Molecular regulation of human hematopoietic stem cells
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Chapter 1

Introduction
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Investigators that are referenced in the introduction. Font size correlates to the number of authorships.
Chapter 1. Introduction

1.1 Maintenance of the hematopoietic system

1.1.1 The hematopoietic hierarchy

The blood system consists of many different cell types that are specialized to perform various functions. For example, B- and T-cells of the lymphoid lineage cooperate to fight infections through recognition of non-self antigens, macrophages of the myeloid lineage phagocytose invading micro-organisms, and red blood cells of the erythroid lineage are responsible for transport of oxygen and carbon dioxide throughout the body. Depending on the level of categorization, there are at least 10 different mature cell types. To uphold the immunity, oxygen transport and blood clotting for which the hematopoietic system is responsible, it is estimated that 10 million mature blood cells are produced per second in an adult male (Ogawa, 1993). To maintain this regenerative potential, the blood system is organized as a hierarchy that is sustained by a limited number of long-lived stem cells at the apex. These multipotent stem cells give rise to several, increasingly lineage-restricted progenitor cells that possess the proliferative potential to sustain mature blood cell production. Seminal studies, particularly in the mouse system, have taught us a lot about the organization of hematopoietic stem and progenitor cells and the factors that influence early cell fate decisions. However, further investigation is necessary to improve our understanding of human hematopoiesis during health and disease, and to manipulate hematopoietic stem cells (HSCs) for clinical purposes such as gene therapy and in vitro expansion to improve cord blood transplantation outcomes.

1.1.2 Selective pressures that drive the fundamental organization of hematopoiesis

Hematopoietic cells at all stages of development, from HSCs to specialized mature cells, possess specific properties to optimize their functional performance. Long-term repopulation potential is restricted to HSCs due to their capacity for self-renewal, a cell division in which one or both of the daughter cells have the same developmental potential as the parent cell. This property of self-renewal should be tightly controlled to prevent uncontrolled proliferation, and accordingly, the HSC pool has additional properties to ensure a life-long blood supply while minimizing the risk of malignant transformation. HSCs utilize specialized programs to prevent damage from reactive oxygen species (ROS) (Ito et al., 2004; Tothova et al., 2007) and DNA mutations (Milyavsky et al., 2010; Yahata et al., 2011). HSCs enter long periods of rest or quiescence, which is thought to protect against replication-associated damage that could lead to oncogenesis (Li, 2011). As well, HSCs are endowed with great regenerative potential and are activated upon injury to increase production of progenitor cells that re-establish homeostasis of the blood system (Wilson et al., 2008). Progenitor cells have a different set of properties. During early differentiation of HSCs, self-renewal is lost and cells start proliferating. Active prolif-
eration likely results in more rapid accumulation of damage, but progenitors do not have the self-renewal capacity to propagate this damage indefinitely. Multipotency is gradually lost and progenitor cells become more lineage-restricted, as they expand to sustain day to day production of terminally differentiated cells. Since terminally differentiated cells have a short lifespan, any mutations that arose during their development will be purged quickly, preventing the accumulation of multiple mutations. On the other hand, mutations that are acquired by a self-renewing HSC are fixed and will persist as long as the HSC clone itself. Over time, accumulation of mutations that confer a proliferative advantage at the level of HSCs can lead to oncogenic transformation (Shlush et al., Nature, in press). A pool of highly potent but largely quiescent HSCs may serve as a reservoir that has specific properties to avoid damage accumulation, whereas progenitors carry the proliferative burden to continuously replenish various mature blood cell types.

1.2 Historical perspective on studying HSCs

1.2.1 Identification of HSCs

Red blood cells were first observed under a microscope in 1658 by the Dutch naturalist Jan Swammerdam and first illustrated by Antoni van Leeuwenhoek (Leeuwenhoek, 1695) (Figure 1). Almost two centuries later, the French physician Alfred Donné discovered platelets in 1842. In 1843, Gabriel Andral and William Addison described leukocytes (Hajdu, 2003). A Russian biologist astutely postulated that these various blood elements arise from a single primitive stem cell (Maximow, 1909). In the 1950s, experimental advances were made that would eventually unveil the origin of different blood cell types. First, lead shielding of the spleen in mice was shown to improve hematopoietic recovery and survival after irradiation (Jacobson et al., 1950). Shortly thereafter, it was shown that intravenous injection of a bone marrow suspension into syngeneic animals improved hematopoietic regeneration after irradiation (Lorenz et al., 1951). Using a donor mouse from a strain with an unusually small chromosome enabled the distinction between cells from donor and recipient mice (Ford et al., 1956). Irradiated recipient mice were injected with donor cells, and the donor cells replaced most of the bone marrow up to 49 days after injection, indicating that hematopoietic recovery after irradiation was mediated by donor derived cells rather than a humoral factor.

The key experiments to establish the existence of hematopoietic stem cells were performed during the 1960s. In Toronto, James Till and Ernest McCulloch detected macroscopic spleen colonies in irradiated mice that were transplanted with donor cells, providing the basis for the first assay to enumerate multipotent hematopoietic progenitor cells (Till and McCulloch, 1961) (Figure 2). Characterization of spleen colony forming units (CFU-S) established all the major stem cell principles that we use today. By irradiating donor cells to induce unique chromosomal markers, it was shown that spleen colonies were derived from a single cell (Becker et al., 1963). CFU-S showed at least some self-renewal potency, as one-third of spleen colonies isolated at day 10 contained CFU-
S progenitors (Siminovitch et al., 1963). The demonstration that colonies, clonally derived from a CFU-S progenitor, could contain erythroid and granulocyte lineages, established the concept of a multilineage progenitor, which was called a hemopoietic stem cell (Wu et al., 1967). By the end of this transformative decade, it was established that blood production is maintained by HSCs, which are characterized by their high capacity for self-renewal, proliferation and multipotential differentiation.

