FUNCTIONAL HETEROGENEITY OF ADULT MOUSE BONE MARROW HEMATOPOIETIC STEM CELLS

by

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Abstract

The mammalian blood-forming system sustains physiologically required levels of mature blood cells by supporting their continuous generation from a rare population of undifferentiated, self-sustaining pluripotent hematopoietic "stem" cells (HSCs). Throughout adult life HSCs are located primarily in the bone marrow. Traditionally, the study of HSCs within larger populations of cells has hampered the direct observation of any unique differentiation or self-renewal properties that might distinguish individual members of the HSC compartment. To circumvent this, I analyzed the number and types of progeny generated from single purified HSCs both in cultures initiated with a single cell and in irradiated mice injected with a single cell. In a first set of experiments of this type, I demonstrated that two growth factor cocktails with the same mitogenic and anti-apoptotic activity on HSCs in vitro could have remarkably disparate effects on their concomitant self-renewal behaviour, even within the span of a single cell cycle. In addition, I used high-resolution video monitoring of single purified HSCs cultured in microwell arrays to identify cellular features that were associated with HSC self-renewal in vitro. These parameters include longer cell-cycle times than those of their differentiating progeny and an absence of uropodia on the majority of cells within the clone during the final 12 hours of culture. When combined, these parameters improved by a factor of 2-3-fold the identification of clones found to contain daughter HSCs with longterm in vivo reconstituting ability. Finally, from longitudinal and serial WBC analyses performed on a large number of recipients of single purified HSCs, I found that the adult HSC compartment could be resolved into 4 HSC subtypes, 2 of which stably
and autonomously propagate their initial unique patterns of WBC reconstitution through many self-renewal divisions *in vivo*. I also found that, *in vitro*, HSCs could rapidly acquire less competitive *in vivo* reconstitution programs although remarkable symmetry was retained in the reconstitution programs acquired by the daughter HSCs generated in the first 4 days *in vitro*. These findings provide evidence of intrinsically determined heterogeneity in the differentiation and self-renewal properties of individual HSCs.
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<th>Description</th>
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<tr>
<td>2-ME</td>
<td>2-betamercaptoethanol</td>
</tr>
<tr>
<td>APC</td>
<td>Allophycocyanine</td>
</tr>
<tr>
<td>ATRA</td>
<td>All-trans retinoic acid</td>
</tr>
<tr>
<td>B6</td>
<td>C57Bl/6J</td>
</tr>
<tr>
<td>BM</td>
<td>Bone marrow</td>
</tr>
<tr>
<td>CDK</td>
<td>Cyclin-dependent kinase</td>
</tr>
<tr>
<td>CFC</td>
<td>Colony forming cell</td>
</tr>
<tr>
<td>CFU-S</td>
<td>Colony forming unit-spleen</td>
</tr>
<tr>
<td>CI</td>
<td>Confidence interval</td>
</tr>
