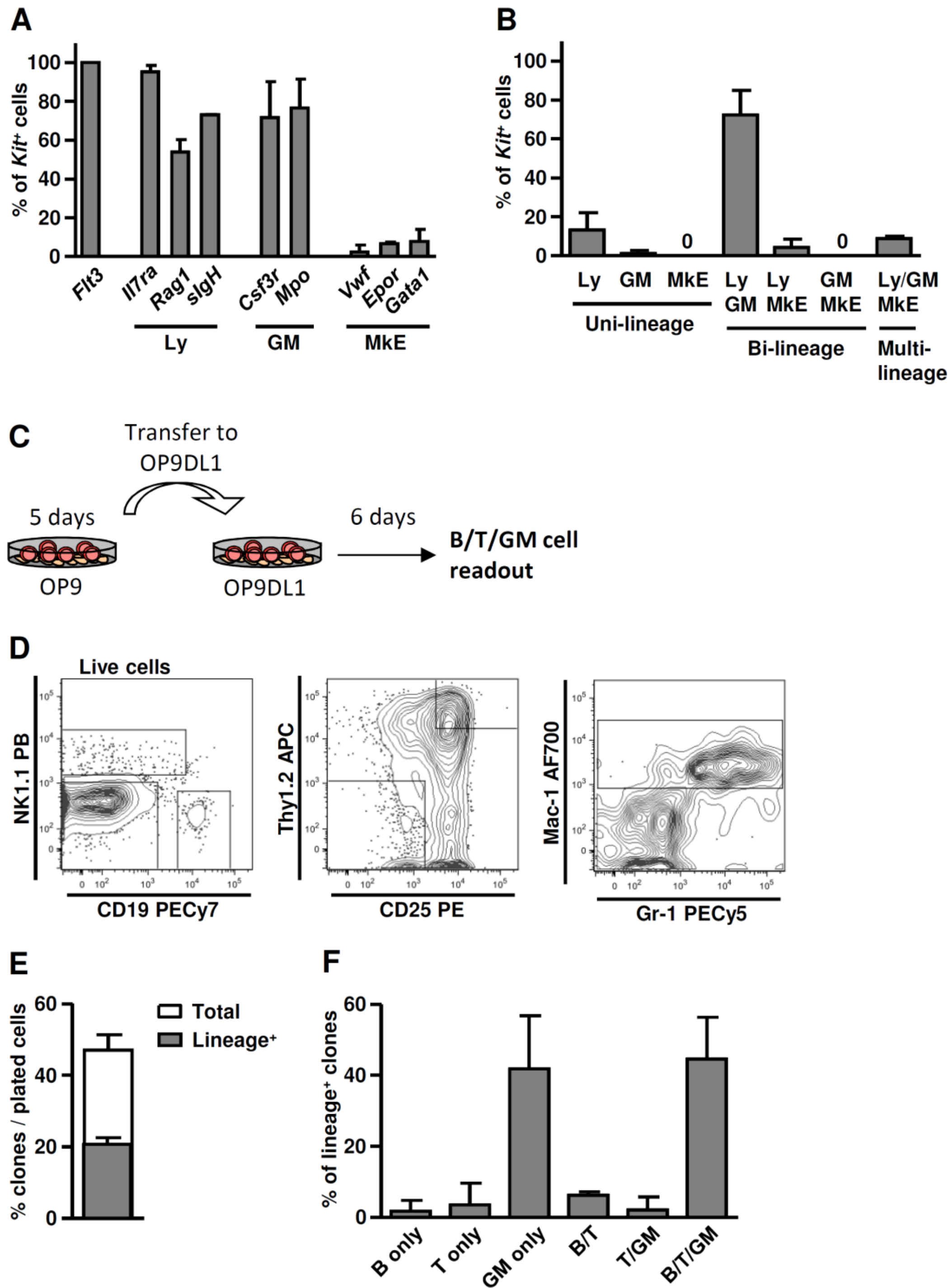


Figure 2.2: Single Lin$^-$CD45$^+$c-Kit$^+$Flt3$^+$IL-7Rα$^+$ E11.5 FL progenitors possess combined lympho-myeloid lineage potentials and gene expression signatures

(A) RT-PCR analysis of single Lin$^-$CD45$^+$c-Kit$^+$Flt3$^+$IL-7Rα$^+$ E11.5 FL cells and (B) combined lineage transcriptional priming patterns based on (A). Mean percentages ± SEM of total *Kit*$^+$ cells (> 93 %), 132 cells analysed, n=2 experiments.

(C) Experimental strategy for the OP9/OP9DL1 switch assay to establish combined lympho-myeloid lineage potentials.

(D) Representative FACS readout of a clone positive for B cell ($CD19^+$), T cell ($Thy1.2^+CD25^+$) as well as GM ($Mac\text{-}1^+$) lineages.

(E) Cloning frequency (white bar) and clones scored as positive for B cell, T cell and/or myeloid lineages. Mean percentages ± SEM, 237 cells analysed, n=3 experiments.

(F) Lineage composition of clones derived from single $Lin^-CD45^+c\text{-}Kit^+Flt3^+IL\text{-}7R\alpha^+$ E11.5 FL progenitors. Mean percentages ± SEM, 237 cells analysed, n=3 experiments.

2.2.2. Lympho-myeloid restricted progenitors emerge before the first definitive HSCs

Previously lymphoid progenitors were detected only after the emergence of definitive HSCs suggesting their stem cell origin and affiliation with definitive haematopoiesis. The herein identified lympho-myeloid progenitors were present in the E11.5 foetal liver. At this time the whole embryo has been suggested to contain only about 3 definitive stem cells, which are possibly but unlikely the origin of $Lin^-CD45^+c\text{-}Kit^+Flt3^+IL\text{-}7R\alpha^+$ lympho-myeloid progenitors. One day earlier in development at E10.5 the entire embryo is thought to contain less than one definitive stem cell (Kumaravelu et al., 2002). Accordingly, while significant long term reconstitution potential and therefore definitive HSCs were detected in E12.5 foetal liver, no long term reconstitution was achieved when whole foetal liver cells from E10.5 or E11.5 embryos were transplanted in the present study (Fig. 2.3).

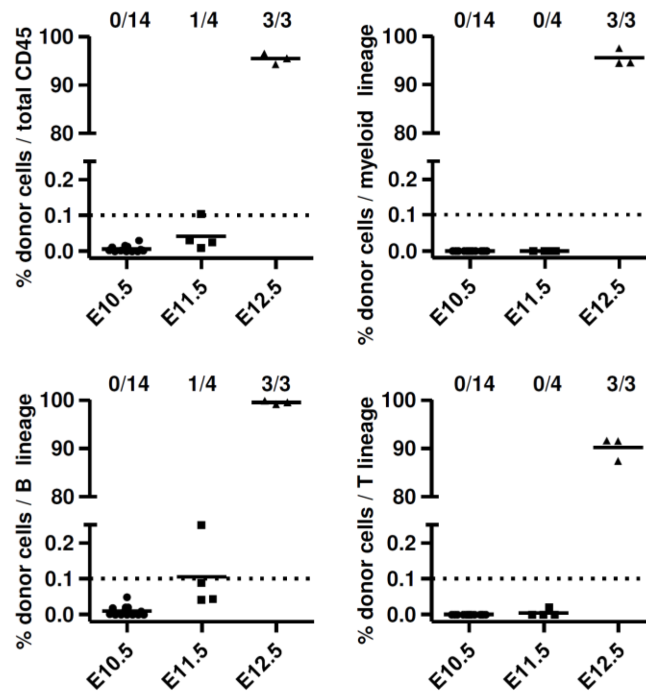


Figure 2.3: Long term repopulation definitive HSCs are not detected in the foetal liver before E12.5

Unfractionated FL cells from E10.5 (1 total FL/recipient), E11.5 (4 total FLs/recipient) and E12.5 (3-5 total FLs/recipient) embryos were transplanted intravenously into lethally irradiated adult wild type recipients together with 250,000 congenic adult support bone marrow cells. Peripheral blood reconstitution was evaluated at 16 weeks post transplantation. Donor reconstitution out of total $CD45^{+}$ cells or the indicated lineages. Dotted line – detection background set at 0.1 % donor reconstitution. Numbers indicate frequencies of mice reconstituted.

To further evaluate the potential pre-definitive emergence of lympho-myeloid progenitors, foetal liver haematopoiesis was investigated at E10.5. To more efficiently purify these progenitors at this early stage a *Rag1*-GFP reporter line was applied (Kuwata et al., 1999). Direct comparison confirmed that $Lin^{-}CD45^{+}c\text{-}Kit^{+}Flt3^{+}IL\text{-}7R\alpha^{+}$ progenitors have highly overlapping expression patterns for Flt3 and *Rag1*-GFP with more than 90 % of cells being positive for both markers (Fig. 2.4).

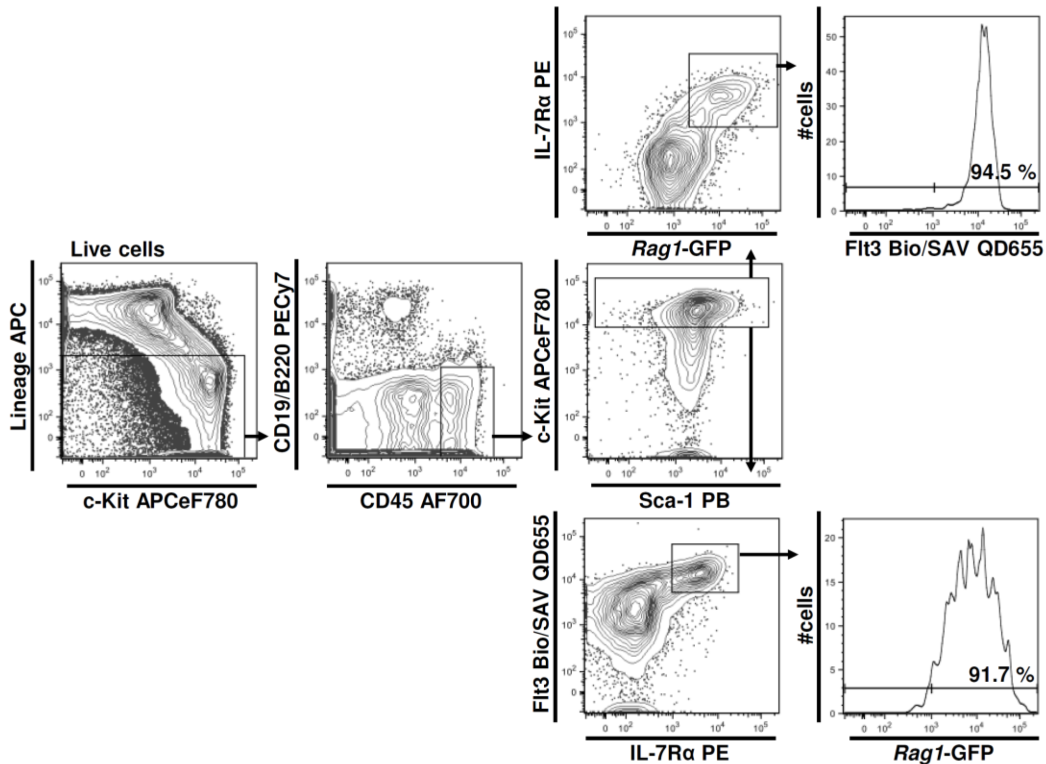


Figure 2.4: Overlapping expression of Flt3, IL-7Rα and *Rag1*-GFP in foetal liver progenitors

Flow cytometry analysis of the expression patterns of Flt3, IL-7R and *Rag1*-GFP in Lin^- $CD45^+$c-Kit^+ E11.5 FL progenitors. Mean percentages out of parent population, total 12 embryos investigated.

Lin^-CD45^+c-Kit^+IL-7Rα^+*Rag1*$^+$ represented 0.03 % of total E10.5 foetal liver cells (Fig. 2.5A). Detailed characterisation of their lineage potentials *in vitro* revealed the lympho-myeloid restricted identity of IL-7Rα^+*Rag1*$^+$ progenitors, comparable to their $Flt3^+$IL-7Rα^+ counterparts in the E11.5 foetal liver (Fig. 2.5B-G). More than 50 % of single Lin^-CD45^+c-Kit^+IL-7Rα^+*Rag1*$^+$ cells robustly produced myeloid cells in liquid cultures (Fig. 2.5B). Morphology analysis confirmed the derived clones to be of macrophage as well as granulocyte identity (Fig. 2.5C). Moreover, IL-7Rα^+*Rag1*$^+$ progenitors completely lacked the ability to produce any Mks in liquid cultures or erythroid colonies when cultured in semisolid medium (Fig. 2.5D, E), while they sustained B as well as T potentials as

revealed by OP9/OP9DL1 stroma co-cultures and flow cytometry (Fig. 2.5F, G). Thus, lympho-myeloid restricted progenitors are already present at the time of stem cell emergence and therefore develop independently of definitive HSCs.

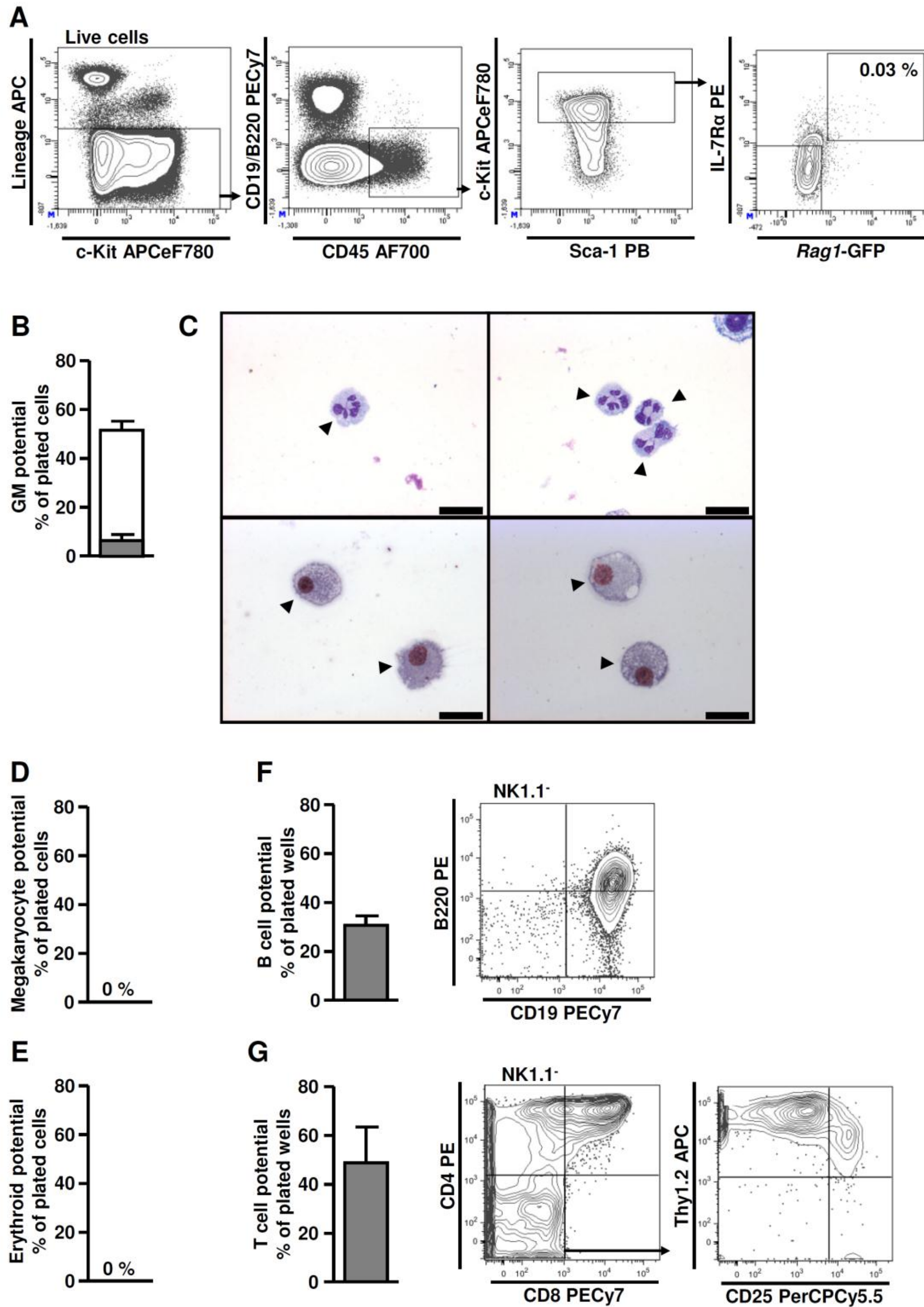


Figure 2.5: Lympho-myeloid restricted progenitors emerge before the first definitive haematopoietic stem cells

(A) Sort profiles for the purification of $Lin^{-}CD45^{+}c\text{-}Kit^{+}IL\text{-}7R\alpha^{+}$*Rag1*$^{+}$ progenitors from E10.5 (30-38 somite pairs) FL of a *Rag1*-GFP reporter strain. Mean percentage of total FL cells, n=5 experiments.

(B) GM lineage potential in liquid culture of single $Lin^{-}CD45^{+}c\text{-}Kit^{+}IL\text{-}7R\alpha^{+}$*Rag1*$^{+}$ E10.5 FL progenitors in 60 well terasaki plates. Total colonies (white bar), colonies covering ≥ 10 % of well (grey bar). Mean percentages ± SEM, total 198 cells analysed, n=5 experiments.

(C) Morphology analysis of myeloid cells derived from $Lin^{-}CD45^{+}c\text{-}Kit^{+}IL\text{-}7R\alpha^{+}$*Rag1*$^{+}$ E10.5 FL progenitors. Representative granulocytes (upper panel) and macrophages (lower panel) as indicated by arrows, scale bar 25 µm.

(D) Mk lineage potential of single $Lin^{-}CD45^{+}c\text{-}Kit^{+}IL\text{-}7R\alpha^{+}$*Rag1*$^{+}$ E10.5 FL progenitors in liquid culture in 60 well terasaki plates. Mean percentage, total 154 cells analysed, n=3 experiments.

(E) Erythroid potential of single $Lin^{-}CD45^{+}c\text{-}Kit^{+}IL\text{-}7R\alpha^{+}$*Rag1*$^{+}$ E10.5 FL progenitors in semisolid medium. Mean percentage, total 212 cells analysed, n=3 experiments.

(F) B cell potential of $Lin^{-}CD45^{+}c\text{-}Kit^{+}IL\text{-}7R\alpha^{+}$*Rag1*$^{+}$ E10.5 FL progenitors in OP9 stromal co-culture. Representative FACS plot of a $NK1.1^{-}B220^{+}CD19^{+}$ B cell clone. Mean percentages ± SEM, total 224 cells analysed, 4 cells/well, n=3 experiments.

(G) T cell potential of $Lin^{-}CD45^{+}c\text{-}Kit^{+}IL\text{-}7R\alpha^{+}$*Rag1*$^{+}$ E10.5 FL progenitors in OP9DL1 stromal co-culture. Representative FACS plot of a $NK1.1^{-}CD4^{+}CD8^{+}Thy1.2^{+}CD25^{+}$ clone (positive clones expressed at least CD4, CD8 or Thy1.2 and CD25). Mean percentages ± SEM, total 220 cells analysed, 4 cells/well, n=3 experiments.

The foetal liver does not de novo generate haematopoietic cells and lacks haematopoietic activity until it is first colonized by progenitors derived from other sites at embryonic day 9 (Delassus and Cumano, 1996; Houssaint, 1981; Kumaravelu et al.,

2002). Amongst other tissues the yolk sac gives rise to haematopoietic cells and has been described as site for the emergence of early primitive haematopoiesis (Palis and Yoder, 2001). Therefore it was explored whether lympho-myeloid progenitors can be found in the yolk sac as early as embryonic day 9.5. Already at this stage $Lin^-CD45^+c\text{-}Kit^+IL\text{-}7R\alpha^+$*Rag1*$^+$ progenitors were found to constitute 0.02 % of total yolk sac cells and expressed IL-7Rα as well as *Rag1*-GFP at levels comparable to the E10.5 foetal liver (Fig. 2.6A). Investigation of transcriptional priming demonstrated that these cells expressed lymphoid (*Flt3*, *Il7ra*, *sIgH*) and myeloid (*Csf3r*, *Mpo*) genes at high frequencies, whereas Mk/E (*Vwf*, *Epor*, *Gata1*) genes were largely absent. Notably, close to 80 % of single E9.5 yolk sac progenitors presented a solely lympho-myeloid gene signature, suggesting their restriction to these lineages (Fig. 2.6B, C). Flow cytometric analysis of whole concepti showed the complete absence of IL-7Rα as well as *Rag1*-GFP expression at E8.5, which is in agreement with the first detection of lymphoid potential at E9 as previously reported (Fig. 2.6D) (Godin et al., 1995; Yoder et al., 1997). Thus, $Lin^-CD45^+c\text{-}Kit^+IL\text{-}7R\alpha^+$*Rag1*$^+$ lympho-myeloid restricted progenitors emerge after embryonic day 8.5 and can be first found in the yolk sac. However, since circulation has already been established at this point and haematopoietic progenitors can freely travel through the embryo their site of origin remains elusive.

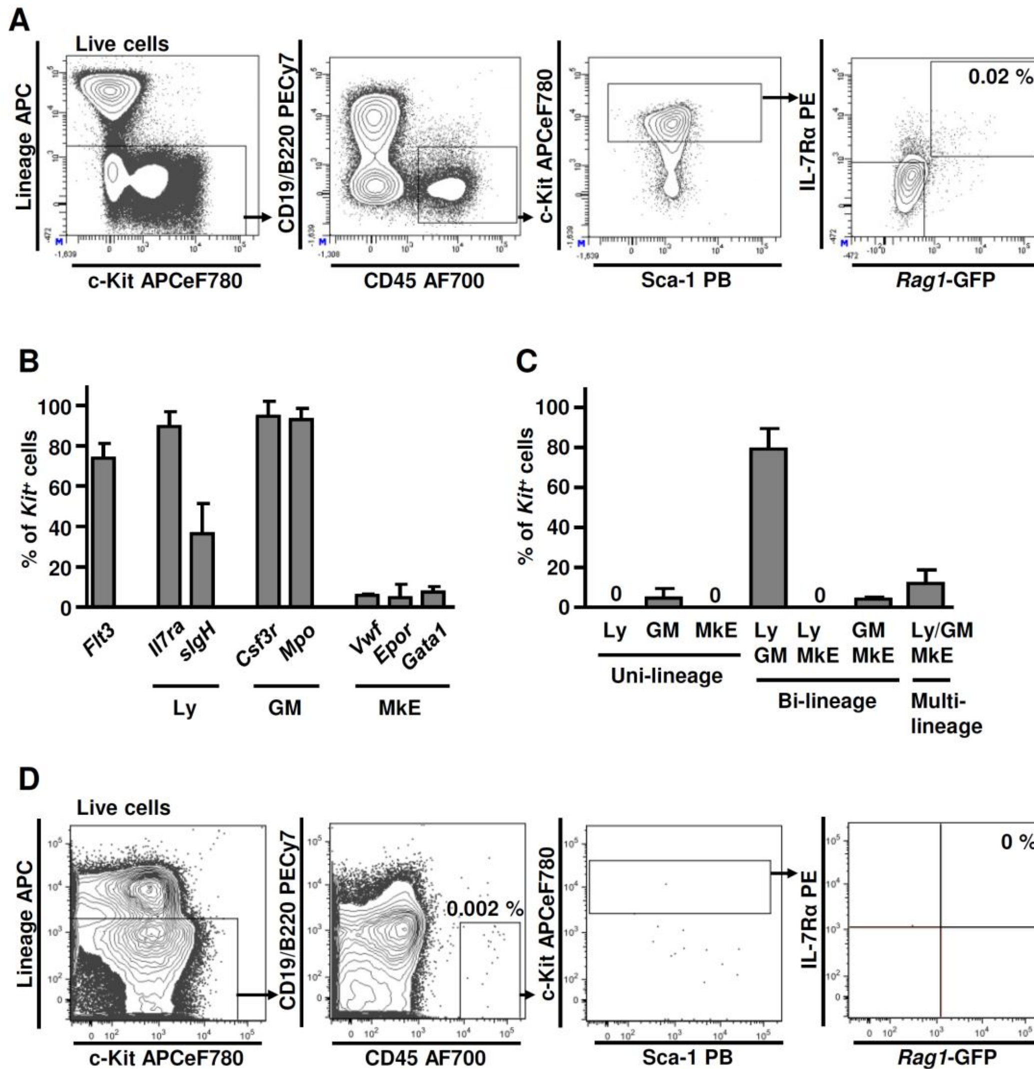


Figure 2.6: Progenitors with lympho-myeloid transcriptional priming can be found already at E9.5 in the yolk sac

(A) FACS purification of Lin$^-$CD45$^+$c-Kit$^+$IL-7Rα$^+$*Rag1*$^+$ progenitors from E9.5 (15–26 somite pairs) YS of a *Rag1*-GFP reporter strain. Mean percentages of total cells, n=4 experiments.

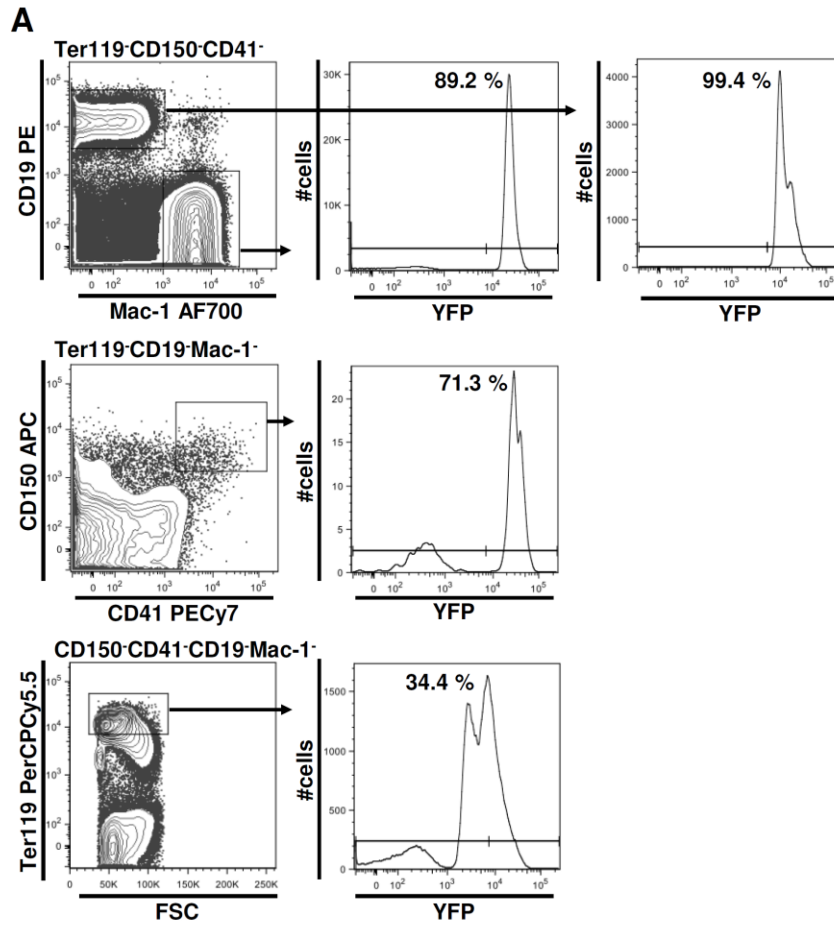
(B) Single cell RT-PCR analysis of Lin$^-$CD45$^+$c-Kit$^+$IL-7Rα$^+$*Rag1*$^+$ E9.5 YS progenitors and (C) combined lineage transcriptional priming patterns based on (A). Mean percentages ± SEM of total *Kit*$^+$ cells (> 91 %), total 53 cells analysed, n=2 experiments.

(D) Analysis of IL-7Rα and *Rag1*-GFP expression in E8.5 (4-11 somite pairs) conceptus. Mean percentage of total cells, n=2 experiments.