After experimental identification of a multilineage stem cell with self-renewal potential, development of an assay to specifically detect HSCs proved a difficult task. In vitro culture assays using semisolid medium were developed to detect and quantify CFU-C progenitor cells of different lineages (Bradley and Metcalf, 1966; Pluznik and Sachs, 1965), but these colonies had little self-renewal potential to generate secondary colonies. The possibility of a common progenitor for CFU-S and lymphoid cells was anticipated (Wu et al., 1968), and several lines of evidence indicated the existence of an undifferentiated multipotent stem cell, higher in the hierarchy than CFU-C or CFU-S. First, the proliferative potential of CFU-S was widely variable (Magli et al., 1982; Siminovitch et al., 1963). Second, measurements of CFU-S did not correlate well with long-term hematopoietic recovery upon irradiation (Harrison, 1972; Kretchmar and Conover, 1970). Third, careful examination of CFU-S colonies showed that they were devoid of lymphoid cells, establishing that cells detected by the CFU-S assay had restricted differentiation options (Abramson et al., 1977; Paige et al., 1981). Fourth, elimination of proliferating cells by 5-FU revealed the presence of non-cycling stem cells that were more primitive than CFU-S (Hodgson and Bradley, 1979). To detect a precursor to both the CFU-C and CFU-S, new assays had to be developed. Long-term in vivo repopulation analysis was gaining traction (Harrison, 1980). Using chromosomal tracking, the existence of multipotent stem cells with long-term in vivo potential was indicated (Abramson et al., 1977). Retroviral marking and insertion site analysis provided a more refined experimental approach to follow progeny of clonal HSC, and conclusively estab-
Chapter 1

1.2.1 Published that multipotent long-term HSC give rise to both myeloid and lymphoid lineages (Dick et al., 1985; Keller et al., 1985). To the present day, long-term multilineage repopulation of a host animal is the best measure of HSC activity. The next leap forward would come from the ability to prospectively isolate stem and progenitor cell types from the bone marrow, which will be detailed below.

1.2.2 Early assays to study human hematopoiesis

The main advantages of using mice over humans for research include the availability of blood and bone marrow cells, the possibility to transfer cells between animals and other experimental manipulations including genetic modification to generate mouse models. As a result, the study of human hematopoiesis is often preceded by pioneering work in the mouse. Although the general structure of hematopoiesis is conserved between mice and humans and even many molecular components function similarly, substantial species-specific differences exist, as described in more detail in the discussion of this thesis. It is vital to also study human cells, especially to learn about human diseases or design new therapies. Inspired by in vitro assays of mouse bone marrow derived CFU-C, the conditions to grow human CFU-C in semi-solid medium were established (Metcalf, 1977; Pike and Robinson, 1970). A more primitive cell than the CFU-C could be detected by the long-term culture initiating cell (LTC-IC), which entails culturing primitive human hematopoietic cells on adherent feeder layers to continuously produce CFU-C over weeks in liquid culture (Gartner and Kaplan, 1980). These conditions were optimized and the LTC-IC assay became a widely used surrogate to assess human HSCs (Sutherland et al., 1991). However, as with the mouse CFU-S, in hindsight we now know that these early assays to detect primitive hematopoietic cells did not efficiently detect multipotent HSCs, but rather more proliferative progenitors (Hao et al., 1996). Assessment of the quintessential functional property of HSCs, to generate all blood lineages for an extended period of time, required in vivo analysis.

1.2.3 Reconstitution of the human blood system in a mouse

The first barrier to engraft human cells in a mouse is rejection by the mouse immune system. An initial solution came from a mouse model of severe combined immunodeficiency (SCID). Infants that suffer from SCID have a highly compromised immune system due to the absence of functional T-cells, and without bone marrow transplant, this debilitating disease...
leads to recurring infections and death. SCID mice are homozygous for a mutation in the Prkdc gene and are unable to recombine the B- and T-cell receptor regions, leading to blocked development of adaptive immunity (Bosma et al., 1983; Fulop and Phillips, 1990; Kirchgessner et al., 1995). The reduced immune system function prompted several groups to transplant human cells into these mice. Human peripheral blood leukocytes could survive and proliferate in the SCID mouse for at least six months (Mosier et al., 1988). Transplantation of human fetal tissue supported sustained engraftment of B- and T-cells, indicating the presence of stem or progenitor cell activity (McCune et al., 1988). Because lymphoid cells are long-lived, it is problematic to ascertain HSC engraftment based on sustained lymphocyte production. Myeloid cells on the other hand have a more rapid turnover, therefore, serial evaluation of myeloid engraftment decisively established an in vivo stem cell assay for human hematopoietic cells (Kamel-Reid and Dick, 1988). Many mouse cytokines do not bind to human cells, prohibiting multilineage differentiation of human cells in the mouse environment. To resolve this, SCID mice were infused with interleukin-3, granulocyte-macrophage colony stimulating factor and/or erythropoietin after transplantation of human bone marrow cells (Lapidot et al., 1992), leading to the detection of multipotential myeloid and erythroid progenitors in engrafted mice even 4 months after transplantation. B-cells were simultaneously detected, fulfilling two key criteria of HSCs: long-term and multilineage engraftment. These studies laid the foundation to experimentally assess human HSC activity.

Since the initial engraftment of human HSCs in vivo, ever improving xenograft models have been instrumental in the investigation of human hematopoiesis and HSCs. One major step forward was crossing the SCID mice to the nonobese diabetic (NOD) strain which has a deficit of NK cells, absence of circulating complement and defective antigen presenting cells (Shultz et al., 1995). The gene that is responsible for support of higher human engraftment in the resultant NOD-scid mouse is Sirpa (Takenaka et al., 2007). Sirpa is a transmembrane protein expressed on myeloid cells, and phagocytosis by macrophages is inhibited when Sirpa binds to CD47. NOD mice have a Sirpa variant that recognizes human CD47, which is ubiquitously expressed on hematopoietic cells. When Sirpa on mouse macrophages binds CD47 on human cells, the probability that human cells are engulfed by mouse macrophages is reduced (Jaiswal et al., 2009). Another major improvement of mouse xenograft models was the development of NOD-scid mice with a deletion of the IL-2R common γ chain, or NSG mice. The IL2-R common γ chain is required for signaling of IL-2, IL-4, IL-7, IL-9, IL-15 and IL-21 cytokines, and NSG mice have a complete deficiency in B-, T- and NK cells (Shultz et al., 2005). Transplantation of human cells into NSG mice gives more robust engraftment compared to NOD-scid mice, and allows for detection of human B-cells, T-cells, NK cells, granulocytes, monocytes, megakaryocytes as well as HSC and progenitor fractions (Figure 3) (McDermott et al., 2010). In addition, NSG mice are more long-lived than NOD-scid.
mice and resistant to developing thymic lymphoma, enabling long-term in vivo analysis of human HSC activity. Robust engraftment of NSG mice was established upon injection of single highly purified human HSCs (Notta et al., 2011). Newer generations of humanized mice express human cytokines such as TPO, IL-3, GM-CSF and others that are not cross-reactive, to optimize differentiation and maturation of human hematopoietic cells (Ito et al., 2012). Thus, steady improvements to immune-deficient mouse models have handed us a sensitive and accurate assay for in vivo analysis of human HSCs.