<tr>
<td>CLP</td>
<td>Common lymphoid progenitor</td>
</tr>
<tr>
<td>CMP</td>
<td>Common myeloid progenitor</td>
</tr>
<tr>
<td>CRU</td>
<td>Competitive repopulating unit</td>
</tr>
<tr>
<td>FACS</td>
<td>Fluorescence activated cell sorting</td>
</tr>
<tr>
<td>FGF</td>
<td>Fibroblast growth factor</td>
</tr>
<tr>
<td>FITC</td>
<td>Fluorescein isothiocyanate</td>
</tr>
<tr>
<td>FL</td>
<td>Fetal liver</td>
</tr>
<tr>
<td>Flt3L</td>
<td>Fms-like tyrosine kinase 3 ligand</td>
</tr>
<tr>
<td>G-CSF</td>
<td>Granulocyte-colony stimulating factor</td>
</tr>
<tr>
<td>GM</td>
<td>Granulocytes/monocytes</td>
</tr>
<tr>
<td>GM-CSF</td>
<td>Granulocyte/macrophage-colony stimulating factor</td>
</tr>
<tr>
<td>GPI</td>
<td>Glucose phosphate isomerase</td>
</tr>
<tr>
<td>HBSS</td>
<td>Hank’s balanced salt solution</td>
</tr>
<tr>
<td>HF</td>
<td>HBSS plus 2% fetal bovine serum</td>
</tr>
<tr>
<td>Ho</td>
<td>Hoechst 33342</td>
</tr>
<tr>
<td>HSC</td>
<td>Hematopoietic stem cell</td>
</tr>
<tr>
<td>IGF</td>
<td>Insulin-like growth factor</td>
</tr>
<tr>
<td>IL</td>
<td>Interleukin</td>
</tr>
<tr>
<td>IMDM</td>
<td>Iscove’s modified Dulbecco’s medium</td>
</tr>
<tr>
<td>KSL</td>
<td>cKit&lt;sup&gt;+&lt;/sup&gt;Scal&lt;sup&gt;+&lt;/sup&gt;Lin&lt;sup&gt;−&lt;/sup&gt;</td>
</tr>
<tr>
<td>Acronym</td>
<td>Description</td>
</tr>
<tr>
<td>---------</td>
<td>-------------</td>
</tr>
<tr>
<td>LDL</td>
<td>Low density lipoproteins</td>
</tr>
<tr>
<td>LIF</td>
<td>Leukemia inhibitory factor</td>
</tr>
<tr>
<td>Lin</td>
<td>Lineage markers</td>
</tr>
<tr>
<td>LTC-IC</td>
<td>Long-term culture-initiating cell</td>
</tr>
<tr>
<td>LTRC</td>
<td>Long-term reconstituting cell</td>
</tr>
<tr>
<td>M-CSF</td>
<td>Macrophage-colony stimulating factor</td>
</tr>
<tr>
<td>MHC</td>
<td>Major histocompatibility complex</td>
</tr>
<tr>
<td>MPP</td>
<td>Multipotent progenitor</td>
</tr>
<tr>
<td>PcG</td>
<td>Polycomb group</td>
</tr>
<tr>
<td>PE</td>
<td>Phycoerythrin</td>
</tr>
<tr>
<td>PRC1</td>
<td>Polycomb repression complex 1</td>
</tr>
<tr>
<td>RAR</td>
<td>Retinoic acid receptor</td>
</tr>
<tr>
<td>RBC</td>
<td>Red blood cell</td>
</tr>
<tr>
<td>Rho</td>
<td>Rhodamine 123</td>
</tr>
<tr>
<td>SA</td>
<td>Streptavidin</td>
</tr>
<tr>
<td>SF</td>
<td>Steel factor</td>
</tr>
<tr>
<td>SP</td>
<td>Side population</td>
</tr>
<tr>
<td>STRC</td>
<td>Short-term reconstituting cell</td>
</tr>
<tr>
<td>TCR</td>
<td>T-cell receptor</td>
</tr>
<tr>
<td>TGF-β</td>
<td>Transforming growth factor beta</td>
</tr>
<tr>
<td>TNF</td>
<td>Tumour necrosis factor</td>
</tr>
<tr>
<td>TPO</td>
<td>Thrombopoietin</td>
</tr>
<tr>
<td>W41</td>
<td>C57Bl/6J&lt;sup&gt;W41/W41&lt;/sup&gt;</td>
</tr>
<tr>
<td>WBC</td>
<td>White blood cell</td>
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For your foresight in agreeing to take on an enthusiastic but completely clueless greenhorn from a no-name university with no funding.
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To my wife Mollie, whose
unwavering encouragement and
support made this all possible
Co-Authorship Statement

