The Biology of Hematopoietic Stem Cells: transgenic approaches to Dissect Native and Perturbed Hematopoiesis

Säwén, Petter

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The Biology of Hematopoietic Stem Cells:
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Petter Säwén

LUND UNIVERSITY

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To be defended on October 5, 2017, at 0900 in Segerfalk lecture hall, BMC A10,
Lund, Sweden

Faculty opponent
Fernando Camargo, PhD
Hematopoiesis is defined as the ongoing production of blood cells. As most mature blood cells are relatively short-lived and require continuous replacement, hematopoiesis is characterized by an extraordinary turnover rate with production of trillions of new blood cells every day. To cope with the enormous proliferation required to generate sufficient numbers of blood cells to maintain homeostasis, the hematopoietic system is hierarchically organized within the bone marrow. Scarce hematopoietic stem cells (HSCs) reside at the top of this hierarchy. More abundant and increasingly developmentally restricted and proliferating progenitor cells, that massively amplify hematopoietic cell generation, reside further down in the hierarchy. HSC function is typically evaluated using transplantation experiments, which offers quantitative and qualitative information on their self-renewal and multilineage differentiation potential. In this setting, potent long-term multilineage contribution can be observed from even single HSCs. After transplantation of myeloblated hosts, HSCs are forced to proliferate extensively to rebuild the hematopoietic system. In sharp contrast, native HSCs display very low proliferation rates. Emerging data has highlighted fundamental differences between hematopoiesis as seen after transplantation compared to that in steady state. Therefore, analysis of native hematopoiesis in models that allows for evaluation in unperturbed settings is necessary.

In article 1 we characterize HSC and progenitor proliferation dynamics in the steady state and following several types of induced stress. Whereas transplantation promoted sustained, long-term proliferation of HSCs, both cytokine-induced mobilization and acute depletion of selected blood cell lineages elicited very limited recruitment of HSCs to the proliferative pool. In addition, coupling of proliferation history with gene expression analysis on single cells led to identification of subtypes of HSCs that have distinct molecular signatures and differ drastically in their reconstitution potentials.

The Mx1-Cre mouse strain is the most commonly used conditional gene-knockout strain in experimental hematology. The Mx1 promoter is activated by endogenous interferon release that is induced by injection of polyinosinic-polycytidylic acid (poly I:C). However, interferon is also released as a part of the inflammatory response. In Article 2, we highlight pitfalls associated with the CreERT2 mediated lineage tracing, a model that can label close to 100 % of adult HSCs in a highly specific manner. We show that apart from blood cells with a known fetal origin, HSC contribution to all blood cell lineages is robust and occurs via a hierarchy of defined intermediate progenitor cells. Our experiments reveal that the time course of regeneration for distinct blood lineages varied substantially. Myeloid lineage cells were generated from HSCs more rapidly than lymphoid cells, with platelets and their corresponding progenitor cells emerging first. Therefore, adult HSCs are active contributors to all lineages of adult hematopoiesis in the steady state.

In summary, we have highlighted features with experimental systems/procedures that are used in experimental hematology and have explored hematopoiesis and HSC biology in models that allows evaluation of unperturbed hematopoiesis.

Key words: Hematopoietic stem cells, Hematopoiesis, Proliferation, Lineage Tracing, Mx1-Cre
The Biology of Hematopoietic Stem Cells: Transgenic Approaches to Dissect Native and Perturbed Hematopoiesis

Petter Säwén
On the cover: Image showing the mCherry signal in the femoral bone marrow cavity in an adult mouse that was labeled with Histone2B-mCherry nine days before imaging.

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Coverphoto by: Talia Velasco

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For Kajsa, Hilda, Ivar, Joar and Elvira
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Original Papers and Manuscripts

Papers included in this thesis

Paper I

Mitotic History Reveals Distinct Stem Cell Populations and Their Contributions to Hematopoiesis.
Säwén P, Lang S, Mandal P, Rossi DJ, Soneji S, Bryder D.

Paper II

Potential Pitfalls of the Mx1-Cre System: Implications for Experimental Modeling of Normal and Malignant Hematopoiesis.
Velasco-Hernandez T, Säwén P, Bryder D, Cammenga J.

Paper III

Adult Hematopoietic Stem Cells Contribute Actively to Myeloerythroid Hematopoiesis in Steady State.
Manuscript
Papers not included in this thesis

**Hematopoietic stem cells are intrinsically protected against MLL-ENL-mediated transformation.**

**MLL-ENL-mediated leukemia initiation at the interface of lymphoid commitment.**
Ugale A, Säwén P, Dudenhöffer-Pfeifer M, Wahlestedt M, Norddahl GL, Bryder D.

**Critical modulation of hematopoietic lineage fate by Hepatic Leukemia Factor**
Martin Wahlestedt, Vasileios Ladopoulos, Isabel Hidalgo, Manuel Sanchez Castillo, Rebeca Hannah, Petter Säwén, Haixia Wan, Monika Dudenhöffer-Pfeifer, Mattias Magnusson, Gudmundur L Norddahl, Berthold Göttgens and David Bryder

*Manuscript*
Abstract

Hematopoiesis is defined as the ongoing production of blood cells. As most mature blood cells are relatively short-lived and require continuous replacement, hematopoiesis is characterized by an extraordinary turnover rate with production of trillions of new blood cells every day. To cope with the enormous proliferation required to generate sufficient numbers of blood cells to maintain homeostasis, the hematopoietic system is hierarchically organized within the bone marrow. Scarce hematopoietic stem cells (HCSs) reside at the top of this hierarchy. More abundant and increasingly developmentally restricted and proliferating progenitor cells, that massively amplify hematopoietic cell generation, reside further down in the hierarchy. HSC function is typically evaluated using transplantation experiments, which offers quantitative and qualitative information on their self-renewal and multilineage differentiation potential. In this setting, potent long-term multilineage contribution can be observed from even single HSCs. After transplantation of myeloablated hosts, HSCs are forced to proliferate extensively to rebuild the hematopoietic system. In sharp contrast, native HSCs display very low proliferation rates. Emerging data has highlighted fundamental differences between hematopoiesis as seen after transplantation compared to that in steady state. Therefore, analysis of native hematopoiesis in models that allows for evaluation in unperturbed settings is necessary.

In article 1 we characterize HSC and progenitor proliferation dynamics in the steady state and following several types of induced stress. Whereas transplantation promoted sustained, long-term proliferation of HSCs, both cytokine-induced mobilization and acute depletion of selected blood cell lineages elicited very limited recruitment of HSCs to the proliferative pool. In addition, coupling of proliferation history with gene expression analysis on single cells led to identification of subtypes of HSCs that have distinct molecular signatures and differ drastically in their reconstitution potentials.

The Mx1-Cre mouse strain is the most commonly used conditional gene-knockout strain in experimental hematology. The Mx1 promoter is activated by endogenous interferon release that is induced by injection of polyinosinic:polycytidylic acid (poly I:C). However, interferon is also released as a part of the inflammatory response. In Article 2, we highlight pitfalls associated with the Mx1-Cre system. Transplantation of cells where Mx1-Cre activation is required for gene knockout resulted in high rates of spontaneous gene deletion. In
addition, poly I:C administration introduced alterations to the hematopoietic stem and progenitor cell (HSPC) compartment. Collectively, this study emphasize that proper controls are crucial when modeling gene deletion with the *Mx1-Cre* system.

A model with limited HSC contribution to native hematopoiesis has been proposed. This is in sharp contrast to the continuous contribution of HSCs to hematopoiesis after transplantation. In the work leading up to article 3 we set out to explore HSC contribution to native hematopoiesis by evaluation of blood cell generation from HSCs in native adult hematopoiesis. For this we used Fgd5-CreERT2 mediated lineage tracing, a model that can label close to 100% of adult HSCs in a highly specific manner. We show that apart from blood cells with a known fetal origin, HSC contribution to all blood cell lineages is robust and occurs via a hierarchy of defined intermediate progenitor cells. Our experiments reveal that the time course of regeneration for distinct blood lineages varied substantially. Myeloerythroid cells were generated from HSCs more rapidly than lymphoid cells, with platelets and their corresponding progenitor cells emerging first. Therefore, adult HSCs are active contributors to all lineages of adult hematopoiesis in the steady state.

In summary, we have highlighted features with experimental systems/procedures that are used in experimental hematology and have explored hematopoiesis and HSC biology in models that allows evaluation of unperturbed hematopoiesis.
### Abbreviations

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<th>Abbreviation</th>
<th>Definition</th>
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<tr>
<td>4-OH TAM</td>
<td>4-OH Tamoxifen</td>
</tr>
<tr>
<td>5-FU</td>
<td>5-fluorouracil</td>
</tr>
<tr>
<td>AGM</td>
<td>Aorta-gonads-mesonephros</td>
</tr>
<tr>
<td>BAC</td>
<td>Bacterial artificial chromosome</td>
</tr>
<tr>
<td>BrdU</td>
<td>Bromodeoxyuridine</td>
</tr>
<tr>
<td>C. elegans</td>
<td>Caenorhabditis elegans</td>
</tr>
<tr>
<td>CFSE</td>
<td>Carboxyfluorescein succinimidyl ester</td>
</tr>
<tr>
<td>CFU-E</td>
<td>Colony forming unit-erythroid</td>
</tr>
<tr>
<td>CFU-S</td>
<td>Colony forming unit-spleen</td>
</tr>
<tr>
<td>CMP</td>
<td>Common myeloid progenitor</td>
</tr>
<tr>
<td>CLP</td>
<td>Common lymphoid progenitor</td>
</tr>
<tr>
<td>Cre</td>
<td>Cre recombinase</td>
</tr>
<tr>
<td>dHSC</td>
<td>Definitive hematopoietic stem cell</td>
</tr>
<tr>
<td>DNMT</td>
<td>DNA methyltransferase</td>
</tr>
<tr>
<td>DOX</td>
<td>Doxycycline</td>
</tr>
<tr>
<td>E(X)</td>
<td>Embryonic day (X)</td>
</tr>
<tr>
<td>EoMP</td>
<td>Eosinophil mast cell progenitor</td>
</tr>
<tr>
<td>ER</td>
<td>Estrogen receptor</td>
</tr>
<tr>
<td>ERT2</td>
<td>Mutated estrogen receptor T2</td>
</tr>
<tr>
<td>FACS</td>
<td>Fluorescence activated cell sorting</td>
</tr>
<tr>
<td>Floxed</td>
<td>DNA sequence that is flanked by 2 LoxP sites</td>
</tr>
<tr>
<td>G-CSF</td>
<td>Granulocyte colony-stimulating factor</td>
</tr>
<tr>
<td>GM</td>
<td>Granulocyte-monocyte</td>
</tr>
<tr>
<td>GMLP</td>
<td>Granulocyte-monocyte-lymphoid progenitor</td>
</tr>
<tr>
<td>GMP</td>
<td>Granulocyte-monocyte progenitor</td>
</tr>
<tr>
<td>H2B</td>
<td>Histone 2B</td>
</tr>
<tr>
<td>HOX</td>
<td>Homeobox</td>
</tr>
<tr>
<td>HCT</td>
<td>Hematopoietic cell transplantation</td>
</tr>
<tr>
<td>HPC</td>
<td>Hematopoietic progenitor cell</td>
</tr>
<tr>
<td>HSC</td>
<td>Hematopoietic stem cell</td>
</tr>
<tr>
<td>HSPC</td>
<td>Hematopoietic stem and progenitor cell</td>
</tr>
<tr>
<td>KrasG12D</td>
<td>Activating Kras mutation G12D</td>
</tr>
<tr>
<td>LK</td>
<td>Lineage+, c-kit positive</td>
</tr>
<tr>
<td>LMPP</td>
<td>Lymphoid-primed multipotent progenitor</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
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<tr>
<td>--------------</td>
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<tr>
<td>LPS</td>
<td>Lipopolysaccharide</td>
</tr>
<tr>
<td>L'S'</td>
<td>Lineage+ and Sca1 positive</td>
</tr>
<tr>
<td>LSK</td>
<td>Lineage-, Sca1 positive, c-kit positive</td>
</tr>
<tr>
<td>LSL</td>
<td>LoxP-STOP-LoxP</td>
</tr>
<tr>
<td>M2rt-TA</td>
<td>M2 reverse tetracycline transactivator</td>
</tr>
<tr>
<td>MegE</td>
<td>Megakaryocyte and erythroid</td>
</tr>
<tr>
<td>MEIS1</td>
<td>Mouse ecotropic Integration site 1</td>
</tr>
<tr>
<td>MkP</td>
<td>Megakaryocyte progenitor</td>
</tr>
<tr>
<td>MPP</td>
<td>Multipotent progenitor</td>
</tr>
<tr>
<td>NK</td>
<td>Natural killer</td>
</tr>
<tr>
<td>pDC</td>
<td>Plasmacytoid dendritic cell</td>
</tr>
<tr>
<td>preGM</td>
<td>Pre granulocyte-monocyte</td>
</tr>
<tr>
<td>preCFU-E</td>
<td>Pre colony forming unit-erythroid</td>
</tr>
<tr>
<td>PRC1</td>
<td>Polycomb repressive complex 1</td>
</tr>
<tr>
<td>Sca1</td>
<td>Stem cell antigen 1</td>
</tr>
<tr>
<td>SCF</td>
<td>Stem cell factor</td>
</tr>
<tr>
<td>TAM</td>
<td>Tamoxifen</td>
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<tr>
<td>Tet</td>
<td>Tetracycline</td>
</tr>
<tr>
<td>TetOP</td>
<td>Tetracycline operon</td>
</tr>
<tr>
<td>TLR</td>
<td>Toll-like receptor</td>
</tr>
<tr>
<td>TF</td>
<td>Transcription factor</td>
</tr>
<tr>
<td>NMP</td>
<td>Neutrophil monocyte progenitor</td>
</tr>
<tr>
<td>YFP</td>
<td>Yellow fluorescent protein</td>
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Background