1.2.4 Characterization of hematopoietic stem and progenitor cells in the mouse

As established by chromosome and retroviral insertion site tracking in the mouse, hematopoiesis is sustained by stem cells that give rise to more committed downstream progeny. These progenitor cells proliferate and differentiate further into mature blood cells. To truly understand the organization of stem and progenitor cells at the apex of this hierarchy, assays that can read out the proliferative and lineage potential of cells have to be combined with the ability to prospectively isolate different cell fractions. A remarkable technology was developed in the 1970s that enabled cell separation based on their fluorescent properties (Bonner et al., 1972), termed fluorescence activated cell sorting (FACS) or flow cytometry. Initial attempts to purify HSCs with FACS used parameters such as wheat germ agglutinin binding (Visser et al., 1984), uptake of the dye rhodamine 123 (Bertoncello et al., 1985) and Hoechst 33342 efflux (Goodell et al., 1996). In 1988, mouse HSCs were greatly enriched from bone marrow cells using a combination of cell surface marker to sort cells that were negative for differentiation markers and positive for Sca-1 and Thy-1 (Spangrude et al., 1988). To this day, sorting cells based on a variety of transmembrane proteins is the most widely used method to purify stem and progenitor cells (Figure 3). Fluorescently labeled antibodies bind to these markers on the cell surface and can be detected by different lasers while cells pass through a liquid stream. Based on their fluorescent properties, droplets containing single cells are given an electrostatic charges and the cell is deflected into one of several containers by charged plates. Current flow cytometers routinely fractionate thousands of viable cells per second based on expression of more than ten surface markers. When this technology is combined with clonal analysis of lineage and proliferative potential, it provides a powerful approach to dissect the hierarchical relationship between phenotypically distinct cell fractions.

Separation of mouse stem and progenitor cells led to the proposal of a relatively simple bifurcation model (Weissman et al., 2001). Long-term stem cells give rise to short-term stem cells with reduced self-renewal potential (Morrison and Weissman, 1994; Spangrude et al., 1988), followed by a split into the common lymphoid progenitor (CLP) and the common myeloid progenitor (CMP). The CLP gives rise to B- and T-cells whereas the common myeloid progenitor splits into granulocyte-macrophage progenitors (GMP) and megakaryocyte/erythrocyte progenitors (MEP) (Akashi et al., 2000; Kondo et al., 1997). Since this model was proposed, it
has become clear that early hematopoiesis does not always strictly follow this branching scheme. For example, clonal analysis has revealed the existence of rare bipotent B-macrophage progenitors in the mouse bone marrow (Montecino-Rodriguez et al., 2001). Improved assays to detect megakaryocyte and erythroid cells have challenged the existence of a CMP, as megakaryocyte/erythroid progenitors may be directly downstream of a multipotent progenitor (Adolfsson et al., 2005). As well, further fractionation of HSCs is revealing profound heterogeneity in this compartment. Prospective isolation of long-term HSCs with the ability to support life-long hematopoiesis and “intermediate-term” HSCs that persist for 6-8 months before becoming extinct, indicates that the stability of self-renewal mechanisms is the foremost determinant of long-term HSC function (Benveniste et al., 2010). Finally, recent studies show that self-renewing HSCs can be divided into different lineage-biased classes and even self-renewing lineage-restricted cells have been described (Benz et al., 2012; Gekas and Graf, 2013; Morita et al., 2010; Muller-Sieburg et al., 2012; Yamamoto et al., 2013). New cell types that are found as a result of these sophisticated clonal analyses should be accommodated in revised models of the stem and progenitor cell hierarchy. However, the essence of the hematopoietic hierarchy that sustain blood in the mouse is largely captured.

1.2.5 Delineation of the human hematopoietic stem and progenitor cell hierarchy

The first marker that was found to enrich for multipotent human progenitors was CD34 (Civin et al., 1984). By isolating CD34+ cells from cord blood, human HSCs can be purified from roughly 1 in a million to 1 a thousand cells. However, CD34+ cord blood is highly heterogeneous, as it contains stem cells as well as many different progenitors. CD90 was identified as a marker that further enriches for human HSCs in combination with CD34 (Baum et al., 1992). As well, CD38 and CD45RA were found to be excluded from the HSC compartment (Bhatia et al., 1997; Lansdorp et al., 1990). These studies culminated in further analysis of the lineage depleted (Lin−) CD34+CD38− fraction, where CD45RA−CD90+ cells were found to be most enriched for HSC activity (1 in 10 cells), whereas the CD45RA−CD90− fraction mostly contains short-term multipotent progenitors (MPPs) (Majeti et al., 2007). Still, this sorting strategy did not allow for purification of homogeneous cell populations. The first steps that HSCs take towards differentiation concur with a gradual decrease in self-renewal potential, which is reflected by incremental reductions in the length of time for which the cell can generate multilineage engraftment. More precise separation of long-term HSCs
from short-term HSCs or MPPs with reduced repopulation potential would enable investigation of the processes that regulate the stability of self-renewal. The latest addition of markers that enrich for human HSCs is CD49f, an integrin that enabled engraftment of single Lin−CD34+CD38−CD45RA−CD90+RhoLo+CD49f+ cells (Notta et al., 2011). As well, the MPP was more precisely defined. These studies have pinpointed the human HSC and opened up the possibility of investigating their molecular constituents with increasing precision.