Chapter 2:
This article was co-first-authored with Dr. Naoyuki Uchida, a post-doctoral fellow who I worked with in Dr. Connie Eaves’ lab during the years 2000 to 2003.

Dr. Uchida identified the original purification strategy and designed the limiting dilution, single cell, and serial transplantation repopulation experiments and the single cell LTC-IC assays. I developed the single cell culture and cell cycle kinetic measurement strategies, discovered the reduced frequency of sex-mismatched sublethally irradiated recipients and designed the doublet injection experiments.

The LTC-IC experiments were performed exclusively by Dr. Uchida. The single cell cultures, cell cycle kinetic measurements, and doublet transplantations were performed exclusively by me. All other aspects of the research were performed jointly.

The LTC-IC analysis, along with the majority of the peripheral blood chimerism analysis was performed by Dr. Uchida, with technical assistance from K. Lyons and F. Leung. I analyzed all of the single cell culture data and all of the doublet transplant data.

Dr. Uchida wrote an initial rough draft of the manuscript and Connie Eaves and I were responsible for the remainder of the manuscript preparation.

Chapter 3:
This article was co-first-authored with John Ramunas, a senior research technician from the lab of Dr. Eric Jervis, Department of Chemical Engineering, University of Waterloo, Waterloo, ON, Canada. We worked together on this project between 2004 and 2006.

Mr. Ramunas, with assistance from his colleague April Blaycock, designed and constructed the time-lapse camera system, the silicon microwell array culture system, the single cell manipulation apparatus, and programmed the algorithms for tracking individual cells within the cultures. Together with David Kent, I improved the efficiency of the Lin-Rho-SP sort with the addition of CD45 and designed the in vivo assays. Collectively we developed a live-cell shipping system.
David Kent and I performed the initial cell harvest and purification in Vancouver, plus injected single cell transplants as controls. Purified cells were then sent to Mr. Ramunas in Waterloo and imaged for 4 days, after which individual clones were harvested and sent back to Vancouver. Together with David Kent, I then transplanted the clones into individual recipients and with the assistance of Lindsay McCaffrey, subsequently collected and analyzed their peripheral blood at regular intervals for donor reconstitution levels.

The analyses of the time-lapse images were performed by Mr. Ramunas, Erin Szumsky, Liam Kelly, and Kristen Fam. Lindsay McCaffrey and I performed the peripheral blood analysis of all recipients. The remainder of the analysis was performed jointly.

I generated most of the figures and tables, and Mr. Ramunas, David Kent, Connie Eaves, Eric Jervis, and I were involved in all other aspects of manuscript preparation.

Chapter 4:

The work presented in this chapter was the culmination of many experiments and much data collection, distillation, and analysis over a period of several years, from 2002 until 2006. I designed the experiments, with intellectual input from David Kent. Transplants of single cells and in vitro clones were performed by David Kent and me. Assistance with peripheral blood sampling, staining, and FACS analysis, was provided by Melisa Hamilton, Lindsay McCaffrey, and Kristin Lyons. Michelle Bowie performed the single cell fetal liver HSC transplants, and Lindsay McCaffrey performed the subsequent PB chimerism analysis. I performed the in-depth data analysis and generated the figures. The manuscript was primarily prepared by me, with significant input from Connie Eaves and David Kent.
Chapter 1

Introduction

1.1. Hematopoiesis and hematopoietic stem cells (HSCs).

The bloodstream of an average adult mouse contains more than 10 billion blood cells. The majority of these are enucleated erythrocytes. The other specialized "end" cells of the blood system include T and B lymphocytes, natural killer cells, dendritic cells, neutrophilic, basophilic, and eosinophilic granulocytes, monocytes (which mature into macrophages), mast cells and megakaryocytes which fragment into platelets. Many of these are critical for survival of the organism. The lifespan of these various types of mature blood cells ranges from a few hours or days (neutrophils) to a few months (erythrocytes) to many years (memory T-cells). As a result, ~100 million mature blood cells need to be produced each day throughout the entire lifetime of the mouse. The process by which these cells are produced is called hematopoiesis.