2.2.3. Lympho-myeloid restricted *Rag1*$^+$ progenitors contribute to embryonic lympho- as well as myelopoiesis *in vivo*

Current haematopoietic roadmaps have largely been established based on the characterisation of lineage potentials *in vitro* or the ability of *in vivo* repopulation. However, the thereby observed lineage potentials might not necessarily reflect the physiological lineage contribution of the respective progenitors during homeostasis *in vivo*. In fact several recent studies suggest that the *in vitro* lineage potentials of multiple progenitors do not match their physiological lineage output. In particular this has been demonstrated for lympho-myeloid restricted *Rag1*-GFP positive LMPPs in the adult, that although sustaining myeloid potential *in vitro* almost exclusively generate lymphoid cells in steady state haematopoiesis, as well as *Flt3* and *Il7ra* expressing CLPs, that can give rise to some myeloid cells in culture, but only contribute to lymphopoiesis *in vivo* (Ehrlich et al., 2011; Luc et al., 2008a; Schlenner et al., 2010; Schlenner and Rodewald, 2010). Thus, while the herein identified embryonic progenitors have the ability to give rise to lymphoid as well as myeloid but not Mk/E lineages *in vitro*, it remained elusive whether they have the same role during homeostasis. Therefore *in vivo Rag1*-Cre fate mapping was applied to establish the physiological lineage contribution of *Rag1*$^+$ embryonic progenitors. With this system all *Rag1*-Cre expressing cells as well as their downstream progeny are labelled by the expression of yellow fluorescent protein (YFP). Initially *Flt3*-Cre fate mapping was applied to validate the efficient labelling of all haematopoietic lineages in the embryo. As previously reported for the adult, *Flt3* expression was initiated in multipotent progenitors and YFP-labelled mature lineages include B lymphoid, myeloid, Mk and erythroid cells (Fig. 2.7A) (Buza-Vidas et al., 2011). When analysing fluorescent reporter lines by flow cytometry, transfer of reporter protein during the preparation cell preparation can lead to unspecific cell labelling. To account for such background labelling CD45.2$^+$ YFP labelled test cells were processed and analysed together with CD45.1$^+$ wild type bone marrow cells, of which up to 5.8 % adsorbed YFP protein, and hence misleadingly appeared as YFP expressing cells (Fig. 2.7B). The

evaluation of adult bone marrow as well as E11.5 and E14.5 foetal liver cells by *Rag1*-Cre fate mapping demonstrated that nearly all mature B cells were derived from *Rag1*$^{+}$ positive progenitors. In agreement with the lympho-myeloid progenitor studies *in vitro* *Rag1*$^{+}$ foetal liver progenitors did not give rise to any Mk or erythroid cells. However, while adult Mac-1^{+} myeloid cells remained largely unlabeled, during embryonic haematopoiesis as much as 36 % of E11.5 and 16 % of E14.5 Mac-1^{+} foetal liver cells developed through a *Rag1* positive stage (Fig. 2.7C, D, E). Interestingly, the contribution of *Rag1*$^{+}$ progenitors to the myeloid lineage successively declined with age (Fig. 2.7F). Since Mac-1 expression in the foetal liver is not completely restricted to cells of the myeloid lineage, the myeloid identity of Mac-1^{+} cells was confirmed by FACS purification and morphology analysis. YFP labelled Mac-1^{+} E14.5 foetal liver cells contained almost exclusively myeloid cells (Fig. 2.7G, H, I). When applying *Rag1*-Cre fate mapping for the evaluation of *in vitro* colony forming potentials, *Rag1*$^{+}$ progenitors accounted for almost 20 % of GM colony forming units in E14.5 foetal liver. Moreover, while foetal liver progenitors readily gave rise to erythroid colonies, they appeared to be YFP negative, confirming that *Rag1*$^{+}$ progenitors do not contribute to erythropoiesis (Fig. 2.7J).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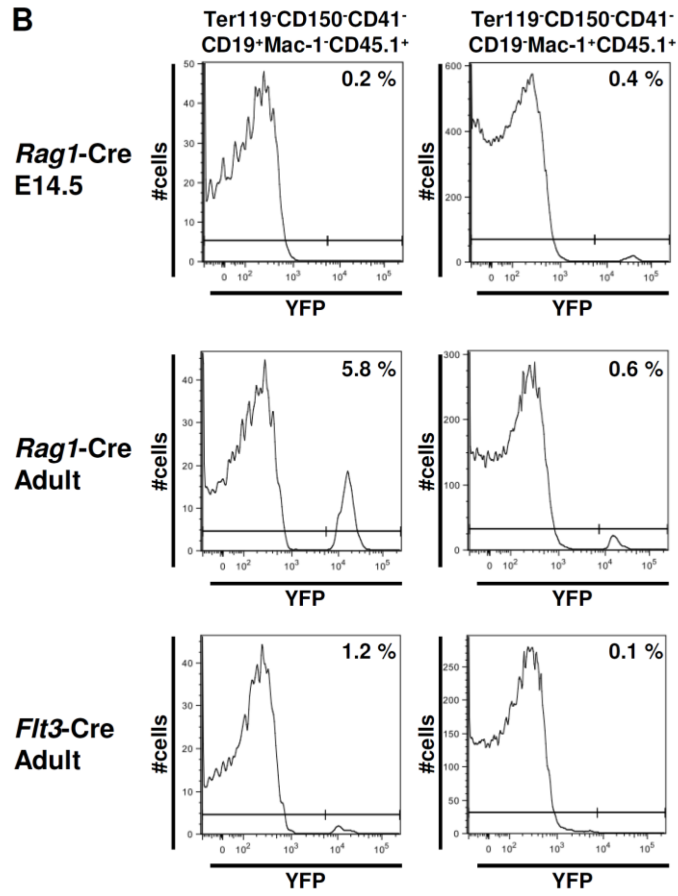
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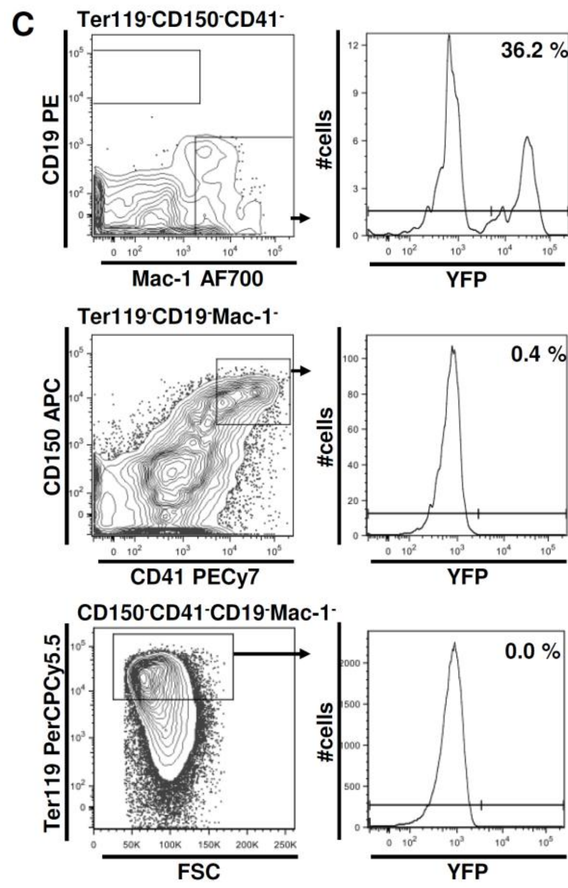
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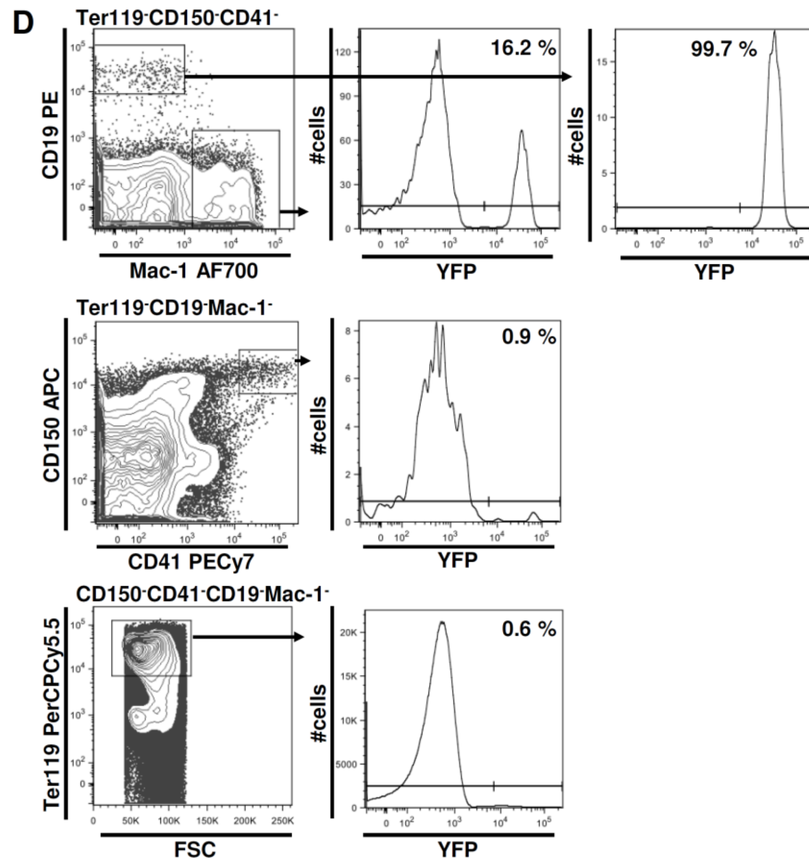
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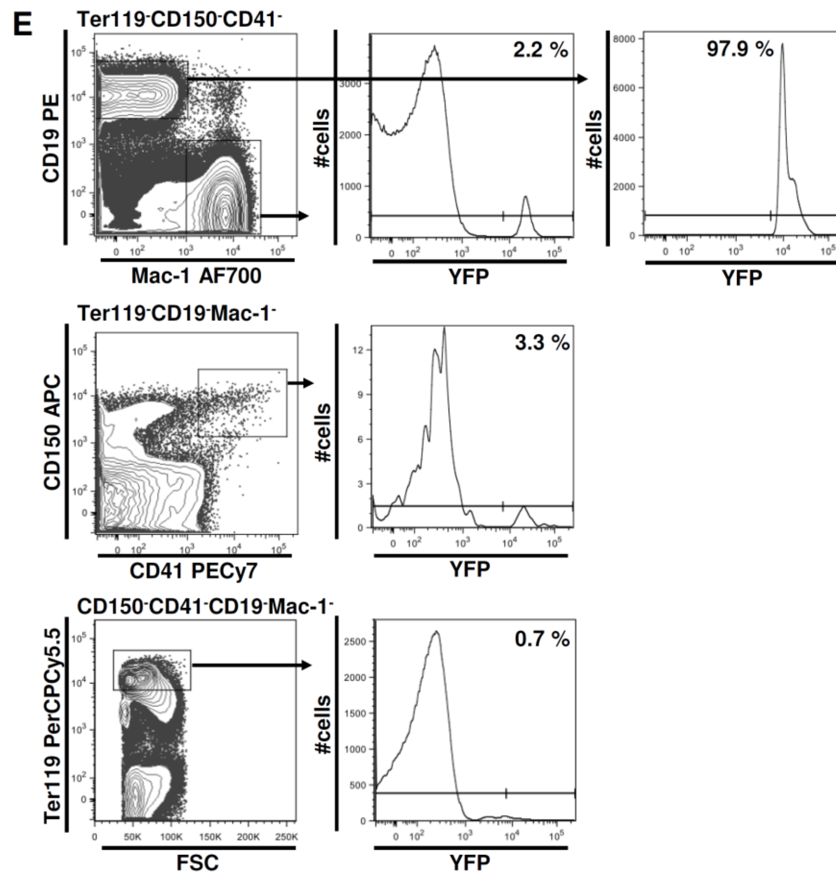
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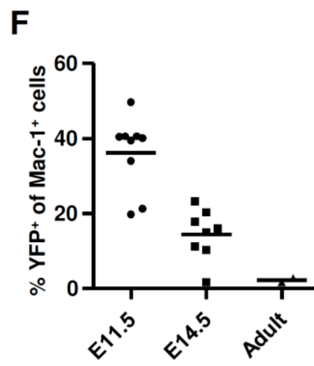
CD41 PECy7
3.3 %
CD150⁻CD41⁻CD19⁻Mac-1⁻
Ter119 PerCPCy5.5
FSC
0.7 %


F
% YFP⁺ of Mac-1⁺ cells
60
40
20
0
E11.5
E14.5
Adult

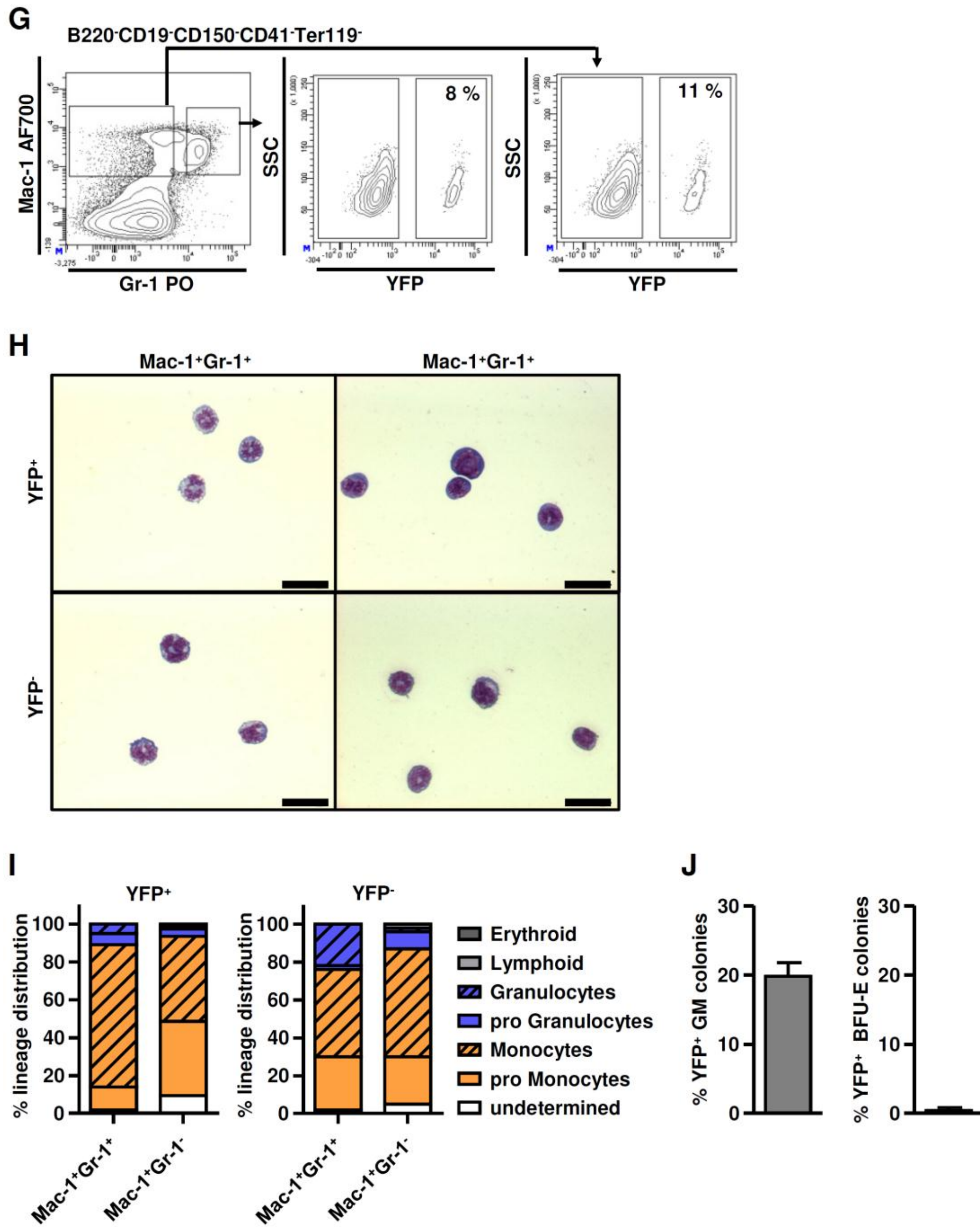


Figure 2.7: *Rag1* expressing progenitors contribute to embryonic myelopoiesis

(A) YFP expression analysis of *Flt3-Cre*$^{tg/+}$*R26R*$^{eYFP/+}$ adult BM for CD19$^+$ (Ter119$^-$CD150$^-$CD41$^-$Mac-1$^-$) B cells and Mac-1$^+$ (Ter119$^-$CD150$^-$CD41$^-$CD19$^-$) myeloid cells (top panels), CD150$^+$CD41$^+$ (Ter119$^-$CD19$^-$Mac-1$^-$) megakaryocytic cells (middle panel) and Ter119$^+$ (CD150$^-$CD41$^-$CD19$^-$Mac-1$^-$) erythroid cells (lower panel). Mean percentages YFP$^+$ cells out of parent population, 2 replicates of n=1 mouse in 1 experiment.

(B) Background levels of YFP in CD19$^+$ (Ter119$^-$CD150$^-$CD41$^-$Mac-1$^-$) B cells (left) and Mac-1$^+$ (Ter119$^-$CD150$^-$CD41$^-$CD19$^-$) myeloid cells (right) for analysis of *Rag1*-

Cre$^{tg/+}$*R26R*$^{eYFP/+}$ E14.5 FL (n=3 mice in 2 experiments) and adult BM (n=2 mice in 1 experiment) as well as *Flt3-Cre*$^{tg/+}$*R26R*$^{eYFP/+}$ adult BM (n=1 mouse). In order to determine unspecific YFP binding/signal, CD45.2$^+$ experimental samples were mixed with YFP negative CD45.1$^+$ bone marrow cells. Mean percentages YFP$^+$ cells out of parent population.

(C - E) *Rag1-Cre*$^{tg/+}$*R26R*$^{eYFP/+}$ (C) E11.5 FL (n=9 FL in 3 experiments), (D) E14.5 FL (n=8 FL in 2 experiments) and (E) adult BM (> 8 weeks old, n=2 mice in 1 experiment) cells were analysed by flow cytometry for expression of YFP in CD19$^+$ (Ter119$^-$CD150$^-$CD41$^-$Mac-1$^-$) B cells and Mac-1$^+$ (Ter119$^-$CD150$^-$CD41$^-$CD19$^-$) myeloid cells (top panels), CD150$^+$CD41$^+$ (Ter119$^-$CD19$^-$Mac-1$^-$) megakaryocytic cells (middle panels) and Ter119$^+$ (CD150$^-$CD41$^-$CD19$^-$Mac-1$^-$) erythroid cells (lower panels). B cells were absent from E11.5 FL. Mean percentages YFP$^+$ cells out of parent populations.

(F) Contribution of YFP$^+$ cells to total myeloid cells in individual E11.5 FL, E14.5 FL and adult BM. Summary of data presented in (C), (E) and (F).

(G – I) Verification of myeloid identity of Mac-1$^+$Gr-1$^-$ and Mac-1$^+$Gr-1$^+$ adult BM cells. (G) FACS purification of YFP$^+$ and YFP$^-$ subsets. Mean percentages of parent populations, n=4 FLs in 2 experiments. (H) Examples of morphological analysis of sorted populations after May-Grunwald Giemsa stain. (I) Morphological distribution of cell types within the YFP$^+$ and YFP$^-$ subsets of Mac-1$^+$Gr-1$^-$ (Ter119$^-$CD150$^-$CD41$^-$CD19$^-$B220$^-$) and Mac-1$^+$Gr-1$^+$ (Ter119$^-$CD150$^-$CD41$^-$CD19$^-$B220$^-$) cells sorted from *Rag1-Cre*$^{tg/+}$*R26R*$^{eYFP/+}$ E14.5 FL. Mean percentages, n=2 experiments.

(J) Fraction of YFP$^+$ out of total GM and erythroid colonies from unfractionated E14.5 *Rag1-Cre*$^{tg/+}$*R26R*$^{eYFP/+}$ FL in semisolid medium. Mean percentages ± SEM, GM: n=12 FLs in 3 experiments, total > 1000 colonies, erythroid: n=9 FLs in 3 experiments, total 218 colonies.

2.2.4. Lympho-myeloid progenitors progressively upregulate the lymphoid transcriptional program while down-regulating Mk/E genes

Lympho-myeloid restricted E11.5 Lin$^-$CD45$^+$c-Kit$^+$Flt3$^+$IL-7Rα$^+$ foetal liver or E9.5 Lin$^-$CD45$^+$c-Kit$^+$IL-7Rα$^+$*Rag1*$^+$ yolk sac progenitors were further characterised by quantitative gene expression analysis and compared to the respective Flt3$^-$IL-7Rα$^-$ or IL-7Rα$^-$*Rag1*$^-$ populations, which were thought be heterogeneous and are likely to contain multipotent progenitors. As expected all investigated Mk/E genes (*Klf1*, *Epor*, *Gata1*, *Gata2*) were markedly down regulated or completely absent in E11.5 as well as E9.5 lympho-myeloid progenitors (Fig. 2.8A). Overall both populations maintained the expression of selected myeloid genes (*Csf1r*, *Csf2rb*, *Csf3r*, *Mpo*) in agreement with their sustained myeloid lineage potentials (Fig. 2.8B). Lymphoid genes (*Ikzf1*, *Flt3*, *Il7ra*, *Rag1*, *Rag2*, *sIgH*), that were almost undetectable in E11.5 Lin$^-$CD45$^+$c-Kit$^+$Flt3$^-$IL-7Rα$^-$ foetal liver or E9.5 Lin$^-$CD45$^+$c-Kit$^+$IL-7Rα$^-$*Rag1*$^-$ yolk sac populations, showed a strong upregulation in lympho-myeloid progenitors (Fig. 2.8C). Thus, the transition from heterogeneous or multipotent embryonic progenitors towards the Flt3$^+$IL-7Rα$^+$ or IL-7Rα$^+$*Rag1*$^+$ lympho-myeloid restricted stage is associated with similar alterations to gene expression programs as the differentiation from adult HSCs to LMPPs (Fig. 2.8A-C). Further, while myeloid gene expression was largely sustained at the same level at E9.5 and E11.5 lympho-myeloid progenitors, lymphoid genes were upregulated between these two developmental stages (Fig. 2.8C).

The embryonic thymus is first seeded at around embryonic day 11 (Owen and Ritter, 1969). Since adult thymus seeding progenitors have been suggested to be lympho-myeloid restricted by this work and others, it was investigated whether embryonic lympho-myeloid restricted progenitors express genes associated with thymus seeding (Bell and Bhandoola, 2008; Benz and Bleul, 2005; Sambandam et al., 2005). Transcripts of the chemokine receptors CCR7 and CCR9, which are required for the homing of TSPs to the thymus, were expressed in embryonic lympho-myeloid progenitors.

Expression was initiated at E9.5 but strongly upregulated towards E11.5, when thymus seeding has already been initiated (Zlotoff et al., 2010). Further, E11.5 Lin$^-$CD45$^+$c-Kit$^+$Flt3$^+$IL-7Rα$^+$ foetal liver or E9.5 Lin$^-$CD45$^+$c-Kit$^+$IL-7Rα$^+$*Rag1*$^+$ yolk sac progenitors expressed key regulators of T cell development such as *Notch1* and its downstream target genes *Hes1* as well as *Gata3* (Fig. 2.8D) (Radtke et al., 2004; Rothenberg, 2012). Thus, embryonic lympho-myeloid restricted progenitors express key genes associated with thymus seeding and qualify as initial thymus seeding progenitors.

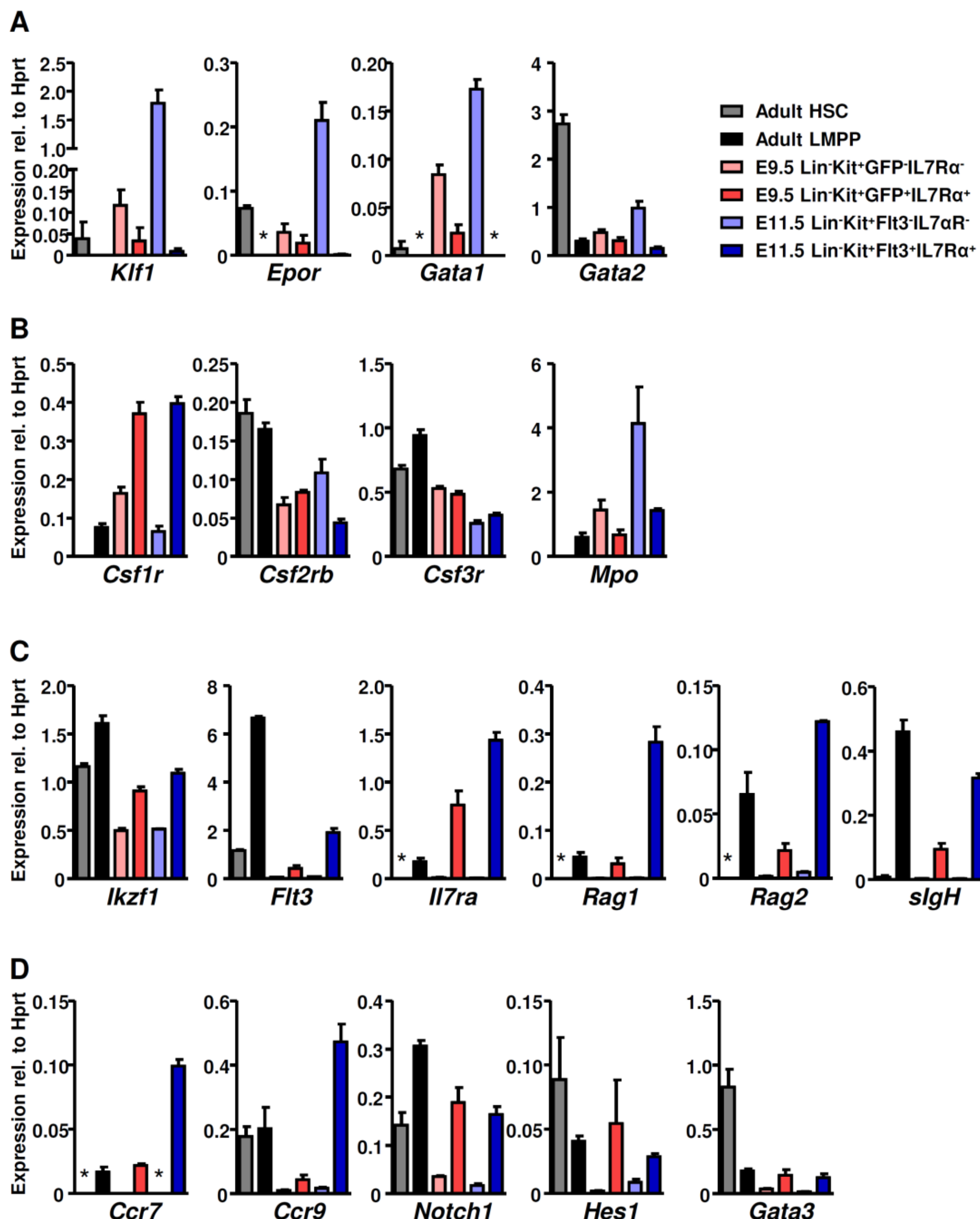


Figure 2.8: Early lympho-myeloid progenitors express specific transcriptional signatures

Quantitative gene expression analysis of $Lin^{-}CD45^{+}c\text{-}Kit^{+}IL\text{-}7R\alpha^{+}$*Rag1*$^{+}$ E9.5 yolk sac and $Lin^{-}CD45^{+}c\text{-}Kit^{+}Flt3^{+}IL\text{-}7R\alpha^{+}$ E11.5 FL progenitors compared to their $Lin^{-}CD45^{+}c\text{-}Kit^{+}IL\text{-}7R\alpha^{-}$ *Rag1*$^{-}$ and $Lin^{-}CD45^{+}c\text{-}Kit^{+}Flt3^{-}IL\text{-}7R\alpha^{-}$ counterparts as well as adult HSCs and LMPPs.

(A) Mk/E, (B) myeloid and (C) lymphoid genes. (D) Genes related to thymus seeding and T cell development. Mean ± SEM, expression relative to *Hprt*, 3 experiments, 2-3 replicate samples per experiment, 25 cells per sample, * not detected.

2.3. Discussion

The adult LMPP constitutes the first lineage restricted cell of the lymphoid-myeloid pathway. However, its lineage characteristics remain disputed in the field and recent studies have challenged its physiological role in myelopoiesis (Schlenner and Rodewald, 2010). Moreover it remains elusive whether the same lymphoid-myeloid restriction process occurs in the embryo and how early that can be observed. In this chapter lympho-myeloid lineage restriction was explored in early embryonic haematopoiesis. *In vivo* contribution of embryonic lympho-myeloid progenitors to lymphoid as well as myeloid lineages was elucidated using *Rag1*-Cre fate mapping

The earliest steps of lymphoid commitment in the adult have been the subject of major research efforts over the last decade. Although several studies have provided compelling evidence, that the generation of LMPPs and the loss of Mk/E potential constitute the first stage of the lymphoid pathway (Adolfsson et al., 2005; Arinobu et al., 2007; Lai and Kondo, 2006; Mansson et al., 2007), the contradicting classical model of haematopoiesis, with the CLP as earliest lymphoid progenitor, is still supported in the literature (Orkin and Zon, 2008; Seita and Weissman, 2010). A lympho-myeloid

commitment pathway has also been suggested for foetal haematopoiesis, where progenitors with T, B and myeloid potential were identified (Cumano et al., 1992; Kawamoto et al., 1997, 1998; Lacaud et al., 1998; Lu et al., 2002; Mebius et al., 2001). These data led to the first proposal of the myeloid model of haematopoiesis (Katsura and Kawamoto, 2001). However, this model has been questioned by earlier findings of unilineage restricted embryonic T or B progenitors as earliest lymphoid committed cells during development (Kawamoto et al., 2000). The present study provides compelling evidence of a lympho-myeloid restricted progenitor in the embryo. Detailed experiments proved that E11.5 foetal liver progenitors with the $Lin^{-}CD45^{+}c\text{-}Kit^{+}Flt3^{+}IL\text{-}7R\alpha^{+}$ phenotype possess robust B, T as well as myeloid lineage potentials at the single level. At the same time they lacked Mk/E potentials and expressed a lympho-myeloid restricted transcriptional signature. The identification of such progenitor in the embryo, similar to the LMPP in adult haematopoiesis, provides general support for the myeloid based as opposed to the classical roadmap.

Since lymphoid progenitors were first detected long after the emergence of the first definitive haematopoietic stem cells, it was previously thought that lymphopoiesis would only emerge as part of definitive haematopoiesis. This work established the lympho-myeloid progenitor not only at E11.5, just after the first definitive stem cells can be found in the foetal liver, but traced it back to the E10.5 foetal liver or the E9.5 yolk sac. Therefore, lympho-myeloid restricted progenitors emerge prior to and independent of definitive HSCs. Analysis of E8.5 concepti established the complete absence of IL-7Rα or *Rag1*-GFP positive cells from the embryo, therefore suggesting their emergence at around E9. Although lympho-myeloid progenitors were first identified in the yolk sac, their actual site of emergence remains elusive since circulation is established at around E8, after which all haematopoietic cells are readily transported through the entire embryo. The first multipotent cells have been found in the yolk sac as well as the embryonic body at E9 (Cumano et al., 1996; Cumano et al., 2001; Godin et al., 1995;

Godin et al., 1999; Palis and Yoder, 2001). Unlike definitive stem cells, these immature or pre-HSCs lack the ability to long term reconstitute irradiated adult wild type recipients, but have been shown to be multipotent. Furthermore, particularly cells isolated from the yolk sac at E9 can reconstitute neonatal recipients, and E10 pre-HSCs successfully reconstituted immune deficient recipients (Yoder et al., 1997). Thus, pre-HSCs are likely to emerge before the first lympho-myeloid restricted progenitors and might serve as their direct precursors.

Current models of haematopoiesis including the classical as well as the myeloid based model rely largely on the prospective isolation of haematopoietic progenitors, which are characterised *in vitro* as well as *in vivo* through non-physiological reconstitution assays. The ongoing controversy as regards the haematopoietic road map is therefore partly due to insufficient information on the lineage contributions of different progenitors during steady state haematopoiesis (Schlenner and Rodewald, 2010). In fact recent studies specifically questioned the physiological relevance of the myeloid potential identified in lymphoid or lympho-myeloid progenitors in the adult (Ehrlich et al., 2011). Fate mapping experiments using reporters for *Il7ra* as well as *Rag1*, two markers present on a subset of adult LMPPs, suggested that progenitors positive for these early lymphoid genes do not contribute to myelopoiesis (Schlenner et al., 2010; Welner et al., 2009). In the present study *Rag1*-Cre fate mapping confirmed this finding for the adult; however, *Rag1*$^{+}$ progenitors produced significant numbers of myeloid cells during embryonic haematopoiesis. In agreement with the *in vitro* lineage potential data and molecular evaluation of lympho-myeloid progenitors in E9.5 yolk sac or E10.5, E11.5 foetal liver, *Rag1*$^{+}$ progenitors did not produce any Mk or erythroid cells. Thus, this study unequivocally demonstrates that early lymphoid-myeloid progenitors not only have the potential to produce myeloid cells *in vitro* but also actively contribute to embryonic myelopoiesis during steady state conditions *in vivo*. The successively decreasing contribution of *Rag1*$^{+}$ progenitors to myelopoiesis with progressing age, or the lymphoid

restriction of adult *Il7ra*$^+$ cells shown by others, is likely due to a changing expression pattern of these markers between embryonic and adult haematopoiesis. Although a subset of adult LMPPs expresses *Rag1* or *Il7ra*, the majority of cells lack expression of these genes (Adolfsson et al., 2005; Luc et al., 2008a). Moreover, *Rag1*$^+$ LMPPs have significantly reduced myeloid potentials *in vitro* compared to their negative counterparts (Luc et al., 2008a). The evaluation of steady state myelopoiesis of LMPPs therefore requires the utilisation of specific markers that cover the entire LMPP population.

The process of thymus seeding and the identity of the thymus seeding progenitor have not been identified for adult or embryonic haematopoiesis (Bhandoola et al., 2007). However, based on recent studies, including this work, the adult LMPP is the currently best candidate thymus seeding progenitor. It not only resembles the lineage potentials of the ETP as earliest cell in the thymus but, as opposed to the CLP, also has superior proliferative potential suggesting its function as upstream precursor (Allman et al., 2003; Bhandoola et al., 2007; Kondo et al., 1997; Schwarz and Bhandoola, 2004). During embryonic development thymus seeding is initiated at E11.5 with progenitors most likely originating from the foetal liver (Owen and Ritter, 1969). Since the herein identified lympho-myeloid progenitors already emerged before E9.5 and could be found in the foetal liver by E10.5, they are potential candidates for the first thymus seeding at E11.5 as well as the maintenance of embryonic thymopoiesis thereafter. This was supported by the detection of genes important for thymus seeding in both E11.5 and E9.5 lympho-myeloid progenitors. *Ccr7* and *Ccr9* expression, that has been shown to be important for migration to the thymus in the adult, might enable lympho-myeloid progenitors to enter the thymic anlage, which is not yet vascularised at the time of thymus seeding (Mori et al., 2010). In addition early lympho-myeloid progenitors were already primed for the expression of Notch receptor and several downstream target genes including *Hes1* as well as *Gata3*. Both transcription factors are important for the generation of ETPs and play a key role during the first T lineage commitment steps (Hendriks et al., 1999;

Hosoya et al., 2009; Tan et al., 2005; Ting et al., 1996; Wendorff et al., 2010). Thus, lympho-myeloid progenitors already express a molecular profile that potentially allows them to not only enter the thymus, but to receive and intergrade Notch signalling for the initiation of T cell development.