Apart from separating multipotent stem and progenitor cells by their ability to sustain the blood system for an extended period of time, the definition of lineage restricted progenitors is important to understand how lineage commitment occurs. To distinguish between one cell with the ability to differentiate into multiple lineages or cells with differing properties coexisting within a population, lineage potential must be assessed at a single cell level. However, progenitors lack the proliferative capacity of stem and multipotent progenitor cells, prohibiting their clonal assessment in vivo. Thus, characterization of progenitor lineage potential at a single cell level relies on in vitro assays. Myeloid and erythroid differentiation can be studied in CFU-C assays. At present, lymphoid development can only be read out using coculture assays, where a layer of adherent cells supports differentiation into B-, T- or NK cells (La Motte-Mohs et al., 2005). Conditions have been established on MS-5 mouse stromal cells supplemented with cytokines to allow clonal analysis of myeloid, B-cell, NK cell and mixed lymphomyeloid potential (Doulatov et al., 2010; Gan et al., 1997; Hao et al., 1996; Yoshikawa et al., 1999). Since in vitro assay do not always correlate to cellular output in vivo (Richie Ehrlich et al., 2011), results must be validated by transplantation. Conforming to these requirements, lymphoid-restricted progenitors, CMPs, GMPs and MEPs were identified within the human CD34+CD38+ progenitor compartment based on the expression of CD45RA, CD10, and CD123 (IL-3Ra) (Galy et al., 1995; Manz et al., 2002). Recently, a single sorting scheme with standardized testing of lineage potential brought clarity and a relatively comprehensive model of early differentiation in the human system (Figure 4) (Doulatov et al., 2010). Using seven markers, the CD34+CD38+ HSC-enriched and CD34+CD38 progenitor fractions were split into seven subsets, which were functionally assessed at the single cell level in vitro and validated by transplantation in vivo. Identification of a multi-lymphoid progenitor (MLP) that gives rise to all lymphoid cell types as well as some myeloid lineages but not megakaryocyte or erythroid cells required a revision of the human hierarchy model. Together with transcriptional analysis that places the MLP as a hybrid cell that contains elements of stem cell as well as lineage-associated programs, it is now established that the first step of commitment is not rigid separation of lymphoid versus myeloid restriction (Doulatov et al., 2012; Goardon et al., 2011; Laurenti et al., 2013). Instead, differentiation is a gradual process of increasing commitment to a certain lineage while other programs are extinguished. It should become apparent in Chapter 3 of this thesis, that the ability to prospectively separate functionally distinct progenitor cell types provides a foundation upon
Figure 4. Current model of lineage differentiation in the human hematopoietic hierarchy. HSC, hematopoietic stem cell; MPP, multipotent progenitor; MLP, multilymphoid progenitor; ETP, early T-cell progenitor; B/NK, B- and NK-cell progenitor; proB, pro-B cell; CMP, common myeloid progenitor; GMP, granulocyte macrophage progenitor; MEP, megakaryocyte erythrocyte progenitor (Doulatov et al., 2012).
which we rely to identify processes and molecules that regulate early lineage commitment.

1.3 Processes that govern HSC maintenance and differentiation

1.3.1 Quiescence and cell cycle regulation

To maintain the blood system throughout the lifetime of an organism, HSCs must have the proliferative potential for long-term production of clonal descendant cells, which depends on their ability to self-renew. However, the HSC self-renewal probability decays with a logistic kinetic over the lifespan of a normal HSC clone (Sieburg et al., 2011). The decline in HSC self-renewal ability is hastened by proliferative stress, suggesting that, like normal somatic cells, HSC can undergo a finite number of divisions (Harrison et al., 1990; Hayflick, 1965; Orford and Scadden, 2008). To prevent exhaustion of the HSC pool, HSCs are in a quiescent state and get activated occasionally (Takizawa et al., 2011). It is estimated that human HSCs divide on average once every 40 weeks (Catlin et al., 2011). As well, the quiescent nature of HSCs is thought to protect them from acquiring mutations that could lead to malignancy (Lobo et al., 2007; Park and Gerson, 2005). Thus, quiescence is an integral part of HSC biology, connected to their ability to sustain the blood system throughout life while minimizing the risk of malignant transformation.

Early evidence for the dormant nature of HSC came from the observation that drugs that preferentially eliminate proliferating cells, such as 5-FU, spare non-cycling stem cells with a high capacity to regenerate the blood (Hodgson and Bradley, 1979). As well, separation of cells based on cell cycle activity by Hoechst 33342 and rhodamine 123 indicated that long-term HSCs with the highest proliferative potential resided in the resting fraction of BM cells (Goodell et al., 1996; Spangrude and Johnson, 1990). More recently, the relationship between HSC function and cell cycle was strengthened by the observation that the divisional history of HSCs is negatively correlated to their functional output, and by the demonstration that the quiescent G₀ fraction contains most of the long-term engraftment potential (Passegué et al., 2005; Punzel and Ho, 2001). To investigate the kinetics of cell cycle entry within the HSC pool, 5-bromo-2-deoxyuridine (BrdU) incorporation studies were performed, and it was suggested that a relatively homogeneous pool of HSCs regularly enters the cell cycle, and the entire mouse HSC pool turns over every few weeks (Cheshier et al., 1999; Kiel et al., 2007). However, a more detailed analysis whereby BrdU retention and the H2B-GFP genetic mouse model were used as complimentary approaches, shed a different light on proliferative characteristics of the HSC pool (Foudi et al., 2009; Wilson et al., 2008). Considerable heterogeneity was found, and dormant HSCs, which are estimated to divide every 145 days, harbor the majority of long-term multilineage repopulation potential. Moreover, it was shown that dormant HSCs enter the cell cycle in response to injury to proliferate and restore homeostasis (Essers et al., 2009). Chronic activation of HSCs by IFNα impaired their function, supporting the link between the proliferative
history of HSCs and their self-renewal potential. Cumulatively, these studies have provided great insight into the dynamics of the HSC pool and the importance of maintaining a quiescent population to sustain the blood system throughout life.