Maintenance of internal stability in biological systems, homeostasis, is paramount for preservation of life. To maintain homeostasis, organisms are structured at different levels. In mammals, one level of structure consists of the cooperation between different tissues/organs – so-called organ systems. One such organ system is the cardiovascular system that is responsible for the continuous circulation of blood to all areas of the body. The basic components of the cardiovascular system include the blood system (hematopoietic system), the heart and the blood vessels. The blood system is a highly regenerative system with distinct cell types and fluid plasma that together mediate a range of essential functions to the organism. For instance, red blood cells (erythrocytes) are responsible for oxygen transport throughout the body, while cell fragments called platelets (thrombocytes) are responsible for blood coagulation. Moreover, the hematopoietic system contains white blood cells (leukocytes) that constitute the immune system and protect against foreign pathogens. The leukocytes can be further subdivided into cells that are part of the innate or adaptive immune system. Innate immune cells function as a “first line of defense” against harmful agents [1], and include macrophages, neutrophils, basophils, eosinophils, natural killer (NK) cells and certain T cell subsets. The adaptive immune system mounts a delayed, more specific immune response to pathogens, and is constituted primarily of B and T cells. Due to their short-lived nature, most mature cells in the hematopoietic system are continuously replaced by newly generated blood cells in an ongoing process called hematopoiesis.

The Discovery of Hematopoietic Stem Cells

In 1658, 68 years after the invention of the microscope, red blood cells were the first cells to be described as components of the blood by the Dutch naturalist Jan Swammerdam. It took another almost 200 years before white blood cells were also identified in 1843 [2, 3]. Although these pioneering studies identified crucial components of blood, it was not until after the 2nd world war, in the 1950s, that hematopoietic research started to generate results that explained how blood cells were formed. Observations of survivors of the nuclear bombings of Hiroshima and
Nagasaki in 1945 revealed that despite surviving the initial explosion, many of them died at later time points by hematopoietic marrow failure caused by exposure to gamma irradiation [4]. Later studies in mice revealed that the hematopoietic failure could be rescued by shielding the spleen from irradiation [5] or by injection of spleen or bone marrow cells from non-irradiated donors [6, 7]. The discovery that injection of cells could save mice from irradiation induced lethality suggested that the bone marrow and the spleen contained proliferating cells that can generate blood cells, and established hematopoietic cell transplantation (HCT) as an assay for evaluation of the “rescue-potential” of cells. Experiments by Till and McCulloch identified a linear relationship between the number of transplanted bone marrow cells and survival of irradiated recipient mice [8]. In following experiments, the same researchers identified splenic nodules that contained proliferating cells of donor origin in recipient mice. These colonies were initiated by cells, referred to as colony forming unit-spleen (CFU-S), and the numbers of transplanted cells and CFU-S displayed a linear relationship [9]. In an elegant follow up study, using sub-lethal irradiation of cells prior to transplantation to introduce cell-specific chromosomal marking, Till and McCulloch showed that the vast majority of cells in individual spleen colonies stemmed from one individual cell and thus had a clonal origin [10]. In addition, when CFU-S cells were transplanted into irradiated secondary hosts they could give rise to new CFU-S [11], demonstrating that the initial CFU-S could self-perpetuate through a process called self-renewal [11]. CFU-S colonies in these experiments only contained cells of erythroid, megakaryocytic and myeloid lineages. However, later studies revealed that CFU-S also harbored lymphoid potential [12], suggesting existence of a common precursor cell for all hematopoietic lineages. Although CFU-S colonies were initially thought to be derived from hematopoietic stem cells (HSCs), CFU-S could not maintain hematopoiesis in the long-term and subsequent experiments revealed that CFU-S colonies are derived from hematopoietic progenitor cells (HPCs) rather than from HSCs [13]. It was not until the 1980s that bone marrow cells capable of generating all hematopoietic lineages for extended periods of time were first observed in experiments based on the same principle of individual genetic marking as those performed by Siminovitch et al. on CFU-S [11]. Donor cells were individually marked by retroviral genetic integration and subsequently transplanted. Identification of common genetic marks in different blood lineages revealed the presence of common clones with multilineage capacities [14-16]. Collectively, these studies strongly indicated the existence of cells with long-term multilineage differentiation potential and ability to self-renew, the defining properties of HSCs (Figure 1).

The lack of HSC-enriching markers made the study of purified HSCs challenging. Technological advancements, such as Fluorescence-Activated Cell Sorting (FACS) and generation of specific antibodies against various cell surface proteins have influenced experimental hematology immensely. Prospective
isolation of increasingly homogenous populations of hematopoietic stem and progenitor cells (HSPCs) has facilitated delineation of the hematopoietic system. Advancements of protocols for HSC identification have revealed HSCs as very rare cells within the bone marrow and have resulted in successful isolation of single HSCs capable of serial long-term multilineage reconstitution [17].

**Figure 1.** Defining properties of HSCs; Self-renewal ability and multilineage differentiation potential.

**Hematopoiesis**

Hematopoiesis is defined as the ongoing production of all blood cellular components. As most mature blood cells are relatively short-lived and require continuous replacement, hematopoiesis is characterized by an extraordinary turnover rate with production of trillions of new blood cells every day [18]. To cope with the enormous proliferation required to generate sufficient numbers of blood cells and maintain homeostasis, the hematopoietic system is hierarchically organized (Figure 2) within the bone marrow [19]. Scarce HSCs reside at the top of the hematopoietic hierarchy with increasingly abundant and proliferating
progenitor cells downstream in the hierarchy that massively amplify hematopoietic
cell generation. Progress downstream in the hematopoietic hierarchy is associated
with increasingly restricted self-renewal ability and differentiation potential.

The adult hematopoietic hierarchy

FACS-based isolation along with development of functional assays have generated
increasingly detailed insights into the hematopoietic hierarchy (Figure 2) [19]. No
single, easily accessible, marker has been identified for identification of HSCs. Instead, combinations of surface markers are used to prospectively isolate HSPCs.
Adult HSCs and most early progenitors are devoid of mature lineage marker
expression, but express the cell surface markers stem cell antigen 1 (Sca1) and the
tyrosine kinase receptor c-kit, so called LinSca1c-kit+ (LSK) cells [20-23]. The
frequency of HSCs within the LSK population has been estimated to approximately one in 30 [19]. LSK cells can be further enriched for HSCs with the
surface markers CD34, Flt3, CD48 and CD150; HSCs being positive for CD150
and negative for CD34, Flt3, and CD48 [17, 24, 25].

Differentiation without cell division would quickly deplete the HSC pool. Therefore, HSC differentiation is thought to be accompanied by cell division. When HSCs divide they can either undergo a symmetric cell division, generating
two HSCs (self-renewal; HSC amplification) or two non-HSCs (differentiation;
HSC loss) or an asymmetric cell division generating one HSC and one
differentiated cell. Differentiation of HSCs into multipotent progenitor (MPP)
cells is accompanied by loss of self-renewal. Like HSCs, MPPs display low
proliferation rates, and can be separated from HSCs by loss of CD150 expression
and gain of CD34 expression [24, 26]. Further fractionation of MPPs has revealed
distinct subsets that differ in proliferation rates and lineage potential [27].
Alternative differentiation routes have been proposed where differentiation of
HSCs through an MPP state may not be mandatory or even the primary
differentiation route [28].

Lineage commitment downstream of MPPs remains controversial. In one
model, further differentiation involves differentiation through either a common
myeloid progenitor (CMP) or a common lymphoid progenitor (CLP). CMPs have
the capacity to form myeloid, megakaryocytic and erythrocyte progeny, while
CLPs form NK, B and T cells [29, 30]. CMPs have subsequently been found to be
a highly heterogeneous population containing several types of lineage committed
progenitors [31]. More recently lymphoid-primed multipotent progenitors (LMPP)
were identified as LSK cells, located downstream of MPPs, that express the CD34
and Flt3 markers [32]. LMPPs are phenotypically highly overlapping with the later
identified granulocyte monocyte lymphoid progenitors (GMLPs). Both subsets
harbor myeloid and lymphoid potential but lack robust megakaryocyte and
erythroid (MegE) potential [33]. This suggests a common differentiation route for granulocytes, monocytes and lymphoid cells that is separate from MegE differentiation. Downstream of GMLPs, the prevailing view has been that cells become committed to either granulocytes-monocyte (GM) differentiation via preGM progenitors, or to lymphoid differentiation via CLPs. MegE cells are believed to be generated from HSCs and MPPs via MegE restricted preMegEs that can generate megakaryocyte progenitors (MkP) and erythroid progenitors (pre-colony-forming-unit-erythroid; preCFU-E, CFU-E), that upon further maturation generate mature platelets or erythrocytes, respectively.

preGM is a functionally heterogeneous population that can be further fractionated based on expression of the transcription factor Gata1 or by expression of CD55 [34]. Functional analysis of Gata1− preGMs revealed that these cells, in addition to megakaryocytes and erythroid cells, had the potential to generate mast cells and eosinophils, two granulocytic cell types, while lacking monocyte-macrophage capacity and harboring only limited neutrophil potential. Conversely, Gata1+ preGMs generated monocytes and neutrophils, no mast cells and limited amounts of eosinophils [34]. These findings question the commonly held view that separation of monocyte-macrophage versus granulocyte lineages occur downstream of preGMs in a common granulocyte-monocyte progenitor (GMP), and instead suggest that the eosinophil and mast cell lineages separate from the monocyte-neutrophil lineages before they segregate from the MegE lineages.