In conclusion this work identified a lympho-myeloid progenitor in the E11.5 foetal liver that expresses Flt3 as well as IL-7Rα, possesses combined B/T/GM potentials and is transcriptional primed for these lineages. This progenitor further lacks Mk and erythroid lineage potentials resembling the adult LMPP. Utilizing a *Rag1*-GFP reporter line it was possible to trace this cell back to the foetal liver at E10.5 and even to the E9.5 yolk sac prior to the emergence of first definitive stem cells or the presence of known lymphoid committed progenitors in the embryo. *Rag1*-Cre fate mapping showed and thereby confirmed the complete restriction of all *Rag1*$^{+}$ progenitors to lymphoid and myeloid but not Mk or erythroid lineages *in vivo* and *in vitro*. In addition *in vivo* *Rag1*-Cre fate mapping demonstrated the physiological contribution of *Rag1*$^{+}$ lympho-myeloid progenitors to steady state myelopoiesis in the embryo. In adult haematopoiesis, however, *Rag1*$^{+}$ progenitors generated almost exclusively cells of the lymphoid lineage.

3. Early thymic progenitors sustain B, T and myeloid lineage potentials

3.1. Introduction

Most haematopoietic lineages develop in the bone marrow, where multipotent haematopoietic stem cells give rise to progenitors that eventually differentiate into mature blood cells. In contrast, T cell development is located in the thymus, which provides a unique microenvironment for the generation of naïve effector T cells. Since the thymus itself does not harbour any stem cells or cells with long term self-renewing potential, thymopoiesis is sustained through regular replenishment by TSPs from the bone marrow via the circulation (Donskoy and Goldschneider, 1992; Scollay et al., 1986). Several candidate TSPs such as LMPPs, CLPs or other lymphoid restricted progenitors as well as HSCs have been identified in the bone marrow and can be found in circulating blood (Adolfsson et al., 2005; Bhandoola et al., 2007; Kondo et al., 1997). However, as yet it has not been possible to identify and characterise the lineage potentials of the TSP, which is likely to resemble the lineage potentials of the most primitive cell in the thymus.

ETPs have been shown to possess strong T lymphoid potential but also have the potential to generate NK cells, dendritic cells and myeloid cells. ETPs have also been suggested to retain B cell potential, although this has only been detected at very low frequencies of approximately 1/30 or 1/500 (Benz and Bleul, 2005; Sambandam et al., 2005), whereas other studies have completely failed to detect any B cell potential (Balciunaite et al., 2005b). B cell potential in the thymus has been suggested by

experiments using mice deficient for Notch, the key driver of T cell commitment. In these studies large numbers of B cells were found in the thymus of Notch-deficient animals, which would be compatible with ETPs undergoing B cell commitment in the absence of Notch signalling (Radtke et al., 1999; Wilson et al., 2001). However, in this setting it cannot be excluded that B cells enter the thymus from the periphery independent of ETPs (Feyerabend et al., 2009). In fact, B cells respond to CCR7 as well as CCR9 signalling (Bowman et al., 2000), which plays an important role in attracting haematopoietic progenitors to the thymus (Zlotoff et al., 2010). Two laboratories recently investigated the lineage potentials of adult ETPs at the single cell level. These studies independently demonstrated that a large fraction of ETPs have combined T cell as well as granulocyte/monocyte potential, whereas B cell potential was not detected (Bell and Bhandoola, 2008; Wada et al., 2008). This suggested that the ETP is T/myeloid restricted and accordingly points to a T/myeloid restricted TSP. However, such a cell has not yet been found in the bone marrow and all candidate TSPs have significant B cell potential. Thus, the lineage potentials of ETPs and the identity of the TSP remain a controversy in the field. The thymus seeding by HSCs is also possible; however Mk/E potential has never been investigated in the ETP population.

Neonatal thymopoiesis has the highest rate of T cell production. The number of early progenitors with B cell potential is also most abundant at that stage (Ceredig et al., 2007; Taub and Longo, 2005). Therefore this work evaluates the lineage potentials of neonatal ETPs, and shows that these cells possess significantly higher B cell potential than adult ETPs. Furthermore neonatal ETPs have lost Mk potential but retain T, myeloid as well as B cell potential. Combined T/B/myeloid potential in ETPs is demonstrated using two different experimental approaches *in vitro*.

3.2. Results

3.2.1. Neonatal ETPs sustain high B cell potential but lack Mk potential

Although most recent studies failed to detect any B cell potential in adult ETPs, others were able to observe B cell generation at a very low frequency in ETPs isolated based on Flt3 or *Ccr9* (Bell and Bhandoola, 2008; Benz and Bleul, 2005; Sambandam et al., 2005; Wada et al., 2008). This study therefore focussed on the $Flt3^{hi}$ fraction of ETPs. Since the neonatal thymus has been shown to contain increased B cell potential (Ceredig et al., 2007) $Lin^{-}CD4^{-}CD8^{-}CD25^{-}c\text{-}Kit^{hi}Flt3^{hi}$ ETPs were FACS purified from the c-Kit enriched fraction of neonatal thymocytes (Fig. 3.1A). B and T cell potential of neonatal ETPs as well as adult ETPs was evaluated using OP9 or OP9DL1 stroma co-cultures and flow cytometry analysis (Fig. 3.1B, C). In agreement with previous findings single adult $ETPFlt3^{hi}$ produced significant numbers of T cells. However, the vast majority of cells lacked B cell potential , whereas adult LMPPs robustly generated T as well as B cells in the same experiments as previously shown (Adolfsson et al., 2005). However, when $Lin^{-}CD4^{-}CD8^{-}CD25^{-}c\text{-}Kit^{hi}Flt3^{hi}$ ETPs were isolated from neonatal mice, the frequency of B cell potential increased at least 10-fold to more than 30 %, while T cell clones were almost doubled to over 50 %, showing the increased proliferative potential at this earlier stage of development. Moreover, the B cell potential in neonatal ETPs was entirely confined to the $Flt3^{hi}$ fraction, whereas the T cell generation from $Flt3^{hi}$ and $Flt3^{-}$ ETPs was comparable (Fig. 3.1D). To exclude the possibility that the B cell potential was derived from any stem cells being present in the purified $ETPFlt3^{hi}$ population, the Mk potential of these cells was evaluated and compared to $LSKFlt3^{-}$ bone marrow progenitors, which are known to generate Mk colonies in culture (Adolfsson et al., 2005). While more than 50 % of $LSKFlt3^{-}$ derived colonies contained Mks, Mk potential was completely absent from $ETPFlt3^{hi}$ or $ETPFlt3^{-}$ thymocytes (Fig. 3.1E).

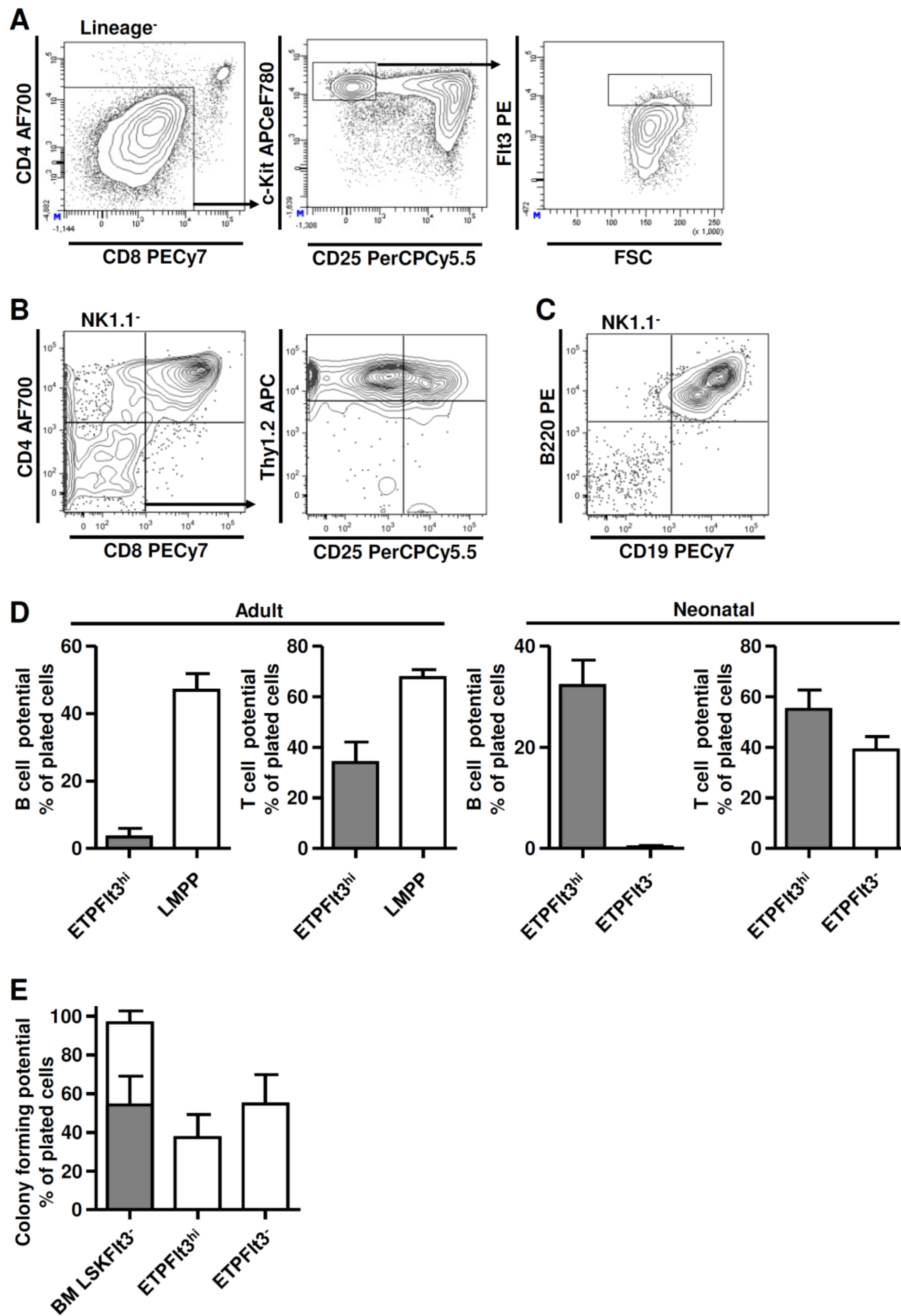


Figure 3.1: Single lineage potentials of ETPs

(A) ETPs of neonatal mice were FACS purified from the c-Kit enriched fraction of thymocytes based on the cell surface marker definition $Lin^-CD4^-CD8^-c\text{-}Kit^{hi}CD25^-Flt3^{hi}$. The Flt3 sorting gate was typically set based on Flt3 background staining on the DN2 ($Lin^-CD4^-CD8^-c\text{-}Kit^{hi}CD25^+$) population, that is negative for Flt3.

(B, C) Representative flow cytometry readouts of (B) B cell clones from OP9 or (C) T cell clones from OP9DL1 stroma co-cultures. B cell clones were defined as NK1.1$^-$ B220$^+$CD19$^+$. Positive T cell clones were gated negative for NK1.1 and expressed CD4 and CD8 or the combination of Thy1.2 and CD25.

(D) Lymphoid lineage potentials evaluated by co-culture on OP9 or OP9DL1 stroma. B and T cell potential of adult ETPFlt3^{hi} cells compared to adult LMPPs (left panel). B and T cell potential of neonatal ETPFlt3^{hi} cells compared to ETPFlt3$^-$ cells (right panel). Mean percentages ± SEM of plated cells at 1 cell/well, adult: ETPFlt3^{hi} n=3, LMPP n=16, neonatal: ETPFlt3^{hi} B cell n=12, T cell n=11, ETPFlt3$^-$ B cell n=14, T cell n=8 experiments.

(E) GM and Mk potential of ETPFlt3^{hi}, ETPFlt3$^-$ and BM LSKFlt3$^-$ progenitors. Total frequency accounting for colonies containing GM, Mk or both cell types (white bars) and frequencies of colonies containing Mks (grey bars). Mean percentages ± SEM of plated cells at 1 cell/well, BM LSK Flt3$^-$, ETPFlt3^{hi} n=4, ETPFlt3$^-$ n=3 experiments.

3.2.2. ETPs from neonatal mice are B/T/myeloid restricted multipotent progenitors

While the above experiments clearly established the presence of B cell potential in phenotypic ETPFlt3^{hi} cells from neonatal mice, it could not be ruled out that this was due to contaminating B lineage restricted progenitors. Therefore to conclusively demonstrate the presence of cells with combined B, T as well as myeloid potential, it was required to evaluate all three lineage potentials in single ETPs. Previously it was shown that T and myeloid potential could be detected in the same culture conditions (Bell and Bhandoola, 2008; Wada et al., 2008). However, the generation of B and T cells under the same conditions proved to be impossible. *In vitro* T lineage differentiation requires the strong activation of Notch signalling through its ligand DLL1 on OP9DL1 stroma, which at the same time leads to suppression of B lineage potential (Pui et al., 1999). Two different strategies involving the early separation of progeny from single ETPs for cultivation in B or T/myeloid promoting conditions were used to overcome this problem. Studies with

LMPPs had shown that B, T and myeloid potentials could be read out using the OP9/OP9DL1 switch assay (Mansson et al., 2007). Single ETPFlt3^{hi} were cultured on OP9 stroma for initial expansion. Subsequent transfer of half the primary clone onto OP9DL1 stroma enabled the readout of T together with myeloid potential, whereas the other half was transferred to a new layer of OP9 stroma for the readout of B lineage potential (Fig. 3.2A). Using this strategy more than 10 % of single ETPFlt3^{hi} gave rise to B as well as T cell colonies, while 2 % of all cells also produced myeloid cells (Fig. 3.2B). Furthermore, a second strategy was developed to detect the combined B, T and myeloid potential from ETPs. Single ETPFlt3^{hi} were cultured over night until the occurrence of the first cell division. Subsequently the paired daughter cells were separated by micromanipulation and transferred to OP9 stroma co-cultures for B lineage readout and OP9DL1 stroma co-cultures for detection of T and myeloid lineage potentials (Fig. 3.2C). In agreement with the switch technique over 10 % of manipulated cells were positive for B as well as T potentials and approximately 2 % also generated myeloid cells (Fig. 2D). Cells with combined lineage potentials gave rise to T cells positive for CD4, CD8, Thy1.2 and/or CD25, myeloid cells positive for F4/80 and Mac-1 as well as CD19 and B220 positive B cells (Fig. 3.2E, F). Although in both assays lineage potentials were generally detected at lower frequencies compared to single readouts, these results undoubtedly established the existence of multipotent ETPFlt3^{hi} cells with combined B, T and myeloid potential in the neonatal thymus. Comparing both strategies, readout frequencies from the switch technique regard all plated cells, whereas the paired daughter cell technique only selects dividing cells for manipulation, secondary culture and readout, and thereby potentially enriches for a subset of ETPFlt3^{hi} cells.

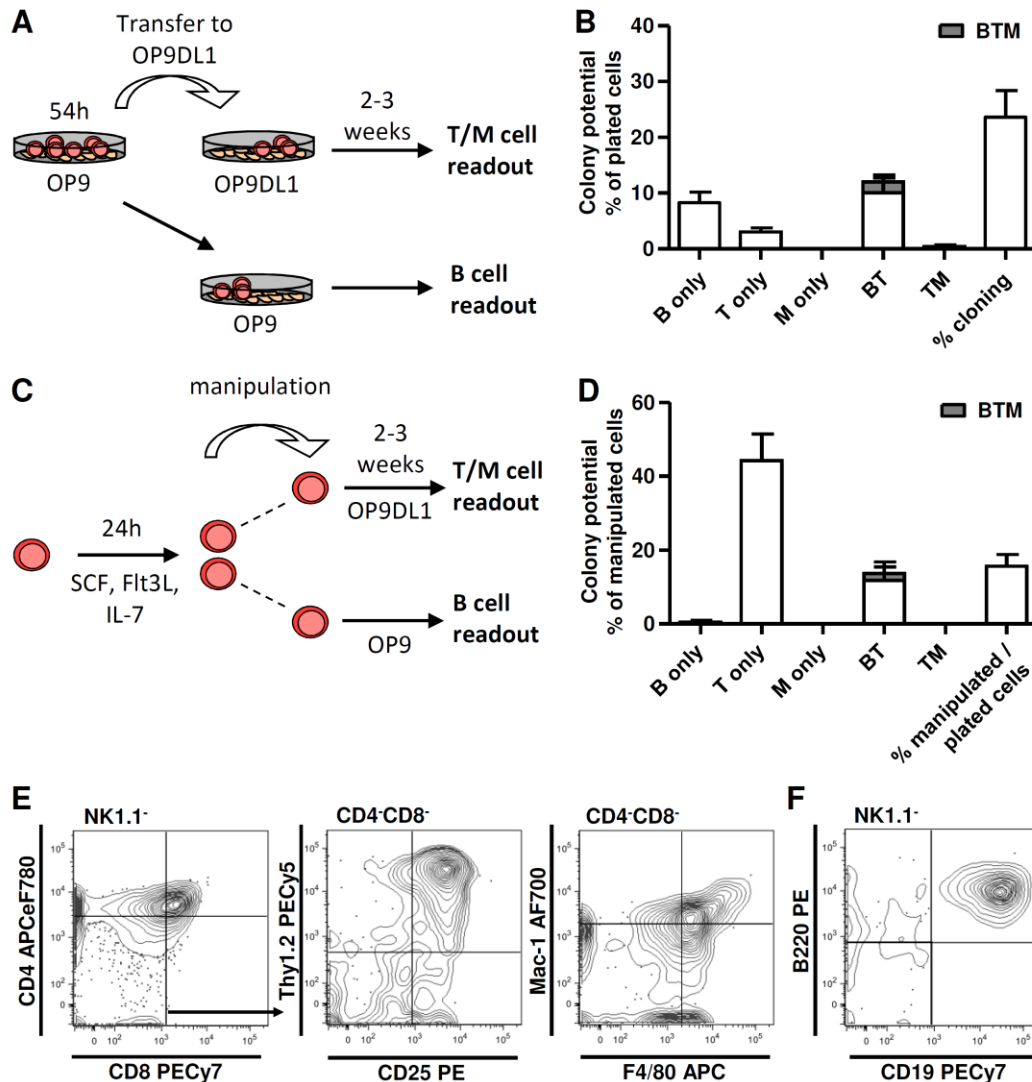


Figure 3.2: Multilineage potentials of ETPs

Analysis of multi-lineage potentials of single ETPFlt3^{hi} using the PDC or switch techniques.

(A) Experimental layout and (B) combined B, T and myeloid lineage potentials evaluated using the OP9/OP9DL1 switch technique. Frequency of B/T/myeloid colonies amongst colonies with BT potential (grey bars). Myeloid (M), B cell (B), T cell (T). Mean percentages ± SEM of plated single cells, n=4 experiments.

(C) Experimental layout and (D) combined B, T and myeloid lineage potentials determined using the paired daughter cell technique. Frequency of B/T/myeloid colonies amongst colonies with BT potential (grey bars). Myeloid (M), B cell (B), T cell (T). Mean percentages ± SEM of manipulated single cells, n=5 experiments.

(E, F) Representative FACS profiles of a (E) T and myeloid colony from OP9DL1 stromal co-culture or (F) B cell colony from OP9 stromal co-culture of a single ETPFlt3^{hi} after separation using the PDC technique. T cell clones were defined as Nk1.1^{-} and expressed CD4 and CD8 or the combination of Thy1.2 and CD25, whereas myeloid cells were gated negative for NK1.1, CD4, CD8 but expressed Mac-1, F4/80. B cell clones were defined as NK1.1^{-}B220^{+}CD19^{+}.

3.3. Discussion

Recently two studies by independent laboratories stated that the majority of ETPs have the ability to produce cells of T as well as myeloid lineages, but lack B cell potential (Bell and Bhandoola, 2008; Wada et al., 2008). However, since others previously also detected low levels of B cell potential in ETPs, and studies of Notch deficient mice suggested the presence of B cell potential in the thymus, the identity of the earliest cell in the thymus remained controversial (Benz and Bleul, 2005; Radtke et al., 1999; Sambandam et al., 2005; Wilson et al., 2001). By characterising ETPs purified from neonatal thymi this work unequivocally demonstrated ETPs to sustain B, T, as well as myeloid potential at the single cell level. Moreover it showed that ETPs entirely lack the potential to generate Mks and thereby suggests that the thymus is not seeded by HSCs. This was confirmed by studies beyond this thesis which showed that ETPs completely lacked erythroid potential. Furthermore by transplantation of whole neonatal thymi into irradiated recipients it was shown that the thymus lacks any long term reconstitution potential and therefore does not harbour HSCs (Luc et al., 2012).

The identification of combined B, T and myeloid potential in ETPs also enabled for the first time to draw the connection to a potential TSP in the bone marrow. Candidate TSPs include CLPs, the earliest lymphoid cell of the classical model, as well as LMPPs, which have the same lineage potentials as the newly characterised ETPs (Adolfsson et al.,

2005; Kondo et al., 1997). Both cell populations have been isolated from peripheral blood supporting their potential transfer to the thymus via circulation (Umland et al., 2007). Although CLPs retain low myeloid potential, their low proliferative capacity led to the conclusion that ETPs must develop through a CLP independent pathway (Allman et al., 2003; Ehrlich et al., 2011). In contrast LMPPs have superior proliferative potential to ETPs and qualify as their upstream precursors (Bhandoola et al., 2007). Further evidence for the LMPP as candidate thymus seeding progenitor came from global gene profiling analysis, which highlighted the similarities between the LMPP and ETP populations (Luc et al., 2012).

The switch assay for readout of combined B, T and myeloid lineages was described previously and was successfully applied in this study with slight modifications (Mansson et al., 2007). Herein, a new strategy for multilineage potential readout based on the micromanipulation and separation of paired progenitors was developed. This enabled the successful detection of combined B, T and myeloid lineages in neonatal ETPFlt3^{hi} cells at frequencies comparable to the switch assay. The paired daughter strategy selects for dividing cells and is therefore potentially biased towards a subset of cells. However, the separation of test cells' progeny at the two cell stage, which have the highest probability to resemble the test cells' lineage potentials, makes the assay applicable to a broad range of combined lineages, even if they require culture conditions that immediately suppress the opposite lineage potential. Moreover, the application of this assay is not necessarily restricted to the haematopoietic system.

In conclusion this work established a novel assay for the detection of combined B, T and myeloid lineages and demonstrated ETPs to possess all three potentials at the single cell levels. Together with the experimental data reported by Luc et al. this work suggests a TSP with B, T and myeloid but not Mk/E potential and points to the LMPP as a candidate for such a cell.

4. Megakaryocyte potential in the Lineage$^-$c-Kit$^+$ Sca-1$^+$ bone marrow compartment

4.1. Introduction

The first two chapters of this thesis provide novel insights into the development of the lymphoid pathway, which is accompanied by the loss of Mk/E potential as one of the first lineage restriction events downstream of HSCs. The following chapter investigates the development of the Mk lineage.

Classical as well as revised models of haematopoiesis describe the generation of unilineage restricted as well as common Mk/E progenitors via a CMP dependent route (Luc et al., 2008b). First Mk/E progenitors were identified in the Lin$^-$IL-7Rα$^-$Sca-1$^-$c-Kit$^+$ fraction of bone marrow cells based on the absence of FcγR and CD34, and were thought to develop directly from the FcγR$^-$CD34$^+$ classical CMP (Akashi et al., 2000). Mk restricted cells were first purified based on the expression of CD41 as well as CD9 (Nakorn et al., 2003). Although these cells are restricted to the Mk lineage, they generate only small Mk colonies consisting of few cells *in vitro* and give only very low platelet reconstitution *in vivo*. This suggests CD41$^+$CD9$^+$ progenitors have limited proliferative activity and are positioned far down in the haematopoietic hierarchy (Nakorn et al., 2003). Recently Pronk and colleagues developed a novel staging that enables the purification of various progenitors of myeloid/Mk/E lineages. Here, Mk progenitors are defined by the expression of CD150 and CD41 within the Lin$^-$Sca-1$^-$c-Kit$^+$ fraction of bone marrow cells (Pronk et al., 2007). Similar to CD9$^+$CD41$^+$ MkPs these cells possess little proliferative potential after transplantation and often produce only one mature

megakaryocyte in culture. These findings were confirmed by Ng and colleagues who identified progenitors using a CD150^{+}CD9^{+} phenotype of which a large fraction also expressed CD41 (Ng et al., 2012). However, Ng and colleagues also detected *in vivo* erythroid potential indicating an Mk/E progenitor, although this was not evaluated at the single cell level.

In all studies described above the LSK fraction of bone marrow cells has not been investigated for Mk progenitors based on the assumption that this population only contains stem and multipotent progenitor cells (Okada et al., 1992). However, in accordance with the early initiation of lymphoid lineage restriction and the purification of the LMPP, a recent study also identified a new CMP within the LSK compartment based on the expression of a reporter for GATA1 (Arinobu et al., 2007). Unlike the classical CMP which was later suggested to be rather a mix of MEPs and GMPs, around 40 % of *Gata1*$^{+}$ CMPs generate mixed myeloid/Mk/E colonies *in vitro* (Nutt et al., 2005; Rieger et al., 2009b). In these studies less than 30 % of phenotypic HSCs, that are known to generate high Mk output in culture, gave rise to colonies containing Mks, suggesting that the used conditions preferentially promote GM differentiation. However, in the same conditions over 80 % of new CMPs generated Mk containing clones demonstrating the high Mk potential within the population. About half of those colonies exclusively contained Mks, which would be compatible with the presence of Mk restricted progenitors within the *Gata1*$^{+}$ CMP population (Arinobu et al., 2007).

The potential existence of a very early Mk progenitor is further supported by the fact that cells of the Mk lineage possess many similarities with stem cells (Huang and Cantor, 2009). Of the key extrinsic regulators, TPO is critical for both Mk differentiation and HSC maintenance (Alexander et al., 1996; Gurney et al., 1994; Kimura et al., 1998; Qian et al., 2007). Many surface markers for the purification of phenotypic HSC in the adult as well as the embryo such as CD150, CD41 or the TPO receptor Mpl, are also expressed

on Mks and their precursors (Kiel et al., 2005; Mikkola et al., 2003; Pronk et al., 2007). The transcription factor *Runx1* plays an essential role in the development of first definitive HSC in the embryo but is equally important for the maturation of Mks (Ichikawa et al., 2004; Ichikawa et al., 2008; North et al., 2002; Okuda et al., 1996). Additional shared transcription factors include GATA2, that has overlapping functions with GATA1 in megakaryopoiesis, as well as MEIS1, that is specifically required for embryonic Mk development (Azcoitia et al., 2005; Hisa et al., 2004). A primitive Mk progenitor could therefore serve as the missing link between highly proliferative HSCs and the known rather mature Mk progenitors with little proliferative capacity.

Both the generation of pure Mk colonies from early LSK bone marrow progenitors as well as the similarity between Mks and stem cells is compatible with the existence of an early Mk progenitor that potentially develops through a CMP independent pathway. Herein, further evidence is provided for the presence of a potential Mk progenitor within the LSKCD150^{+}CD48^{+} population. The use of a novel GATA1 reporter enabled the enrichment of Mk potential within the *Gata1^{pos}* (III) fraction as shown by functional as well as molecular analysis. Moreover, in support of the alternative model of lineage commitment, the residual Mk potential detected in the LMPP population was significantly enriched in the LMPPs' small *Gata1^{+}* subset.

4.2. Results

4.2.1. GATA1 is highly expressed in a subset of primitive bone marrow progenitors

The utilisation of a *Gata1*-GFP reporter facilitated the identification of the earliest CMP currently known (Arinobu et al., 2007). However, a large fraction of *Gata1^{+}* bone marrow progenitors in these experiments still gave rise to pure Mk colonies suggesting that this CMP population might contain unilineage restricted Mk progenitors. This was further evaluated in the current study. The previously used GATA1 reporter, which

encompasses a promoter region with 3 DNA hypersensitive sites, does not contain all known haematopoietic regulatory elements and might not truly represent *Gata1* transcript expression throughout all haematopoietic stages (Drissen et al., 2010; Jasinski et al., 2001; McDevitt et al., 1997; Suzuki et al., 2009). Therefore herein a new mouse line that was generated using a bacterial artificial chromosome (BAC) containing all known *Gata1* regulatory elements was utilized (Fig. 4.1A). In initial flow cytometry experiments bone marrow myeloid/MK/E progenitors of this new *Gata1*-EGFP reporter line were analysed according to a staging developed by Pronk and colleagues (Pronk et al., 2007). In agreement with previous quantitative gene expression experiments, Mk and erythroid progenitors expressed high levels of *Gata1*-EGFP, whereas only small fractions of GMPs as well as preGMs were positive for *Gata1* (Fig. 4.1B) (Pronk et al., 2007). Next the LSK bone marrow subset, that mostly consists of stem and multipotent progenitor cells (Okada et al., 1992), was subdivided based on the expression of CD150, CD48 and CD34 and analysed for *Gata1* expression. While only a minor fraction of LMPPs (4.0 %) and CD150^{-}CD48^{+} multipotent progenitors (10.6 %) expressed *Gata1*-EGFP, significant fractions of CD150^{+}CD48^{-} (30.3 %) or CD150^{+}CD48^{-}CD34^{-} (22.5 %) phenotypic stem cells were *Gata1*$^{+}$ although at a low mean fluorescence intensity (MFI). The highest expression levels of *Gata1* were detected in the relatively uncharacterised CD150^{+}CD48^{+} LSK subset, where 74.0% of cells expressed the EGFP reporter (Fig. 4.1C). Conversely the LSK*Gata1*$^{+}$ fraction, that represents the Gata1^{+} CMP described by Arinobu and colleagues, was enriched for CD150^{+} cells but in particular CD150^{+}CD48^{+} progenitors (total LSK 5.2 %, LSK*Gata1*$^{+}$ 21.3 %) (Arinobu et al., 2007). Within the LSK*Gata1*$^{-}$ fraction the CD150^{+}CD48^{+} population was almost absent (2.1 %) (Fig. 4.1D).