The importance of cell cycle regulation for HSC homeostasis has prompted much investigation into cell-intrinsic molecular mechanisms that control HSC proliferation. Genetic mouse models where one or more genes are deleted by homologous recombination have unveiled many genes that influence HSC biology and quiescence, which is the subject of several recent reviews (Li, 2011; Rossi et al., 2012). When cyclins and cyclin-dependent kinases (CDKs) form complexes to phosphorylate Rb and thereby inhibit Rb function, the transcription factor E2F is released to stimulate cell cycle entry. The importance of this pathway for HSC expansion during embryonic development is shown by deletion of members of the cyclin D/Cdk4 complex, which results in anemia and decreased HSCs numbers and repopulation ability (Kozar et al., 2004; Malumbres et al., 2004). In the adult, simultaneous deletion of the Rb family members Rb, p107 and p130 results in hyperproliferation and expansion of mutant HSCs, but their reconstitution potential is impaired upon transplantation (Viatour et al., 2004). The cyclin-dependent kinase inhibitors p21 (Cdkn1a), p27 (Cdkn1b) and p57 (Cdkn1c) are negative regulators of proliferation. Whereas loss of p21 only affects HSC quiescence or function under stress conditions (van Os et al., 2007) and p27 deletion affects progenitor cells but not HSCs (Cheng et al., 2000), loss of p57 decreases the quiescence and self-renewal capacity of adult HSCs (Matsumoto et al., 2011; Zou et al., 2011). Another pathway important for regulation of HSC proliferation is phosphatidylinositol 3-kinase (PI3K)-Akt signaling. PI3K phosphorylates PIP2, whereupon Akt is activated leading to cell growth through mTOR inactivation, proliferation through FoxO inhibition, and inhibition of apoptosis through Mdm2. Pten negatively regulates this pathway by dephosphorylating PIP3. Loss of the Pten or FoxO genes results in a significant depletion of the HSC pool due to enhanced HSC activation, and Pten knockout mice progress to develop leukemia (Tothova et al., 2007; Yilmaz et al., 2006; Zhang et al., 2006). Deletion of factors that are less obviously related to cell cycle control, but also result in HSC hyperproliferation, is frequently associated with hematopoietic defects. Examples are the transcription factors Gfi1, E2A and PU.1 (Hock et al., 2004; Semerad et al., 2009; Staber et al., 2013). Together, these studies established that sustained HSC function and self-renewal depends on many intrinsic factors that promote HSC quiescence.

The HSC environment or niche also plays a major role in HSC maintenance. This was first appreciated in mice with mutated membrane-bound stem-cell factor (SCF) (Barker, 1997; McCulloch et al., 1965), since an HSC defect was found not to be intrinsic, but rather caused by changes in the HSC niche. Binding between SCF and its receptor c-Kit, expressed on HSCs, plays a role in HSC self-renewal and bone marrow retention (Driessen et al., 2003; Ogawa et al., 1991). Cytokines such as TPO, TGFβ, SCF and IFNα regulate the intracellular localization of cell cycle machinery including p57, such that HSCs
maintain their quiescent state in the niche (Tesio and Trumpp, 2011). For a long time, investigation of the HSC niche was held back by difficulties such as the encasement of the bone marrow in bone and the lack of specific markers for mesenchymal and stromal cells. As conditional mouse models and imaging tools for investigating the niche improve, most HSCs appear to occupy a perivascular rather than an endosteal niche, and Cxcl12 expression in the perivascular region supports HSC quiescence and function (Ding and Morrion, 2013; Greenbaum et al., 2013; Hanoun and Frenette, 2013; Kunisaki et al., 2013). In summary, quiescence is an integral part of HSC biology and profoundly integrated with self-renewal. HSCs display heterogeneous levels of quiescence/proliferation, and dormancy segregates with primitiveness. Both cell-intrinsic and niche factors play essential roles in maintaining quiescence to protect HSCs from exhaustion and potentially malignant transformation.

Almost all studies into cell cycle dynamics of the HSC pool are performed in the mouse. The lifespan of laboratory mice is 2-3 years, yet they are more susceptible to leukemia than humans are in 70-80 years (Rangarajan and Weinberg, 2003). This indicates that human HSCs have evolved antineoplastic mechanisms that are not present in the mouse, some of which likely impact regulation of proliferation. More studies are needed in human HSCs, as these represent a more clinically relevant population. In Chapter 2 of this thesis we describe a novel layer of cell cycle regulation by post-transcriptional control of PI3K/AKT signaling that is conserved from mouse to human HSCs.

1.3.2 Transcriptional control of HSC differentiation

HSCs need to coordinate many processes simultaneously to uphold their self-renewal, differentiation potential and responsiveness to extracellular signals. Transcription factors are well positioned to perform a wide variety of functions due to their ability to regulate many target genes. Therefore, it is not surprising that many transcription factors that are essential for HSC homeostasis have been identified. In particular, the process of differentiation is accompanied by extensive transcriptional changes. Globally, a distinction can be made between transcription factors that are required for differentiation into MEPs, GMPs, B/NK cell progenitors and early T-cell progenitors (Mercer et al., 2011a; Orkin and Zon, 2008). One of the most striking illustrations of a strictly lineage-associated transcription factor is the requirement for GATA1 in erythropoiesis (Ferreira et al., 2005). GATA1-deficiency leads to a block in erythroid cell maturation and embryo death by anemia, whereas other tissues remain unaffected (Pevny et al., 1991; Takahashi et al., 1997). Direct antagonism between the transcription factors GATA1 and PU.1 segregates MEP from GMP, as GATA1 promotes MEP commitment whereas high PU.1 directs a cell towards a myeloid fate (Liew et al., 2006; Rekhtman et al., 1999; Rhodes et al., 2005; Zhang et al., 1999). PU.1 regulation is complex, as high levels direct a myeloid fate, low levels allow MEP commitment through GATA1, but intermediate levels are required for lymphoid differentiation (DeKoter and Singh, 2000; Singh et al., 1999). Together with Ikaros, which is required for lymphoid lineage differentia-
tion, these factors act in multipotent progenitors to establish early branch points (Arinobu et al., 2007; Wang et al., 1996). Once lineage potential is restricted to the lymphoid lineage, a different set of transcription factors directs B-cell differentiation. This transcriptional network is extensively studied and serves as a paradigm for the transition from an early progenitor (CLP) to a specified precursor (pre-B cell). Identified by mouse knockout models, factors that are required for B-cell specification are E2A (Bain et al., 1997; Zhuang et al., 1994), Ebf1 (Lin and Grosschedl, 1995), Pax5 (Urbanek et al., 1994), Bcl11a (Liu et al., 2003), and Foxo1 (Dengler et al., 2008). These factors are sequentially upregulated, interdependent by cooperation, and activate a network of genes that incite B-cell commitment and maturation (Lin et al., 2010). Recently, investigation of transcriptional dynamics in human progenitors revealed that transcriptional programs are shared across lineage-potential boundaries (Laurenti et al., 2013), providing a supportive environment for interactions between transcriptional programs associated with different lineages.