Combined MegE and granulocyte (eosinophil, mast cell) potential in Gata1 expressing preGMs challenges the suggestion of an early segregation of MegE and myeloid lineages, and highlights the hematopoietic hierarchy as an evolving model rather than a set framework portraying hematopoietic differentiation (Figure 2). Continuous efforts aimed at elucidating differentiation trajectories and cellular constituents of the hematopoietic system will likely refine this model further.
**Hematopoietic stem cell development**

Establishment of the hematopoietic system during ontogeny is characterized by two distinct waves referred to as primitive and definitive hematopoiesis (Figure 3). In the mouse embryo, primitive hematopoiesis generates the first blood cells at embryonic day 7-7.5 (E7-7.5) in the extra-embryonic yolk sac [35, 36]. Primitive hematopoiesis is transient and generates mainly red blood cells. These are distinct from later-generated red blood cells by their presence of a large nucleus and an embryonic form of hemoglobin [37, 38]. Primitive hematopoiesis is not believed to produce cells that contribute to adult hematopoiesis. Following primitive hematopoiesis, a second wave of definitive hematopoiesis gives rise to the first
definitive HSCs (dHSCs), defined by their ability to self-renew and long-term multilineage reconstitute irradiated hosts after transplantation (Figure 3). The first evidence of the emergence of embryonic dHSCs came from studies of chimeric quail-chicken embryos [39]. dHSCs were later confirmed also in mouse embryos where the aorta-gonads-mesonephros (AGM) region was proposed as the site for de novo dHSC generation (at approximately E10.5) [40, 41]. Additional sites where dHSCs are generated have subsequently been suggested, including the placenta [42, 43], vitelline/umbilical arteries [44, 45], the embryonic head [46] and the yolk sac [47-49] (Figure 3). Fetal liver, which is the major site for hematopoiesis in the developing embryo, is seeded at E9.5 by developmentally restricted HPCs that generate fetal hematopoietic cells [50, 51]. However, it is not until E11 that the first dHSCs, capable of long-term reconstitution of irradiated hosts can be found within the fetal liver [41]. dHSC expand massively in the fetal liver during E12-E16 [52] after which they begin their migration to the bone marrow, where they can be found in adult mice. During the first 3-4 weeks after birth, bone marrow HSCs retains some fetal dHSC characteristics before switching to adult HSCs characteristics [53].

Figure 3. Scheme depicting early hematopoietic development. A wave of primitive hematopoiesis is followed by definitive hematopoiesis. Proposed developmental origins of dHSCs are indicated. Hematopoietic cells seed the fetal liver where dHSCs expands before colonizing the bone marrow, which is the major hematopoietic organ during adult life. AGM, aorta-gonads-mesonephros, E: Embryonic day, dHSC: definitive hematopoietic stem cell.
Fetal liver dHSCs differ extensively from their counterparts in the adult bone marrow with regards to their surface marker phenotype and, most strikingly, their high proliferation rates [54]. Despite that high proliferation of adult HSCs is typically associated with a lower long-term reconstitution capacity, fetal HSCs outperform adult HSCs after transplantation. Additionally, fetal and adult HSCs show distinct differentiation potentials [55]. Although most mature hematopoietic cell types are continuously maintained by adult HSCs, some subsets are only generated during fetal hematopoiesis, including tissue resident macrophages such as Langerhans cells (skin) and microglia (brain), and lymphoid peritoneal B1a B cells and epidermal Vγ3δ T cells. Maintenance of these cells is primarily upheld by homeostatic proliferation in the tissues where they reside [56-60].

Aging hematopoiesis is associated with functional impairments, such as increased incidence of myeloid diseases (for instance leukemia) and anemia, as well as decreased potential to mount adaptive immune responses [61]. A hallmark of hematopoiesis in aged subjects is a skewed output of mature effector cells. Upon transplantation of aged HSCs into young recipients, donor HSCs generate myeloid biased reconstitution patterns at the expense of lymphoid cell reconstitution [62-65]. Age-associated alterations of HSCs are believed to be mostly intrinsic to aged HSCs as reciprocal transplantation of young HSCs into aged recipients confers normal reconstitution patterns [63]. However, extrinsic factors regulating HSC aging have also been reported [66]. The aged hematopoietic system also associates with skewed frequencies of HSPCs, where lymphoid progenitors are severely decreased while myeloid progenitor frequencies are increased. The aged HSC compartment is greatly expanded and display impaired performance in competitive transplantation experiments compared to young HSCs [63, 67, 68]. Reports of transcriptional changes in old HSCs are in line with the observed myeloid skewing seen from aged HSCs [63, 69, 70]. Collectively, studies of hematopoiesis in aged subjects indicate that impairment of the aged hematopoietic system is primarily a consequence of cell-intrinsic, age-associated alterations to HSCs.

Regulation of HSCs and Hematopoiesis

To maintain life-long hematopoiesis cells within the hematopoietic system needs to be able to respond to challenges and at the same time avoid exhaustion. HSCs are constantly faced with different fate-options including entry into cell-division, apoptosis or differentiation. To maintain a proper balance between available fate-options, HSPCs and are regulated by a complex network of both intrinsic and extrinsic regulators that are discussed in the section below.
The HSC niche

The existence of a three-dimensional local tissue-microenvironments (niche), located in the bone marrow where the HSCs reside, was postulated by Schofield in 1978 [13]. For many years, technical difficulties hindered elucidation of the niche that maintain and regulate HSCs. However, in the last decades, dissection of structures and cells that regulate HSCs locally has intensified, resulting in the proposed existence of two distinct niches with different supportive functions; an endosteal niche, lining the trabecular bone that supports HSC quiescence [13, 71], and a perivascular niche at sinusoidal blood vessels that regulates HSC proliferation and differentiation [24]. Recent progress has favored a perivascular niche as the primary locale for adult quiescent HSCs [72-75]. HSC niches are constituted by multiple cell types that contribute to regulation of HSCs, including endothelial and mesenchymal Stromal cells [24, 76] as well as megakaryocytes, sympathetic nerves, macrophages, osteoclasts and non-myelating Schwann cells (Figure 4) [24, 77-81]. Niche cells can regulate HSCs by secreting cytokines and growth factors. For example, osteoblasts secrete Thrombopoietin and Angiopoietin 1 that regulate HSC quiescence and bind to HSC-expressed Thrombopoietin and Tie2 surface receptors, respectively [82, 83]. Endothelial cells in the perivascular HSC niche express stem cell factor (SCF) that signals through the c-kit tyrosine kinase receptor, that is expressed on all HSPCs. Genetic deletion of SCF from endothelial cells has been shown to deplete HSCs, whereas SCF deletion in hematopoietic cells had no effect on HSC function or frequency [84].
Regulation of HSCs by inflammatory signaling

Inflammation can regulate HSCs via emerging response signals. Inflammation is triggered by pathogen infection or injury and is characterized by an inflammatory response elicited by components of the hematopoietic system. Inflammation is triggered by defined cytokines that have strong effects on both immune effector cells and HSCs. Haas et al. reported that stem cell like megakaryocytic progenitors, that are phenotypically similar to HSC, can effectively generate platelets. These cells are quiescent but primed for platelet generation and are activated in response to acute inflammation [85]. Interferons are inflammatory cytokines that are released in response to several pathogens, such as bacteria and viruses. Interferons have been shown to directly stimulate HSC proliferation [86-88], while another cytokine, granulocyte colony-stimulating factor (G-CSF), disrupts the HSC niche and mobilizes HSCs from the bone marrow [89, 90]. HSCs express multiple pattern recognizing Toll-like receptors (TLR), which recognize pathogens directly. TLR-signaling play key roles in activation of the innate immune system and ligation by for example Lipopolysaccharide (LPS) to TLRs enhances HSPC proliferation and activate HSCs [91-93]. Chronic exposure to LPS or inflammatory cytokines leads to HSC exhaustion highlighted by compromised reconstitution after transplantation and impaired self-renewal ability [86, 94].

Studies of HSCs during inflammation is complicated by phenotypic alterations induced by inflammatory signals. For example, Ly6a, the gene encoding for the cell surface protein Sca1, is regulated by an interferon responsive DNA element resulting in increased Sca1 expression after exposure to interferons [95]. Similarly, c-kit expression is reduced in response to treatment with the chemotherapeutic 5-fluorouracil (5-FU) [96]. In summary, inflammatory signaling regulates HSCs and can change their immunophenotype; hence, care must be taken in experiments with possible ongoing inflammatory signaling (discussed in article 2).

Intrinsic regulation of hematopoiesis

While the microenvironment is clearly important for HSC regulation, intrinsic genetic regulators and their interactions are also crucial determinants of HSC fate. Transcription factors (TFs) are proteins that control gene expression. TFs are instrumental in regulating HSC differentiation programs and ~50 different TFs have been implicated in HSC regulation directly or indirectly [97]. These include Stem cell leukemia/TAL1 (SCL/TAL1), which is instrumental in fetal HSC genesis and in adult megakaryocyte/erythroid development [98], and HOX genes, that in many cases are preferentially expressed in HSCs and are proposed to regulate HSC self-renewal [99, 100]. The HOX co-factor MEIS1 have also been implicated in HSC regulation, where its loss results in increased HSC cycling and
subsequent HSC exhaustion [101]. Many additional TFs, which are often involved in downstream lineage commitment, are also important for HSC maintenance, including C/EBPA, E2A, IKAROS, MYC and PU.1 [102-106]. Other types of HSC-regulators have also been identified, for example regulators of apoptosis such as the anti-apoptotic gene BCL2 [107, 108] and cell cycle inhibitors and their regulators, including the transcriptional repressors GFI1 and retinoblastoma and the cycline-dependent kinase inhibitors p21 and p18 [109-112].

Epigenetic changes are stable modifications that lead to a reshaped overall structure of a DNA sequence without altering the underlying DNA sequence, and make up another form of intrinsic regulation to HSCs. Epigenetic modifications include DNA methylation and various alterations to core histone proteins. Histones are stable proteins that form nuclear structures, which pack DNA by wrapping DNA stretches into units called nucleosomes. DNA methylation is typically associated with repression of gene expression while histone modification generally promote gene activation [113]. Epigenetic regulators play crucial roles in the regulation of HSC self-renewal and differentiation by modulating gene expression patterns. For example, the DNA methyltransferases DNMT3a and DNMT3b and the chromatin modifying polycomb repressive complex 1 and 2 (PRC1 and PRC2) have been shown to regulate HSCs self-renewal [114-116]. In addition, epigenetic changes have been linked to age-associated alterations of HSCs [117-119] as well as to malignant hematopoiesis and leukemia [120].