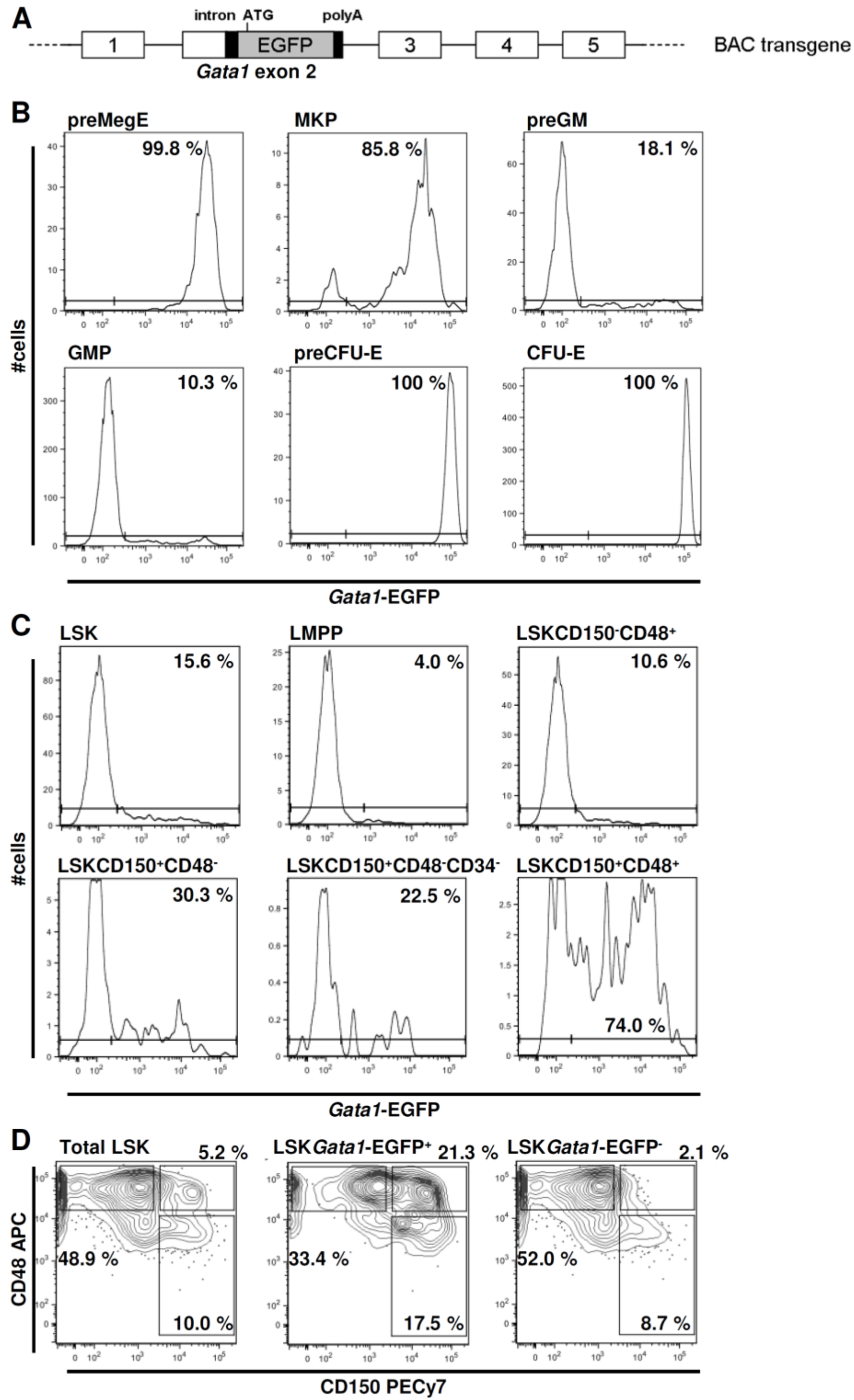


Figure 4.1: Evaluation of *Gata1*-EGFP reporter expression in bone marrow progenitors

(A) Diagrammatic representation of the *Gata1*-EGFP BAC transgenic reporter construct.

(B) *Gata1* reporter expression in myeloid/Mk/E progenitors; preMegE (Lin⁻c-Kit⁺Sca-1⁻CD41⁻Fcyr⁻CD150⁺CD105⁻), MkP (Lin⁻c-Kit⁺Sca-1⁻CD41⁺CD150⁺), preGM (Lin⁻c-Kit⁺Sca-

1$^-$CD41$^-$Fcyr$^-$CD150$^-$CD105$^-$) GMP (Lin$^-$c-Kit$^+$Sca-1$^-$CD41$^-$Fcyr$^+$), preCFU-E (Lin$^-$c-Kit$^+$Sca-1$^-$CD41$^-$Fcyr$^-$CD150$^+$CD105$^+$), CFU-E (Lin$^-$c-Kit$^+$Sca-1$^-$CD41$^-$Fcyr$^-$CD150$^-$CD105$^+$). Mean percentages of *Gata1*$^+$ cells for all analysed mice, n=4 mice in 1 experiment.

(C) *Gata1* reporter expression in bone marrow stem and progenitor cell populations; LMPP (LSKFlt3$^+$), LSKCD150$^-$CD48$^+$ (multipotent progenitors), LSKCD150$^+$CD48$^-$ (CD34$^-$) (HSCs), LSKCD150$^+$CD48$^+$ (largely undefined population). Mean percentages of *Gata1*$^+$ cells for all analysed mice, n=4 mice in 1 experiment.

(D) Distribution of CD150 and CD48 expression of total LSK bone marrow stem and progenitor cells (left panel), LSK *Gata1*$^+$ cells (middle panel) as well as LSK *Gata1*$^-$ cells (right panel) based on (C). Mean percentages out of parent population for all analysed mice, n=4 mice in 1 experiment.

4.2.2. Enrichment of primitive Mk progenitors in the LSKCD150$^+$CD48$^+$*Gata1*^{pos} population

Since the highest frequencies as well as levels of *Gata1* expression were observed in the long term stem cell containing LSKCD150$^+$CD48$^-$ subsets, and in particular the CD150$^+$CD48$^+$ population was enriched for *Gata1*$^+$ CMPs, the *in vitro* Mk potential of these cells was evaluated in comparison to CD150$^-$CD48$^+$ multipotent progenitors (Arinobu et al., 2007; Kiel et al., 2005). As expected CD150$^+$CD48$^-$ stem cells generated mostly mixed colonies that contained Mks together with other, potentially myeloid cells, whereas the CD150$^-$CD48$^+$ fraction that contains a high frequency of LMPPs did not produce significant numbers of Mk cells. In strong contrast nearly all colonies derived from CD150$^+$CD48$^+$ cells consisted of a large proportion of Mks. Most notably around 40 % of these colonies had pure Mk content, which would be compatible with the presence of Mk restricted progenitors in that population. Moreover, as expected from an Mk progenitor these cells produced Mks faster than the more primitive stem cells (Fig. 4.2A). Therefore further efforts were focussed on the subdivision and characterisation of

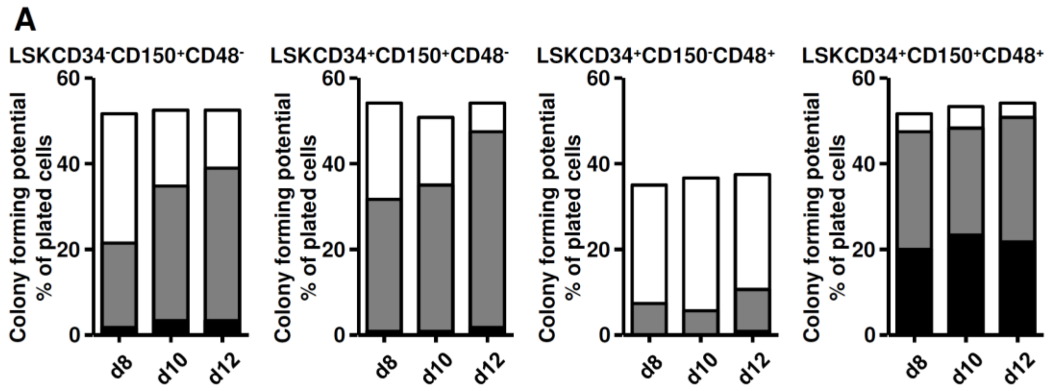
the CD150$^+$CD48$^+$ population using the *Gata1* reporter, which had previously been shown to enrich for Mk potential (Arinobu et al., 2007). *Gata1*-EGFP positive subpopulations that expressed 5 different levels of *Gata1* (*Gata1*^{low} (I), *Gata1*^{int} (II), *Gata1*^{pos} (III), *Gata1*^{hi} (IV) and *Gata1*^{max} (V)) or were *Gata1* negative (*Gata1*$^-$ (0)) were purified by FACS (Fig. 4.2B). Increasing *Gata1*-EGFP levels were accompanied by the gradual loss of T lymphoid potential, although T potential was not entirely lost even at the highest *Gata1*-EGFP levels (Fig. 4.2C). Subfractionation of the CD150$^+$CD48$^+$ population also allowed for further enrichment of Mk potential and the highest Mk production was observed from the *Gata1*^{pos} (III) population. More than 50 % of all colonies derived from single *Gata1*^{pos} (III) cells contained only Mks (Fig. 4.2D). Similarly quantitative gene expression showed the highest expression of Mk associated genes in the *Gata1*^{pos} (III) as well as *Gata1*^{hi} (IV) populations (Fig. 4.2E). While the loss of T lymphoid potential and increasing levels of *Gata1* expression were as expected associated with downregulation of stem cell genes, myeloid associated genes were maintained at consistent levels (Fig. 4.2F, G). Interestingly, erythroid genes were strongly upregulated only in the *Gata1*^{max} (V) population (Fig. 4.2H). Thus, in agreement with *Gata1* levels observed in downstream unilineage committed progenitors, intermediate levels of *Gata1* expression were associated with the Mk lineage, while the highest *Gata1* expression was found in cells with erythroid gene priming (Fig. 4.1B). As expected the level of *Gata1* transcript correlated well with the *Gata1*-EGFP reporter expression (Fig. 4.2I).

4.2.3. The LSKCD150$^+$CD48$^-$*Gata1*$^+$ population does not contain long term reconstituting stem cells

Low levels of *Gata1* transcripts have previously been detected in phenotypic haematopoietic stem cell populations. GATA1 deletion has no functional impact on stem cells and *Gata1* expression has been interpreted as transcriptional priming (Gutierrez et al., 2008; Hu et al., 1997; Mansson et al., 2007). In the present study intermediate levels

of *Gata1*-EGFP reporter expression were detected in more than 10 % of LSKCD150$^+$CD48$^-$ phenotypic stem cells (Fig. 4.2B) (Kiel et al., 2005). This was accompanied by small changes in the gene expression profile, most notably the downregulation of all four evaluated stem cell genes, indicating the potential loss of HSC function in that population (Fig. 4.2E-I). To further investigate whether *Gata1* expression is in fact initiated in HSCs, *Gata1*-EGFP positive and negative LSKCD150$^+$CD48$^-$ were transplanted into lethally irradiated recipients for the evaluation of their long term reconstitution potential. While *Gata1*$^-$ stem cells gave high levels of reconstitution of all lineages after 22 weeks, no reconstitution was observed from *Gata1*$^+$ cells (Fig. 4.2J). Thus, these experiments demonstrate that long term reconstituting HSCs do not express detectable levels of the *Gata1* reporter.

A



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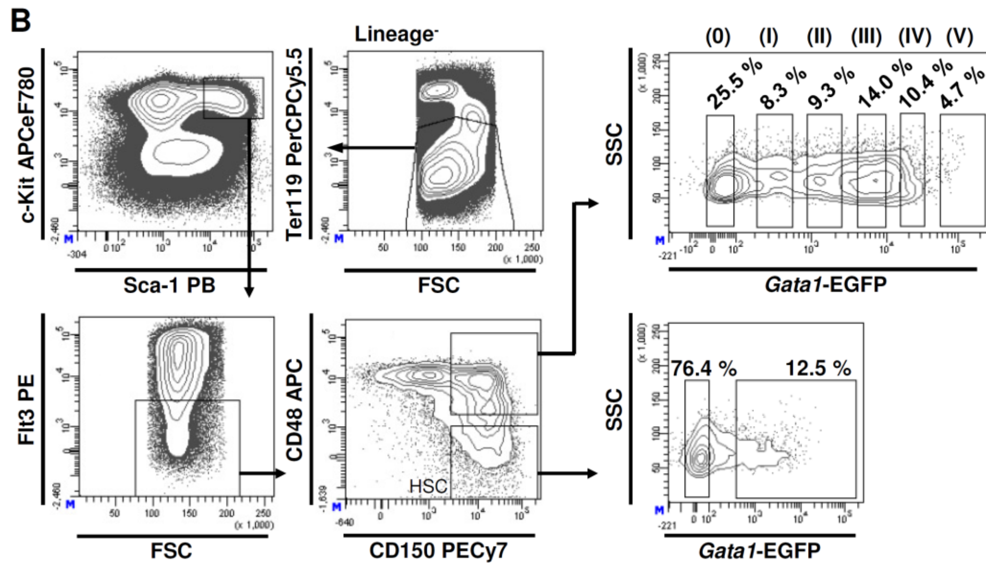

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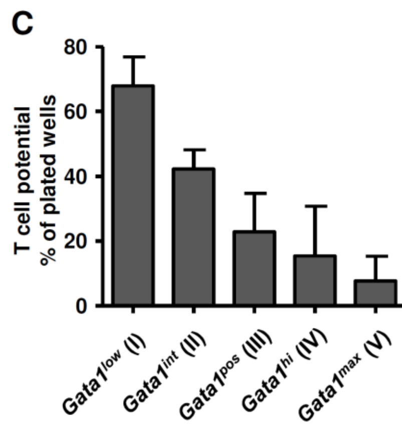

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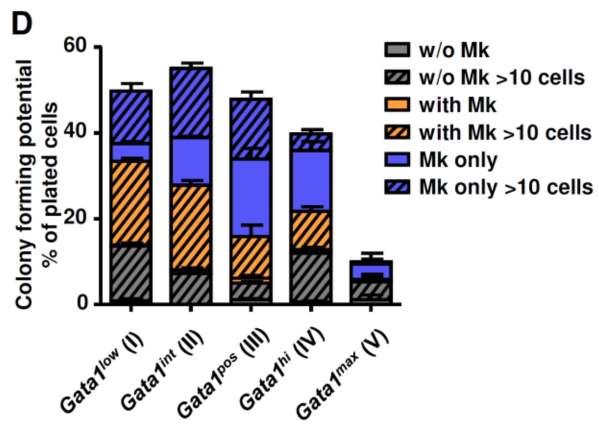
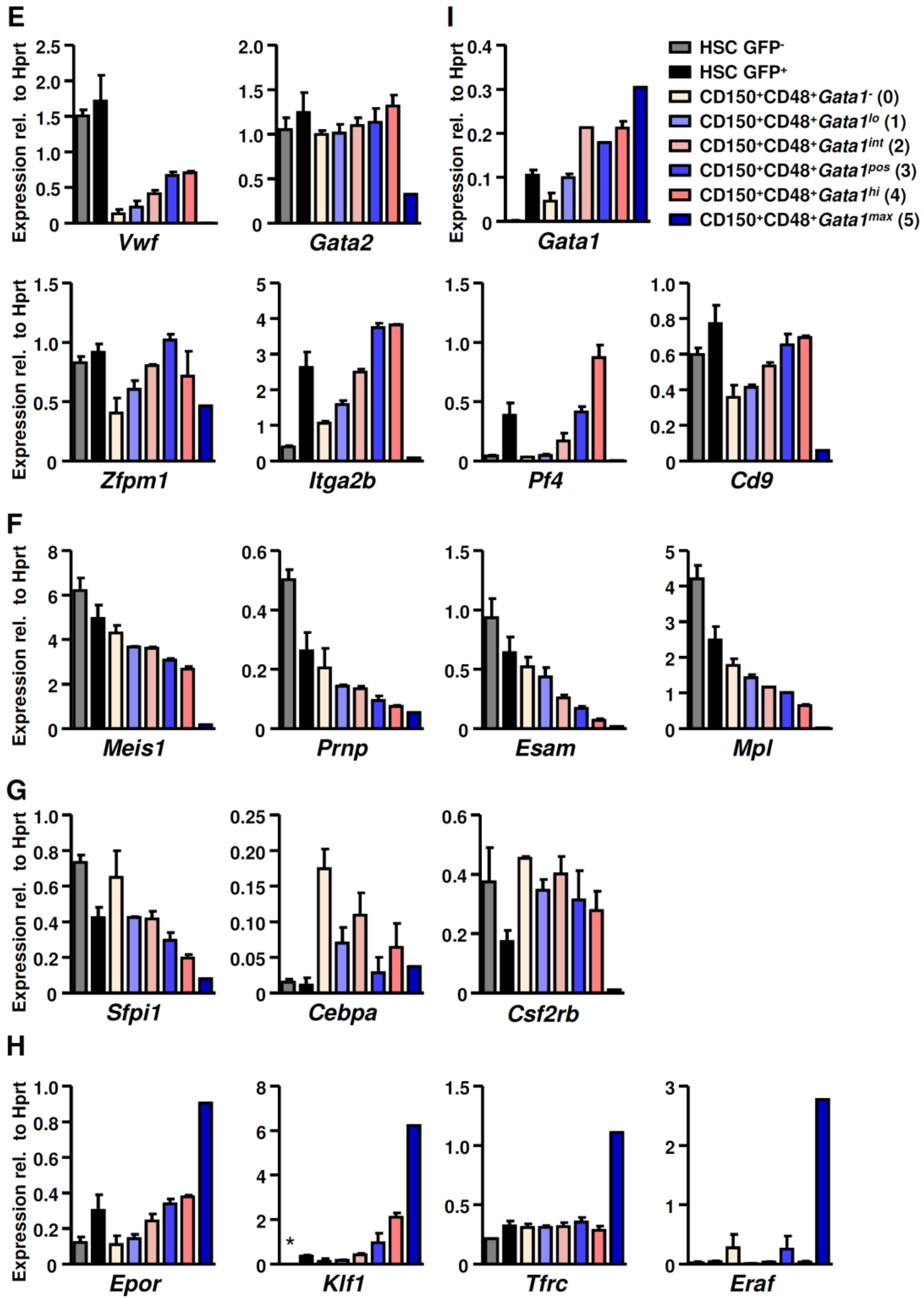




E
I
HSC GFP-
HSC GFP+
CD150+CD48+Gata1- (0)
CD150+CD48+Gata1lo (1)
CD150+CD48+Gata1int (2)
CD150+CD48+Gata1pos (3)
CD150+CD48+Gata1hi (4)
CD150+CD48+Gata1max (5)
Expression rel. to Hprt
Vwf
Gata2
Gata1
Zfpm1
Itga2b
Pf4
Cd9
F
Meis1
Prnp
Esam
Mpl
G
Sfpi1
Cebpa
Csf2rb
H
Epor
Klf1
Tfrc
Eraf

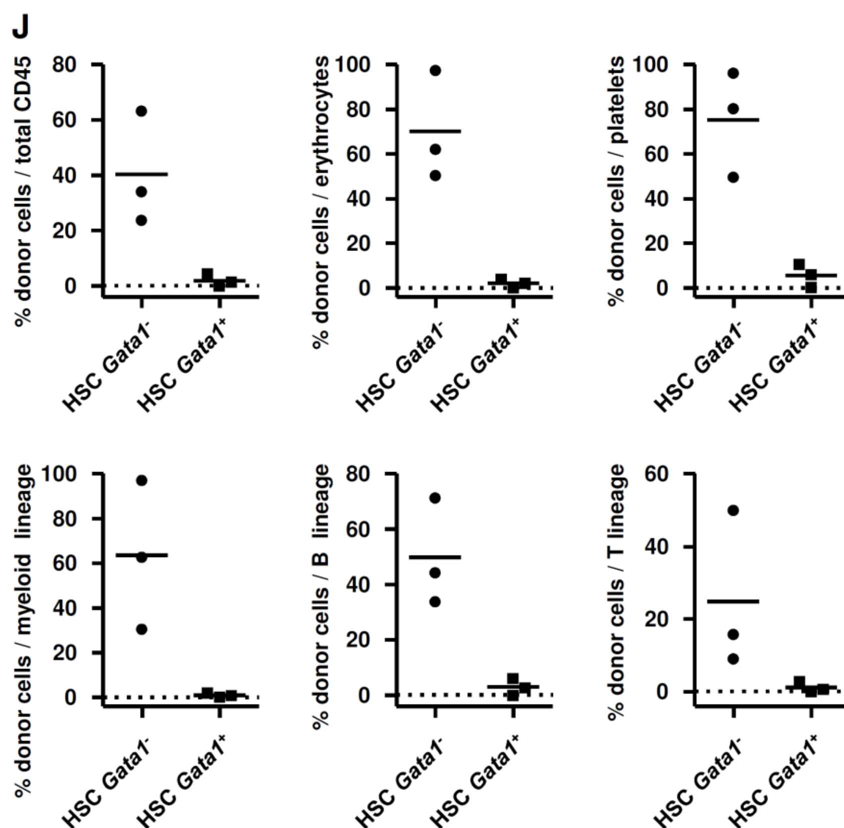


Figure 4.2: *Gata1*-EGFP expressing LSKCD150⁺CD48⁺ BM progenitors have restricted lymphoid but high megakaryocyte potential

(A) The LSK fraction of bone marrow progenitors was subdivided using CD150, CD48 and CD34. Mk potential was evaluated in liquid culture. LSKCD34⁻CD150⁺CD48⁻ or LSKCD34⁺CD150⁺CD48⁻ (HSCs), LSKCD34⁺CD150⁻CD48⁺ (multipotent progenitors), LSKCD150⁺CD48⁺ (largely undefined population). Total colonies (white bars), colonies containing Mks (grey bars) and pure Mk colonies (black bars). Frequencies out of plated cells, n=1 experiment.

(B) Sort profiles for the purification of *Gata1* subsets of LSKCD150⁺CD48⁺ BM progenitors as well as *Gata1* positive and negative phenotypic HSCs (defined as LSKCD150⁺CD48⁻). Mean percentages out of parent population from n=5 (for LSKCD150⁺CD48⁺ populations) or n=2 (for HSC populations) independent experiments.

(C) *In vitro* analysis of T lymphoid potential. For each population FACS purified progenitors were manually plated at the equivalent of 4 cells per well onto 12 wells of

OP9DL1 stroma. All colonies were analysed for the content of T lymphoid cells defined as cells positive for the surface markers CD4, CD8 or Thy1.2 and CD25. Mean percentages ± SEM of plated wells, n=3 independent experiments.

(D) *In vitro* analysis of Mk potential in liquid culture. 120 FACS purified progenitors were manually plated in 60 well terasaki plates at the equivalent of 1 cell per well. Colonies were read out at day 12 of culture. Mean percentages ± SEM of plated cells, n=3 independent experiments.

(E-I) Quantitative gene expression analysis of HSC and LSKCD150^{+}CD48^{+} subsets. 100 cells were directly sorted into PCR lysis buffer and analysed on a 48.48 Fluidigm BioMark dynamic array.(E) Mk genes, (F) stem cell genes, (G) myeloid genes, (H) erythroid genes, (I) *Gata1*. Data is shown as expression relative to *Hprt*. Mean relative expression ± SEM, n=2, for CD150^{+}CD48^{+}*Gata1*^{max} n=1 replicates in 1 experiment, * not detected.

(J) 22 weeks *in vivo* peripheral blood reconstitution after transplantation of 100 FACS purified LSKCD150^{+}CD48^{-} HSCs together with 250,000 congenic whole BM cells (dotted line, detection level at 0.1 % donor reconstitution). Each data point represents one individual mouse, n=1 experiment.

4.2.4. *Gata1*$^{+}$ LMPPs retain significant Mk potential

Much controversy about the haematopoietic roadmap, and whether the earliest fate decision divides myeloid/Mk/E versus lymphoid lineages according to the classical model, or lympho-myeloid versus myeloid/Mk/E lineages according to the alternative or myeloid based model, is based on the LMPPs residual but small Mk/E potential (Adolfsson et al., 2005; Forsberg et al., 2006). Since the *Gata1*-EGFP reporter enriched for Mk potential in the LSKCD150^{+}CD48^{+} population it was also applied to subdivide the LMPP. While less than 1 % of all LMPPs expressed *Gata1*-EGFP, they comprised a significant proportion of the LMPPs' Mk potential. Out of 120 cells plated only one FACS purified *Gata1*$^{-}$ LMPPs gave rise to a colony that contained Mks, whereas 24 Mk clones

were derived from the same number of *Gata1*$^{+}$ LMPPs (Fig. 4.3A, B). Next *Gata1*$^{-}$ LMPPs were analysed by short term *in vivo* reconstitution and compared to total LMPPs according the experiments described by Forsberg and colleagues. By analysing platelet reconstitution at 16 days after transplantation of 500 or 2500 LMPPs they detected up to 3 % contribution to this lineage (Forsberg et al., 2006). Herein, the analysis of platelet as well as erythroid reconstitution 14 days after transplantation of 1000 cells into irradiated hosts revealed a contribution of about 1 % to both lineages from total LMPPs or *Gata1*$^{-}$ LMPPs (Fig. 4.3C). Thus, although the majority of Mk colonies were derived from *Gata1*$^{+}$ LMPPs *in vitro*, the *in vivo* analysis showed that even *Gata1*$^{-}$ LMPPs retained low levels of residual Mk and E potentials. Transplantation of the LSKCD150^{+}CD48^{+} in the same experiments resulted in high platelet and erythroid reconstitution, supporting the high Mk but also the presence of erythroid potential in this population (Fig. 4.3C).

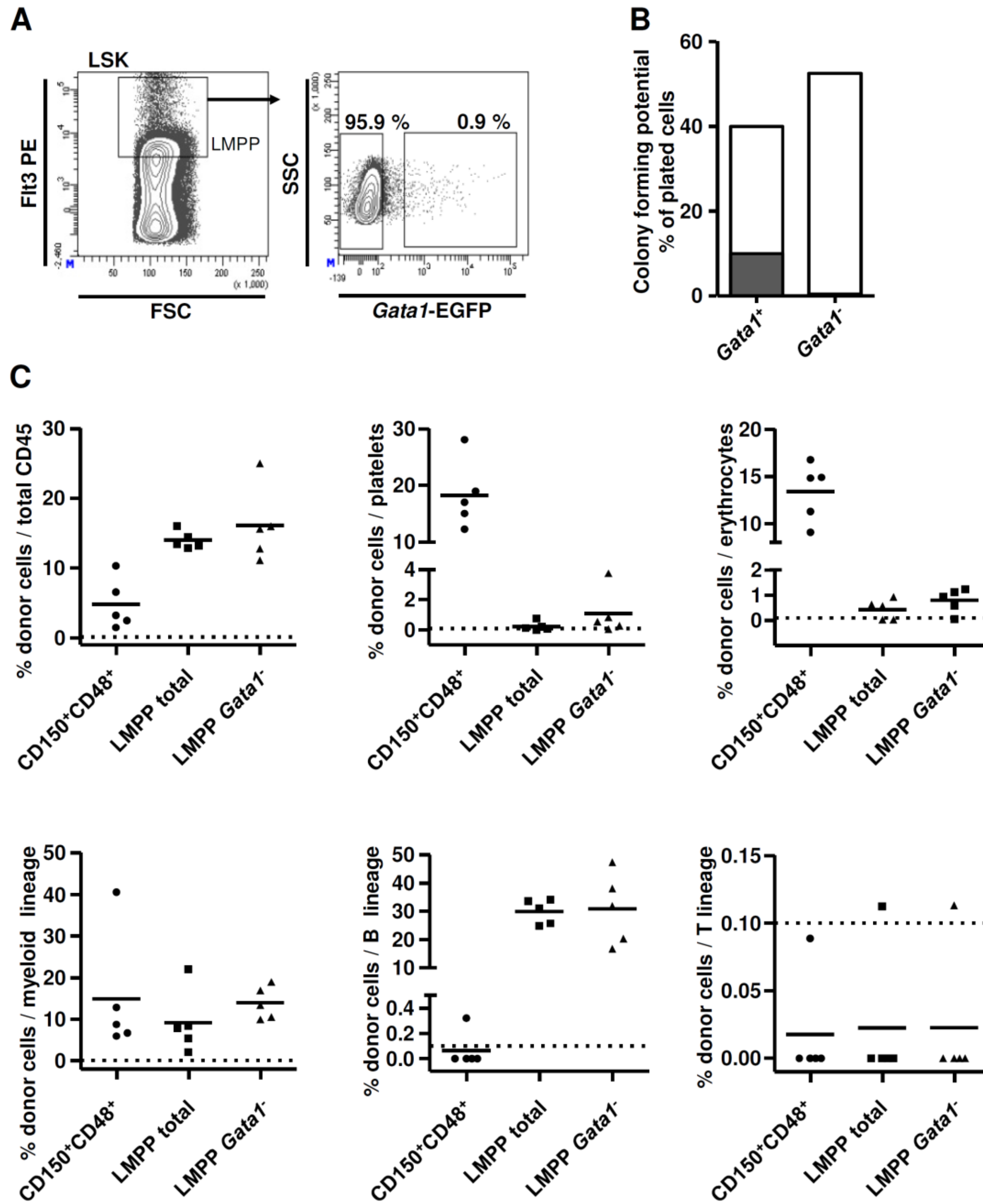


Figure 4.3: *Gata1*$^+$ LMPPs possess increased megakaryocyte potential

(A) Sort profiles for the purification of *Gata1*$^+$ and *Gata1*$^-$ subsets of LMPPs defined as the 25 % of LSK cells with the highest Flt3 expression. Mean percentages of parent population, n=2 experiments.

(B) *In vitro* colony potential. 120 FACS purified LMPPs were manually plated in 60 well terasaki plates at the equivalent of one cell per well. Total colonies (white bars) and colonies containing megakaryocytes (grey bars). Mean percentage of plated cells, n=2 experiments.

(C) Day 14 *in vivo* peripheral blood reconstitution. 1000 FACS purified LSKCD150^{+}CD48^{+} progenitors or LMPPs (total or *Gata1*$^{-}$) were transplanted together with 250,000 congenic unfractionated BM cells (dotted line, detection level 0.1 % donor reconstitution). Each data point represents 1 transplanted mouse, n=1 experiment.

4.3. Discussion

Unilineage restricted Mk progenitors are thought to develop through a CMP, and previously identified Mk progenitors are rather mature, have little proliferative potential and produce only small colonies. However, several lines of experimental data are compatible with the existence of an early more primitive Mk restricted cell. This work explored this potential Mk progenitor within the LSKCD150^{+}CD48^{+} compartment of a *Gata1*-EGFP reporter mouse.

Previously studies aiming to purify Mk progenitors have focussed on the Lin^{-}Sca-1^{-}c-Kit^{+} compartment of bone marrow cells and identified Mk restricted progenitors that have low proliferative potential suggesting they are situated at a late stage of haematopoiesis (Nakorn et al., 2003; Ng et al., 2012; Pronk et al., 2007). The present work explored Mk potential in the LSK compartment of early stem and multipotent progenitors and suggests the existence of an early potentially Mk restricted progenitor. Results reported by Arinobu and colleagues already pointed to such cell (Arinobu et al., 2007). Herein further evidence was provided that this putative Mk progenitor resides in the CD150^{+}CD48^{+} subset and expresses intermediate to positive (*Gata1*^{pos}) levels of *Gata1*-EGFP. Residing within the LSK compartment and expressing *Gata1* this population is most likely a subset of the *Gata1*$^{+}$ CMP described by Arinobu and colleagues. Although CD150^{+}CD48^{+}*Gata1*^{pos} cells expressed high levels of Mk specific genes, priming of both myeloid and erythroid genes was detected. Further the detection of residual T cell potential, although declining towards higher *Gata1*-EGFP expression levels, is

compatible with heterogeneity of the CD150$^+$CD48$^+$*Gata1*^{pos} population. This potential heterogeneity could be explored through single cell transcriptional priming analysis, which would also give further insight on whether the CD150$^+$CD48$^+$*Gata1*^{pos} population contains indeed Mk progenitors.

In agreement with the importance of GATA1 for erythroid development very high levels of erythroid genes were detected in the CD150$^+$CD48$^+$*Gata1*^{max} population (Fujiwara et al., 1996; Mancini et al., 2012; Takahashi et al., 1997). Although the *Gata1*^{pos} subset expressed much lower levels, erythroid transcripts were still detected. Since erythroid potential was not evaluated in this study, it remains unclear to what extent it is sustained in *Gata1*^{pos} cells and whether this population might contain pure Mk progenitors, Mk/E progenitors or a mix of both. The expression of *Zfpm1* (FOG1), recently proposed as master regulator of Mk/E commitment, would suggest the presence of high numbers of MkE progenitors (Mancini et al., 2012). Arguing against this CD150$^+$CD48$^+$*Gata1*^{pos} cells expressed high levels of the Mk associated genes *CD9* and *Itga2b* (CD41), which are only expressed at low levels on previously identified preMegEs (Nakorn et al., 2003; Pronk et al., 2007).