Hematopoiesis is sustained by a rare population of cells called hematopoietic stem cells (HSCs). HSCs have the ability to produce all types of mature blood cells (i.e., they are pluripotent) but also generate daughter cells with the same pluripotentiality through many cell divisions (i.e. they can self-renew). However, it now appears that the irreversible loss of pluripotency is a highly complex process that may span many cell divisions and be accompanied by reproducible phenotypic changes. This has led to the concept of HSCs with indefinite and intrinsically limited self-renewal activity and a forced reliance on a combination of phenotypic markers and functional (progeny output)
endpoints to distinguish pluripotent cells with lifelong blood cell producing ability. Operationally, HSCs are assessed by transplanting them into irradiated recipients and revealing their ability to produce detectable numbers of lymphoid and myeloid progeny for at least 4 months in the recipients. Because this is an experimental description of a conceptually defined cell, it is important to note that the cells identified as HSCs in such transplantation assays may vary depending on the exact endpoint chosen to infer their initial presence. For example, these may involve differences in when and how donor-derived blood cell production is measured, the level and/or types of differentiated cells required to infer origin from HSCs, and whether or not limiting dilution strategies are employed. Although there is clearly irrefutable evidence from retroviral marking studies\(^1\)\(^2\) and single cell transplants\(^3\)\(^4\) that pluripotent hematopoietic cells with extensive self-renewal activity exist, the widespread use of different criteria for HSC identification in functional assays has clouded their detailed characterization (see section 1.1.4 and Table 1.1 below for further details).

1.1.1 Evolution of HSC concept, detection, and quantification

In the early 1950’s, it was realized that extracts from the bone marrow or spleen could protect mice from radiation-induced death\(^5\). However, in the following years, it was hotly debated whether this protective effect was humoral or cellular in origin. The use of transplants of cytologically distinct donor cells allowed this issue to be resolved in the mid 1950’s and established that the bone marrow contained cells able to repopulate the
hematopoietic system of irradiated mice. 30 years later, it was finally proven through clonal marking experiments that the lifelong reconstitution of the hematopoietic system in transplanted irradiated mice was attributable to the activity of a small number of long term repopulating cells (LTRCs) capable of self-renewal and multi-lineage differentiation.

Not surprisingly, functional assays for quantifying primitive hematopoietic cells and methods for their differential purification were developed and modified in parallel with conceptual advances in understanding how their properties changed during their early differentiation. Functional readouts of the various primitive cell types were originally necessary because all shared an identical, “blast” or undifferentiated morphology. Even as strategies were developed to prospectively identify the various primitive hematopoietic cell types, the functional aspect of these assays remained particularly important since the phenotypic identifiers were fortuitously discovered rather than representing known elements of the stem cell status of HSCs and subsequent studies showed that most of those used are also unstably tied to HSC status (discussed in more detail in section 1.1.2.1).

The spleen colony assay, originally described by Till and McCulloch, was the first quantitative functional assay described for primitive hematopoietic cells and provided a unique tool for testing and refining many of the basic concepts of stem cell biology, including stem cell self-renewal, multipotentiality, commitment and differentiation. Till and McCulloch found that when irradiated mice were transplanted with small numbers of bone marrow cells, discrete macroscopic nodules formed in the spleens of the recipients and the number of these detected was linearly related to the
number of bone marrow cells injected. Histological examination of these nodules revealed that they were composed of proliferating undifferentiated cells as well as differentiated cells of the granulocytes/macrophages and erythroid lineages. Because of the linear relationship between cell dose and nodule count, it was hypothesized that the nodules represented the clonal progeny of rare cells present in the original inoculum.

The clonal nature of these nodules was later established using cytogenetic markers. Subsequent experiments demonstrated that the cell of origin, which they termed a colony-forming unit-spleen (CFU-S) could generate all myeloid lineages and shared a common origin with a cell that also had lymphoid potential.