**Lineage priming**

In addition to expression of TFs and regulators that maintain HSCs, lineage affiliated genes that become increasingly higher expressed during differentiation have been found to be expressed at low levels in HSCs [121, 122]. This phenomena, commonly termed “lineage priming”, suggests that these cells have not yet committed to any specific developmental path and has been proposed to reflect multipotency of HSCs [123]. Studies using single-cell transplantations of HSCs revealed that subsets of HSCs show specific lineage biased reconstitution patterns that were either myeloid biased, lymphoid biased or balanced, perhaps reflecting transplantation of HSC clones with different lineage priming [124]. Reconstitution experiments, where also platelet generation was evaluated, revealed platelet-biased HSCs that express the platelet-lineage associated protein von Willebrand factor and showed a megakaryocyte-affiliated gene expression pattern [125]. Generally, HSCs express more myeloid and MegE associated transcripts compared to lymphoid associated transcripts, suggesting that the HSC compartment per se is naturally myeloid biased. Furthermore, Comparisons of old and young HSCs by single-cell RNA sequencing and clonal transplantation experiments have revealed a relative expansion of megakaryocyte-biased HSCs,
with a coinciding loss of lymphoid biased, myeloid biased and balanced HSCs upon aging [69].

**Lineage development**

Several TFs and regulators that dictate specific differentiation fates of progenitors downstream of HSCs have been identified. PU.1 and GATA1 have been proposed to act as antagonists during early erythroid development where GATA1 promotes erythropoiesis while PU.1 stimulates myelopoiesis in multipotent progenitors [126]. However, the importance of GATA1-PU.1 antagonism in early myeloid lineage choice has been challenged, instead these TFs have been suggested to execute and reinforce lineage choice once made [127].

Maturation toward the platelet or erythroid lineages from bipotent MegE progenitors depends on an antagonistic TF relationship between KLF1, which promotes erythropoiesis, and FLI1, that promotes megakaryopoiesis [128]. Development into myeloid or lymphoid lineages from multipotent progenitors (GMLP/LMPP) depends on the magnitude of PU.1 expression, with a higher PU.1 expression favoring myeloid differentiation [129]. Further myeloid development of preGMs/GMPs into macrophage or neutrophil lineages depends on PU.1 and C/EBPA regulation of an antagonistic relationship between GFI1 and EGR, where GFI1 promotes neutrophil and EGR macrophage differentiation [130, 131].

Lymphoid differentiation is specified in PU.1 expressing cells, that are capable of lymphomyeloid differentiation, and requires IKAROS expression. IKAROS is believed to promote GFI1 expression that in turn suppress the expression of PU.1 and, thereby, myeloid fate [132, 133]. Further lymphoid development into B cells from the CLP depends on an E2A-EBF-PAX5-FOXO1 transcriptional program while E2A and GATA3 expression and Notch signaling support T cell development [134, 135].

**Hematopoietic Stem Cell Transplantation**

HCT transplantation is tremendously important both clinically and in experimental hematology. Historically, transplantation has been the mainstay of research on HSPC biology in vivo. In HCTs, prospectively isolated hematopoietic cell populations are most commonly transplanted by injection into the bloodstream of hosts that have been myeloablated with irradiation (Figure 5). Mice or humans that are exposed to lethal doses of irradiation succumb to hematopoietic failure caused by depletion of vital mature cells; for example, maintenance of sufficient levels of platelets is necessary to prevent internal bleedings [136]. However, transplantation
of non-irradiated bone marrow can save lethally irradiated animals [7]. Purified HSCs alone are not able to produce mature blood cells rapidly enough to ensure survival after lethal irradiation. Therefore, it is critical to co-transplant HPCs to ensure survival in the short-term. Transplantation of HSCs is however critical for long-term survival. Competitive transplantation measures the functional potential of HSCs against a set number of co-transplanted HSCs (usually whole bone marrow that includes both HPCs and HSCs). Long-term and multilineage reconstitution potential of competitively transplanted HSCs is evaluated by analysis of peripheral blood cell reconstitution kinetics and bone marrow chimerism at least 16 weeks after transplantation. Self-renewal ability of HSCs is evaluated in serial transplantations where bone marrow cells from primary recipient mice are transplanted into secondary recipients followed by HSC chimerism analysis (Figure 5). To distinguish donor cells from host and competitor cells after transplantation, congenic mice, that express CD45.1 and/or CD45.2 on the cell surface, are routinely used. However, only leukocytes express the CD45 antigen and therefore analysis of reconstitution of the platelet and erythrocyte lineages has most often been omitted in transplantation experiments.

Figure 5. Schematic depicting hematopoietic stem cell transplantation followed by evaluation of the key characteristics of hematopoietic stem cells; durable self-renewal and long-term multilineage potential.

Transplantation - in vivo – but non-physiological

The transplantation procedure exposes HSCs to several non-physiological circumstances. Isolation of HSCs from their resident niches in the bone marrow is commonly followed by purification of HSCs, often involving extensive preparations followed by FACS. This workflow exposes HSCs to in-vitro mechanical handling, which is potentially destructive [137]. After isolation, cells are injected into the blood stream of irradiated recipient animals, where they are exposed to a distorted cytokine milieu caused by massive cell death and an inflammatory response [138]. Transplanted HSCs are next forced to circulate, exit circulation and migrate to their niche in the bone marrow. Only HSCs that are able to colonize new niches can be evaluated by transplantation. Under physiological conditions, HSCs are perhaps never exposed to the massive proliferation pressure they encounter when they are forced to rebuild the obliterated hematopoietic system after transplantation into irradiated recipients. Instead, their native
residence is in a protected environment where adult HSCs display very low proliferation rates [139, 140].

Substantial efforts have been aimed at limiting the confounding factors that pre-conditioning confers to evaluation of HSCs in transplantation. Sub-lethal doses of irradiation relieve some of the stress inflicted by lethal irradiation. Mice strains that have defective c-kit signaling have been used as recipients of the transplanted graft. Such strains allow engraftment of HSCs without prior irradiation, although, c-kit signaling impairments in recipients introduces new confounding factors [141-143]. Depletion of HSCs, by antibodies or restricting dietary valine, has also been proposed as possible strategies to allow engraftment without prior irradiation [144-147].

Post- vs. Pre-transplantation hematopoiesis

Methods utilizing in vivo labeling of hematopoietic cells that do not rely on transplantation have recently been adopted and developed for analysis of hematopoiesis in native conditions (see chapters on lineage tracing and proliferation assays, pages 33-39). For example, histone labeling allows for long-term tracking of proliferation history in HSPCs by evaluation of cell-division dependent dilution of label, while lineage tracing in native conditions have begun to resolve HSC contributions to native hematopoiesis.

Single HSC transplantation experiments have exposed substantial heterogeneity in the HSC pool [124, 148-151] and transplantations of individually “barcoded” HSCs have allowed analysis of many HSC-reconstitution patterns simultaneously in the same recipient [152, 153]. Such experiments have revealed oligoclonal HSC contribution to post-transplantation hematopoiesis, with low numbers of contributing HSCs at any given time point. In sharp contrast, lineage tracing and evaluation of lineage relationships and kinetics of cell generation in an unperturbed hematopoietic hierarchy has revealed that native blood production is highly polyclonal, with many thousands of clones contributing simultaneously [154, 155]. In article 1, we show that transplantation/reconstitution alters HSC behavior long-term after reconstitution of the blood system. This suggests that the underlying behavior of transplanted HSPCs is altered even after normal blood-values are restored, perhaps permanently. Collectively, fundamental differences of hematopoiesis post- and pre-transplantation highlight a need for analysis of hematopoiesis in models that allows for evaluation in unperturbed settings.

The Cre/loxP System

The mouse is a powerful experimental model in medical research. Reasons for this includes the ability to introduce a DNA sequence (so called transgene) into a
specific site in the germline genome of mice. This has traditionally been done by homologous recombination and is called gene targeting. Gene deletion/disruption (knockout) is one outcome of gene targeting. Here, a gene is made non-active because of mutation or deletion, allowing examination of phenotypes resulting from knockout of the target gene. Gene knock-in is another outcome of gene targeting; here, an endogenous DNA-sequence (e.g. gene) is replaced with a transgene that is inserted into the genome. Knock-in of reporter genes in various loci has generated models that allow monitoring of where and when genes of interest are expressed. A further developed form of gene knockout is conditional knockout. With this technique, specific genes can be targeted at specific times rather than being deleted in cells from the beginning of life.

**Cre/loxP recombination**

Cre recombinase (Cre)/loxP recombination is an experimental system that is frequently used in biomedical research as it enables conditional activation or deletion of specific genes at precise time points and/or in specific cells/tissues. Cre is a site-specific recombinase enzyme derived from the bacteriophage P1. The Cre enzyme recognizes distinct short DNA sequences, called loxP-sites, between which it recombines DNA segments (so-called “floxed” regions). Recombination leads to inversion or deletion of the floxed DNA segment depending on the orientation of the loxP sites (Figure 6). Cre expression is driven by a promoter, which initiates transcription of Cre in all cells where the promoter is active. This allows targeting of Cre to specific cells/tissues by selection of a Cre-promoter that is active in the cell type of interest. The Cre/loxP system was originally developed and used in yeast and mammalian cell lines [156, 157] before it was adopted for use in transgenic mouse models [158, 159], with Cre expressed from cell type specific promoters [160, 161].

![Figure 6. Cre/loxP recombination leading to deletion of a floxed gene.](image)
Even though Cre/Lox recombination deletes (or inverts) the DNA sequence between loxP sites, strategies have been developed that allows activation of gene expression by Cre-mediated recombination [162, 163]. In the most widespread strategy, a STOP cassette that is flanked by loxP sites (often referred to as a lox-STOP-lox or LSL cassette) is deleted by recombination, thereby ceasing transcription inhibition of a target gene. The transgene that harbor the LSL cassette antecedently to the target gene is commonly targeted to the ubiquitously expressed ROSA26 locus, conferring expression of the target gene in all cells where Cre excises the STOP cassette (Figure 7).

Continuous expression of Cre, without temporal control of recombinase activity, precludes studies of gene knockouts that present embryonically lethal phenotypes. In addition, Cre activity through ontogeny can generate unwanted, complex phenotypes, resulting from secondary compensatory effects to gene-deletion in early development. This can make interpretations of adult knockout phenotypes difficult. To overcome such issues, Klaus Rajewsky’s group developed transgenic mice where expression of Cre could be conditionally activated at a given time during ontogeny [164]. In the generated mouse-strain, expression of a Cre transgene is controlled by the inducible Mx1-promoter (Mx1-Cre mice) that is involved in defense against viral infections. Expression from the Mx1-promoter is silent in healthy mice but can be transiently activated to high levels of transcription upon exposure to high levels of interferon α or β (Figure 8) [165].
Further development of inducible forms of Cre has addressed issues with promiscuous “leaky” expression from the Mx1-promoter and has enabled stricter temporal control over the recombinase activity then can be achieved with the Mx1-Cre system. One commonly used, inducible, form of Cre is a chimeric protein where Cre recombinase is fused with the ligand-binding domain of the estrogen receptor (ER) [166]. The CreER chimeric protein is retained in the cytoplasm until Tamoxifen ([TAM] or its metabolite 4-OH TAM) binds to the receptor part of CreER. 4-OH TAM binding allows translocation of CreER to the nucleus where Cre can recombine between loxP sites (Figure 9). CreERT2 is a more recently modified form of CreER that is extra sensitive to exogenously administrated TAM but is insensitive to endogenous estrogen [167].
Studying Native Hematopoiesis

Studies of native and perturbed hematopoiesis are necessary for establishing how and why hematopoiesis is altered after challenge. Hematopoietic challenges include hematological malignancies like anemia and leukemia, as well as infections and clinical treatments, such as cytokine induced HSC mobilization, chemotherapy, irradiation and transplantation, and combinations of these regimens. It is clear that hematopoiesis is disrupted by several of these states/treatments. However, the mechanisms for regeneration of hematopoiesis and niches after injury are largely unknown. In addition, much of the knowledge gained from experimental hematology is derived from transplantation experiments. Generation of long-term multilineage progeny exclusively from HSCs after transplantation has argued for a critically important role of HSCs in maintenance of homeostasis of the hematopoietic system. However, fundamental differences between post- and pre-transplantation prompt investigation of hematopoiesis in more unperturbed conditions. Development of transgenic approaches that allows dissection of unperturbed hematopoiesis has generated insights into how the hematopoietic system is maintained and has led to re-examination of a critical role for HSCs in maintenance of adult hematopoiesis [154, 155, 168].