Further purification using additional surface markers or transcriptional reporters that are more specific to the Mk lineage will be required to prospectively identify the Mk progenitor potentially present in the LSKCD150$^+$CD48$^+$ population. Since the herein used *Gata1*-EGFP reporter only yielded insufficient enrichment of Mk progenitors, replacement or combination with a reporter for a Mk specific gene would be needed. Although the large overlap of the Mk transcriptional program with stem cells complicates the search for unique regulators, several candidates have been described. GATA2 has a partly redundant role with GATA1 but is thought to be more Mk specific (Huang and Cantor, 2009). Similarly, FOG1 that has been shown to be important for Mk and E lineages and has been reported as Mk associated transcription factor (Mancini et al.,

2012; Tsang et al., 1997a; Tsang et al., 1997b). Additional purification could also be achieved by the use of surface markers such as CD41, which expression is shared with HSCs, and in particular CD9, which has been successfully used to identify more mature Mk restricted progenitors (Nakorn et al., 2003; Ng et al., 2012).

Since the discovery of the LMPP multiple reports have confirmed its existence in support of the alternative model of haematopoiesis. In addition to high Flt3 expression LMPPs have been isolated based on the expression of transcriptional reporters for the genes of PU.1 or Ikaros as well as absence of the surface markers VCAM1 or Mpl (Adolfsson et al., 2005; Arinobu et al., 2007; Lai and Kondo, 2006; Luc et al., 2008a; Yoshida et al., 2006). However, controversy in the field persists around the LMPPs residual Mk/E potential that was estimated to about 3 % for Flt3^{hi} LMPPs (Forsberg et al., 2006). Flt3^{hi}Vcam1^{-} LMPPs retained Mk/E potential at a frequency of 1 %, while absence of Mpl enabled further reduction to roughly 1/1000 cells (Lai and Kondo, 2006; Luc et al., 2008a). PU.1 positive LMPPs did not give rise to any Mks or erythroid cells. However, this analysis was performed in a less efficient colony assay that only detected cells of Mk or E lineages in 30 % of stem cell derived colonies (Arinobu et al., 2007). All above markers identify only a small subset of LMPPs with very low Mk/E potential. Thereby the majority of LMPPs is disregarded, which presents a disadvantage for studies that require consideration of the entire LMPP population such as mutation screenings or the identification of other distinct subsets. Herein, *Gata1*-EGFP has been demonstrated to mark a small subset of LMPPs that sustains the majority of Mk potential *in vitro*. Although *Gata1*$^{-}$ negative LMPPs were not completely free of Mk potential, they did represent the majority of the LMPP population.

In conclusion the results of this study are compatible with the existence of a primitive Mk restricted progenitor in the LSKCD150^{+}CD48^{+} bone marrow compartment. Functional and molecular data supported the enrichment of Mk potential in the *Gata1*^{pos} fraction of a

novel *Gata1*-EGFP reporter line. However, the LSKCD150^{+}CD48^{+}*Gata1*^{pos} population retained low T lymphoid potential as well as considerable myeloid potential and further purification will be required to prospectively identify this putative Mk progenitor. Finally, using this *Gata1*-EGFP reporter the residual Mk potential sustained in LMPPs could be enriched in a small *Gata1*$^{+}$ subset, which facilitates a better purification of LMPPs in the *Gata1*$^{-}$ fraction.

5. Paired daughter fate mapping of megakaryocyte lineage biased haematopoietic stem cells

5.1. Introduction

According to the classical road map, haematopoiesis is maintained throughout the life span of an organism by a small but homogeneous population of long term HSCs that sit atop a hierarchy of short lived stem and progenitor cells. However, several lines of evidence have recently emerged which suggest that the most primitive stem cell population is functionally heterogeneous, containing cells capable of generating overlapping but distinct lineage outputs. Retrospective analysis after transplantation of single LT-HSCs revealed distinct reconstitution patterns with predominantly myeloid cells from some cells, whereas other LT-HSCs more efficiently reconstituted lymphoid lineages. Although both lymphoid and myeloid biased cell types yielded long term reconstitution over several months, myeloid biased HSCs appeared to be more primitive, giving slower initial reconstitution in primary hosts and more efficiently reconstituted secondary hosts (Benz et al., 2012; Challen et al., 2010; Dykstra et al., 2007). Prospective enrichment of myeloid or lymphoid primed stem cells was achieved by purification of subsets of the side population, that is highly enriched for HSCs (Challen et al., 2010). Similarly the stem cell population could be subdivided by using differential expression of the surface marker CD150, where the highest levels of CD150 expression marked the most primitive myeloid biased stem cells (Morita et al., 2010). Using a BAC transgenic EGFP reporter for von Willebrand factor (*Vwf*), a gene associated with the Mk lineage, which is heterogeneously expressed in otherwise phenotypically homogeneous LT-HSCs, it was possible to further subdivide the primitive CD150^{+} stem cell fraction.

While *Vwf*$^{+}$ as well as *Vwf*$^{-}$ HSCs give long term reconstitution of myeloid as well as lymphoid lineages in primary as well as secondary recipients, confirming their primitive HSC identity, *Vwf*$^{+}$ stem cells gave significantly higher platelet reconstitution. *Vwf*$^{+}$ HSCs are enriched for a Mk associated transcriptional program, indicating that this population is both a transcriptionally and functionally biased subset of the primitive stem cell population. Moreover, *Vwf*$^{+}$ stem cells can give rise to their *Vwf*$^{-}$ counterparts but not *vice versa*, and have therefore been suggested to reside at the apex of the haematopoietic hierarchy (Sanjuan-Pla A., Nerlov C., Jacobsen S.E. manuscript submitted).

HSCs have been proposed to be maintained through asymmetric self-renewal divisions, resulting in the production of one daughter HSC as well as one multipotent progenitor (Morrison and Kimble, 2006). This process would ensure the maintenance of the stem cell pool, while at the same time downstream progenitors and eventually mature blood cells are replenished. However, despite of ample evidence for asymmetric stem cell self-renewal in other model organisms or cellular systems, asymmetric stem cell divisions are yet to be conclusively demonstrated in the mammalian haematopoietic system (Knoblich, 2008). Due to technical limitations it is to date not possible to track haematopoietic stem and progenitor cells with the required resolution *in vivo* (Lo Celso et al., 2009; Xie et al., 2009). Similarly, although time lapse microscopy allows continuous and high resolution tracking, cells can only be phenotypically characterised and therefore not conclusively identified (Eilken et al., 2009; Rieger et al., 2009a; Wu et al., 2007). The functional and molecular analysis of paired progeny is therefore the most promising technique for the evaluation of HSC division. Several studies investigated the lineage potentials of paired progeny from prospectively purified HSCs using *in vitro* colony assays. Although only myeloid/Mk/E lineages were analysed in these experiments, a high degree of asymmetric readouts were observed suggesting an underlying asymmetric cell division. However, due to the high heterogeneity of observed

readouts and the nature of the used colony assays, that cannot detect all lineage potentials with absolute efficiency, asymmetric division could not be proven (Ema et al., 2000; Takano et al., 2004).

Vwf^{+} and *Vwf*$^{-}$ HSCs have been extensive functionally as well as molecularly characterised at the population level (Sanjuan-Pla A., Nerlov C., Jacobsen S.E. manuscript submitted). However, the single cell composition of both populations remains largely unknown. Therefore this work evaluated single *Vwf*$^{+}$ and *Vwf*$^{-}$ stem cells and their earliest lineage restriction through *in vitro* paired daughter fate mapping. Single *Vwf*$^{+}$ HSCs generated increased numbers of Mk colonies and more frequently expressed Mk associated transcripts. Detailed analysis of paired progeny suggests that Mk potential is more often symmetrically inherited from *Vwf*$^{+}$ HSCs compared to the *Vwf*$^{-}$ counterparts. Moreover, analysis of lineage priming between daughter cells revealed the asymmetric expression of key lineage determinants and stem cell regulators.

5.2. Results

5.2.1. Increased Mk colony generation and lineage priming in single *Vwf*$^{+}$ HSC and their immediate progeny

Previous experiments demonstrated that *Vwf*$^{+}$ stem cells are biased for Mk differentiation *in vivo* and are characterised by increased expression of a Mk-lineage transcriptional program when compared with their *Vwf*$^{-}$ counterparts (Sanjuan-Pla A., Nerlov C., Jacobsen S.E. manuscript submitted). However, in these experiments HSCs were only analysed at the population level, and it remains undetermined if all or only a fraction of *Vwf*$^{+}$ cells show an increased propensity for Mk generation. Therefore in this study lineage potentials and transcriptional lineage priming of *Vwf*$^{+}$ and *Vwf*$^{-}$ HSC were evaluated at the single cell level. Moreover, in order to assess the lineage fate of *Vwf*$^{+}$

and *Vwf*$^{-}$ HSC their immediate progeny, daughter cells resulting from a single cell division *in vitro* were included in the analysis (Fig. 5.1). *Vwf*$^{+}$ and *Vwf*$^{-}$ stem cells were purified according to the LSKCD150^{+}CD48^{-} phenotype (Fig. 5.2A). The phenotype used here was slightly different from the study by Sanjuan-Pla and colleagues, who defined HSCs as LSKCD150^{+}CD48^{-}CD34^{-} (Sanjuan-Pla A., Nerlov C., Jacobsen S.E. manuscript submitted). In agreement with the hierarchical position downstream of *Vwf*$^{+}$ stem cells, *Vwf*$^{-}$ HSC gave rise to Mks marginally faster than *Vwf*$^{+}$ HSCs (Fig. 5.2B). However, as expected Mk biased *Vwf*$^{+}$ HSC produced Mks at a higher frequency for the last readout time point at day 15 (Fig. 5.2C). At the same time both populations had a comparable overall cloning frequency and produced similar numbers of T cell colonies (Fig. 5.2C, D, G, H). Higher numbers of Mk-containing colonies were also observed from daughters of *Vwf*$^{+}$ HSCs compared to their *Vwf*$^{-}$ counterparts, although at a lower overall frequency (Fig. 5.2E, H). Daughter cells from *Vwf*$^{+}$ as well as *Vwf*$^{-}$ HSCs more efficiently produced T cells suggesting that the initial culture period and cell division makes paired daughters more responsive to survival and growth signals in OP9DL1 co-cultures (Fig. 5.2F, G).

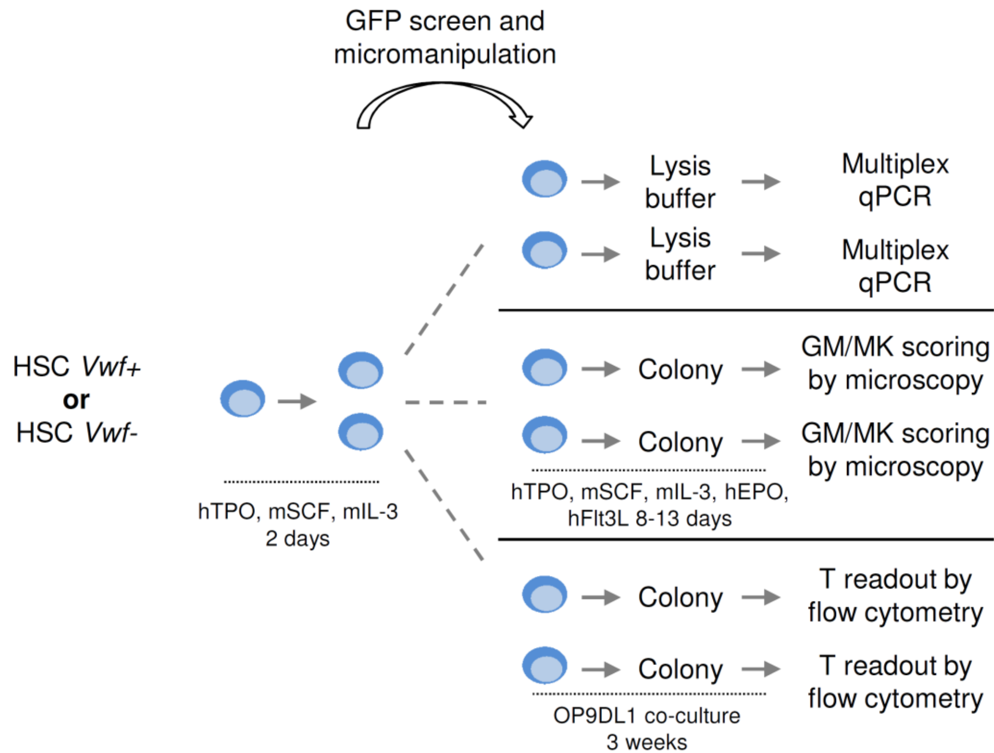
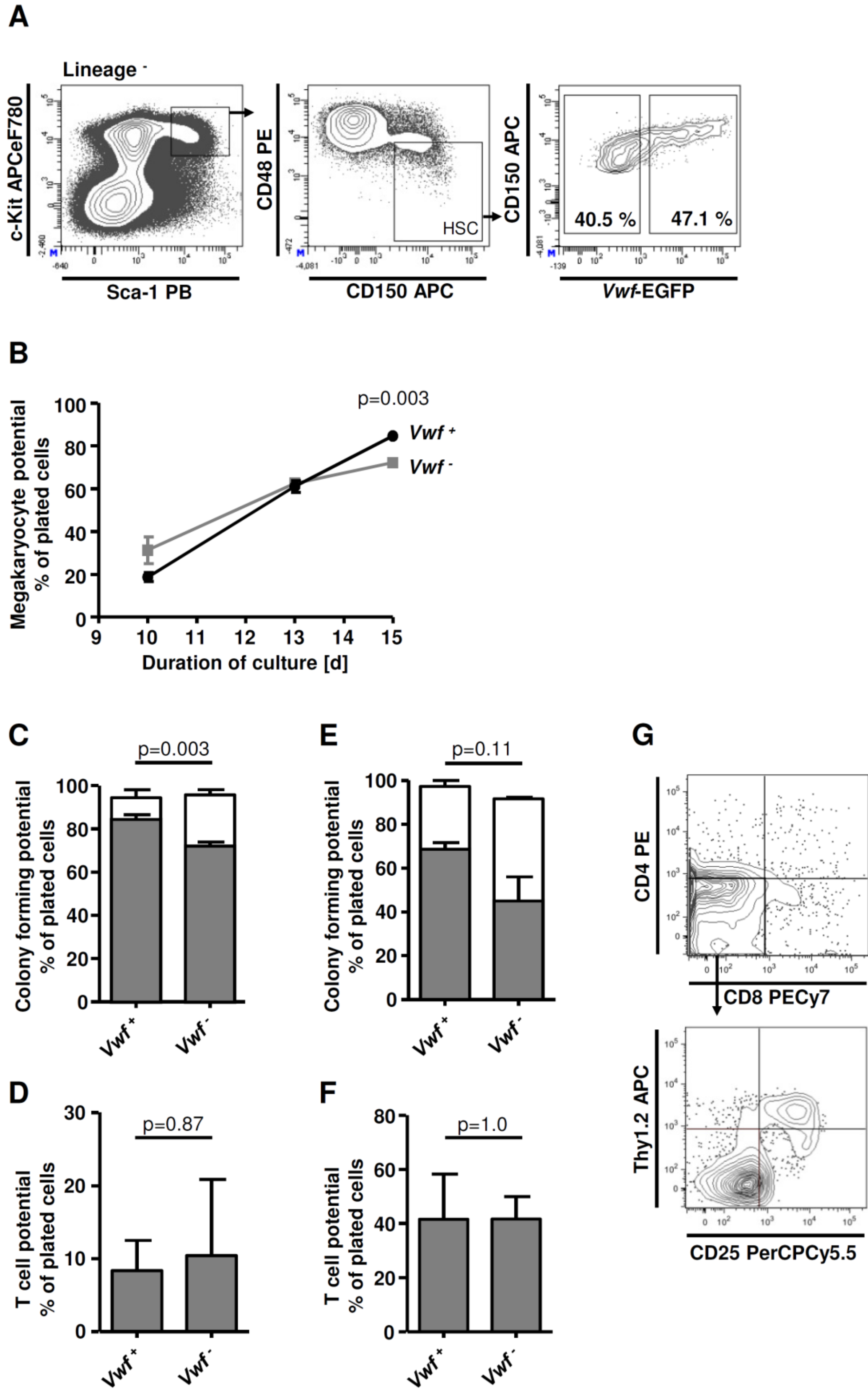


Figure 5.1: Paired daughter fate mapping

Experimental set up for paired daughter fate mapping of haematopoietic stem cells. Single *Vwf*$^{+}$ or *Vwf*$^{-}$ cells were FACS purified and cultured for 2 days with hTPO, mSCF and mIL-3. Colonies were screened for EGFP expression by microscopy and single cells from clones containing exactly two daughters were transferred into lysis buffer for quantitative PCR analysis or secondary culture for readout of GM/Mk or T cell potentials.


A
Lineage -
c-Kit APCeF780
Sca-1 PB
CD48 PE
CD150 APC
HSC
CD150 APC
40.5 %
47.1 %
Vwf-EGFP
B
p=0.003
Vwf +
Vwf -
Megakaryocyte potential
% of plated cells
Duration of culture [d]
C
p=0.003
Colony forming potential
% of plated cells
Vwf +
Vwf -
E
p=0.11
Colony forming potential
% of plated cells
Vwf +
Vwf -
G
CD4 PE
CD8 PECy7
Thy1.2 APC
CD25 PerCPCy5.5
D
p=0.87
T cell potential
% of plated cells
Vwf +
Vwf -
F
p=1.0
T cell potential
% of plated cells
Vwf +
Vwf -

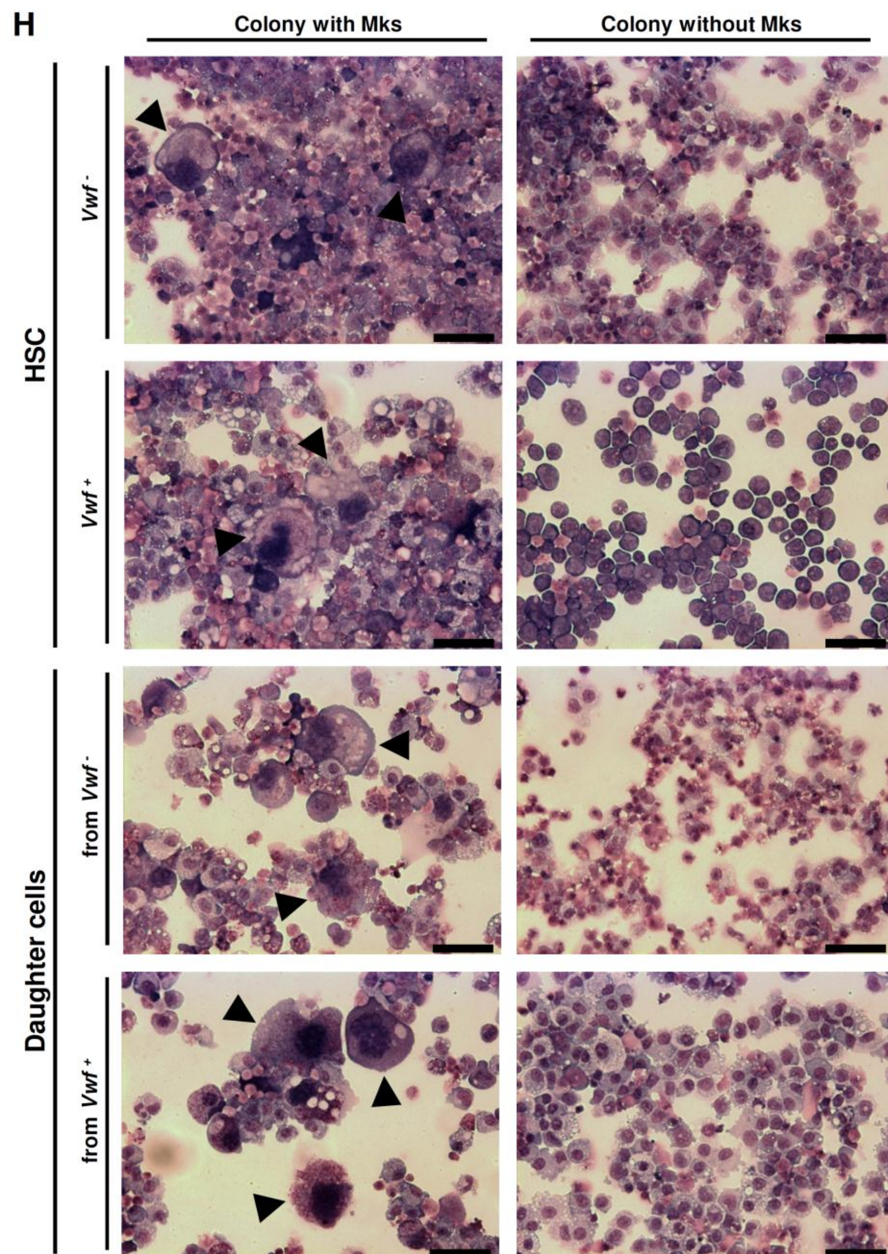


Figure 5.2: Lineage potential analysis of *Vwf*⁺ and *Vwf*⁻ HSCs and their immediate progeny

(A) FACS purification of *Vwf*⁺ and *Vwf*⁻ HSC from the *Vwf*-EGFP reporter mouse strain. Mean percentages of parent population, n=3 experiments.

(B) Kinetics of the emergence of Mks in colonies derived from *Vwf*⁺ and *Vwf*⁻ HSCs. Mean frequency of Mk colonies ± SEM out of plated single cells , n=3 experiments, ** p<0.01.

(C – D) Colony forming potential of FACS purified (C) single *Vwf*$^{+}$ or *Vwf*$^{-}$ HSCs and (D) paired daughters of those. Total colony frequency (white bars) and colonies containing Mks (grey bars). Mks were confirmed by direct microscopic analysis of cultures as well as expression of the *Vwf*-EGFP reporter. Mean frequency of Mk colonies ± SEM out of plated single cells, n=3 experiments.

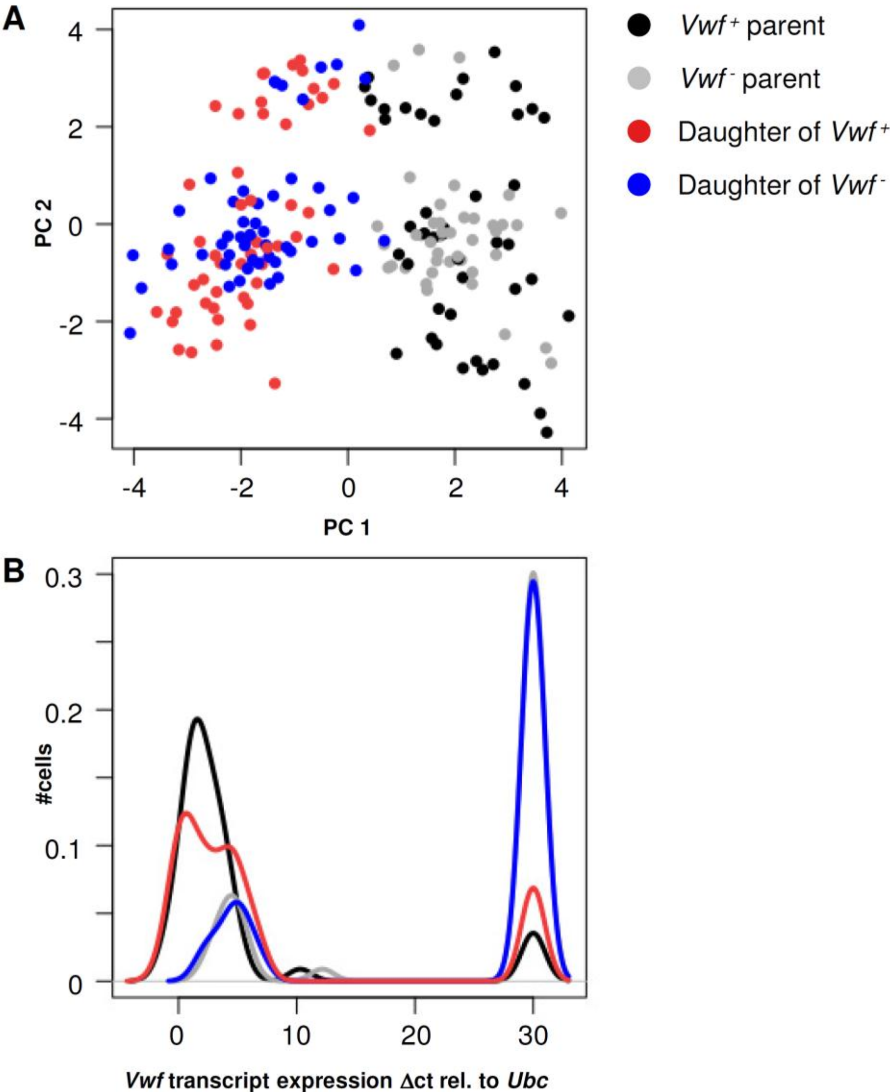
(E – F) T lymphoid potential of FACS purified (E) single *Vwf*$^{+}$ or *Vwf*$^{-}$ HSCs and (F) paired daughters of those. T cell colonies were defined by the expression of at least CD4, CD8 or Thy1.2 and CD25 surface markers. Mean frequency of Mk colonies ± SEM out of plated single cells, n=2 experiments.

(G) Representative FACS plots of a T cell colony derived from a single paired daughter cell. Positive colonies expressed CD4, CD8 and/or Thy1.2 and CD25.

(H) Confirmation of presence or absence of Mks by May-Grunwald Giemsa stain and morphology analysis in day 15 colonies from *Vwf*$^{+}$ or *Vwf*$^{-}$ HSCs or in day 13 colonies from daughters of those. Examples of Mks are indicated by arrows, scale bar 25 µm.

To further characterise *Vwf*$^{+}$ as well as *Vwf*$^{-}$ HSC and their immediate progeny at the molecular level, single cell quantitative gene expression analysis using a gene set covering all lineages was performed. Principle component analysis (PCA) revealed that a distinct difference in gene expression signatures is apparent between parent HSCs and their progeny. Gene expression signatures of *Vwf*$^{+}$ and *Vwf*$^{-}$ subsets were, however, very heterogeneous and largely overlapping, suggesting no or only marginal differences in lineage priming (Fig. 5.3A). Detailed analysis for lineage specific genes confirmed very heterogeneous gene expression profiles for single cells within each population. Both *Vwf*$^{+}$ and *Vwf*$^{-}$ stem cells expressed low levels of lymphoid genes. Only *Ikzf1* (Ikaros), which is not restricted to the lymphoid lineage but has previously been shown to be important for initiation of lymphoid priming in HSCs is more frequently expressed (Ng et al., 2009). Similarly, myeloid/erythroid as well as genes associated with stem cell function were expressed at comparable frequencies between *Vwf*$^{+}$ and

Vwf$^-$ HSCs. However, in agreement with their increased Mk colony formation, *Vwf*$^+$ HSCs were enriched for a number of Mk related transcripts such as *Neo1*, *Sdpr*, *Gpr64*, *Clu*, *Zfpm1* (FOG1), *Itga2b* (CD41) as well as *Mycn*. Upon cell division lineage profiles significantly changed as already indicated by PCA analysis. Unexpectedly daughter cells expressed many out of the tested stem cell genes (*Tek*, *Prnp*, *Hoxb4*, *Bmi1*, *Ly6a (*Sca-1*), Cd34*, *Evi1*, *Cdkn1c*) at higher frequencies than their precursors, although some stem cell genes such as *Cd34* have been shown to be induced in culture by previous studies (Sato et al., 1999). The expression of erythroid and myeloid genes remained largely constant. An upregulation of lymphoid transcripts (*Tcfe2a*, *sIgH*, *Ikzf1*) was observed in the daughter cells, which likely makes them more responsive to survival and growth signals in OP9DL1 co-cultures and results in increased T cell readouts (Fig. 5.2F). The expression pattern of Mk associated genes remained largely constant during cell division with *Vwf*$^+$ daughters being more Mk primed than the *Vwf*$^-$. Strong upregulation, however, was observed for *Itgb3* (CD61) in both daughter populations (Fig. 5.3C). The expression of *Vwf*, as a transcript associated with the Mk lineage, correlated well with the EGFP reporter expression for stem cells as well as their progeny. Almost all daughter cells maintained (90.4 %) or only marginally changed (6.1 %) EGFP reporter expression compared to their HSC precursors (Fig. 5.3B).


A
B
Vwf+ parent
Vwf- parent
Daughter of Vwf+
Daughter of Vwf-
PC 1
PC 2
#cells
Vwf transcript expression Δct rel. to Ubc

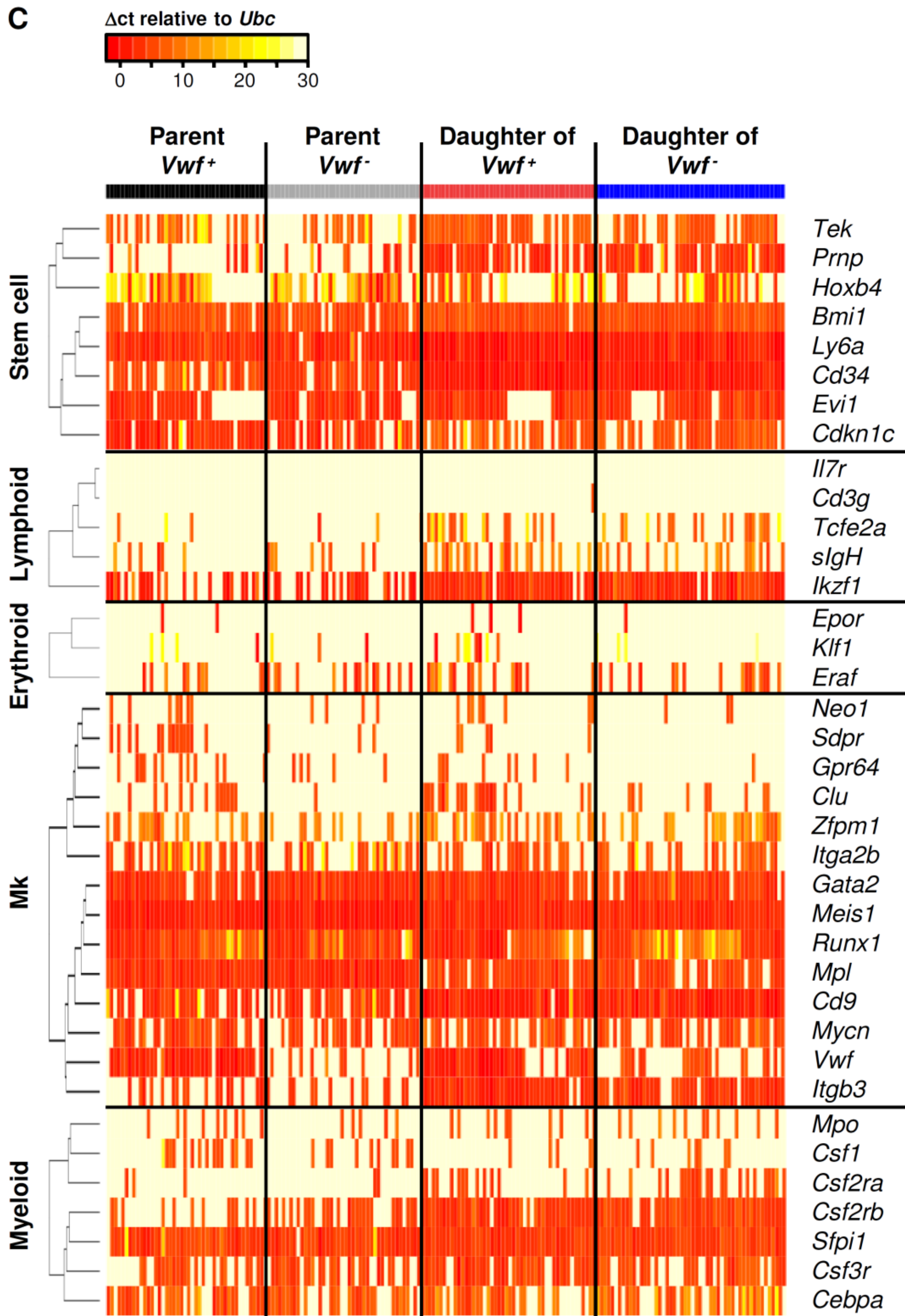


Figure 5.3: Transcriptional lineage priming of HSCs and their immediate progeny

Quantitative expression analysis of *Vwf*$^+$ (45 cells) and *Vwf*$^-$ (45 cells) HSCs, as well as immediate progeny of *Vwf*$^+$ (48 cells) and *Vwf*$^-$ (52 cells) HSCs for a list of 48 transcripts covering genes associated with different haematopoietic lineages, stem cells and

housekeeping genes. Data are presented as ΔC_t with expression of each gene normalised to the expression of *Ubc.*

(A) Principle component analysis (PCA) based on all genes in all cells with the exception of housekeeping genes and genes with high background in non-template controls. Each data point represents one individual cell, and the data are combined from cells isolated and analysed in three individual experiments.