Indeed, there is extensive crosstalk between lineage-associated transcription factors. In addition to activating genes required for B-cell development, E2A, Ebf1 and Pax5 repress alternative lineage genes (Ikawa et al., 2004; Lukin et al., 2011; Nechanitzky et al., 2013). Accordingly, pro-B cells of which maturation is blocked by deletion of E2a, Ebf1 or Pax5 gain the ability for long-term in vitro expansion and show a wide range of differentiation abilities (Ikawa et al., 2004; Nutt et al., 1999; Pongubala et al., 2008; Rolink et al., 1999). In Chapter 3 of this thesis, we extend this knowledge by showing interaction between the lymphoid differentiation program and human HSC self-renewal. In summary, HSC differentiation is guided by transcription factors that regulate early commitment decisions through the coordinated initiation and inhibition of programs that are required for cell specification and maturation.

Although the most obvious function of lineage-associated transcription factors lies in lineage commitment, many of them are expressed at low levels in the HSC compartment. An early study that described low-level expression of lineage-related genes in uncommitted primitive cells, or lineage priming, reported coexpression of lineage markers such as MPO and β-globin in single cells of a multipotential cell line (Hu et al., 1997). Promiscuous expression of lineage-associated genes gained further support from the observation that myeloid and erythroid genes were coexpressed in prospectively isolated single CMPs, whereas B- and T-cell markers were coexpressed in CLPs (Miyamoto et al., 2002). However, the validity of these studies could still be debated due to impurity of the cell populations that were used. Conclusive evidence for priming in the HSC came from a lineage tracing model where lysozyme expression, a gene highly expressed in granulocytes and macrophages, was linked to an irreversible EYFP reporter (Ye et al., 2003). Upon bone marrow transplantation, EYFP expression was found in all lineages, establishing that (1) HSCs express lineage-associated genes and (2) this does not impair long-term repopulation potential. Whether this low-level expression of lineage-related genes in uncommitted HSCs has a function is not well under-
stood, although the maintenance of multipotency and rapid response to demands of mature blood cell production are thought to rely on lineage priming (Orkin, 2003; Orkin and Zon, 2008). The promiscuity of expressing multilineage genes may be the result of HSCs keeping open chromatin available for transcription factors to engage a differentiation program in response to intrinsic or extrinsic cues. Thus, lineage commitment involves extensive silencing of inappropriate genes, and should not be seen as the imposition of a differentiation program on an otherwise blank slate.

1.3.3 Epigenetic regulators and micro-RNAs

Broadly defined, epigenetics is the study of changes in cellular behavior due to factors other than the underlying DNA sequence. I will discuss two aspects of this field in relation to HSCs, namely, the heritable epigenetic information contained within chromatin, and gene expression regulation by microRNAs. Chromatin consists of DNA as well as histones and other proteins that compact DNA in the cell nucleus. Molecular groups can be attached to these components, for example, DNA methylation by DNMT proteins or histone modification by Polycomb Repressive Complexes (PRC) can alter gene expression. Bmi-1, of the PRC1 complex, is involved in repression of many targets by stimulating ubiquitin ligase activity (Sauvageau and Sauvageau, 2010; Sparmann and Van Lohuizen, 2006). Loss of Bmi-1 results in a severe proliferative defect in lymphoid cells (van der Lugt et al., 1994). This in part established through Bmi-1 mediated silencing of the Ink4a/Arf tumor suppressor locus, since lymphocyte counts can be restored by Ink4a/Arf removal (Jacobs et al., 1999). The proliferation and frequency of HSCs is reduced in adult bone marrow of Bmi-1 deficient mice, and serial transplantation shows a complete loss of self-renewal (Park et al., 2003). In a genetic mouse model of leukemia, Bmi-1 deletion had a limited effect on initial leukemia development but self-renewal of leukemia stem cells was abrogated, as shown by serial transplantation (Lessard and Sauvageau, 2003). Since the Ink4a/Arf locus does not account for all the effects of Bmi-1 deletion, additional mechanisms through which this epigenetic modifier enables proliferation and self-renewal of HSCs are being investigated (Chagraoui et al., 2011; 2006; Chatoo et al., 2009). In human HSCs, BMI-1 over expression increases self-renewal as read out by serial transplantation and can cooperate with BCR-ABL in the initiation of leukemia (Rizo et al., 2010; 2008). In summary, the chromatin modifier Bmi-1 is a prime example of a versatile epigenetic factor that is essential for the proliferation and self-renewal capacity of HSCs.

Improved technologies for genome-wide evaluation of epigenetic marks are beginning to unveil coordinated regulation of DNA methylation during HSC differentiation and aging. In a multipotent progenitor cell line, a substantial fraction of lymphoid and myeloid lineage-associated genes was poised for activation by methylation marks in enhancer regions (Mercer et al., 2011b). This data supports that global epigenetic lineage priming in HSCs precedes commitment. In addition, extensive differences in DNA methylation were found between young and old HSCs, potentially through decreased expression of
PRC2 complex components (Beerman et al., 2013; Chambers et al., 2007). Further analyses of global chromatin modifications will likely improve our understanding of coordinated gene expression regulation in hematopoietic stem and progenitor cells during homeostasis, aging and disease.

MicroRNAs provide a post-transcriptional mechanism of coordinated gene expression control. MicroRNAs represent a class of over 2,500 small non-coding RNAs that repress protein expression through microRNA binding to complementary sequences embedded primarily within the 3’ UTR of target mRNA, with each microRNA typically suppressing multiple mRNA targets (Bartel, 2009; Kozomara and Griffiths-Jones, 2011). This targeting is carried out in coordination with the RNA-induced silencing complex (RISC) and results in both destabilization and translational inhibition of the target mRNAs (He and Hannon, 2004). Whereas the regulation of any individual target is often minor, coordinated repression of several targets linked within functional pathways and signaling networks can lead to profound overall changes in cell identity. Through coordinated silencing or derepression of gene expression programs, microRNAs have been shown to play a major role in cell fate determination in lineage-restricted hematopoietic progenitor cells (Havelange and Garzon, 2010). Recently, the functional significance of microRNA in HSCs is beginning to be explored. Deletion of Dicer, a principal factor for microRNA biogenesis, induces apoptosis of hematopoietic stem and progenitor cells and impairs the ability to repopulate the blood system upon transplantation, suggesting that HSC function depends on microRNAs (Guo et al., 2010). Enforced expression of miR-125a expanded the HSC pool more than 8-fold by serial limiting dilution analysis in vivo. The miR-125a homolog miR-125b, which has the same seed region, also has profound effects on normal and malignant hematopoiesis through down-regulation of pro-apoptotic genes (Shaham et al., 2012). Over expression of miR-125b enhances mouse HSC function in competitive and serial transplantation experiments, and can lead to lymphoid lineage skewing or AML development depending on expression levels (O’Connell et al., 2010; Ooi et al., 2010). Over expression of miR-125b also enhanced human HSC engraftment, although serial transplantation was not performed. Overall, these studies show that microRNAs can have profound effects on HSC self-renewal, proliferation, differentiation and apoptosis. However, much remains to be discovered regarding microRNAs in hematopoiesis.