Figure 9. Schematic illustration over CreER mediated recombination. CreER is expressed in all cells where its promoter is active but can only enter the nucleus and recombine after binding to 4-OH Tamoxifen. Therefore recombination activity is conditional and can be controlled by administration of Tamoxifen.
Proliferation assays

Most adult HSCs reside in a quiescent state. Upon differentiation HSCs enter HPC stages that are characterized by extensive proliferation [169]. Proliferative status is correlated with functional capacity of HSCs after transplantation, where cycling HSCs display impaired reconstitution potentials [170]. However, it is possible that analysis of adult HSCs in active cell cycle is selecting for HSCs that initiated differentiation and thus have lost key HSC properties [171]. In addition, cell cycle analysis only gives a snapshot illustration of the fraction of cells that reside in different cell cycle phases at the time of analysis. Therefore, evaluation of proliferation history should be advantageous for studies aimed at elucidating a possible link between proliferation and HSC potential.

Bromodeoxyuridine (BrdU) is a thymidine analogue that has been extensively used for detection of proliferating cells. Analysis of incorporation of BrdU in DNA [172, 173] or dilution of BrdU after DNA-labeling [139, 174] can be used to evaluate proliferation history in HSCs both short- and long-term. Generally, results from such investigations have supported low proliferation rates of HSCs. Detection of BrdU in cells is however incompatible with prospective isolation of viable cells as it requires fixation of cells prior to analysis. This together with reports of BrdU-induced toxicity that directly affects proliferation [139, 175, 176] has highlighted the need for further development of proliferation-quantification tools.

Labeling techniques that allow for prospective isolation of viable cells after label-evaluation has been developed [177, 178]. In an approach published by Takizawa et al., LSK cells were labeled with the cell-membrane permeable fluorescent dye carboxyfluorescein succinimidyl ester (CFSE) resulting in stable labeling due to intracellular coupling of CFSE to lysine residues. In this approach cells are labeled in vitro, prior to transplantation into non-conditioned recipient mice [177]. CFSE label is subsequently diluted between daughter cells upon cell division and CFSE label retention can thus be used for discrimination of cells based on their divisional history (Figure 10). The in vitro labeling of cells associated with this method does however preclude investigations of cellular proliferation of cells at steady state conditions. To overcome such obstacles, in vivo biotin labeling was developed as a method that allows for investigation of cellular proliferative history in steady state [178].

Biotin labels surface membrane proteins and is diluted upon division, but allows for labeling without isolating cells ex vivo and preserves cell viability. Biotin labeling does however suffer from poor resolution due to non-proliferation-
associated label dilution and is therefore not suitable for evaluation of proliferation in long-term experiments [178].

**Histone-2B Labeling**

Recent development of transgenic mouse models that transiently express fluorescent protein-coupled Histone-2B (H2B) has facilitated more reliable evaluations of long-term steady state proliferation history [179-181]. Natural and modified H2B proteins are very stable, allowing for long-term tracking of label retention. After a labeling period, when labeled H2B proteins are produced and incorporated into nucleosomes, a chase period is initiated when no more H2B labeled protein is produced. During the chase period, H2B label is divided between daughter cells upon cell division and the level of label retention is therefore correlated to the proliferative history of the cell (Figure 10).

![Chase Period – Label Dilution](image)

**Figure 10.** Schematic outline depicting the principle of H2B-label dilution. Cells are labeled during a labeling period. During a following chase period label is divided among daughter cells, and thereby diluted, upon cell division.

Both Tetracycline (Tet)-ON [182] and Tet-OFF [139, 171] H2B labeling systems have been adapted for use in mice. In Tet-OFF systems H2B labeling is continuous until Tetracycline (or a Tetracycline derivate like Doxycycline [DOX]) is administrated, whereas in Tet-ON systems H2B labeling is induced (ON) only when DOX is administrated. Which cells that are H2B labeled is decided by the promoter/locus that controls labeling. Both ubiquitous (ROSA26) and HSPC-specific models have been used for H2B-labeling, however, so far, no HSC-specific system.

Despite reports of transgene expression “leakage” in a H2B Tet-OFF system [183] evaluations of label retention in Tet-ON and Tet-OFF systems have
generated similar results in regard to HSC proliferation [139, 182]. Multiple reports on proliferation after H2B labeling have now confirmed that HSCs are slowly dividing cells in steady state [139, 140], in addition, it has been established that also immature multipotent progenitor cells are slowly dividing [27, 140].

Further studies of H2B-label retention have revealed sex specific proliferation rates in HSPCs that are dependent on sex hormones [184] and proliferation history has been correlated with HSC surface phenotypes and reconstitution potentials [171]. Recently Bernitz et al. used a HSPC-selective Tet-OFF labeling system to evaluate very long-term divisional history in adult and aged HSCs [185]. From these experiments, it was concluded that HSCs can count and remember their self-renewal divisions, however, no mechanism for how this is achieved was presented.

H2B-labeling has several advantages over previously used assays for evaluation of proliferation. This system allows labeling without perturbation to the hematopoietic system and, importantly, enables prospective isolation of cells based on proliferation history. Collectively this has made evaluation of H2B-label retention the new standard for the study of proliferation in mice.

**Lineage tracing**

In lineage tracing techniques, single cells are labeled with heritable marks that are passed on to their progeny and can thus be used to evaluate common origins of generated cells. Lineage tracing can be used to evaluate for example proliferation kinetics and progeny founder (Figure 11). Lineage tracing has classically been used to study development and embryonic origin of various tissues and cell types, but is being increasingly applied for adult stem cell research.

![Figure 11. Schematic depiction of the principle of label tracing. Cells are labeled with heritable marks that are passed on to their progeny. Contribution of an initially labeled population of cells to distinct cell types can be followed over time.](image-url)
The first lineage tracing studies were performed at the turn of the 18th century by direct observation of cells under a microscope. Developmental biologists studied lineage relationships in leech and nematode embryos by light microscopy culminating in determination of the fate of every cell in the C. elegans embryo [186]. Lineage tracing by direct observations is only possible in species with few cells or in transparent embryos that are accessible. When direct observation of cells is not possible lineage tracing requires labeling of cells in a way that ensures that the label is transmitted to all progeny of a labeled cell. This results in progression of label from the originally marked cells to their descendants (Figure 11). Developmental biologists have studied neural development in chicken and frog embryos with use of lipid soluble dyes that label the cell membrane [187, 188] and DNA or histone label that is divided among daughter cells upon cell division has been used to track the fate of progeny from infrequently dividing stem cell populations [180, 189, 190].

*Genetic labeling*

In recent years, genetic marking of cells has become a common practice for lineage tracing studies. In one early study of HSC fate after transplantation into irradiated recipient mice Lemischka et al. genetically marked cells using a c-myc retroviral vector [16]. Transplantation of cells from one embryo or animal to another is one form of lineage tracing that presents many options for distinguishing between host and donor cells and therefore allows fate mapping of transplanted cells. However, a major disadvantage is that cells may not behave the same after transplantation as in homeostasis (see discussion in chapter on transplantation, pages 26-28) [140, 154, 191].

In hematopoietic research, the Cre/loxP system is the most commonly used method for labeling cells for lineage tracing. A variety of mouse strains where Cre is expressed from different promoters have been used to visualize distinct mature hematopoietic cell types and for fate mapping in specific cell lineages. Most of these strains are based on constitutive expression of Cre under control of a promoter that display a lineage restricted expression pattern [192-195]. Several Cre strains that mark progenitors downstream of HSCs have proven useful for elucidation of the in-vivo differentiation potential of the targeted progenitors. For example, IL7ra-Cre mice allowed investigation of development downstream of the IL7ra expressing CLP [196]. Boyer et al. generated an Flt3-Cre lineage tracing mouse model where CLPs and more immature progenitors just downstream of HSC are efficiently labeled. In this strain Flt3-Cre labeled cells in all lymphoid and myeloid lineages suggesting that development of all hematopoietic cell types involves progression through an Flt3 expressing progenitor [197].

Label tracing studies have been used to elucidate when and where HSCs emerge in a developing embryo. Using a model where an enhancer of SCL that is responsible for SCL expression within early HPCs where used to drive CreER
expression, Göthert et al. pulse labeled E10.5-E11.5 embryos and found labeling of adult HSCs, implying that adult HSCs originate from an embryonic stage [198]. Similarly, Samokhvalov et al. labeled embryonic Runx1 expressing cells before onset of circulation to show that Runx1 is essential for the emergence of HSCs and that its expression at E7.5 is limited to the yolk sac [49, 199]. These cells were later shown to give rise to both primitive and definitive hematopoiesis in the fetal liver. Additionally, approximately 10 % of adult HSCs were labeled in this model, suggesting that the yolk sac can contribute substantially to definitive hematopoiesis [49].

**Multicolor reporters**

Lineage tracing with multicolor reporter constructs was pioneered with the development of the “Brainbow” construct [200]. In this system, recombination at multiple loxP sites enables combinatorial expression of multiple fluorescent proteins. Multicolor reporter models are useful for lineage tracing in solid tissues, because the underlying tissue-architecture can be preserved while clonal composition is observed by microscopy. In a further developed Brainbow system (Brainbow 2.1), cells are stochastically labeled with one of four possible fluorophores. The Brainbow 2.1 system was used in the “Confetti mouse” to investigate stem cell-derived clones within the intestinal crypt [201]. Brainbow technology was also applied in a recent study aimed at dissecting the clonal dynamics in hematopoiesis and the associated epigenetic memory in HSC clones [202]. In this work, a “HUe” mouse model with approximately 20 tandemly integrated fluorescent reporter cassettes was generated. After recombination, stochastic expression of one out of 4 possible fluorescent proteins from each cassette can in theory generate $10^3$ distinct color combinations, which was proposed to allow for assessment of individually labeled clones. However, in mice where multiple clones are actively contributing to hematopoiesis, identification of cells that constitute clones is dependent on flow cytometric analysis of simultaneous expression of multiple fluorescent proteins that will be expressed at different levels and have overlapping emission spectra. Based on these concerns, it is difficult to envision the exactness in the system, thereby raising concerns of the resolution that can be achieved in the HUe system. Analysis of clonal dynamics in this model suggested that native hematopoiesis is composed of a few major clones that persist and additional clones that emerge, disappear or expand. Furthermore, as labeling in the HUe system is ubiquitous, it precludes direct investigations of HSCs and their contributions without transplantation.