(B) *Vwf* transcript expression in each of the four populations analysed, where EGFP signals were detected in HSCs by flow cytometry and in daughter cells by fluorescence microscopy. ΔC_t values for transcripts with $\Delta C_t > 30$ were treated as undetected transcripts and set to 30.

(C) Expression of transcripts associated with stem cells (8 genes), lymphoid lineage (5 genes), erythroid lineage (3 genes), Mk lineage (14 genes) and myeloid lineage (7 genes). ΔC_t values for transcripts with $\Delta C_t > 30$ were treated as undetected transcripts and set to 30.

5.2.2. *Vwf*$^+$ HSC maintain high Mk potential through frequent symmetric cell division

Vwf$^+$ HSCs generate Mk containing colonies at higher frequencies than their negative counterparts, a difference that is maintained in their immediate progeny. In order to investigate how lineage potentials are propagated during cell division paired progenitors were analysed in detail. Although T lymphoid potential could only be read out from about 40 % of progeny from *Vwf*$^+$ as well as *Vwf*$^-$ HSCs, the majority of T cell potential was segregated symmetrically from both populations (Table 5.1). The analysis of GM and Mk potentials revealed different division patterns for *Vwf*$^+$ and *Vwf*$^-$ stem cells. While 80 % of *Vwf*$^+$ HSCs symmetrically segregated GM and Mk potentials, only about 64 % of *Vwf*$^-$ HSC divided symmetrically. Moreover, 60 % of *Vwf*$^+$ daughter pairs maintained Mk potential, whereas this was reduced to 34 % for *Vwf*$^-$ cells. Conversely, symmetrical loss

of Mk potential was detected for 29 % of daughter pairs from *Vwf*$^{-}$ HSCs, compared to 20 % for the *Vwf*$^{+}$ subset. The asymmetric segregation of Mk potential was observed for 13 % *Vwf*$^{+}$ HSCs. Interestingly this was more frequently detected for *Vwf*$^{-}$ HSCs, of which 22 % divided asymmetrically (Table 5.2). Thus, these results suggest that *Vwf*$^{+}$ HSC maintain the potential to generate Mk colonies through symmetric segregation. *Vwf*$^{-}$ HSC not only often lose Mk potential through symmetric cell division but also show more frequent asymmetric cell divisions. Mk commitment, associated with the loss of GM potentials, was rarely observed and at higher frequencies for *Vwf*$^{-}$ HSCs (5 %) compared to *Vwf*$^{+}$ HSCs (4 %) (Table 5.2). Therefore these results suggest that the Mk bias of *Vwf*$^{+}$ HSC is a result of increased maintenance of Mk potential rather than early Mk commitment.

Table 5.1: T lymphoid potential of paired daughter cells

Parent cell	T cell potential		No. of pairs	
	One	The other		
Vwf^{+}	T	T	6	37.5%
	neg.	neg.	7	43.8%
	total symmetric pairs		13	81.3%
	T	neg.	3	18.8%
	total pairs		**16**	**100.0%**
Vwf^{-}	T	T	5	33.3%
	neg.	neg.	8	53.3%
	total symmetric pairs		13	86.7%
	T	neg.	2	13.3%
	total pairs		**15**	**100.0%**

The table displays the total number of pairs investigated in 2 independent experiments.

Table 5.2: GM/Mk potentials of paired daughter cells

Parent cell	Differentiation potential		No. of pairs	
	One	The other		
Vwf$^+$	GMMK	GMMK	31	56.4%
	GM	GM	11	20.0%
	MK	MK	2	3.6%
	total symmetric pairs		44	80.0%
	GMMK	GM	7	12.7%
	GMMK	neg.	2	3.6%
	GM	neg.	1	1.8%
	MK	neg.	1	1.8%
	total asymmetric pairs		11	20.0%
	total pairs		**55**	**100.0%**
Vwf$^-$	GMMK	GMMK	17	28.8%
	GM	GM	17	28.8%
	MK	MK	3	5.1%
	neg.	neg.	1	1.7%
	total symmetric pairs		38	64.4%
	GMMK	GM	11	18.6%
	GMMK	neg.	2	3.4%
	GM	MK	2	3.4%
	GM	neg.	4	6.8%
	MK	neg.	2	3.4%
	total asymmetric pairs		21	35.6%
	total pairs		**59**	**100.0%**

The table displays the total number of pairs investigated in 3 independent experiments.

5.2.3. Key lineage determinants are asymmetrically expressed in paired daughter cells

Several previous studies have aimed to address the long standing question of whether haematopoietic cells and in particular stem cells are able to divide asymmetrically by analysis of paired progeny/daughters arising from cell division. Due to high heterogeneity of colony readouts it has not yet been possible to obtain conclusive results (Ema et al., 2000; Suda et al., 1984a; Takano et al., 2004). While in this study it was valid to make comparisons between *Vwf*$^+$ and *Vwf*$^-$ subsets of HSCs, it was not possible to sufficiently address the question of asymmetric cell division, and in agreement to previous reports highly heterogeneous colony readouts were obtained. Although the use

of fate reporters could help to overcome this problem, in the current study attempts to utilise *Vwf*-EGFP as indicator for Mk commitment were not successful, and additional candidate genes are likely needed to investigate this question. In order to find new potential reporters for *in vitro* paired daughter fate mapping a total of 50 daughter pairs from *Vwf*$^{+}$ as well as *Vwf*$^{-}$ stem cells were analysed for asymmetric expression of lineage specific genes. The most frequent differentially expressed genes include the key lineage determinants *Cebpa*, which has been suggested to instruct myeloid fate, *Zfpm1*, important for Mk/E lineage commitment, as well as *Tcfe2a* a regulator of early lymphoid specification (Dias et al., 2008; Iida et al., 2008; Jones et al., 2009; Kee, 2009; Mancini et al., 2012; Mercer et al., 2011; Semerad et al., 2009; Wolfler et al., 2010). High asymmetry was also detected for a group of genes with specific roles for stem cell maintenance and function including *Hoxb4* and *Cdkn1c* (Antonchuk et al., 2002; Bjornsson et al., 2003; Brun et al., 2004; Hills et al., 2011; Matsumoto et al., 2011). The transcript encoding *Numb*, which has been associated with asymmetric expression in paired daughter cells and is asymmetrically distributed during cell division was also shown to have highly asymmetric expression between pairs of daughter cells in the present study (Wu et al., 2007). In addition asymmetric expression was observed for *sIgH*, an untranslated lymphoid specific transcript produced by the Ig locus prior to rearrangement (Fig. 5.4). Although there was no asymmetric expression of overall lineage programs detected in paired daughter cells the differential expression of key lineage determinants or stem cell genes could trigger the initiation of the downstream lineage fate. Most interestingly reporters for these genes could be used to monitor such fate decisions in paired daughter cells and help to address the long standing question of asymmetric division of HSCs.

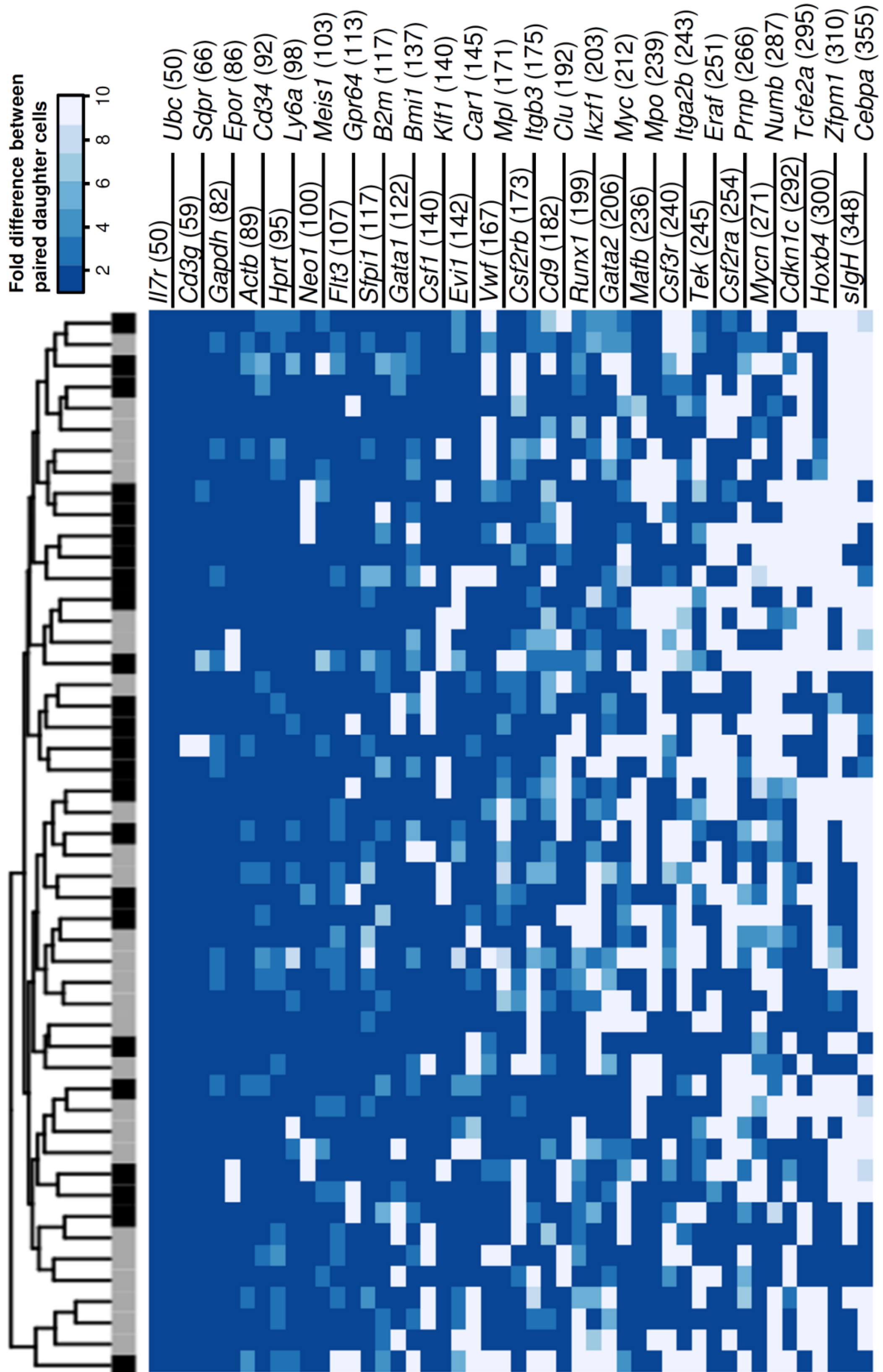

Fold difference between paired daughter cells
2
4
6
8
10
Il7r (50)
Ubc (50)
Cd3g (59)
Sdpr (66)
Gapdh (82)
Epor (86)
Actb (89)
Cd34 (92)
Hprt (95)
Ly6a (98)
Neo1 (100)
Meis1 (103)
Flt3 (107)
Gpr64 (113)
Sfpi1 (117)
B2m (117)
Gata1 (122)
Bmi1 (137)
Csf1 (140)
Klf1 (140)
Evi1 (142)
Car1 (145)
Vwf (167)
Mpl (171)
Csf2rb (173)
Itgb3 (175)
Cd9 (182)
Clu (192)
Runx1 (199)
Ikzf1 (203)
Gata2 (206)
Myc (212)
Mafb (236)
Mpo (239)
Csf3r (240)
Itga2b (243)
Tek (245)
Eraf (251)
Csf2ra (254)
Prnp (266)
Mycn (271)
Numb (287)
Cdkn1c (292)
Tcfe2a (295)
Hoxb4 (300)
Zfpm1 (310)
sIgH (348)
Cebpa (355)

Figure 5.4: Asymmetric gene expression in paired progeny

Analysis of differential expression within daughter pairs from *Vwf*$^{+}$ and *Vwf*$^{-}$ HSC. The heatmap displays the fold difference of expression for each gene between two daughter cells of the same pair calculated based on the $\Delta\Delta C_t$ of the respective pair relative to *Ubc*. Fold differences >10 were set to 10. Genes were ranked according to the cumulative fold difference within all daughter pairs. Numbers in brackets indicate the cumulative fold change for each gene. Daughter pairs of which one or both cells expressed less than 30 % of all genes were excluded from the analysis. The colour bar at the top of the heatmap indicates the phenotype of the parent cell. Daughter pairs derived from *Vwf*$^{+}$ HSC (black) and *Vwf*$^{-}$ HSC (grey).

5.3. Discussion

Mk biased *Vwf*$^{+}$ HSC have been studied as a population but their functional and molecular heterogeneity has not been explored in single cells. Asymmetry of stem cell divisions has been subjected to much speculation but remains to be conclusively proven. In this chapter *Vwf*$^{+}$ and *Vwf*$^{-}$ HSCs and their immediate progeny were analysed at the single cell level in an attempt to evaluate the heterogeneity within the population in particular as well as the symmetric versus asymmetric characteristics of HSC cell divisions in general.

The *Vwf*$^{+}$ subset of HSCs has been shown to represent a Mk primed stem cell population that preferentially gives rise to platelets in the blood after competitive transplantation of 50 purified cells. Since previous experiments were performed on the population level, they do not address the potential inherent heterogeneity of *Vwf*$^{+}$ stem cells (Sanjuan-Pla A., Nerlov C., Jacobsen S.E. manuscript submitted). In addition recent reports suggest that HSCs can be classified as having four different, hierarchically related subsets, each with distinct, cell intrinsic lineage reconstitution bias.

These studies accounted only for lymphoid and myeloid lineage outputs, but nevertheless suggest a high heterogeneity within the HSC population (Dykstra et al., 2007; Morita et al., 2010). The present work showed that *Vwf*$^{+}$ HSCs contain an increased frequency of cells that give rise to Mk containing colonies, while total colony potential or T cell generation were comparable to *Vwf*$^{-}$ HSCs. In agreement *Vwf*$^{+}$ and *Vwf*$^{-}$ cells expressed similar frequencies of genes associated with myeloid, lymphoid and erythroid lineages as well as stem cell genes, whereas most but not all *Vwf*$^{+}$ stem cells expressed a higher number of Mk associated genes. The results outlined in this chapter confirm previous *in vivo* reconstitution experiments, where *Vwf*$^{+}$ and *Vwf*$^{-}$ stem cells similarly reconstituted myeloid and lymphoid lineages, whereas *Vwf*$^{+}$ cells generated significantly higher platelet reconstitution (Sanjuan-Pla A., Nerlov C., Jacobsen S.E. manuscript submitted). In addition, the present study suggests that this increased platelet output is the result of a higher frequency of Mk primed cells in the *Vwf*$^{+}$ population.

There have been a number of insights which suggest a molecular or regulatory relationship between HSCs and the Mk lineage. Both have a critical requirement for the megakaryocytopoietic cytokine TPO, and express an overlapping set of surface markers as well as key transcription factors. This close phenotypic relationship led to the hypothesis that HSCs might be able to directly commit to Mk restricted progenitors via a so far unknown cellular pathway (Huang and Cantor, 2009). Therefore the Mk primed *Vwf*$^{+}$ HSCs could serve as the candidate population capable of such commitment. In support of this, increased Mk priming was also observed in the direct progeny of *Vwf*$^{+}$ HSCs. Detailed analysis of daughter pairs revealed that *Vwf*$^{+}$ HSCs are more prone to symmetric cell division associated with maintenance of Mk and GM potentials, whereas *Vwf*$^{-}$ HSCs more frequently lost Mk potential in one or both of the daughter cells. Therefore this data suggests that the increased Mk colony output *in vitro* as observed in the present study, or the previously observed Mk reconstitution bias of *Vwf*$^{+}$ HSC, is due

to the better maintenance of Mk potential during cell division rather than higher frequencies of Mk commitment.

Although all stem cells by definition carry the potential to generate lymphoid cells, single cell transplantation experiments demonstrated that a fraction of HSCs give only low level lymphoid reconstitution *in vivo* (Dykstra et al., 2007; Morita et al., 2010). Similarly in this study, although LSKCD150^{+}CD48^{-} phenotypic HSC represent a highly enriched stem cell population, only about 10 % of single cells produced T cells in culture. However, in line with results from previous *in vivo* reconstitution experiments, no difference in T cell generation was observed between *Vwf*$^{+}$ or *Vwf*$^{-}$ HSC. The T cell readout from immediate progeny of both stem cell populations increased to 40 %, which was also accompanied with the upregulation of lymphoid transcripts. Thus, the one day culture period for generation of paired progenitors was sufficient for the cells to activate a lymphoid program that makes them significantly more responsive to the survival and growth signals present in T cell promoting cultures. This stands in agreement with previous reconstitution experiments, where the fraction of HSCs that lacked lymphoid output was reduced from 27 % to 7 % when the cells were cultured for 4 days before transplantation (Dykstra et al., 2007). Interestingly, in the present study 80 % of T cell readouts from progeny were symmetrical with more than 30 % of daughter pairs generating T cells. Therefore, intrinsic ability to generate T cells in culture must be already present in the parent cells and, in support of an underlying symmetric cell division, is symmetrically propagated to the daughters.

The evaluation of lineage potentials in functional assays performed in the present study generally supports the existence of symmetric stem cell divisions in culture. In agreement with previous reports asymmetric readouts, particularly asymmetric Mk colony formation, were also frequently observed. However, due to the heterogeneity of the readouts obtained in the present as well as previous studies and the nature of

utilised culture assays, it cannot be ruled out that these asymmetries are the result of inefficient potential detection from one of the two daughter cells in a respective pair (Ema et al., 2000; Takano et al., 2004). In further support of symmetric cell division detailed analysis for differential expression of lineage associated transcripts showed no overall trends for asymmetry. However, the present study only evaluated a very limited number of genes and global gene expression data would allow more definitive conclusions. The frequency of asymmetric expression for the here analysed genes was very heterogeneous. While some transcripts, including housekeeping genes, were detected at uniform levels, other genes including the key lineage determinants *Cebpa*, *Zfpm1* and *Tcfe2a*, the stem cell genes *Cdkn1c* and *Hoxb4* as well as *Numb* and *sIgH* were highly differentially expressed. *Cebpa* is a key regulator of the GM lineage, while *Zfpm1* and *Tcfe2a* are important for Mk/E and lymphoid lineages, respectively (Dias et al., 2008; Iida et al., 2008; Jones et al., 2009; Kee, 2009; Mancini et al., 2012; Mercer et al., 2011; Semerad et al., 2009; Wolfler et al., 2010). Interestingly, a recent study specifically reported the interaction between *Cebpa* and *Zfpm1* as determinant for GM versus Mk/E lineage choice downstream of CMPs (Mancini et al., 2012). Whether both factors have a similar role in stem cells will have to be evaluated in future investigations. The product of *Tcfe2a* has been extensively described in the regulation of B cell development where it initiates a cascade of transcription factors including EBF and PAX5 that eventually leads to B cell restriction (Busslinger, 2004; Kee, 2009). However, *Tcfe2a* has also been implicated in the development of other lymphoid lineages and most interestingly has recently been shown to promote the development of LMPPs (Dias et al., 2008). Thus, although within one cell division there was no overall lineage specific pattern of transcriptional priming observed, *Cebpa*, *Tcfe2a* and *Zfpm1* might initiate lineage programs that become more evident further downstream. Therefore future analyses should in addition to paired daughters also include granddaughter cells or even later progeny that might present more global changes in lineage programs. The stem cell genes *Hoxb4* and *Cdkn1c* have been associated with HSC regulation. *Hoxb4* has been

shown to be specifically important for stem cell proliferation and expansion, while *Cdkn1c* has an important role in HSC maintenance and promotes stem cell quiescence (Antonchuk et al., 2002; Bjornsson et al., 2003; Brun et al., 2004; Hills et al., 2011; Matsumoto et al., 2011). Thus, their differential expression in stem cell derived paired daughters points to an asymmetric self-renewal division. Importantly the screening for asymmetry genes in the present study also detected *Numb*, which has been implicated in asymmetric cell division by several other studies before, therefore validating this experimental approach (Wu et al., 2007). *Numb* has been described as an adaptor protein and inhibitor of Notch signalling with important functions in neuronal development; however, its specific function in asymmetric cell division in haematopoiesis remains largely unknown (Guo et al., 1996; Wakamatsu et al., 1999). Surprisingly, the list of most asymmetric genes also included *sIgH*, a transcript that indicates lymphoid priming but has no reported function itself (Magor et al., 1999). Therefore its asymmetric expression is most likely caused by one or more other lymphoid regulators, but nevertheless it might indicate lymphoid differentiation. Most asymmetric genes described in this study might not only be key regulators of lineage commitment or stem cell self-renewal, but could be utilised as reporters for future fate mapping studies. Thereby such reporters could serve as early fate indicators in paired progenitors, enrich for a certain cellular lineage fate, and thereby overcome the problem of heterogeneous readouts observed in the current and previous studies (Ema et al., 2000; Takano et al., 2004). Eventually such experiments could prove the existence of asymmetric stem cell divisions.

In conclusion, this study demonstrated *Vwf*$^{+}$ HSCs to have superior Mk colony forming potential and to express higher frequencies of Mk associated transcripts compared to *Vwf*$^{-}$ HSCs. This confirms previous findings of an *in vivo* Mk reconstitution bias as well as increased Mk priming of the *Vwf*$^{+}$ stem cell population. Analysis of paired daughter cells suggests that Mk biased *Vwf*$^{+}$ HSCs better maintain Mk potential during cell

division in a multipotent state rather than directly committing to the Mk lineage. Finally, although most results supported symmetric stem cell division, detailed transcriptional priming analysis detected the asymmetric expression of key lineage determinants and stem cell regulators. These genes might serve as candidate reporters for future studies of stem cell division by paired daughter fate mapping.

6. Discussion

The alternative, or myeloid based, model of lineage commitment describes the bifurcation of lympho-myeloid and myeloid-Mk-E lineage pathways as the earliest commitment event and has been supported by multiple reports over the last decade, including the prospective identification of LMPPs and CMPs as the corresponding primitive progenitor populations. However, several aspects of this lineage commitment process are poorly understood. Little is known about the emergence of primitive stem and progenitor cells during embryonic development; similarly, the early fate decisions made in primitive stem and progenitor cells in adult haematopoiesis requires further investigation. The studies described in this thesis attempted to evaluate the early lympho-myeloid pathway; its emergence in the embryo as well as its progression in neonatal thymopoiesis. Moreover, this study sought to elucidate early megakaryopoiesis and aimed to trace the fate of haematopoietic stem cells, as the origin of both lympho-myeloid as well as myeloid-Mk-E pathways, in the adult.

6.1. Embryonic lymphopoiesis is initiated prior to the emergence of definitive HSCs

Embryonic haematopoiesis emerges in two distinct waves, a primitive wave that appears around embryonic day 7 and is transient, as well as the definitive wave that originates from definitive HSCs, is first established at day 10.5 and maintained throughout life (Dzierzak and Speck, 2008). The majority of the first haematopoietic cells are of erythroid nature, but cells of the myeloid as well as Mk lineage are also part of primitive haematopoiesis (Medvinsky et al., 2011; Palis et al., 1999). Lymphoid cells on the other

hand have only been detected at later stages during development, and are therefore thought to be part of definitive haematopoiesis and derived from definitive HSCs. Similarly lympho-myeloid restricted cells that were able to generate B and/or T cells as well as macrophages have only been retrospectively identified at day E12 or at later stages in the foetal liver (Cumano et al., 1992; Kawamoto et al., 1997; Lacaud et al., 1998; Lu et al., 2002; Mebius et al., 2001). The present work established and for the first time prospectively isolated such lympho-myeloid progenitors from the E11.5 foetal liver, and conclusively demonstrated its lympho-myeloid restriction at the functional as well as molecular level (Chapter 2).

Several lines of evidence suggest the existence of lymphoid potential in the embryo at much earlier stages and even before the emergence of first definitive HSCs. Immature or pre-definitive HSCs, that are not able to reconstitute irradiated adult hosts and therefore do not qualify as definitive HSCs, are multipotent and can generate lymphoid cells in culture or when transplanted into immunocompromised or neonatal recipients (Bertrand et al., 2005; Cumano et al., 1996; Cumano et al., 2001; Godin et al., 1995; Kieusseian et al., 2012). Low level transcripts of the lymphoid regulators IL-7Rα and RAG1, that are expressed in adult LMPPs but not HSCs, can already be detected in the embryo at the time of definitive stem cell emergence, suggesting the activation of the lymphoid pathway at that early stage (Adolfsson et al., 2005; Hu et al., 1997; Kawamoto et al., 2000; Mombaerts et al., 1992; Ng et al., 2009; Peschon et al., 1994a; Yokota et al., 2006). Moreover, a recent study reported the purification of progenitors with T lineage potential from the E9.5 yolk sac. These cells generated mature T cells in culture or *in vivo* after transplantation (Yoshimoto et al., 2012). In agreement with the above results by using IL-7Rα and *Rag1* as markers this study identified a lympho-myeloid restricted progenitor in the E10.5 foetal liver as well as the E9.5 yolk sac that has properties similar to its E11.5 equivalent (Chapter 2). This lympho-myeloid progenitor is likely derived from pre-definitive HSCs and might serve as the precursor for the described T cell progenitor

in the E9.5 yolk sac. As such it would be the earliest known embryonic progenitor of the lymphoid pathway.

6.2. Lympho-myeloid progenitors significantly contribute to steady state myelopoiesis in the embryo

The findings in this study clearly establish the existence of a lymphoid-myeloid restricted progenitor in the embryo resembling the adult LMPP, that has been confirmed by multiple reports since its first discovery (Adolfsson et al., 2005; Arinobu et al., 2007; Lai and Kondo, 2006; Luc et al., 2008a; Mansson et al., 2007; Yoshida et al., 2006). Nevertheless the LMPP as well as the alternative model of haematopoiesis remain challenged by studies supporting the classical haematopoietic roadmap, which is still accepted in the literature (Akashi et al., 2000; Boyer et al., 2011; Forsberg et al., 2006; Kondo et al., 1997). In particular recent studies have questioned the physiological role of LMPPs in myelopoiesis (Schlenner et al., 2010; Schlenner and Rodewald, 2010; Welner et al., 2009). While it has been unequivocally demonstrated that LMPPs readily give rise to myeloid cells in *ex-vivo* cultures, their contribution to the myeloid lineage under steady state conditions *in vivo* remains less certain (Adolfsson et al., 2005; Schlenner and Rodewald, 2010). *In vivo* fate mapping experiments with *Il7rα* or *Rag1*, which are both expressed by adult LMPPs, observed only a minor contribution of labelled and therefore *Il7rα*$^+$ or *Rag1*$^+$ cell derived myeloid cells (Schlenner et al., 2010; Schlenner and Rodewald, 2010; Welner et al., 2009). However, these markers are only expressed by a small fraction of adult LMPPs, which also have low myeloid potential *in vitro* (Luc et al., 2008a). On the contrary all embryonic lympho-myeloid progenitors are positive for IL-7Rα as well as *Rag1*. In this study *Rag1* fate mapping demonstrated significant contribution of *Rag1*$^+$ foetal lympho-myeloid progenitors to embryonic steady state myelopoiesis. In agreement with previous studies myeloid contribution decreased in older embryos and labelled myeloid cells were barely if at all detected in the adult

(Chapter 2). This might however reflect the restriction of *Rag1* expression within the lympho-myeloid progenitor population during aging rather than a reduction of myeloid contribution of by these cells. A definitive prove of LMPP derived steady state myelopoiesis in the adult will require fate mapping studies using more LMPP specific markers, which might be identified through global gene profiling.

6.3. ETPs and lympho-myeloid progenitors as potential link between foetal liver, bone marrow and thymus

The final stages of T cell development take place in the thymus that provides a specific microenvironment and cell extrinsic signals for the generation of naïve effector T cells. Thymopoiesis is maintained through constant replenishment with progenitors from the bone marrow (Donskoy and Goldschneider, 1992; Scollay et al., 1986). Although several candidate TSPs have been proposed its identity remains as yet unknown (Bhandoola et al., 2007). This study characterised the ETP as the earliest cell in the thymus, that is likely to resemble the cellular characteristics of the TSP. ETPs sustain B, T as well as myeloid potential at the single cell level and therefore resemble the LMPP in the adult bone marrow as well as the herein identified embryonic lympho-myeloid progenitor (Chapter 2, 3). Moreover, molecular characterisation and comparison of all 3 populations supports adult LMPPs as well as embryonic lympho-myeloid progenitors as potential TSPs (Chapter 2) (Luc et al., 2012).

6.3.1. ETPs sustain B, T as well as myeloid lineage potentials

ETPs are defined as the earliest T cell progenitors in the thymus and in addition to T cell potential they have been shown to generate NK cells, dendritic cells as well as myeloid cells. ETPs have also been shown to produce B cells *in vitro* as well as *in vivo*, although this has only been detected at very low frequencies (Benz and Bleul, 2005; Sambandam et al., 2005). The presence of B cell potential in the thymus has been supported by

studies of mice deficient for Notch signalling. The absence of Notch as key driver of T cell and inhibitor of B cell development resulted in accumulation of large numbers of B cells in the thymus (Radtke et al., 1999; Wilson et al., 2001). Accordingly, ETPs might give rise to B cells rather than commit to T cell development. However, in this setting it could not be excluded that B cells entered the thymus from the periphery. Moreover, two recent studies that investigated the lineage potentials of ETPs at the single cell level detected a large fraction of these cells to have T as well as myeloid potentials, but failed to observe any B cell generation (Bell and Bhandoola, 2008; Wada et al., 2008). By investigating ETPs from neonatal mice, this study demonstrated at the single cell level that ETPs possess T and myeloid potentials but also sustain significant B cell potential (Chapter 3). In addition ETPs were shown to lack Mk potential, suggesting that the thymus is not seeded by HSCs, and pointing towards the LMPP, that resembles the ETP's lineage potentials, as a candidate TSP. In experiments beyond this thesis work it was confirmed that ETPs lack Mk as well as erythroid potentials and detailed global gene expression analysis indicated its lympho-myeloid restriction (Luc et al., 2012).