Initial evidence of heterogeneity amongst cells identified as CFU-S came from experiments showing that CFU-S with differing self-renewing capacities could be physically separated. Later, it was discovered that CFU-S could be subdivided into different classes according to the speed with which they produced detectable spleen colonies and their durability once formed. Colonies that appeared between 7 and 9 days post-transplant were transient, predominantly composed of one lineage, and had limited self-renewal activity. Conversely, colonies that were present on day 14 were not visible within 7-9 days, became larger by day 14, contained cells of multiple lineages, as well as significant numbers of secondary CFU-S.

Although CFU-S were equated with HSCs for almost twenty years, the concept of a “pre-CFU-S” cell was hypothesized many years before convincing experimental evidence of a distinct population of this type was obtained. This came from the use of Rhodamine 123 staining and counterflow centrifugal elutriation, respectively, to separate CFU-S from long-term repopulating cells that were able to subsequently
generate daughter CFU-S. Assays for the qualitative and quantitative assessment of LTRCs are described in detail in section 1.1.4.

1.1.2 HSC purification

Around the same time, a bone marrow subpopulation including primitive hematopoietic cells was prospectively isolated using flow cytometric techniques by selecting cells that lacked any of a panel of cell surface markers expressed on differentiated hematopoietic cells (so-called lineage or lin markers) but at the same time expressed the newly christened "stem cell antigen – 1", or Sca-1\(^{21}\). This strategy was subsequently refined by the additional selection of cells expressing c-kit\(^{22}\). Nevertheless, both the Lin-Sca-1+ and Lin-Sca-c-kit+ (KSL) populations were shown to contain both LTRCs and CFU-S, suggesting that the populations were heterogeneous. Later experiments showed that the KSL population could be further fractionated into functionally distinct subpopulations, using cell surface markers such as CD34\(^3\), CD27\(^{23}\), flk2/flt3\(^{24,25}\), endoglin/CD105\(^{26}\), SLAM family receptors CD150 and CD244\(^{27}\), EPCR/CD201\(^{28}\), and \(\alpha\)-2 integrin/CD49b\(^{29}\), or by differences in staining with the vital dyes, Rhodamine-123\(^{30}\) or Hoechst 33342\(^{31,32}\). Cells with the ability to efflux Hoechst 33342 are often visualized using two emission wavelengths, giving rise to the characteristic side population or SP phenotype\(^{33}\), which we have combined with Rhodamine efflux activity\(^{19,34}\) in a sorting strategy not reliant on either Sca-1 or c-kit\(^{35}\).
Using these latter approaches, cells with long-term, multilineage reconstituting ability can now be routinely isolated at purities of >20% by a variety of strategies.

1.1.2.1 Limitations of phenotypic identifiers of HSCs:

In spite of the identification of phenotypes of adult mouse bone marrow cells that are almost exclusive for HSCs, this relationship is known to break down under a variety of circumstances. Thus the use of these phenotypes as surrogate indicators of HSCs can lead to false conclusions because non-HSCs expressing the same marker profile are mistaken for HSCs and HSCs with a different marker profile are missed. For example, murine mast cells and their precursors lack classical hematopoietic lineage markers and co-express c-kit and Sca-1, making them phenotypically indistinguishable from KSL cells, and hence are commonly assumed to be HSCs when HSCs are not, in fact, present\(^36\). Many stem cell markers are expressed differently in quiescent (steady-state) HSCs versus activated HSCs, including those present in developing hematopoietic tissues (fetal liver), or mobilized in adults by cytokine administration or after myelosuppressive treatments with drugs or radiation, or when exposed to growth factors in culture. Examples of markers that show such lability in their expression on HSCs include CD34\(^37\), CD38\(^38\), Hoechst and Rhodamine efflux activity\(^39\), endoglin/CD105\(^40\), and Mac-1\(^41\).

However, recent reports suggest the existence of some markers that may be stable in both steady-state and activated HSCs, including SLAM family markers\(^42\), endomucin\(^43\), and EPCR (David Kent, personal communication). Recently, two FACS-based strategies have
been described that allow the isolation of fetal liver HSCs at >10% purity\textsuperscript{44,45}. Perhaps by using these strategies as a starting point, further experiments will result in the development of a purification strategy that robustly identifies activated HSCs.