**HSC lineage tracing**

Evaluation of labeled cells at different time points after labeling can be used to estimate label progression from the initially labeled cells into their progeny (Figure 11). From such data, it is possible to estimate generation kinetics and
contribution to distinct cell types. Analysis of label progression in adult mice is preferably done after inducible labeling, where the initial labeling of cells can be controlled. Models with constitutive Cre expression are therefore less suitable for analysis of dynamics of hematopoiesis. In addition, constitutively active Cre strains that label only adult HSCs have been unavailable. Hence, lineage tracing studies from HSCs have used inducible labeling of HSCs by various versions of CreER.

_Tie2_ is a transmembrane tyrosine-protein kinase receptor that is expressed in both adult and embryonic HSCs. Taking advantage of the HSC specificity, Busch et al. used the _Tie2_ locus to drive inducible expression of a modified form of CreER (MerCreMer) in a reporter mouse strain [155]. Differentiation monitoring after HSC labeling revealed striking differences between fetal and adult HSC contribution to hematopoiesis. Downstream label progression from fetal liver HSCs was very rapid, reaching an almost complete label-equilibrium between HSCs and HPCs and mature blood cells at one week after birth, revealing robust generation of all investigated hematopoietic cell types by fetal HSCs. In sharp contrast, label progression from adult HSCs into their proposed progeny was extraordinarily slow. Label progression from adult HSCs was however increased after perturbation of hematopoiesis by injection of the cytostatic 5-FU, that kills dividing cells and causes transient leukopenia in the blood. From these results, it was concluded that adult HSCs are only rarely active in unperturbed hematopoiesis but can be activated by hematopoietic stress.

Direct comparisons of label progression between fetal and adult HSCs after Tie2<sup>MerCreMer</sup> labeling is not straightforward as the frequency of initially labeled HSCs are very different in fetal and adult HSCs. After in utero TAM injection, at E10.5, on average 25 % of fetal HSCs were labeled, while multiple TAM injections in adult mice labeled less than 1 % of the HSC pool. Very low labeling of adult HSCs could make label tracing in adult mice unreliable as a very low frequency of initially labeled HSCs may aggravate or preclude evaluation of label progression into certain cell types because the fraction of labeled cells is too low for reliable evaluation. More importantly, initial labeling of a small fraction of HSCs is potentially the result of biased labeling of a subset of HSCs. Label tracing after biased HSC labeling will reflect the behavior of the labeled subset of HSCs, which may not reflect the general behavior of the HSC pool. These considerations need to be taken into account when the results discussed above are evaluated [155].

A recent lineage tracing study by Sawai et al. evaluated steady state dynamics of adult HSC contribution to hematopoiesis using a Pdzk1ip1-CreER mouse model to specifically label HSC [203]. By sampling of serial bone marrow biopsies, the authors revealed an expansion of the initially 30 % labeled HSC fraction over time, and concluded that a subset of HSCs that self-renew more than the average HSC pool are labeled by Pdzk1ip1-CreER. Robust label progression was noted.
into all investigated peripheral blood and HPC populations. In contrast, cell types that are thought to emerge during embryonic hematopoiesis, like B1a B cells, brain microglia and Langerhans cells, harbored only minor fractions of labeled cells confirming their primarily fetal origin. Finally, Sawai et al. proposed that adult HSCs continuously contribute to all hematopoietic cell lineages in steady state. This conclusion seems incompatible with the conclusions from Busch et al. [155]. However, the observed differences could very well be explained by labeling of distinct HSC subsets.

A novel mouse model that allows fate mapping after clonal labeling of cells in-situ was recently developed in Fernando Camargo’s lab [154]. In this system, inducible mobilization followed by genome reintegration of a transposome generates heritable, cell-specific, DNA “scars” that can be utilized as cell-specific barcodes. This system is driven by the ROSA26 locus, hence, both differentiated and immature hematopoietic cells are barcoded. When the clonal composition in mature peripheral blood cells was monitored over time, granulocyte production was revealed to be highly polyclonal and originate from distinct sets of progenitors that are successively activated to generate granulocytes. Comparison of clonal compositions between blood lineages revealed only a few granulocyte clones that were present in multiple lineages. Instead, most clones were detectable only at one sampling time point. When barcodes in HSPCs and previously analyzed blood cells were compared, surprisingly few overlapping tags were detected in HSCs and mature blood cells or HPCs. In contrast, multipotent progenitor cells readily shared tags with myeloid progenitors and mature blood cells. This generated the conclusion that HSC contribute only sparsely to granulopoiesis in unperturbed hematopoiesis; rather, successive recruitment of thousands of lineage-restricted and multipotent clones drive hematopoiesis. The conclusion from the work of Sun et al. has been challenged and potential caveats of this experimental approach, like “leaky” secondary transposone mobilization and detection of false positive integration sites have been pointed out [203].
Mitotic History Reveals Distinct Stem Cell Populations and Their Contributions to Hematopoiesis.
Petter Säwén, Stefan Lang, Pankaj Mandal, Derrick J. Rossi, Shamit Soneji, David Bryder

Background to Article 1

Most mature hematopoietic cells are short-lived cells in need of continuous replenishment during an individual’s lifetime. The hematopoietic system is therefore characterized by high cellular output and vigorous proliferation. This proliferation capacity is utilized in clinical HCT. For such, HSPCs are commonly harvested from the peripheral blood of donors following cytokine-induced mobilization of HSPCs from the bone marrow. Harvested HSPCs are subsequently transplanted into patients that have been preconditioned with chemotherapy and/or irradiation. HCTs are tremendously important in the treatment of many blood cell malignancies, including leukemia. Little is however known about potential long-term adverse effects on hematopoiesis derived from mobilization of cells from the bone marrow or from the extensive proliferation that is required by the transplanted graft to rebuild the hematopoietic system. To evaluate such effects evaluations of HSCs and hematopoiesis with non-invasive methods is necessary (See chapter on studying native hematopoiesis, pages 32-39).

The first experiments that linked proliferation activity to HSC potency revealed that actively cycling cells (identified by a high DNA content) in HSC enriched Lineage marker negative and Sca1+ (L’S+) adult bone marrow possess lower reconstitution capacities compared to non-cycling cells [170]. Competitive transplantation experiments of LSK cells at different cell cycle phases confirmed that adult HSCs typically reside in the G0/G1 phase of the cell cycle [172, 204] with a sharp reduction in reconstitution capacity when recruited to active cycling [204-206].
Development of assays where proliferation history can be followed without disruption of cells has facilitated studies of HSC proliferation in the steady state (see chapter on proliferation assays, pages 33-35). By combining results from long-term H2B-GFP and BrdU label retention studies with computational modeling, Wilson et al. concluded that in the steady state about 20% of HSCs are deeply dormant, dividing only approximately 5 times over the lifetime of a mouse [139]. Furthermore, this study reported that dormant HSCs could be reversibly activated into cell cycle upon exposure of hematopoietic stressors, such as 5-FU treatment, G-CSF mobilization or exposure to BrdU. Foudi et al. also demonstrated, using a Tet-ON H2B labeling model, that HSCs divide very infrequently and that the most potent reconstituting HSCs are confined to the most dormant HSCs [182].

HSCs are most commonly identified by complex multi-parameter flow cytometry. Refinements of HSC identification-strategies has been ongoing for decades and has allowed study of increasingly pure populations of HSCs. Isolation based on high H2B label retention can substantially enrich for primitive HSCs in mouse bone marrow but cannot be used alone to identify HSCs [182]. Despite substantial efforts for purification of HSCs phenotypically equivalent prospective HSCs show heterogeneous behavior with purities of sorted HSCs typically under 50% [17, 24, 28, 124, 150, 151, 153, 207]. Single-cell index sorting has made it possible to review the full flow cytometry phenotypes of individually sorted cells [208]. This knowledge allows prospective correlation of differences in surface marker expression, and combinations of such, with functional and transcriptional single-cell data. Index sorting can be used to refine existing isolation protocols for hematopoietic cell types and has revealed gating strategies that enhance existing HSC purification protocols further [207]. In article 1 we combine analysis of proliferation history with index sorting and single-cell gene expression to dissect HSC heterogeneity and identify HSC subsets with distinct potentials.

**Summary of Article 1**

In this study, we explored proliferation dynamics of HSPCs in steady state and after exposure to various stressors to the hematopoietic system by use of a DOX-inducible Tet-ON Histone-2B-mCherry (H2B-mCherry) mouse model. Establishment of this model revealed high, homogenous, labeling of HSPCs after a labeling period, when DOX was administrated, of one week. Previous reports of fast proliferation kinetics among myeloid progenitors were confirmed in analysis of H2B-mCherry label retention one week after the end of the labeling period (chase) (Figure 10) [169]. Further evaluations of label retention after longer periods of chase (up to 56 weeks) confirmed low proliferation rates of HSCs in
steady state [139, 182]. Interestingly, MPPs, believed to be located immediately downstream of HSCs in the hematopoietic hierarchy (Figure 2), exhibited label retention levels comparable to those seen in HSCs. In contrast, GMLPs showed faster proliferation kinetics than HSC or MPPs, which was further elevated in 16 months old mice, while HSCs and MPP proliferation was not significantly increased in aged mice.

Following establishment of steady state proliferation dynamics among HSPCs we moved on to investigate effects of hematopoietic stressors on HSPC proliferation behavior. To investigate how HSC proliferation is affected by transplantation we transplanted bone marrow from H2B-mCherry mice into lethally irradiated wild type recipients. After reconstitution of the blood system, donor derived cells were labeled and label retention in donor HSCs was subsequently analyzed after a chase period. Although recipient mice had regained normal and stable peripheral blood values at the time of H2B-mCherry labeling, proliferation kinetics of HSCs were elevated up to 4-5 months post transplantation. In addition, the increased proliferation rate of HSCs was further enhanced upon serial transplantation.

Mobilization has been suggested to be interlinked with HSPC proliferation [204]. Therefore, we set out to study proliferation kinetics in HSPCs after cyclophosphamide/G-CSF-induced mobilization. HSPCs were successfully mobilized from the bone marrow of previously H2B-mCherry labeled mice, resulting in an almost complete depletion of MPPs from both the spleen and the bone marrow. Label retention analysis in mobilized mice revealed increased proliferation of both splenic and bone marrow HSPCs. Next, we investigated the effects of selective depletion and subsequent recovery of mature blood cells on proliferation kinetics of HSPCs. By injections of cell-depleting antibodies, directed specifically against mature B or myeloid cells, we successfully depleted cells of the respective lineages from the peripheral blood in previously labeled mice. Label retention analysis revealed no alterations to HSPC proliferation kinetics after B-cell depletion, whereas depletion of Gr1+ myeloid cells increased the proliferation kinetics of MPPs and GMLPs while proliferation in HSCs was unaffected.