The relevance of microRNAs in human hematopoietic cells is poorly understood. Several studies have profiled microRNA expression in human hematopoietic progenitor subsets (Georgantas et al., 2007; Merkerova et al., 2010). However, in silico methods to predict microRNA function is insufficient to gain biological insight due to the high rate of false positive and false negative hits of microRNA target prediction algorithms. Insight into microRNA function in human hematopoietic cells requires gain- and loss of function experiments. miR-126 expression and activity is high in mouse and human HSCs, and is downregulated during early commitment (Gentner et al., 2010). Repopulating HSCs were highly enriched in the miR-
126 high population, sorted using a lentiviral reporter. In Chapter 2 of this thesis, we describe how miR-126 maintains quiescence in mouse and human HSCs.

1.3.4 Signaling pathways

The activity of signaling pathways is intricately regulated to maintain HSC function. Several pathways have been implicated in stem cell biology and for a comprehensive oversight of Smad, Notch, Hedgehog, Wnt and Polycomb signaling in HSCs, the reader is referred to a recent review series (Luis et al., 2012b). The complexity of signaling pathway control in HSCs will be discussed using two examples: Wnt and Notch signaling.

Wnt signaling can be subdivided in canonical and non-canonical pathways; most research has focused on canonical Wnt signaling (Clevers, 2006). In the absence of a Wnt signal, the cytoplasmic destruction complex, which is composed of Axin, GSK3β, CK1 and other components, phosphorylates β-catenin leading to its degradation by the proteasome. In the nucleus, Wnt target genes are repressed because the Tcf/Lef transcription factors are bound by Groucho. Wnt signaling is initiated by binding of a Wnt ligand to a Frizzled receptor, which then forms a complex with Lrp5/6. Activity of the pathway is highly enhanced by the Lgr5/R-spondin interaction (de Lau et al., 2011). Wnts induce phosphorylation of Lrp5/6, resulting in the recruitment of the β-catenin destruction complex component Axin. This releases β-catenin from destruction, allowing it to translocate to the nucleus and displace Groucho from the Tcf/Lef transcription factor to promote transcription of Wnt target genes.

Gain- and loss-of-function approaches have been taken to study Wnt signaling in HSCs (Luis et al., 2012a; Staal et al., 2008). Reduced Wnt activity by deletion of β-catenin and its homolog γ-catenin did not have an effect on the ability of HSCs to self-renew and reconstitute the blood cell lineages (Jeannet et al., 2008; Koch et al., 2008). Surprisingly, canonical Wnt signals were still transduced in the absence of these key components, suggesting that the gene targeting approach did not completely abrogate β-catenin function or that another factor may transduce Wnt signals in HSCs (Jeannet et al., 2008). Near complete abrogation of Wnt signaling activity was attained by deletion of Wnt3a (Luis et al., 2009; 2010) or over expression of the Wnt inhibitor Dkk1 in niche cells (Fleming et al., 2008). Both of these approaches strongly and irreversibly impaired HSC repopulation potential. These studies indicate that HSC function requires continuous low-level stimulation of the Wnt pathway, which is established through Wnt3a secretion by niche cells during early development.

Forced expression of β-catenin can either lead to enhanced HSC self-renewal or exhaustion of long-term HSCs (Kirstetter et al., 2006; Reya et al., 2003; Scheller et al., 2006). This apparent discrepancy was resolved using genetic mouse models with 5 gradients of Wnt signaling intensity (Luis et al., 2011). HSC function was enhanced when Wnt signaling was mildly increased over endogenous levels, whereas higher levels impaired HSC repopulation and differentiation. These studies indicate that activity of the canonical Wnt pathway needs to be kept within a narrow window for normal HSC maintenance. Whereas
mildly increased Wnt levels improve HSC function, HSCs are lost when canonical Wnt signaling is either too low or too high. Given the effects of secreted Wnt ligands on HSC development and maintenance, it is perhaps not surprising that deregulated Wnt signaling can contribute to hematological malignancies and leukemia stem cell maintenance (Luis et al., 2012a). Thus, modulating Wnt pathway activity provides opportunities for leukemia treatment, directed differentiation towards blood cells as well as ex vivo culture to expand transplantable HSCs.

Canonical Notch signaling is initiated by binding of one of 5 Notch ligands of the Delta and Jagged families to one of 4 Notch receptors (Pajcini et al., 2011). This leads to proteolytic cleavage of the intracellular domain of the Notch receptor (ICN) by γ-secretase. The ICN translocates to the nucleus where it associates with CSL and forms a complex that activates transcription of target genes such as Hes and Hrt. The outcome of Notch signaling depends on several variables. The particular ligand/receptor pair involved is important, for example, Delta1-Notch2 is less efficient in promoting T-cell and preventing B-cell development compared to Delta4-Notch1 binding (Mohtashami et al., 2010). The Notch pathway can promote or reduce cell differentiation, proliferation and survival depending on the context, timing and dose of Notch signals. Therefore, the role of Notch always has to be investigated in a particular cell type at a particular point in development. As well, the level of Notch activation has to be considered before generalizing observations from gain- and loss-of-function studies.

Notch plays an important role in the generation of HSCs during embryonic development. In the mouse, HSCs develop in the AGM region between E9.5 and E10.5. In Notch1-deficient embryos, HSC generation was impaired (Kumano et al., 2003). Interestingly, γ-secretase inhibitors interfered with HSC development in AGM regions isolated at E9.5, but not E10.5. This indicates that Notch may be specifically important for HSC generation, whereas subsequent proliferation and maintenance may not depend on Notch signaling. Subsequent studies have confirmed the cell-autonomous importance of Jagged1-Notch1 signaling for HSC generation in the AGM (Pajcini et al., 2011).