6.3.2. Adult and foetal lympho-myeloid progenitors are candidates TSPs

Although the exact identity of the TSP remains elusive several candidates including stem cells as well as multipotent and lymphoid restricted progenitors have been proposed (Bhandoola et al., 2007). The detailed characterisation of the ETP that retains B, T as well as myeloid but not Mk/E potentials allows further conclusions about the identity of the TSP (Chapter 3) (Luc et al., 2012). Since the thymus does not contain any long term reconstitution potential and ETPs do not possess any Mk/E potential, the thymus is unlikely seeded by stem cells, unless they undergo immediate commitment upon thymus entry. According to the classical haematopoietic roadmap CLPs give rise to B and T lymphocytes (Kondo et al., 1997). CLPs have been shown to also possess myeloid potential, and therefore qualify as precursors of ETPs (Ehrlich et al., 2011). However, CLPs are less proliferative than ETPs and express lower levels of the primitive markers

c-Kit and Sca-1. Furthermore, in loss of function studies of the transcription factor Ikaros, CLPs were completely absent, whereas ETPs remained largely unaffected, suggesting the CLP independent development of ETPs (Allman et al., 2003). The adult LMPP is therefore the currently best candidate TSP and not only resembles the ETPs lineage potentials but has also been shown to be more primitive and proliferative (Schwarz et al., 2007). Moreover adult LMPPs express the chemokine receptors CCR7 and CCR9 that are required for thymus seeding, and deficiency for both receptors results in severe reductions of ETPs (Lai and Kondo, 2007; Zlotoff et al., 2010). In further support of thymus seeding by LMPPs detailed global transcriptional priming analysis demonstrated a close molecular relation between ETPs and LMPPs (Luc et al., 2012). During embryonic development the thymus is first seeded at around E11.5 most likely by foetal liver progenitors (Owen and Ritter, 1969). The lympho-myeloid progenitors identified herein were already present in the foetal liver at E10.5. Similar to adult LMPPs they were characterised by the expression of transcripts for *Ccr7* as well as *Ccr9* and primed for members of the Notch signalling pathway (Chapter 2). Thus, this lympho-myeloid progenitor is a candidate TSP for initial thymus seeding as well as maintenance of embryonic thymopoiesis.

6.4. Early Mk development in the adult bone marrow

The alternative haematopoietic roadmap implies the early separation of lympho-myeloid and myeloid-Mk-E pathways. The first part of this work investigated the development of the lympho-myeloid lineage, which is accompanied by the loss of Mk/E potentials. The second part evaluated the Mk lineage pathway and provided evidence compatible with the existence of a primitive Mk progenitor. Further it evaluated an Mk biased *Vwf*$^+$ stem cell subset at the single cell level and showed that a significantly higher frequency of *Vwf*$^+$ HSCs generate Mk colonies, express Mk associated genes and *Vwf*$^+$ HSCs are more likely to maintain Mk potential after cell division.

6.4.1. Evidence compatible with the existence of a primitive Mk progenitor in the LSK bone marrow compartment

Unilineage restricted Mk progenitors as well as progenitors restricted to Mk and erythroid lineages have primarily been identified in the c-Kit$^+$ but Sca-1$^-$ bone marrow compartment, which contains less primitive progenitor populations. In particular Mk progenitors that were previously identified based on the expression of combinations of the surface markers CD150, CD41 and CD9 have low proliferative potential (Nakorn et al., 2003; Ng et al., 2012; Pronk et al., 2007). However, several lines of data would be compatible with the existence of a more primitive, more proliferative Mk progenitor that most likely resides in the LSK bone marrow subset of stem and early progenitor cells. Firstly the Mk lineage shares several properties with HSCs, that include common extrinsic regulators such as TPO, but also multiple surface markers and key transcription factors, which suggest a close developmental link between both (Huang and Cantor, 2009). Secondly, the *Vwf*$^+$ Mk biased HSC subset characterised herein could serve as precursor with the ability to give rise to such an early Mk progenitor (Chapter 5). Finally, a recent study identified a new more primitive CMP than previously described using a GFP reporter for *Gata1*. While the majority of CMPs generated mixed colonies containing a combination of myeloid, Mk and erythroid cells, nearly 40 % of all colonies were purely megakaryocytic and potentially derived from Mk restricted progenitors within that population (Arinobu et al., 2007). Herein, a population with high Mk potential that expressed intermediate to positive but not high levels of a new *Gata1*-GFP reporter was shown to be primarily located in the LSKCD150$^+$CD48$^+$ bone marrow compartment. The presence of Mk restricted cells within this population was supported by the expression of Mk associated genes. However, although erythroid transcripts were only expressed at low levels, erythroid potential was not functionally evaluated in this study and it remains possible that these putative Mk progenitors also sustain erythroid potential (Chapter 4). The LSKCD150$^+$CD48$^+$*Gata1*^{pos} population also sustained low T lymphoid as well as significant myeloid potential. Thus, further sub-fractionation using more specific surface

markers or transcriptional reporters will be required to purify this putative primitive Mk progenitor. Promising candidates include the surface markers CD9 or CD41, that are also expressed on known more mature Mk progenitors, or Mk associated transcription factors such as GATA2 or FOG1 (Huang and Cantor, 2009; Mancini et al., 2012; Nakorn et al., 2003; Ng et al., 2012; Tsang et al., 1997a; Tsang et al., 1997b). Furthermore transcriptional lineage priming analysis of single LSKCD150^{+}CD48^{+}*Gata1*^{pos} cells would give additional information about the heterogeneity within the population and the potential presence of Mk restricted cells.

6.4.2. The majority of residual Mk potential in the LMPP is restricted to a small *Gata1*$^{+}$ subset

The discovery of the adult LMPP has been a major contribution to the establishment of the alternative or myeloid based roadmap (Adolfsson et al., 2005). Its existence has been confirmed by multiple studies in the adult and the present work established an equivalent lympho-myeloid progenitor during embryonic haematopoiesis (Chapter 2) (Arinobu et al., 2007; Yoshida et al., 2006). However, many studies have challenged these results in support of the classical haematopoietic roadmap, which is still prevailing in the literature (Boyer et al., 2011; Forsberg et al., 2006). The LMPP has mainly been questioned based on its small residual Mk/E potential suggesting it to be a multipotent rather than lympho-myeloid restricted progenitor. Previous studies purified subsets of LMPPs based on the expression of Mpl or VCAM1, which had significantly reduced Mk/E potentials (Lai and Kondo, 2006; Luc et al., 2008a). However, it remained uncertain whether the majority of original LMPPs contain low Mk/E potentials or all Mk/E potential is confined to a small subset of LMPPs. In this study by using a GFP reporter for *Gata1*, a small fraction of LMPPs was identified to carry the majority of Mk potential. Accordingly, *Gata1*$^{-}$ LMPPs retained only a reduced level of residual Mk potential (Chapter 4). In contrast to previous reports where only small subsets with reduced Mk/E potential were purified, the *Gata1*$^{-}$ LMPP represents nearly the entire original LMPP

population. Purification of the LMPP to a complete absence of Mk/E potential will be subject to future studies and might require a global screen for the identification of new specific markers.

6.4.3. *Vwf*-GFP marks a stem cell subset with superior Mk differentiation potential

The classical haematopoietic road map suggests the existence of a single homogeneous stem cell population with the ability to produce all downstream blood cell lineages. However, retrospective analysis of single cell transplants, and more recently prospective purification of stem cells based on differential expression levels of the surface marker CD150, demonstrated heterogeneity within the HSC population (Benz et al., 2012; Challen et al., 2010; Dykstra et al., 2007; Morita et al., 2010). While HSCs with high levels of CD150 expression were more primitive and preferentially reconstituted the myeloid lineage, lower levels of CD150 were associated with predominant lymphoid reconstitution (Morita et al., 2010). Further subdivision of this $CD150^{hi}$ HSC subset using a reporter for *Vwf* led to the identification of a Vwf^{+} Mk biased stem cell population that gives superior platelet reconstitution *in vivo*, expresses an Mk associated transcriptional signature and is placed upstream of its Vwf^{-} counterpart (Sanjuan-Pla A., Nerlov C., Jacobsen S.E. manuscript submitted). However, Vwf^{+} HSCs were only characterised at the population level and therefore it remained elusive whether or not they represent a heterogeneous population themselves. This study showed that Vwf^{+} HSCs more frequently produce colonies with Mk content in culture when compared to Vwf^{-} HSCs. Moreover most but not all cells expressed a higher number of Mk associated transcripts suggesting different levels of Mk priming within the population.

The similarity between HSCs and cells of the Mk lineage as well as the potential existence of a primitive Mk progenitor led to the hypothesis of a putative direct Mk differentiation pathway from HSCs to Mk committed progenitors (Huang and Cantor, 2009). Mk biased Vwf^{+} HSC could thereby serve as the potential origin of such

commitment pathway. The herein performed analysis of paired progeny indicated that *Vwf*$^{+}$ HSCs more efficiently maintain Mk together with myeloid potentials, while GFP^{-} cells frequently produce pairs of progeny, of which one or both cells have lost the ability to produce Mks. This suggests that *Vwf*$^{+}$ HSCs better sustain Mk potential in a multipotent state rather than undergoing direct Mk commitment. However, the present analysis observed only one HSC division, which was not associated with a distinct change of lineage priming (Chapter 5). A Mk commitment event from HSCs or their progeny could still occur further downstream and its detection might require the observation of multiple generations of daughter cells.

6.5. Asymmetric expression of key lineage determinants in paired progeny of HSCs

In support of primarily symmetric cell divisions of *Vwf*$^{+}$ as well as *Vwf*$^{-}$ stem cells the herein performed transcriptional priming analysis revealed no overall trends or differential expression of lineage programs between paired progenitors. However, this analysis was confined to a small set of lineage associated genes and a more global and comprehensive analysis might be required to detect changing lineage programs. Currently it is not yet possible to obtain high quality global gene expression data from single haematopoietic cells. However, in the near future state of the art techniques such as RNA sequencing should allow this analysis, which has already been achieved for some other cell types (Ramskold et al., 2012).

The analysis of paired progenitors performed herein detected the asymmetric expression of multiple key lineage determinants (*Cebpa*, *Zfpm1*, *Tcfe2a*) as well as stem cell regulators (*Hoxb4*, *Cdkn1c*). The expression of these lineage specific regulators could serve as the first step in a lineage commitment event and as such might instruct lineage choice. In fact *Cebpa* has previously been shown to instruct myeloid fate in early

haematopoietic cells (Wolfler et al., 2010). Similarly, *Zfpm1* and *Tcfe2a* are involved in the early specification of Mk/E or lymphoid lineages and are likely to instruct differentiation towards these lineages (Dias et al., 2008; Kee, 2009; Mancini et al., 2012). Genes encoding other transcription factors that have previously been proposed as key lineage determinants such as GATA1 or PU.1 did not appear to be frequently asymmetrically expressed in the present study (Iwasaki et al., 2003; Nerlov and Graf, 1998). This could reflect that both factors are not involved in early asymmetry or they might function further downstream. The differential expression of *Hoxb4* and *Cdkn1c* might indicate the asymmetric self-renewal of HSCs (Chapter 5). In particular *Hoxb4* has previously been shown to promote stem cell self-renewal and its ectopic expression resulted in *ex-vivo* HSC expansion (Antonchuk et al., 2002).

Asymmetric cell division or more specifically asymmetric self-renewal of HSCs has been proposed to be an important mechanism for simultaneous stem cell maintenance and production of haematopoietic progenitors, but is yet to be conclusively demonstrated. Paired daughter fate mapping has been described as a promising tool for the evaluation of such asymmetric cell divisions. However, although asymmetric colony readouts in the present as well as previous studies would be compatible with the occurrence of asymmetric cell divisions, due to highly heterogeneous readouts these results could not prove the existence of such divisions (Chapter 5) (Ema et al., 2000; Takano et al., 2004). In order to overcome these drawbacks lineage specific reporters could be used to link colony readouts with the fate of paired daughter cells and allow for the detection of a specific lineage commitment event even if it occurs only very infrequently. The herein identified highly asymmetrically expressed key lineage determinants *Cebpa*, *Zfpm1* and *Tcfe2a* are promising candidates for such reporters and might enable the investigation of asymmetric lineage fates of HSCs. Moreover, reporters for the stem cell specific transcripts *Hoxb4* or *Cdkn1c* could serve as markers for self-renewal in such studies (Chapter 5).

6.6. Conclusion

This thesis work provided new insights into early embryonic and adult haematopoietic lineage commitment, explored lympho-myeloid as well as myeloid-Mk-E pathways, and evaluated HSC fate decisions that underlie this lineage bifurcation. Thereby it specifically showed:

An early lympho-myeloid progenitor exists within embryonic haematopoiesis, emerges prior to the first definitive HSCs and significantly contributes to steady state myelopoiesis *in vivo*.

ETPs possess B, T and myeloid potentials, which has implications for thymus seeding, and points to the LMPP as a putative TSP.

The LSKCD150^{+}CD48^{+}*GATA1*^{pos} bone marrow subset is enriched for Mk potential and possibly contains a putative primitive Mk progenitor.

Vwf^{+} single HSCs show heterogeneous Mk priming and frequently propagate Mk potential during cell division.

Key lineage determinants and stem cell regulators are differentially expressed in paired progeny of HSCs and could serve as reporters for future paired daughter fate mapping studies.

7. Material and Methods

7.1. Mice

7.1.1. Mouse husbandry

The animals used in this study were bred and maintained at University of Oxford Biomedical Services. Animal handling as well as all procedures were performed under the UK Home Office Animals Scientific Procedures Act (1986).

7.1.2. Mouse strains

C57BL/6 (CD45.2) or C57B6SJLCD45 (CD45.1) mice were obtained from University of Oxford Biomedical Services. Mice with *Rag1* driven GFP expression were obtained from N. Sakaguchi (Department of Immunology, Kumamoto University School of Medicine, Japan) (Kuwata et al., 1999) and bred and maintained as $Rag1^{GFP/GFP}$ or $Rag1^{GFP/+}$. For experiments only $Rag1^{GFP/+}$ embryos were used and *Rag1*-GFP was always inherited from the male parent to ensure all GFP expressing cells were embryo derived. $Flt3\text{-}Cre^{tg/+}$ mice were provided by C. Bleul (Department of Developmental Immunology, Max-Planck-Institute of Immunobiology, Freiburg, Germany) (Benz et al., 2008). $Rag1\text{-}Cre^{tg/+}$ mice were obtained from T. Rabbits (MRC Laboratory of Molecular Biology, Cambridge, UK) (McCormack et al., 2003). *Rosa26 (R26R)*$^{eYFP/eYFP}$ mice were generated by S. Srinivas (Department of Genetics and Development, Columbia University, New York, USA) (Srinivas et al., 2001). The *Vwf*-EGFP and *Gata1*-EGFP transgenic mouse lines were provided by C. Nerlov (EMBL, Monterotondo, Italy). Both lines were generated via bacterial artificial chromosome engineering and intracytoplasmic sperm injection. For the *Vwf*-EGFP reporter line two founder lines were generated, reporter expression evaluated

by the Nerlov lab and one line was chosen for all following experiments. For the *Gata1*-EGFP reporter only one founder line was generated. All lines were backcrossed to and maintained on a C57BL/6 genetic background.

7.1.3. Generation of embryos

Embryos for experiments were generated by timed mating over night. The next morning mating was confirmed by detection of the vaginal plug. The morning of vaginal plug detection was set as embryonic day 0.5.

7.1.4. Phenotyping of mouse strains

Transgenic identity of *Vwf*-EGFP and *Gata1*-EGFP transgenic mice was determined by EGFP phenotyping of peripheral blood platelets. Transgenic individuals of both strains have platelets labelled by high expression of EGFP. For blood collection mice were briefly warmed up in a heat box and placed in a mouse restrainer. After a small incision in one of the tail veins, a small drop of blood was collected using a capillary and immediately resuspended in 100 µl PBS. EGFP expression was analysed by flow cytometry. Platelets were identified according to their FSC/SCC profile.

7.1.5. Genotyping of mouse strains

Rag1-GFP, *Flt3-Cre*$^{tg/+}$, *Rag1-Cre*$^{tg/+}$ and *Rosa26 (R26R)*$^{eYFP/eYFP}$ were genotyped by polymerase chain reaction on genomic DNA extracted from ear biopsies. DNA isolation was performed using the Phire Animal Tissue Direct PCR Kit (Thermo Fisher Scientific). Ear biopsies were placed into 20 µl Dilution Buffer plus 0.5 µl DNARelease Additive, mixed, briefly vortexed and incubated at room temperature for 2-5 min followed by 2 min incubation at 98 °C. The genomic DNA containing supernatant was diluted 1:10 with H_2O to a total volume of 200 µl.

7.1.6. Polymerase chain reaction

Specifically optimised PCR reactions were run for each genotype. Usually 1-2 µl pre-diluted genomic DNA sample was added to a total reaction volume of 20-25 µl containing a combination of forward and revers primers (final concentration 0.2-0.5 µM, Invitrogen), 0.2-0.4 µl Taq polymerase (Thermo Fisher Scientific, Invitrogen) and either 10 µl 2x PCR buffer with dNTPs (Thermo Fisher Scientific) or 10x buffer, dNTP mix and magnesium chloride (Invitrogen). PCR reactions were performed using a thermal cycler (Tetrad II 96-Well Alpha Unit, Biorad).

Table 7.1: List of primers for genotyping

Strain	Primer name	Primer sequence
Rag1-GFP	Rag forward	AGGTAGCTTAGCCAACATGG
	Rag reverse	CAACATCTGCCTTCACGTCGATCC
	GFP reverse	GCTCAGGTAGTGGTTGTCGG
Flt3-Cre	Flt3 Cre forward	ACGGAGTCCAGGCAACTTCC
	Flt3 Cre reverse	GAAGCATGTTTAGCTGGCCC
Rag1-Cre	Cre forward	CGTTTTCTGAGCATACCTGGA
	Cre reverse	ATTCTCCCACCGTCAGTACG
Rosa26-YFP	RosaWT forward	GGAGCGGGAGAAATGGATATG
	RosaWT revers	AAAGTCGCTCTGAGTTGTTAT
	YFP forward	CGTAAACGGCCACAAGTTCAG
	YFP reverse	GAACTCCAGCAGGACCATGTG

Table 7.2: List of PCR protocols for genotyping

Strain	PCR protocol
Rag1-GFP	1) 95 °C for 5 min 2) 95 °C for 30 s 3) 55 °C for 45 s 4) 72 °C for 1 min (back to 2, 10 cycles) 5) 95 °C for 30 s 6) 52 °C for 45 s 7) 72 °C for 1 min (back to 5, 35 cycles) 8) 72 °C for 2 min
Flt3-Cre	1) 98 °C for 5 min 2) 98 °C for 10 s 3) 68 °C for 5 s 4) 72 °C for 20 s (back to 2, 34 cycles) 5) 72 °C for 1 min
Rag1-Cre	1) 98 °C for 5 min 2) 98 °C for 5 s 3) 58 °C for 5 s 4) 72 °C for 20 s (back to 2, 30 cycles) 5) 72 °C for 1 min
Rosa26-YFP	1) 98 °C for 5 min 2) 98 °C for 5 s 3) 69 °C for 5 s (decrease by 0.5 °C per cycle) 4) 72 °C for 20 s (back to 2, 15 cycles) 5) 98 °C for 5 s 6) 59 °C for 5 s 7) 72 °C for 20 s (back to 5, 20 cycles) 8) 72 °C for 1 min

7.1.7. Agarose gel electrophoresis

PCR products were evaluated on a tris-acetate EDTA (TAE) buffer (prepared in house) based 2 % agarose gel (Invitrogen). DNA fragments were visualised using ethidium bromide stain (1 drop per 50 ml agarose gel, Dutcher Scientific) on a ultra-violet transilluminator.

7.2. *In vivo* assays

7.2.1. *In vivo* repopulation assay

In all experiments donor versus recipient/competitor white blood cells were detected using mice with C57BL/6 (expression CD45.2) versus C57B6SJLCD45 (expressing CD45.1) background. In experiments with *Gata1*-EGFP transgenic donor mice platelet as well as red blood cell reconstitution was evaluated in addition. For this strain all donor derived platelets and red blood cells were labelled $EGFP^+$ and could be distinguished from $EGFP^-$ recipient/competitor cells. Recipient mice were typically conditioned by two doses of 450 cGy irradiation from a cesium-137 source. Within 24 h FACS purified test cells were injected intravenously in the tail along with 250,000 congenic competitor bone marrow cells in a total volume of 200 µl. After irradiation/transplantation mice were monitored daily on days 4-16 and twice weekly thereafter.

7.2.2. Blood collection for multi-lineage reconstitution analysis

At 16-22 weeks after transplantation for long term reconstitution analysis, or after 14 days for short term repopulation experiments, mice were analysed for multilineage peripheral blood reconstitution. Mice were warmed up in a heat box to increase blood circulation in the tail and placed in a sterilized mouse restrainer. After local sterilization a small incision was made into the tail vein and 100-200 µl peripheral blood were collected (never exceeding 10 % of total blood volume) in EDTA-coated collection tubes (Sarstedt).

7.3. Flow cytometric analysis and cell sorting

7.3.1. Preparation of single cell suspension from thymus tissue

Thymi from neonatal or adult mice were harvested in phosphate buffered saline (PBS, Invitrogen) supplemented with 5 % FCS (Thermo Fisher Scientific, always used as additive to PBS), cleaned from fat tissue as well as blood and disrupted by passing the

tissue through a 70 μm cell strainer (Beckton, Dickenson and Company). Cells were resuspended in a defined volume of PBS with 5 % FCS and viable cells were counted in a Neubauer chamber after live/dead stain using trypan blue (Sigma) supplemented with Zap-OGLOBIN II (Beckman Coulter).

7.3.2. Preparation of single cell suspension from adult bone marrow

Femurs, tibias as well as iliac crests were harvested from individual mice, cleaned from excess muscle tissue and placed into PBS with 5 % FCS. Bones were crushed in a mortar and the bone marrow tissue was disaggregated by pipetting through a 70 μm cell strainer (Beckton, Dickenson and Company). Cells were counted after live/dead stain in a Neubauer chamber using trypan blue (Sigma) supplemented with Zap-OGLOBIN II (Beckman Coulter) or a Sysmex haemocytometer (KX-21N, Sysmex).

7.3.3. Preparation of single cell suspension from foetal haematopoietic tissues

Cell suspensions from E8.5 embryos were obtained by collagenase treatment of whole concepti (yolk sac, allantois and embryo proper combined, without the ectoplacental cone). E9.5 yolk sac was dissected without vitelline and umbilical cord or E10.5 foetal livers were dissected and treated with collagenase (type 1, Sigma) at a final concentration of 0.12 % for 10 min at 37 °C. Treated yolk sacs or E10.5 foetal livers were disaggregated by gentle pipetting. E11.5 or E14.5 foetal livers were dissected and single cell suspension was made using a syringe with 27G or 25G needle. All foetal tissues or cells were always handled in Dulbecco's phosphate buffered saline (DPBS) with calcium chloride and magnesium chloride (Invitrogen) with 10 % FCS.

7.3.4. Preparation of cells from mouse peripheral blood

For evaluation of red blood cells 1 μl peripheral blood was diluted and directly used for antibody staining. For platelet preparation peripheral blood samples were spun down at 1000 rpm for 10 min. The platelet containing supernatant was collected. For preparation

of white blood cells the pallet was typically resuspended in PBS with 1 % FCS to a total volume of 300 µl to 500 µl. For red blood cell sedimentation PBS with 2 % dextran (Sigma) was added at a 1:1 ratio followed by 25 min incubation at 37 °C. The white blood cell containing supernatant was collected, cells were washed and remaining red blood cells were lysed by incubation in ammonium chloride solution (1x, NH_4Cl, Stem Cell Technologies) for 1 min.

7.3.5. c-Kit$^+$ cell enrichment

Prior to sorting of haematopoietic stem and progenitor cells from bone marrow or thymic tissue c-Kit$^+$ cells were enriched by magnetic activated cell sorting according to manufacturer's instructions with minor modifications (MACS, Miltenyi Biotec). Single cell suspensions were spun down and resuspended in PBS with 5 % FCS at a concentration of 100 million cells per 100 µl for bone marrow or 300 million cells per 100 µl for thymus. For blocking of Fc receptors in some experiments cells were incubated with purified anti-FcγR antibody (prepared in house) for 10 min. c-Kit microbeads were added at a ratio of 2.5 µl per 100 µl total sample volume followed by incubation for 20 min at 4 °C or on ice while regularly shaking to avoid cell sedimentation. After a washing step to remove excess c-Kit microbeads samples were resuspended in 3 ml PBS with 5 % FCS, filtered through a cell strainer and applied to a pre-equilibrated MACS LS column on a MACS separator (Miltenyi Biotec). Typically not more than 500 million cells were applied per column and multiple columns were used for larger samples. The column was washed 3 times by adding 3 ml PBS with 5 % FCS to remove c-Kit$^-$ cells, while microbead labelled c-Kit$^+$ cells were retained in the magnetic field of the column. In order to elute the c-Kit$^+$ cell enriched fraction the column was removed from the MACS separator and flushed with 5 ml PBS with 5 % FCS using the plunger provided. Typically cells were counted after live/dead stain in a Neubauer chamber using trypan blue (Sigma) supplemented with Zap-OGLOBIN II (Beckman Coulter) or a Sysmex haemocytometer (KX-21N, Sysmex).

7.3.6. Antibody staining for flow cytometric analysis and sorting

For flow cytometric analysis as well as cell sorting the compensation set up of the flow cytometer was performed using single stained CompBeads (Beckton, Dickenson and Company). Typically 1 µl of the respective antibody was mixed with one drop species specific as well as one drop negative control CompBeads and incubated for 15 min at 4 °C. After one washing step with 1 ml PBS with FCS beads were resuspended in 200 µl PBS with FCS for analysis.

Fluorescence minus one (FMO) controls were typically stained in a total volume of 25 µl. Cells were resuspended in 12.5 µl PBS with FCS containing a 1x dilution of purified anti-FcγR antibody (in house production) and incubated for 10 min at 4 °C. Samples for analysis as well as sorting were typically stained in a total volume of 200-500 µl with higher volumes in some cases, never exceeding a cell concentration of 10 million cells per 100 µl. Accordingly sample cells were typically resuspended in 100-250 µl or half the total staining volume PBS with FCS containing a 1x dilution of purified anti-FcγR antibody (in house production) and incubated for 10 min at 4 °C.

Antibody cocktails were prepared at 2x concentration and half the total staining volume according to table 7.3. For staining FMO or sample cells were mixed with 2x antibody cocktails and incubated for 15 min at 4 °C. For staining panels involving a secondary antibody or biotin/streptavidin staining step FMO and sample cells were washed and subsequently resuspended in a 1x dilution of the respective secondary antibody or streptavidin conjugate followed by 15 min incubation at 4 °C. After washing stained cells were typically resuspended in 50-200 µl PBS with FCS for analysis. For sorting cells were resuspended at or below a concentration of 10 million cells per ml.

For all analysis as well as sorting dead cells were excluded by 4,6-diamidino-2-phenylindole (DAPI; Invitrogen) or 7-amino-actinomycin D (7-AAD, Sigma) staining. Flow cytometry analyses were performed on an LSRII analyser (table 7.4, special order research product, Beckton, Dickenson and Company). Cell sorts were performed on a FACSAriaII cell sorter (table 7.5, special order research product, Beckton, Dickenson and Company). Sort purity as determined by test sorting and reanalysis prior to and after the sample sort was typically above 95 %. Flow cytometry data analysis was performed using FACSDiva (Beckton, Dickenson and Company) or Flowjo (TreeStar).