As a last part of this study we explored whether information on the proliferative history of HSCs can be correlated to identifiable gene expression signatures. HSCs, retaining heterogeneous levels of H2B-mCherry, were index sorted from mice after two or five weeks of chase. Multiplexed single-cell gene expression analysis was subsequently performed on a panel of genes including hematopoiesis-related TFs, cytokine receptors, cell surface markers and cell-cycle-related genes. Clustering of HSCs with similar gene expression profiles identified 4 distinct groups of HSCs. H2B-mCherry label retention values were next probed to all HSCs. This revealed that gene expression could be linked to proliferative history. HSCs that had proliferated the least generally displayed a more primitive gene
expression signature compared to HSCs with higher proliferation kinetics. Furthermore, index markers revealed apparent differential expression of both Sca1 and c-kit between groups. HSCs that had proliferated the most displayed higher values of c-kit and lower values of Sca1 surface expression compared to remaining HSCs. In contrast, HSCs in the least proliferative group demonstrated the lowest c-kit levels. Differential expression of Sca1 and c-kit cell surface markers between molecularly distinct HSC groups allowed us to compare these groups functionally by prospective isolation followed by transplantation into lethally irradiated hosts. Peripheral blood reconstitution analysis revealed that the identified HSC subsets displayed distinct reconstitution potentials. Sca1\textsuperscript{low} HSCs generated virtually no long-term reconstitution, whereas Sca1\textsuperscript{high} HSCs showed robust peripheral blood reconstitution with Sca1\textsuperscript{high}c-kit\textsuperscript{low} HSCs, representing the most slowly dividing HSCs, demonstrating the highest reconstitution capacity.

In summary, this study established an H2B-mCherry labeling mouse model that allows for evaluation of steady state proliferation history. This model was utilized for evaluation of the effect of various hematopoietic stressors on HSPC proliferation. We show that milder stressors enforce proliferation of MPPs rather than HSCs, and conclude that transplantation enforces increased HSC proliferation even after reestablishment of “normal” hematopoiesis. Furthermore, HSC divisional history was linked to distinct surface marker expression patterns, reconstitution potentials, and gene expression profiles.

**Article 2**

**Potential Pitfalls of the Mx1-Cre System: Implications for Experimental Modeling of Normal and Malignant Hematopoiesis.**

Velasco-Hernandez T, Säwén P, Bryder D, Cammenga J.


**Background to Article 2**

Cre/loxP recombination is commonly used for generation of gene knockouts. When temporal control over gene deletion is necessary the conditionally inducible Mx1-Cre system is frequently used (see chapter on the Cre/loxP system pages 28-32). In Mx1-Cre mice, Cre is expressed after interferon binding of the Mx1-promoter. Interferon release is usually induced by injection of the synthetic RNA-analog poly I:C that induces a strong interferon response when administered to mice (Figure 8) [209]. Interferon induction has been suggested to trigger HSC cycling [86, 88] and introduce phenotypic alterations to HSCPs [86]. Endogenous
interferon production can activate the *Mx1* promoter in the absence of poly I:C injection, explaining reports of approximately 10% spontaneous gene deletion in spleens of non-treated *Mx1-Cre*-driven knockout mice [164].

In experimental hematology, transplantation of bone marrow cells from *Mx1-Cre* knockout models is performed for evaluation of effects from gene-deletion only in the hematopoietic system. This is achieved by letting transplanted bone marrow cells reconstitute the irradiation-ablated blood system before inducing gene knockout by administration of poly I:C. However, irradiation causes extensive cell death and is accompanied by massive release of inflammatory cytokines, including interferons, which can potentially activate the *Mx1* promoter (See chapter on hematopoietic stem cell transplantation, pages 26-28). Interferons are normally made and released by cells in response to presence of several pathogens such as viruses, bacteria and parasites. Tumor cells can also induce interferon release by, for example, plasmacytoid dendritic cells (pDC) [210], which depend on FLT-3 signaling for their development [211, 212]. In the FLT-3ITD mouse model, a constitutively activated form of the FLT-3 receptor tyrosine kinase is expressed. We therefore also evaluated pDCs and spontaneous *Mx1-Cre* activation in the context of FLT-3ITD expression.

### Summary of Article 2

In this study, we originally set out to investigate the involvement of hypoxia inducible factor 1α (HIF-1α) in leukemia. For this aim, conditional knockout mice with floxed *Hif-1α* alleles were crossed to *Mx1-Cre* mice. C-kit+ bone marrow progenitor cells from the generated *Mx1-Cre;Hif-1α* flox/flox mice were transduced with retroviruses carrying different oncogenes before transplantation into wild type recipients. Remarkably, a substantial fraction of cells were deleted for *Hif-1α* prior to poly I:C injection [213]. This prompted us to investigate the underlying cause for the observed spontaneous deletion of *Hif-1α*. For this, we crossed YFP<sup>LSL</sup> reporter mice [214] with *Mx1-Cre* mice to generate a strain (*Mx1-Cre-YFP<sup>LSL</sup]*) where (spontaneous) recombination results in YFP expression, that is detectable by flow cytometry. Next, c-kit+ cells from *Mx1-Cre-YFP<sup>LSL</sup>* mice were subjected to transduction of retroviruses encoding fluorescent proteins and/or transplantation after which the rate of spontaneous recombination was determined. Strikingly, YFP expression could be identified in approximately 20% of the non-manipulated cells. *In vitro* culturing for 48 hours or transduction with retroviruses did not significantly increase the percentage of YFP+ cells. By contrast, > 70% of donor cells expressed YFP at 10 weeks after transplantation despite any evident positive selection pressure of recombined cells, indicating that the transplantation procedure *per se* promotes spontaneous recombination in *Mx1-Cre* deletion models.
Previous work revealed that adult mice with a combination of Mx1-Cre; Hif-1α\textsuperscript{floxed/floxed} and FLT-3\textsuperscript{ITD} knock-in showed spontaneous deletion of Hif-1α prior to poly I:C injection [215-217]. To investigate if Hif-1α deletion was initiated already during embryonic development, fetal liver cells from E14.5 embryos were extracted and analyzed \textit{in vitro}. Around 20 % of colonies derived from fetal liver cells showed partial spontaneous deletion of Hif-1α, whereas an astonishing 70 % of colonies from 12-week-old bone marrow cells were deleted for the Hif-1α gene in both alleles. This indicates that the spontaneous excision effect observed in Mx1-Cre mice is cumulative over time. To exclude that the high levels of spontaneous recombination was due to a particularly high sensitivity for recombination of the Hif-1α\textsuperscript{floxed/floxed} allele, FLT-3\textsuperscript{ITD}-Mx1-Cre mice were crossed with Phd2\textsuperscript{floxed/floxed} mice to evaluate spontaneous recombination of another floxed region. In Mx1-Cre-FLT-3\textsuperscript{ITD}-Phd2\textsuperscript{floxed/floxed} strain, over 70 % of colonies expanded from adult bone marrow showed spontaneous deletion of the floxed Phd2 gene. Comparison of the ~70 % deletion rate seen in FLT-3\textsuperscript{ITD}-Mx1-Cre crossed models with the < 20 % spontaneous deletion rate noted in Mx1-Cre-YFP\textsuperscript{LSL} mice indicate that FLT-3\textsuperscript{ITD} expression increases the spontaneous excision effect observed in Mx1-Cre mice. An explanation for the high rate of spontaneous recombination at floxed alleles in FLT-3\textsuperscript{ITD}-Mx1-Cre mice may be promiscuous Mx1-Cre activation caused by increased endogenous interferon levels. Indeed, an increased abundance of interferon-producing plasmacytoid dendritic cells (pDCs) was observed in FLT-3\textsuperscript{ITD} mice [212].

Mx1 promoted expression of Cre is most commonly induced by endogenous interferon release as a response to poly I:C injection. Interferon signaling has been shown to enhance proliferation of HSPCs \textit{in vivo} [86, 88]. To further evaluate proliferation after poly I:C administration, we investigated HSPC proliferation with the H2B-mCherry labeling model we established in article 1. HSPCs from bone marrow in previously labeled H2B-mCherry mice were analyzed for label retention at different time points (1-24 days) after poly I:C or control injections. These analyses revealed no significant increase of proliferation in HSCs after poly I:C injection. In contrast, one and three days after poly I:C injection, GMLPs and more lineage restricted preGMs showed decreased label retention, indicating a possible transient increase in proliferation among these progenitors. However, dramatic phenotypic alterations and skewed frequencies of HSPCs within the bone marrow LSK and LK compartments were noted immediately after poly I:C injection. Therefore, observed changes in HSPC proliferation short-term after poly I:C administration are hard to interpret. HSPC frequencies had returned to baseline values 3 days after poly I:C induction while Sca1 surface expression and H2B-mCherry label retention in HPCs was comparable to control-levels 8 days after poly I:C injection. These results indicate that even though strong Sca1 induction is seen after poly I:C injection, this effect is transient and completely resolved at 8
days post injection, in line with previous reports of Sca1-induction upon interferon administration [86].

Finally, we explored an alternative approach to the Mx1-Cre model for recombination in cells with floxed alleles. In this approach, recombination of a floxed DNA segment was resolved by in vitro incubation with Tat-Cre before transplantation rather than by in vivo transgenic expression of Cre. Tat-Cre is an engineered form of the Cre recombinase enzyme that can diffuse through cell membranes and enter the nucleus where it carries out recombination at loxP sites [218]. Tat-Cre could mediate efficient recombination in control experiments. Next, Tat-Cre-induced recombination was used in a model where expression of an activating Kras mutation (Kras\textsuperscript{G12D}) is hindered by a floxed stop cassette (Kras\textsuperscript{LSL-G12D}). Such mice were crossed with DOX inducible MLL-ENL mice [219] to study Kras\textsuperscript{G12D} in the context of MLL-ENL-driven leukemia. As previously reported [219], transplantation of purified MLL-ENL expressing HSCs does not generate leukemia. However, all recipients of MLL-ENL-Kras\textsuperscript{LSL-G12D} HSCs, which had been subjected to incubation with Tat-Cre prior to transplantation, developed myeloid leukemia within 10 weeks after transplantation, confirming the feasibility of Tat-Cre mediated recombination in in vivo leukemia models.

In summary, we highlight several potential pitfalls of the Mx1-Cre system in this study. First, we report a high spontaneous recombination rate in Mx1-Cre models, especially upon transplantation but also when Mx1-Cre and FLT-3\textsuperscript{ITD} are present together in the genome. Second, we show that injection of poly I:C, a routine procedure for Mx1-promoter activation, transiently alters bone marrow HSPC phenotypes in treated mice. Finally, we propose Tat-Cre treatment as an alternative to Mx1-driven Cre expression for recombination in cells that will be subjected to transplantation or any other ex vivo procedure.
Article 3

Adult Hematopoietic Stem Cells Contribute Actively to Myeloerythroid Hematopoiesis in Steady State.

Background to Article 3

Recent developments of transgenic mouse models have facilitated the study of HSCs in unperturbed hematopoiesis. Non-invasive systems for evaluation of proliferation history have revealed that HSCs proliferate very slowly in steady state [139, 140, 182]. Furthermore, HSC lineage tracing experiments in unperturbed hematopoiesis have generated conflicting conclusions regarding HSC contribution to hematopoiesis in steady state (see chapter about lineage tracing pages 35-39). This prompted us to investigate HSC contribution to steady state hematopoiesis using an HSC-specific $Fgd5^{CreERT2}$ lineage tracing model.