In adult HSCs, several approaches have been taken to assess the effect of reduced Notch signaling. One study found increased HSC differentiation and reduced reconstitution upon genetic and pharmacological inhibition of Notch signaling in HSCs (Duncan et al., 2005). However, deletion of Jagged1 and Notch1 failed to show an effect in HSCs (Mancini et al., 2005). As well, over expression of dominant negative MAML or deletion of CSL/RBPJ, which prevents transcriptional activation by all 4 Notch receptors, did not affect primary or secondary repopulation, nor did it affect hematopoietic recovery from 5-FU treatment (Maillard et al., 2008). These studies indicate that Notch signaling is dispensable for adult HSCs under homeostatic and stress conditions.

Conversely, multiple gain-of-function approaches have shown that Notch signaling can improve HSC function. Exposure of mouse and human stem cell-enriched populations to Delta1 and Jagged1, respectively, increased the repopulation potential
of hematopoietic stem and/or progenitor cells (Karanu et al., 2000; Varnum-Finney et al., 2003). Following these and other studies, it was shown that endothelial cells expressing Notch ligands promoted expansion and prevented exhaustion of mouse HSCs in co-culture assays (Butler et al., 2010). These HSCs possessed serial repopulating ability, showing that Notch signaling can promote long-term HSC expansion. Stimulation of human HSC and progenitor cells with Notch ligands may be clinically applied to accelerate reconstitution after human cord blood transplantation (Delaney et al., 2010).

Studying Notch and Wnt signaling in HSCs is complex, and the cell fate outcome of these pathways can differ depending on the cell type, developmental stage and signal intensity. Still, the knowledge that has been gained over the last two decades has implications for generation of HSCs from pluripotent stem cells, expansion of HSCs for transplantation, and our understanding of leukemia.

1.3.5 Cellular stress factors
Since the blood system depends on the long-term proliferative potential of HSCs, it is essential that the HSC pool is protected from damage accumulation to prevent either loss of function or the initiation of malignancy. The longevity of HSCs comes with exposure to multiple stress stimuli with the potential to generate damaged HSCs. Investigating the response of HSCs to DNA damage has revealed important roles for HSC mutagenesis in leukemic transformation and aging (Jan and Majeti, 2012; Rossi et al., 2007; Rübe et al., 2011). In comparison to committed progenitor cells, mouse HSCs preferentially survived DNA damage and utilized error-prone nonhomologous end joining (NHEJ) to repair DNA breaks (Mohrin et al., 2010). In contrast, human HSC were predisposed to undergo apoptosis upon irradiation-induced DNA damage compared to progenitor cells (Milyavsky et al., 2010). This contrast was initially attributed to the distinct proliferative status of the cells used in the studies – quiescent mouse bone marrow and “proliferating” human cord blood stem cells (although >90% of the CD34+CD38-CD45RA-CD90+ cells used in the latter study are Ki67+) (Blanpain et al., 2011). However, a direct comparison between human and mouse bone marrow revealed that indeed, mouse HSCs are more proficient at NHEJ than their descendants, whereas human HSCs have less NHEJ activity compared to human progenitor cells (Shao et al., 2012). This suggests that the DNA damage response may be intrinsically different between mouse and human HSCs, possibly due to alternative selective pressures that the organisms are subjected to (Rangarajan and Weinberg, 2003). Ionizing radiation causes double stranded breaks upon which γH2AX foci quickly accumulate. Upon p53 inactivation in human HSC, it was shown that reduced self-renewal coincided with increased γH2AX foci (Milyavsky et al., 2010), indicating that DNA damage negatively influences self-renewal. Interestingly, DNA damage induces mouse HSC differentiation (Wang et al., 2012), revealing that commitment to differentiation may be one mechanism by which HSCs prevent long-term propagation if DNA mutations occur. Taken together, these studies illustrate that evolutionary constraints of DNA damage have imposed different DNA repair mech-
anisms on HSCs compared to short-lived progenitor cells. However, this field is still developing and more insight into mutagenesis in stem and progenitor cells is needed. Reactive oxygen species (ROS), potentially damaging molecules that are generated as a byproduct of metabolic processes in the cell, are another source of cellular stress that strongly affects hematopoietic stem and progenitor cells (Rossi et al., 2008). The first indication that ROS signaling is important for HSCs came from the Atm deficient mouse model, which resulted in ROS accumulation that affected HSCs more than progenitor cells (Ito et al., 2004). The defect in Atm deficient HSCs was caused by elevated ROS as treatment with the antioxidative agent N-acetyl-L-cysteine (NAC) restored HSC reconstitution capacity. Elevated ROS caused HSC exit from G0 and exhaustion through p38 MAPK and p16\textsuperscript{Ink4a} induction (Ito et al., 2006). FoxO transcription factors deletion also results in elevated intracellular ROS levels, which led to a NAC-reversible increase in cycling and apoptosis, as well as enhanced short-term yet diminished long-term repopulation activity (Miyamoto et al., 2007; Tothova and Gilliland, 2007; Tothova et al., 2007). Recently, it was shown that replication stress of human HSCs by serial in vivo transplantation leads to elevated intracellular ROS levels and subsequent DNA damage (Yahata et al., 2011). This study established the importance of replication stress and ROS signaling in human HSCs and linked ROS, HSC mutagenesis and aging-induced impairment of HSC function together. Cumulatively, these studies have increased our understanding of HSC adaptability to extrinsic and intrinsic sources of stress. However, a comprehensive understanding of the processes that protect the integrity of the HSC pool to preserve life-long blood cell production is lacking. In Chapter 4 of this thesis, we implicate the Unfolded Protein Response (UPR), a pathway that was not previously associated with HSC or progenitor cell function, as an important component of the human HSC stress response.

1.4 References


Introduction


Chapter 1

through p38 MAPK to limit the lifespan of hematopoietic stem cells. Nature Medicine 12, 446–451.


Introduction

635–643.


Chapter 1


Rhodes, J., Hagen, A., Hsu, K., Deng, M., Liu, T.X.,
Introduction