1.1.3 Classical view of the hematopoietic hierarchy in adult mice

Based on the collective efforts of the past 40+ years, a hierarchical model of hematopoiesis has been proposed (Figure 1.1). HSCs are placed at the top of this hierarchy and besides their extensive self-renewal ability, are thought to give rise to all mature blood cell types. These are continually produced through a series of successive differentiation and amplification steps. The immediate progeny of HSCs are multipotent short-term reconstituting cells (STRCs, also called multipotent progenitor cells, MPPs) that retain full lineage potential yet have a relatively limited capacity for sustaining self-renewal divisions. Since these primitive cells are incapable of prolonged self-renewal, they actively produce cells for only a few weeks or months.

These STRCs, in turn, give rise to oligopotent progenitors, which are more restricted in their developmental potential. This is viewed as a major branching point in the hematopoietic hierarchy with the common lymphoid progenitor (CLP)\textsuperscript{46} giving rise only to mature lymphoid cells and a common myeloid progenitor (CMP)\textsuperscript{47} capable of giving rise only to mature myeloid cells (see figure 1.1). The oligopotent progenitors in turn give rise to more lineage-restricted progenitors from which all of the mature blood cells eventually arise.
Although some unanswered questions remain and exceptions to this described hierarchy exist, most agree that the sequential differentiation of HSCs through progenitors to fully differentiated blood cells generally occurs in this fashion and is generally an irreversible process. Because each differentiation choice is regulated, extensive proliferation (or dampening) can occur at every step, with rates of proliferation increasing in more differentiated progenitors\textsuperscript{48}. This permits a huge potential amplification in the numbers of differentiated progeny that can ultimately arise from a single HSC, but also allows the fine-tuning of the system to respond to ever-changing demands for particular cell types. The hierarchical strategy also demands little proliferation from HSCs themselves, which may explain why they cycle so infrequently and are primarily quiescent\textsuperscript{49}. Avoiding the hazards of constant activation, including the risk of mutations during DNA replication, as well as the mutagenic by-products of active metabolism, allows these cells to contribute to hematopoiesis throughout life, without exhausting or becoming neoplastic.

1.1.4 \textit{in vivo} Assays for HSCs

Investigations of the hierarchical model just described have been greatly facilitated by the introduction of \textit{in vivo} repopulation assays that use prolonged outputs of multiple mature blood cell types as endpoints to allow input HSCs with putative lifelong self-sustaining ability to be specifically discriminated (Figure 1.2). The functional aspect of these assays remains particularly important since most phenotypic identifiers can be
altered under certain conditions (see section 1.1.2.1). Primitive cells are tested via injection into genetically distinct myeloablated (usually irradiated) recipients, along with some source of radioprotective cells. This is essential to ensure short-term survival, since purified test cells may require several weeks to produce functional progeny\textsuperscript{20}. Several months later, the differentiated hematopoietic progeny are analyzed to determine the presence or absence of myeloid and lymphoid cells generated from the test cells originally injected. Over the years, different strategies have been used to distinguish donor chimerism in the recipient mice including detection of sex mismatches, and differences in isoenzyme and cell surface alloantigen expression. When co-injected with measured numbers of cells with predefined hematopoietic activity, the relative competitive ability of the test cells can be measured based on the proportion of donor cells in their hematopoietic tissues, usually the blood. This allows a semi-quantitative measurement of HSC activity, and mathematical descriptions relating competitive repopulation ability with input HSC have been proposed\textsuperscript{40-52}.