Although HSCs are commonly identified by their immunophenotype using complex multi-parameter flow cytometry, recently developed genetic models have enhanced the ability to detect rare HSCs. In these reporter strains, expression of fluorescent proteins is driven from promoters of HSC-specific genes, such as $\alpha$-catulin, Hoxb5, Hoxb4 and Fgd5, which allows single-parameter identification of HSCs [75, 99, 220, 221]. or $Fgd5$ gene was identified as a potential HSC-marker gene by comparison of transcriptomes from more than 40 hematopoietic populations. In addition to expression in HSCs, $Fgd5$ is abundantly expressed in endothelial cells. Transgene knock-in to the Fgd5 locus disrupts endogenous Fgd5 expression and homozygous Fgd5 knock-in mice die during embryonic development, whereas heterozygous Fgd5 knock-in mice display no phenotype. Bone marrow cells identified based solely on Fgd5 reporter expression show robust HSC activity with stem cell activity restricted to the Fgd5 labeled fraction. In addition to Fgd5 reporter mice, an inducible CreERT2-Fgd5 knock-in strain, $Fgd5^{ZsGreen-CreERT2}$ mice, that harbor a fluorescent reporter (ZsGreen) has been developed [220].
Summary of Article 3

In the study “Adult Hematopoietic Stem Cells Contribute Actively to Myeloerythroid Hematopoiesis in Steady State” we set out to perform lineage tracing from adult HSCs in steady state. For this we utilized Fgd5-CreERT2 mice that were developed in Derrick Rossi’s lab [220]. Within the hematopoietic system, Fgd5 is expressed in HSCs and at low levels in scarce immature progenitors. We confirmed HSC specific expression of Fgd5 by comparison of single-cell transcriptome data from 520 SLAM-HSCs (LSKCD150^-CD48^Fgd5^+) and 793 Fgd5-HSCs (Lineage^-Kit^-Fgd5^+) to a large data set of single-cell transcriptome data (11,588 cells) from c-kit^+ bone marrow cells that includes many types of HPCs, but very few HSCs. This comparison revealed that SLAM-HSC and Fgd5^-HSC transcriptomes overlapped almost completely, confirming that Fgd5 expression marks HSCs at a transcriptional level. Next, Fgd5-CreERT2 mice were crossed with Tomato^LSL^ reporter mice to generate a strain (Fgd5-CreERT2-Tomato^LSL^) that allows for lineage tracing from HSCs. HSC specific labeling was confirmed by evaluating the fraction of Tomato labeled bone marrow HSPCs short-term after Tomato labeling by a single TAM injection. 48 hours after labeling 5.7 % of HSCs and 0.2 % of other LSK cells were Tomato labeled, while no other hematopoietic bone marrow cells expressed Tomato.

To investigate the generation dynamics of mature hematopoietic cell types we Tomato labeled HSCs by feeding cohorts of Fgd5-CreERT2-Tomato^LSL^ mice with TAM containing food pellets for 16 weeks. HSCs were completely Tomato labeled (>99 %) after the labeling period and label progression into mature peripheral blood cell lineages was monitored by regular blood sampling both during the labeling period and during the following chase period (up to 32 weeks). Myeloerythroid cells generally acquired label with more rapid kinetics than lymphoid cells. Platelets acquired Tomato label with the fastest kinetics, followed by granulocytes and erythrocytes. Among lymphoid cells, NK cells were labeled faster than B or T cells. T cells showed the slowest labeling kinetics and CD4^+ T cells acquired label faster than CD8^- T cells. In addition to Tomato labeling by continuous administration of TAM containing food, we also “pulse labeled” mice by injecting TAM one or five times on consecutive days. After labeling, mice were chased for up to 32 weeks. During the chase period all mice were regularly bled and evaluation of Tomato label progression into peripheral blood cells revealed similar label progression kinetics as after labeling with TAM food. At the experimental end-point all mice were evaluated for Tomato label in the HSC compartment. The fraction of Tomato^+ HSCs was next correlated to the fraction of Tomato^+ peripheral blood cells at the experimental end-point. This analysis
revealed that 5x injected HSCs more actively generate mature peripheral blood cells than HSCs from 1x injected mice, indicating that labeling of a larger fraction of HSCs (5x TAM) includes labeling of more active HSCs than labeling of a smaller fraction of HSC (1x TAM).

We took advantage of completely HSC labeled Fgd5-CreERT2-TomatoLSL mice by analyzing the input from adult HSCs to reportedly fetal derived hematopoietic subsets. By investigating the fraction of Tomato+ cells from hematopoietic cells types in the epidermis we confirmed a fetal origin of the tissue resident macrophages of the skin, Langerhans cells. We next investigated Tomato label progression into B1a B cells isolated from the peritoneal cavity of completely HSC labeled mice. Only a minor fraction (<10 %) of B1a B cells were Tomato+ confirming their fetal origin. Finally, we investigated Tomato expression in microglia, the tissue resident macrophages of the brain, and in line with their reported fetal origin we did not find any Tomato+ microglia.

At the end-point analysis we investigated the fraction of Tomato+ cells in various HSPC subsets in 1x and 5x TAM injected mice. When fractions of labeled progenitors were related to the fraction of labeled HSCs in individual mice, we noted faster labeling kinetics of progenitors in 5x injected mice than in 1x injected mice, in line with the faster label progression into peripheral blood cells in 5x injected mice. MkPs acquired Tomato label with faster kinetics then lineage restricted erythroid (CFU-E) and myeloid (preGM) progenitors, in agreement with the fast label progression into the platelet lineage. Among LSK-progenitors, LSKCD150−CD48+ cells (MPPs) acquired label with the most rapid kinetics approaching label equilibrium with HSCs after 12 days in 5x injected mice. The rare population of LSKCD150+CD48− cells also reached label-equilibrium with HSCs (after 112 days). By contrast, the much more abundant LSKCD150+CD48− cells (GMLPs) acquired Tomato label with slower kinetics then other LSK cell subsets. Comparisons of percentages of labeled cells between mature peripheral blood cells and their proposed progenitor after different periods of chase revealed that granulocytes and platelets reach label equilibrium with their respective progenitor 25 days after labeling, while label equilibrium between erythrocytes and CFU-Es reached equilibrium with slower kinetics. This suggests a longer maturation time for erythrocytes than for platelets and granulocytes before entry into circulation.

The fast label progression seen into LSKCD150+CD48− cells prompted us to characterize this subset further with regards to lineage potentials/affiliations. First, we performed multiplexed quantative Real Time-PCR to obtain gene expression data from a panel of defined HSPCs including LSKCD150+CD48− cells. Principal component analysis revealed a transcriptional association of LSKCD150+CD48− cells with progenitors of the platelet and erythroid lineages rather than with myeloid progenitors or HSCs. Finally, we functionally evaluated the lineage potentials of LSKCD150+CD48− in vitro. When generated cell types from single-
cell sorted HSCs, GMLPs and LSKCD150^CD48^ cells were compared, higher generation of megakaryocytes and erythrocytes were seen from LSKCD150^CD48^ cells than from HSCs or GMLPs. Collectively, gene expression analysis and cell culture experiments suggest MegE lineage potential/bias of LSKCD150^CD48^ cells.

In summary, we here present the Fgd5-CreERT2 model as a suitable model for lineage tracing studies from adult HSCs. We confirmed initial HSCs specific labeling, and achieved labeling of virtually all HSCs in adult mice. Results from our label tracing studies confirm the conclusion that adult HSCs contribute substantially to adult hematopoiesis but not to fetal derived hematopoietic subsets that was reported by Sawai et al. [203]. Further, this study reveal that platelets are generated from HSC with faster kinetics than other peripheral blood cells and that myeloid cells in general are generated with faster kinetics than lymphoid cells. MPPs are labeled with the fastest kinetics among LSK-progenitors, while LSKCD150^CD48^ cells displayed fast labeling kinetics compared to GMLPs. Furthermore, gene expression analysis and cell culture experiments with LSKCD150^CD48^ cells suggest an association of these cells with the MegE lineages.
Future Studies

Continued efforts aimed at dissecting properties of native hematopoiesis are necessary to maintain hematopoietic research as a fundament for study of adult stem cell biology. In situ labeling of individual HSCs is an interesting avenue for dissection of clonal contributions of HSCs in native hematopoiesis [154]. Future studies in such models could perhaps reach higher resolution by initial labeling of HSCs specifically, while clonal labeling at different stages in life could answer outstanding questions regarding clonal dynamics in aged hematopoiesis. Further studies of H2B-label retention, in HSC specific labeling systems, could be used for lineage tracing in the hematopoietic system as have been done in other tissues [180, 201]. Simultaneous evaluations of H2B label retention and lineage tracing dynamics [203, 222] would be informative and could identify progeny of label-retaining HSCs over time and generate informative results about relationships between proliferation and lineage output/potential. Combined evaluations of native HSC properties and single-cell RNA-sequencing is another exiting avenue for systematic dissection of relationships between transcriptome profiles and function in the hematopoietic system.

Under senare år har det utvecklats musmodeller som tillåter analys av blodsystemet och dess celler utan större påverkan på blodsystemet. I en sådan modell har vi analyserat hur mycket blodstamceller och progenitorer delar sig, dels under normalt blodbildande och dels efter att blodsystemet utsatts för olika typer av stress. Vi kom fram till att efter transplantation ökar frekvensen som blodets stamceller delar sig med även lång tid efter att blodsystemets återbildats. Förflyttning av blodstamceller från benmärgen till blodet påverkade inte hur ofta blodstamceller delar sig. Istället försvinner vissa omogna progenitorer helt från benmärgen efter sådan behandling. Selektiv destruktion av endast B-celler påverkade inte celldelningsfrekvensen hos blodets stamceller eller progenitor celler,
däremot påverkade destruktion av andra mogna blodceller, granulocyter, hur ofta vissa progenitorer delade sig. Sluttligen kopplade vi ihop hur mycket individuella stamceller hade delat sig med analys av uttryck av ett antal gener i samma cell. När blodstamceller delades in i grupper beroende på hur mycket de hade delat sig såg vi att stamceller i olika grupper uttryckte olika gener och de hade också olika potential i transplantationsexperiment.

Att ”knocka ut” gener är en viktig experimentell strategi för att utvärdera specifika generers betydelse i olika sammanhang. ”Knocka ut” betyder ofta att man helt raderar ut den DNA sektion som utgör en viss gen från en cell. Det finns väletablerade musmodeller där man kan styra när radering av en gen ska ske genom att skapa en stark immunreaktion i musen. I artikel 2 har vi undersökt en sådan modell och visat att i denna modell kan man inte helt kontrollera när en gen ska raderas eftersom transplantationstrålning orsakar en immunreaktion som leder till att genen raderas spontant i vissa celler. Vi belyser också andra problem med denna modell. Bland annat förändras sammansättningen av omogna blodstamceller och progenitorer i benmärgen av immunreaktionen som ska kontrollera när gen-knockout ska ske. Sammantaget måste man vara försiktigt när man använder denna modell i transplantationsexperiment och vi föreslår slutligen en alternativ procedur där dessa problem kan undvikas.

vidare upptäckte vi att många av dessa celler är progenitorer till blodplättar och röda blodkroppar. Sammanfattningsvis visar vi i denna studie att blodstamcelled bidrar till bildande av alla typer av blodceller under normala förhållanden men med olika kinetik.

Vi har i dessa studier analyserat blodets stamceller i modeller där de kan studeras med minimal yttre påverkan. Detta har lett till identifiering av subklasser av stamceller såväl som till etablerande av blodets stamceller som kontinuerligt blodcellsbildande.
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References