Table 7.3: List of antibodies for flow cytometry

Antibody conjugate	Clone	Supplier	Application
CD19-PECy7	1D3	BD, eBioscience	Ch. 2, 4, white blood cell stain
CD45.1-PE	A20	BD	Ch. 2, 4, white blood cell stain
CD45.2-AlexaFluor700 (AF700)	104	Biolegend	Ch. 2, 4, white blood cell stain
CD4-APCeF780	RM4-5	eBioscience	Ch. 2, 4, white blood cell stain
CD8a-APCeF780	53-6.7	eBioscience	Ch. 2, 4, white blood cell stain
Gr-1-PacificOrange (PO)	RB6-8C5	Invitrogen	Ch. 2, 4, white blood cell stain
Mac-1-APC	M1/70	Biolegend	Ch. 2, 4, white blood cell stain
NK1.1-PacificBlue (PB)	PK136	Biolegend	Ch. 2, 4, white blood cell stain
B220-PECy7	RA3-6B2	Biolegend	Ch. 2, FL/YS stain
CD19-PECy7	1D3	BD, eBioscience	Ch. 2, FL/YS stain
CD3e-APC	I45-2C11	BD	Ch. 2, FL/YS stain
CD45-AlexaFluor700 (AF700)	30-F11	eBioscience	Ch. 2, FL/YS stain
c-Kit-APCeFluor780 (APCeF780)	2B8	eBioscience	Ch. 2, FL/YS stain
F4/80-APC	BM8	Invitrogen	Ch. 2, FL/YS stain
Flt3 (CD135)-biotin	A2F10	eBioscience	Ch. 2, FL/YS stain
Gr-1-APC	RB6-8C5	BD, Biolegend	Ch. 2, FL/YS stain
IL-7Rα-PE	A7R34	eBioscience	Ch. 2, FL/YS stain
NK1.1-APC	PK136	BD	Ch. 2, FL/YS stain
Sca-1-FITC	E13-161.7	BD	Ch. 2, FL/YS stain
Sca-1-PacificBlue (PB)	E13-161.7	Biolegend	Ch. 2, FL/YS stain
Streptavidin-Qdot655 (QD655)	-	Invitrogen	Ch. 2, FL/YS stain
Ter119-APC	TER119	eBioscience	Ch. 2, FL/YS stain
B220-PETexasRed (PETxR)	RA3-6B2	BD	Ch. 2, Rag1 fate mapping
CD150-APC	TC15-12F12.2	Biolegend	Ch. 2, Rag1 fate mapping
CD19-PE	1D3	BD	Ch. 2, Rag1 fate mapping
CD41-PECy7	MWrag30	eBioscience	Ch. 2, Rag1 fate mapping
Gr-1-PacificOrange (PO)	RB6-8C5	Invitrogen	Ch. 2, Rag1 fate mapping
Mac-1-AlexaFluor700 (AF700)	M1/70	eBioscence	Ch. 2, Rag1 fate mapping
Ter119-PECy5.5	TER119	eBioscence	Ch. 2, Rag1 fate mapping
B220-PE	RA3-6B2	eBioscience	Ch. 2-5, OP9/OP9DL1 readout
CD19-PECy7	1D3	BD, eBioscience	Ch. 2-5, OP9/OP9DL1 readout

CD25-PE	3C7	BD	Ch. 2-5, OP9/OP9DL1 readout
CD25-PerCPCy5.5	PC61	BD BioSciences	Ch. 2-5, OP9/OP9DL1 readout
CD4-AlexaFluor700 (AF700)	RM4-5	eBioscience	Ch. 2-5, OP9/OP9DL1 readout
CD4-APCeFlour780 (APCeF780)	RM4-6	eBioscience	Ch. 2-5, OP9/OP9DL1 readout
CD4-PE	H129.19	Biolegend	Ch. 2-5, OP9/OP9DL1 readout
CD71-PE	RI7217	Biolegend	Ch. 2-5, OP9/OP9DL1 readout
CD8a-PECy7	53-6.7	eBioscience	Ch. 2-5, OP9/OP9DL1 readout
F4/80-APC	BM8	Caltag	Ch. 2-5, OP9/OP9DL1 readout
Gr-1-PECy5	RB6-8C5	Biolegend	Ch. 2-5, OP9/OP9DL1 readout
Mac-1-AlexaFluor700 (AF700)	M1/70	eBioscience	Ch. 2-5, OP9/OP9DL1 readout
NK1.1-PacificBlue (PB)	PK136	Biolegend	Ch. 2-5, OP9/OP9DL1 readout
Ter119-PECy5.5	TER-119	eBioscience	Ch. 2-5, OP9/OP9DL1 readout
Thy1.2-APC	30-H12	Biolegend	Ch. 2-5, OP9/OP9DL1 readout
B220-APC	RA3-6B2	BD	Ch. 3, ETP stain
CD11c-APC	N418	eBioscience	Ch. 3, ETP stain
CD19-APC	1D3	BD	Ch. 3, ETP stain
CD25-PerCPCy5.5	PC61	BD	Ch. 3, ETP stain
CD3e-APC	145-2C11	eBioscience	Ch. 3, ETP stain
CD4-AlexaFluor700 (AF700)	RM4-5	eBioscience	Ch. 3, ETP stain
CD8a-PECy7	53-6.7	eBioscience	Ch. 3, ETP stain
c-Kit-APCeFluor780 (APCeF780)	2B8	eBioscience	Ch. 3, ETP stain
Gr-1-APC	RB6-8C5	eBioscience	Ch. 3, ETP stain
NK1.1-APC	PK136	eBioscience	Ch. 3, ETP stain
Tcrb-APC	H57-597	eBioscience	Ch. 3, ETP stain
Tcrgd-APC	eBioGL3	eBioscience	Ch. 3, ETP stain
B220-purified	RA3-6B2	eBioscience	Ch. 4, HSC and MP stain
CD105-Biotin	MJ7/18	Biolegend	Ch. 4, HSC and MP stain
Mac-1-purified	M1/70	Biolegend	Ch. 4, HSC and MP stain
CD150-APC	TC15-12F12.2	Biolegend	Ch. 4, HSC and MP stain
CD16/32 (FcγR)-PE	93	eBioscience	Ch. 4, HSC and MP stain
CD34-Biotin	RAM34	eBioscience	Ch. 4, HSC and MP stain
CD41-PECy7	MWReg30	eBioscience	Ch. 4, HSC and MP stain
CD4-purified	H129.19	BD	Ch. 4, HSC and MP stain
CD5-purified	53-7.3	eBioscience	Ch. 4, HSC and MP stain
CD8a-purified	53-6.7	eBioscience	Ch. 4, HSC and MP stain
c-Kit-APCeFluor780 (APCeF780)	2B8	eBioscience	Ch. 4, HSC and MP stain
F(ab')2 IgG (H+L)-PECy5	-	Invitrogen	Ch. 4, HSC and MP stain
Gr-1-purified	RB6-8C5	eBioscience	Ch. 4, HSC and MP stain
Sca-1-PacificBlue (PB)	E13-161.7	Biolegend	Ch. 4, HSC and MP stain
Streptavidin-PETexasRed (PETxR)	-	BD	Ch. 4, HSC and MP stain
Ter119-purified	TER-119	eBioscience	Ch. 4, HSC and MP stain
B220-PECy5	RA3-6B2	Biolegend	Ch. 4, HSC and progenitor sort
Mac-1-PECy5	M1/70	Biolegend	Ch. 4, HSC and progenitor sort
CD150-PECy7	TC15-12F12.2	Biolegend	Ch. 4, HSC and progenitor sort
CD48-APC	HM48-1	Biolegend	Ch. 4, HSC and progenitor sort
CD4-PECy5	RM4-5	Biolegend	Ch. 4, HSC and progenitor sort
CD5-PECy5	53-7.3	Biolegend	Ch. 4, HSC and progenitor sort
CD8a-PEcy5	53-6.7	Biolegend	Ch. 4, HSC and progenitor sort
c-Kit-APCeFluor780 (APCeF780)	2B8	eBioscience	Ch. 4, HSC and progenitor sort
Gr-1-PECy5	RB6-8C5	Biolegend	Ch. 4, HSC and progenitor sort
Sca-1-PacificBlue (PB)	E13-161.7	Biolegend	Ch. 4, HSC and progenitor sort
Ter119-PECy5	TER-119	Biolegend	Ch. 4, HSC and progenitor sort
Ter119-PECy5.5	TER-119	eBioscience	Ch. 4, HSC and progenitor sort

CD150-APC	TC15-12F12.2	Biolegend	Ch. 4, platelet stain
CD41-PECy7	MWReg30	eBioscience	Ch. 4, platelet stain
Ter119-PECy5.5	TER-119	eBioscience	Ch. 4, platelet stain
Ter119-PECy5.5	TER-120	eBioscience	Ch. 4, red blood cell stain
B220-PECy5	RA3-6B2	Biolegend	Ch. 5, HSC sort
CD11b (Mac-1)-PECy5	M1/70	Biolegend	Ch. 5, HSC sort
CD150-APC	TC15-12F12.2	Biolegend	Ch. 5, HSC sort
CD48-PE	HM48-1	Biolegend	Ch. 5, HSC sort
CD4-PECy5	RM4-5	Biolegend	Ch. 5, HSC sort
CD5-PECy5	53-7.3	Biolegend	Ch. 5, HSC sort
CD8a-PEcy5	53-6.7	Biolegend	Ch. 5, HSC sort
c-Kit-APCeFluor780 (APCeF780)	2B8	eBioscience	Ch. 5, HSC sort
Gr-1-PECy5	RB6-8C5	Biolegend	Ch. 5, HSC sort
Sca-1-PacificBlue (PB)	E13-161.7	Biolegend	Ch. 5, HSC sort
Ter119-PECy5	TER-119	Biolegend	Ch. 5, HSC sort

Table 7.4: Instrument configuration BD LSRII SORP

Laser	Wavelength	Laser power	Fluorophore	Emission filter
Violet	407 nm	50 mW	Pacific Blue	450/50
			Sytox Blue	525/50
			Pacific Orange	585/42
			QD605	610/20
			QD655	660/40
Blue	488 nm	100 mW	SSC	488/10
			FITC	525/50
Green	532 nm	150 mW	PE	575/25
			PE-Texas Red	610/20
			PECy5	685/35
			PECy5.5 (PerCPCy5.5)	710/40
			PECy7	780/60
Red	640 nm	40 mW	APC	670/14
			Alexa Fluor 700	730/45
			APCeFluor780	780/60

Table 7.5: Instrument configuration BD FACSAriaII SORP

Laser	Wavelength	Laser power	Fluorophore	Emission filter
Violet	407 nm	100 mW	Pacific Blue	450/50
			Sytox Blue	525/50
			Pacific Orange	585/42
			QD605	610/20
			QD655	660/40
Blue	488 nm	100 mW	SSC	488/10
			FITC	525/50
Green	532 nm	150 mW	PE	575/26
			PE-Texas Red	610/20
			PECy5	685/35
			PECy5.5 (PerCPCy5.5)	710/50
			PECy7	780/60
Red	640 nm	40 mW	APC	670/14
			Alexa Fluor 700	730/45
			APCeFluor780	780/60

7.4. *In vitro* evaluation of lineage potentials

7.4.1. *In vitro* evaluation of single lymphoid potentials

For evaluation of single B and T cell potentials, 1-4 haematopoietic stem or progenitor cells were plated on a ~80 % confluent monolayer of OP9 or OP9DL1 stroma, that was prepared one day in advance by plating 2000 OP9 or OP9DL1 stroma cells in each well of a flat bottom 96 well plate. Haematopoietic cells were either sorted directly into each well by automated single cell deposition or bulk sorted into a defined volume of medium followed by manual plating. OP9 co-cultures for the evaluation of B cell potential were performed in a total volume of 200 µl OptiMEM supplemented with 10 % FCS (HyClone, always used for *in vitro* culture), 1 % β-mercaptoethanol (0.1 mM, Sigma-Aldrich), 1 % Penicillin/Streptomycin (PAA), hIL-7 (20 ng/ml, Peprotech), hFlt3L (25 ng/ml, Amgen) and mSCF (25 ng/ml, Peprotech). T cell potential was evaluated by co-culture on OP9DL1 stromal cells, which express the Notch delta like 1 ligand. OP9DL1 cultures were performed in a total volume of 200 µl OptiMEM supplemented with 10 % FCS, 1 % β-mercaptoethanol (0.1 mM, Sigma-Aldrich), 1 % Penicillin/Streptomycin (PAA), hFlt3L (25 ng/ml, Amgen) and mSCF (25 ng/ml, Peprotech). The culture medium was partially changed once a week. Approximately 100 µl of the total culture volume were gently

aspirated from each culture well and replaced by fresh medium as above. After the first week SCF was omitted from the OP9DL1 cultures. Cultures were analysed by flow cytometry at 2-3 weeks. Clones were resuspended, transferred to a round bottom 96 well plate, spun down and resuspended in 12.5 µl PBS with FCS containing a 1x dilution of anti FcγR antibody.

7.4.2. *In vitro* evaluation of combined lineage potentials of foetal liver progenitors

For evaluation of combined B cell, T cell and myeloid potentials foetal liver progenitors were seeded onto OP9 stroma monolayers as described above. OP9 co-cultures were supplemented with hIL-7 (20 ng/ml, Peprotech), hFlt3L (25 ng/ml, Amgen) and mSCF (25 ng/ml, Peprotech). At day 5 of culture haematopoietic clones were transferred to OP9DL1 stroma co-cultures supplemented with hFlt3L (25 ng/ml, Amgen) and cultured for additional 6 days. For readout all clones were transferred to round bottom 96 well plates and resuspended in 12.5 µl 1x FcγR antibody dilution for antibody staining and flow cytometric analysis.

7.4.3. *In vitro* evaluation of combined lineage potentials of ETPs

Combined lineage potentials of ETPs were evaluated in two different ways. For the paired daughter cell (PDC) technique sorted single ETPs were cultured in OptiMEM supplemented with 10 % FCS, 1 % β-mercaptoethanol (0.1 mM, Sigma-Aldrich), 1 % Penicillin/Streptomycin (PAA), hIL-7 (20 ng/ml, Peprotech), hFlt3L (25 ng/ml, Amgen) and mSCF (25 ng/ml, Peprotech) for approximately 24 h or until the first cell division had occurred. Subsequently the two daughter cells were separated by micromanipulation and transferred onto OP9 stroma for evaluation of B cell potential as described above or OP9DL1 stroma for T and myeloid differentiation. The OP9DL1 co-culture was carried out in OptiMEM supplemented with 10 % FCS, 1 % β-mercaptoethanol (0.1 mM, Sigma-Aldrich), 1 % Penicillin/Streptomycin (PAA), mSCF (10 ng/ml, Peprotech), hFlt3L (5 ng/ml, Amgen), hIL-7 (1 ng/ml, Peprotech), hIL-6 (10 ng/ml, Peprotech), mIL-3

(5 ng/ul, Peprotech), hG-CSF (10 ng/ml, Amgen), mGM-CSF (10 ng/ml, Amgen) and hM-CSF (10 ng/ml, Peprotech). For the switch technique cells were directly sorted on OP9 stroma. After 54h approximately 50 % of each cell clone was transferred to OP9DL1 culture for readout of T and myeloid lineages. The second half of the clone remained on OP9 stroma for the readout of the B lineage. Cultures were analysed by flow cytometry at 2-3 weeks. Clones were transferred to round bottom 96 well plates and resuspended in 12.5 µl PBS with FCS containing a 1x dilution of anti FcγR antibody for staining and analysis by flow cytometry.

7.4.4. *In vitro* evaluation of GM, Mk and E potentials in liquid culture

For evaluation of GM, Mk or erythroid potentials foetal liver or adult haematopoietic cells were sorted into a defined volume of medium and 0.5 to 1 cells were plated in a total volume of 20 µl per well in a 60 well terasaki plate (Nunc). Cells were cultured in X-vivo15 with Gentamycin (Lonza) supplemented with 10-20 % FCS and 1 % β-mercaptoethanol (0.1 mM, Sigma Aldrich). For detection of GM potential the medium was supplemented with mSCF (25 ng/ml, PeproTech), hFlt3L (25 ng/ml, Amgen), hTPO (25 ng/ml, PeproTech), mIL-3 (10 ng/ml, PeproTech), hG-CSF (25 ng/ml, Amgen) and mGM-CSF (25 ng/ml, PeproTech). Mk and erythroid potentials were detected by culture in medium supplemented with mSCF (50 ng/ml, PeproTech), hFlt3L (50 ng/ml, Amgen), hTPO (50 ng/ml, PeproTech), mIL-3 (20 ng/ml, PeproTech) and hEPO (5 U/ml, Roche). GM, Mk and erythroid colonies were read out at day 7-10 using an inverted microscope (IX71, Olympus). In some experiments colony readouts were confirmed by evaluation of cytospins after May-Grunwald Giemsa staining or 2,7-diaminofluorene (DAF, Sigma-Aldrich) staining of erythroid colonies (see 7.4.7).

7.4.5. *In vitro* evaluation of Mk potential in semisolid medium

Mk potential of foetal liver progenitors was also evaluated with a Megacult collagen-based assay (Stem Cell Technologies). 200 FACS purified cells were plated in Megacult collagen media supplemented with mIL-3 (10 ng/ml, PeproTech), hTPO (50 ng/ml, PeproTech), hIL-6 (20 ng/ml, PeproTech), hIL-11 (50 ng/ml, Genetics Institute). Mk colonies were read out at day 7 by acetylthiocholiniodide staining according to manufacturer's instructions (Sigma). Culture slides were fixed by incubation in ice cold acetone for 5 min and air dried. The acetylthiocholiniodide solution was prepared by dissolving 100 mg acetylthiocholiniodide in 150 ml 0.1 M sodium phosphate buffer. For preparation of the staining solution 7.5 ml 0.1 M sodium citrate solution, 15 ml 30 mM copper sulphate solution and 15ml 5 mM potassium ferricyanide solution were added to 112.5 ml acetylthiocholiniodide solution. After staining for 4 h slides were fixed in 95 % ethanol for 10 min, rinsed with H_2O and counterstained with Harris' hematoxylin solution for 30 s. Stained slides were rinsed with H_2O, air dried and evaluated using an upright microscope (BX41, Olympus).

7.4.6. *In vitro* evaluation of CFU-GM potentials in semisolid medium

The CFU-GM potential of unfractionated foetal liver progenitors was evaluated using complete methylcellulose (Methocult GF M3434, supplemented with mSCF, mIL-3, hIL-6 and hEPO, Stem Cell Technologies) or methylcellulose base (M3134, Stem Cell Technologies) supplemented with mGM-CSF (5 ng/ml, PeproTech), hFlt3L (10 ng/ml, Amgen), mIL-3 (2 ng/ml, PeproTech) and hG-CSF (10 ng/ml, Amgen). CFU-GM colonies were scored after 7-10 days using an inverted microscope (IX71, Olympus)

7.4.7. *In vitro* evaluation of BFU-E potentials in semisolid medium

For evaluation of erythroid potentials unfractionated or FACS purified foetal liver progenitors were plated in complete methylcellulose (Methocult GF M3434, supplemented with mSCF, mIL-3, hIL-6 and hEPO, Stem Cell Technologies). BFU-E

colonies were scored by morphologic appearance as well as 2,7-diaminofluorene (DAF, Sigma-Aldrich) staining after 7-10 days using an inverted microscope (IX71, Olympus). For DAF staining the DAF stock solution was prepared by dissolving 20 mg DAF powder in 2 ml 90 % glacial acetic acid/H_2O. DAF staining solution was prepared by addition of 0.5 ml DAF stock solution as well as 0.1 ml 30 % hydrogen peroxide to 10 ml 200 mM Tris hydrochloric acid (HCL) (pH 7.5). 1 ml DAF staining solution was added per culture dish and erythroid colonies were scored after 5 min incubation at room temperature but within a total of 30 min. Erythroid cells were identified by the characteristic blue intracellular staining.

7.4.8. *In vitro* evaluation of erythroid potential in co-culture with OP9 stromal cells

Erythroid potential of E11.5 foetal liver progenitors was additionally evaluated in OP9 stromal co-cultures. FACS purified cells were plated on an OP9 stromal monolayer in a total volume of 200 µl OptiMEM supplemented with 10 % FCS, 1 % β-mercaptoethanol (0.1 mM, Sigma-Aldrich), 1 % Penicillin/Streptomycin (PAA), mSCF (50 ng/ml, PeproTech), hTPO (50 ng/ml, PeproTech) and hEPO (5 U/ml, Roche). Colonies were read out at day 7. One half of each colony was analysed by flow cytometry while the second half was used for preparation of cytospins and morphologic identification of erythroid cells after May-Grunwald Giemsa staining.

7.5. Paired daughter cell assay

7.5.1. Imaging platform for manipulation of paired daughter cells

The imaging platform (Image Solutions) is based on an Olympus IX71 inverted microscope equipped with an 120W metal halide Exfo illuminator (Lumen Dynamics), an Evolve 512x512 back illuminated EMCCD camera (Photometrics), motorised filter cube cassette and 4x (0.13 NA), 10x (0.3 NA), 20x (0.5 NA), 40x (0.75 NA), 60x (oil immersion, 1.25 NA), 100x (oil immersion, 1.3 NA) objectives. The platform is controlled

using Image Pro Plus (Image Solutions) on a 64 bit workstation (Acer). The micromanipulation unit consists of a TransferMan NK2 (Eppendorf) equipped with a CellTram vario (Eppendorf). Micropipettes for manipulation were pulled using 1 mm x 100 mm glass capillaries (Narishige) on a P-97 micropipette puller (Sutter Instruments). Micropipettes were bent to 90° on a Bunsen burner gas flame and opened by breaking off the tip using forceps. For cell transfer the micropipette was filled with the respective culture medium.

7.5.2. Culture for generation of paired daughter cells

Single cell sorted haematopoietic stem and progenitor cells were cultured for 2 days using U-shaped 96 well plates in 50 µl StemSpan SFEM (StemCell Technologies) supplemented with 10 % FCS, 1 % β-mercaptoethanol (0.1 mM, Sigma-Aldrich), 1 % Penicillin/Streptomycin (PAA) and cytokines mIL-3 (20 ng/ml, PeproTech), mSCF (50 ng/ml, PeproTech) and hTPO (50 ng/ml, PeproTech). On day 2 colonies were screened for *Vwf*-EGFP reporter expression. Colonies containing exactly 2 daughter cells were separated by micromanipulation and transferred to secondary cultures for evaluation of lineage potentials, or lysis buffer for gene expression analysis. For evaluation of GM/Mk potential cells were cultured in 100 µl StemSpan SFEM (StemCell Technologies) supplemented with 10-20 % FCS, 1 % β-mercaptoethanol (0.1 mM, Sigma-Aldrich), 1 % Penicillin/Streptomycin (PAA) as well as growth factors mSCF (50 ng/ml, PeproTech), hFlt3L (50 ng/ml, Amgen), hTPO (50 ng/ml, PeproTech), mIL-3 (20 ng/ml, PeproTech) and hEPO (5 U/ml, Roche). The morphology of colonies was evaluated on days 8, 11 and 13 after manipulation on an inverted microscope (IX71, Olympus). Megakaryocyte colony content was confirmed by the expression of the *Vwf*-EGFP reporter that is highly expressed in mature megakaryocytes. Some colonies were also evaluated by morphology after May-Grunwald Giemsa staining.

7.6. Gene expression analysis

7.6.1. Non-quantitative multiplex single cell PCR

For multiplex RT-PCR analysis single FACS purified E11.5 foetal liver progenitors were sorted into 96 well PCR plates containing 4 µl lysis buffer and frozen at -80 °C. E9.5 foetal liver progenitors were first bulk sorted and single cells were subsequently micro-manipulated into lysis buffer. Samples were reverse transcribed using gene specific primers in a total volume of 10 µl containing 50 U MMLV reverse transcriptase (Invitrogen). First-round PCR was performed over 35 cycles using gene specific forward primers and 1.25 U Taq polymerase (TaKaRa Bio) in a total volume of 40 µl PCR mix. In second round PCRs 1 µl aliquots of the initial PCR product were subjected to additional 35 amplification cycles using nested gene-specific primers. PCR products were analysed by agarose gel electrophoresis and ethidium bromide staining. Since investigated cells were FACS sorted based on cKit expression, only cells expressing *Kit* were included in the analysis. For quantification of lineage specific gene priming, single cells had to express at least one of the genes associated with a particular lineage to be considered positive for the respective lineage program.

7.6.2. Sample preparation and quantitative PCR

For multiplex quantitative gene expression analysis using the BioMark 48.48 dynamic array (Fluidigm) 1 to 100 cells were either directly sorted or transferred by micromanipulation into 0.2 ml reaction tubes containing 5 µl CellsDirect 2x reaction mix (Invitrogen), 0.1 µl SUPERase 12-In RNAse inhibitor (Ambion), 1.2 µl TE buffer (Sigma), 1.2 µl CellsDirect RT/Taq mix (Invitrogen) and 2.5 µl 0.2x gene specific TagMan assay mix (Applied Biosystems) in a total volume of 10 µl. Reverse transcription and amplification of target transcripts was performed on a thermal cycler (Tetrad II 96-Well Alpha Unit, Biorad) using the following protocol: RT reaction at 50 °C for 15 min, inactivation of reverse transcriptase at 95 °C for 2 min, target gene amplification in

22 cycles of 95 °C for 15 s followed by 60 °C for 4 min. Preamplified cDNA samples were diluted 1:5 in TE buffer (Sigma) to a total volume of 50 µl. Taqman assay and sample mixes were prepared according to manufacturer's instruction and loaded onto a primed 48.48 dynamic array (Fluidigm). The following conditions were used to perform the quantitative PCR reaction: 95 °C for 10 min followed by 40 cycles of 95 °C for 15 s and 60 °C for 60 s. The data were analysed using the BioMark Real-Time PCR Analysis software v2.0 (Fluidigm) and ΔC_t values were calculated by normalising to *Hprt* or *Ubc*.

7.6.3. Data analysis of single cell quantitative gene expression

For all subsequent analysis of single cell gene expression data from *Vwf*-$EGFP^+$ and *Vwf*-$EGFP^-$ HSC and their paired daughters, ΔC_t values relative to the expression of *Ubc* were used. All data analyses were performed using the statistical programming environment, R (version 2.15.0; www.r-project.org). In order to exclude samples that accidently did not receive a cell by cell sorting or micromanipulation, only cells that expressed at least 30 % of all genes were considered for analysis.

PCA analysis was performed on all parent and daughter cells using the R prcomp package. Housekeeping genes (*Ubc, Hprt, B2m, Actb, Gapdh*) were removed from the data set prior to PCA analysis.

For heatmap display of all cell data, the *heatmap.2* function from the R package *gplots* was applied. For clarity of display all ΔC_t values >30 were set to 30 (in most cases where C_t values were greater than 30, the transcript had been arbitrarily assigned a C_t value of 999 by the fluidigm software and was regarded as not detected). For the heatmap display, cells were ordered according to their class and no clustering was applied to the cells. Lineage specific gene sets were prepared and for each lineage programme a heatmap was generated individually.

To establish the nature of asymmetrical gene expression in paired daughter cells $\Delta\Delta C_t$ values were calculated for each gene in each pair. If either cell in a pair expressed less than 30 % of the analysed genes, the entire pair was removed from subsequent analysis. To remove infinite fold differences between pairs, a maximum fold difference was applied, and values >10 were set to 10. This approach was chosen as fold differences were generally much less than 10 or much greater, typically those greater than 10 were infinite. Daughter pairs from *Vwf*-EGFP$^+$ and *Vwf*-EGFP$^-$ HSC were analysed together and pairs were hierarchically clustered. In order to visualise the asymmetric nature of expression of some genes, all genes were ranked based on their cumulative fold difference over all pairs.

Table 7.6: List of TaqMan assays

Gene symbol	**Gene name**	**Assay ID or primer (F, R) / probe (P) sequence**	**Common alt. name**	**Application**
Csf1r	Colony stimulating factor 1 receptor	Mm00432689_m1	*Cd115*	Chapter 2
Csf2ra	Colony stimulating factor 2 receptor alpha	Mm00438331_g1	*Gmcsfra*	Chapter 2
Csf3r	Colony stimulating factor 3 receptor	Mm00432735_m1	*Gcsfr*	Chapter 2
Epor	Erythropoietin receptor	Mm00438760_m1		Chapter 2
Flt3	FMS-like tyrosine kinase 3	Mm00439011_m1		Chapter 2
Gata1	GATA binding protein 1	Mm00484678_m1		Chapter 2
Gata2	GATA binding protein 2	Mm00492300_m1		Chapter 2
Ikzf1	Ikaros family zinc finger 1	Mm00456421_m1	*Ikaros*	Chapter 2
Il7r	Interleukin 7 receptor	Mm00434295_m1		Chapter 2
Klf1	Kruppel-like factor 1	Mm00516096_m1		Chapter 2
Mpo	Myeloperoxidase	Mm00447886_m1		Chapter 2
Rag1	Recombination activating gene 1	F: TGTGGAGCAAGGT AGCTTAGC R: TCATCGGGTGCAG AACTGAAG P: CATGGCTGCCTCC TTG		Chapter 2
Rag2	Recombination activating gene 2	Mm00501300_m1		Chapter 2
sIgH	Sterile immunoglobulin heavy chain transcript	F: GGACTTTGGGATG GGTTTGGTT R: CCCTGGTCCTAGA CATCAGAGTAAT P: CCCAGATGAAGGG CTAC		Chapter 2
Cd9	Cd9 antigen	Mm00514275_g1		Chapter 4
Cebpa	CCAAT/enhancer binding protein (C/EBP), alpha	Mm00514283_s1		Chapter 4
Csf2ra	Colony stimulating factor 2 receptor alpha	Mm00438331_g1	*Gmcsfra*	Chapter 4
Eraf	Erythroid associated factor	Mm04214740_u1	*Ahsp*	Chapter 4

Esam	Endothelial cell specific adhesion molecule	Mm00518378_m1		Chapter 4
Gata1	GATA binding protein 1	Mm00484678_m1		Chapter 4
Gata2	GATA binding protein 2	Mm00492300_m1		Chapter 4
Itga2b	Integrin alpha 2b	Mm00439741_m1	*Cd41*	Chapter 4
Klf1	Kruppel-like factor 1	Mm00516096_m1	*Eklf*	Chapter 4
Meis1	Meis homeobox 1	Mm00487664_m1		Chapter 4
Mpl	Myeloproliferative leukemia virus oncogene	Mm00440310_m1		Chapter 4
Pf4	Platelet factor 4	Mm00451315_g1		Chapter 4
Prnp	Prion protein	Mm00448389_m1		Chapter 4
Sfpi1	SFFV proviral integration 1	Mm00488140_m1		Chapter 4
Tfrc	Transferrin receptor	Mm00441941_m1	*Cd71*	Chapter 4
Vwf	Von willebrand factor homolog	Mm00550376_m1		Chapter 4
Zfpm1	Zinc finger protein, multitype 1	Mm00494336_m1	*Fog1*	Chapter 4
Actb	β-actin	Mm00607939_s1		Chapter 5
B2m	β-2-microglobolin	Mm00437762_m1		Chapter 5
Bmi1	Bmi1 polycomb ring finger oncogene	Mm00776122_gH		Chapter 5
Car1	Carbonic anhydrase 1	Mm00486717_m1		Chapter 5
Cd34	Cd34 antigen	Mm00519283_m1		Chapter 5
Cd3g	Cd3 antigen, gamma polypeptide	Mm00438095_m1		Chapter 5
Cd9	Cd9 antigen	Mm00514275_g1		Chapter 5
Cdkn1c	Cyclin dependent kinase inhibitor 1c	Mm00438170_m1	*p57kip2*	Chapter 5
Cebpa	CCAAT/enhancer binding protein (C/EBP), alpha	Mm00514283_s1		Chapter 5
Clu	Clusterin	Mm00442773_m1		Chapter 5
Csf1	Colony stimulating factor 1	Mm00432688_m1		Chapter 5
Csf2ra	Colony stimulating factor 2 receptor alpha	Mm00438331_g1	*Gmcsfra*	Chapter 5
Csf2rb	Colony stimulating factor 2 receptor beta	Mm00655745_m1		Chapter 5
Csf3r	Colony stimulating factor 3 receptor	Mm00432735_m1	*Gcsfr*	Chapter 5
Epor	Erythropoietin receptor	Mm00438760_m1		Chapter 5
Eraf	Erythroid associated factor	Mm04214740_u1	*Ahsp*	Chapter 5
Evi1	Ecotropic virus integration site 1 protein homolog	Mm00514814_m1		Chapter 5
Flt3	FMS-like tyrosine kinase 3	Mm00439011_m1		Chapter 5
Gapdh	Glyceraldehyde-3-phosphate dehydrogenase	Mm99999915_g1		Chapter 5
Gata1	GATA binding protein 1	Mm00484678_m1		Chapter 5
Gata2	GATA binding protein 2	Mm00492300_m1		Chapter 5
Gpr64	G protein-coupled receptor 64	Mm00724548_m1		Chapter 5
Hoxb4	Homeo box B4	Mm00657964_m1		Chapter 5
Hprt	Hypoxanthine guanine phosphoribosyl transferase 1	Mm00446968_m1		Chapter 5
Ikzf1	Ikaros family zinc finger 1	Mm00456421_m1		Chapter 5
Il7r	Interleukin 7 receptor	Mm00434295_m1	*Ikaros*	Chapter 5
Itga2b	Integrin alpha 2b	Mm00439741_m1	*Cd41*	Chapter 5
Itgb3	Integrin beta 3	Mm00443980_m1		Chapter 5
Klf1	Kruppel-like factor 1	Mm00516096_m1		Chapter 5
Ly6a	Lymphocyte antigen 6 complex locus A	Mm00726565_s1	*Sca1*	Chapter 5
Mafb	V-maf musculoaponeurotic fibrosarcoma oncogene family protein B	Mm00627481_s1		Chapter 5
Meis1	Meis homeobox 1	Mm00487664_m1		Chapter 5
Mpl	Myeloproliferative leukemia virus oncogene	Mm00440310_m1		Chapter 5

Mpo	Myeloperoxidase	Mm00447886_m1		Chapter 5
Myc	Myelocytomatosis oncogene	Mm00487803_m1		Chapter 5
Mycn	V-myc myelocytomatosis viral related oncogene neuroblastoma derived	Mm00476449_m1		Chapter 5
Neo1	Neogenin	Mm00476326_m1		Chapter 5
Numb	Numb gene homolog	Mm00477927_m1		Chapter 5
Prnp	Prion protein	Mm00448389_m1		Chapter 5
Runx1	Runt related transcription factor 1	Mm01213405_m1		Chapter 5
Sdpr	Serum deprivation response	Mm00507087_m1		Chapter 5
Sfpi1	SFFV proviral integration 1	Mm00488140_m1		Chapter 5
SIgH	Sterile immunoglobulin heavy chain transcript	F: GGACTTTGGGATG GGTTTGGTT R: CCCTGGTCCTAGA CATCAGAGTAAT P: CCCAGATGAAGGG CTAC		Chapter 5
Tcfe2a	Transcription factor E2a	Mm01175588_m1		Chapter 5
Tek	Endothelial specific receptor tyrosine kinase	Mm00443242_m1	*Tie2*	Chapter 5
Ubc	Ubiquitin C	Mm01201237_m1		Chapter 5
Vwf	Von willebrand factor homolog	Mm00550376_m1		Chapter 5
Zfpm1	Zinc finger protein, multitype 1	Mm00494336_m1	*Fog1*	Chapter 5

7.7. Statistical analysis

The statistical significance of differences between groups of samples was calculated using the two-tailed Student’s t-test.

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