However, measurements of competitive repopulation ability of populations of cells cannot distinguish between variations in LTRC number and variations in competitive ability per individual LTRC. This important information can only be obtained by injecting single cells, or by utilizing the statistical power of limiting dilution, using the competitive repopulating unit (CRU) assay. This latter assay involves transplantation of varying donor test cell doses into groups of myeloablated recipient mice, along with radioprotective competitor cells. If test cell-derived lymphoid and myeloid progeny are detected, it can be concluded retrospectively that the test cells contained at least one pluripotent long-term repopulating cell. The proportion of negative
animals at each test cell dose is then used to calculate the frequency of CRU in the test cell sample using Poisson statistics and the method of maximum likelihood.

The CRU assay as originally described utilized female competitor cells whose HSC content had been reduced by two serial transplantation cycles, test cells from male donors, and lethally-irradiated female recipients. Donor-derived male myeloid and lymphoid reconstitution was approximated by evaluating BM and spleen cells, respectively, using Southern blot analysis. Refinements of the CRU assay have since been developed. For example, more sensitive PCR-based techniques to detect smaller numbers of donor male cells in a female recipient have been described. Alternatively, erythroid repopulation can be monitored using congenic mouse strains bearing allelic differences in hemoglobin or glucose phosphate isomerase (GPI) genes. Most popular is the use of C57BL/6J (B6) congenic strains that express the CD45.1 or CD45.2 allotypes of CD45 (also known as leukocyte common antigen or Ly5), which is found on the cell surface of all hematopoietic cells except terminally differentiating erythrocytes. Use of such mouse strains allows detection by flow cytometry of donor-derived nucleated blood cells of various types using combinations of fluorochrome-conjugated lineage-specific and anti-CD45.1 or anti-CD45.2 monoclonal antibodies. Preparation of serially-transplanted cells is costly and labour-intensive and has largely been replaced by the use of 1 to 2x10^5 normal BM cells or 1x10^6 Sca1-depleted cells to function as helper cells to ensure short-term hematopoietic recovery of lethally-irradiated recipients and long-term hematopoietic reconstitution even when the grafts do not contain detectable CRU. C57BL/6J-Kit^W^41J (W41) mice, a B6 histocompatible strain with a partial loss of c-kit function through the acquisition of a point mutation, provides a practical
alternative to the use of lethal-irradiation and helper cells. The CRU content of these mice is reduced by about 17-fold\textsuperscript{60} and sub-lethal conditioning of 400 cGy \(\gamma\)-irradiation is sufficient to further compromise endogenous hematopoiesis\textsuperscript{52,61,62}. W41 mice can be partially myeloablated and used as recipients without the need for helper cells, and provide an equivalent competitive environment for individual HSC detection as conventional assays in lethally irradiated mice.

The measurement of long-term multilineage donor reconstitution ability is key to identifying HSC activity in the \textit{in vivo} transplant setting. However, exactly how "long-term multilineage reconstitution" is defined has, over the years, been interpreted in many different ways by different groups. As mentioned, the CRU assay as originally described utilized Southern blots to assess the contribution of male donor cells to the hematopoietic tissues of female recipients. In these experiments, a minimum of 5\% male cells in both the BM and spleen at 5 or 10 weeks post-transplant indicated that one or more CRU was present in the test cells. In subsequent years, flow cytometric based techniques have become the norm. In particular, the advent of the benchtop flow cytometer combined with the utilization of antibodies generated against the alloantigens CD45.1 (Ly5.1) and CD45.2 (Ly5.2) has permitted convenient and effective distinction of donor-derived and host-derived WBCs with the ability to co-stain with antibodies against lineage-specific markers to demonstrate pluripotency of the test cells. Table 1.1 summarizes the criteria used to define long-term HSC activity in various studies and demonstrates the evolution of this definition over time.
1.1.4.1 Limitations and other considerations of \textit{in vivo} HSC assays:

HSCs have two defining characteristics; multipotentiality and self-renewal. However, there are additional requirements that must be met before cells can read out in the \textit{in vivo} assays just described. For example, the ability to home and engraft is an integral part of the definition, since without these abilities they will not read out. An interesting recently described example is the engraftment defect of HSCs in the S/G2/M phases of cell cycle. Because of this engraftment defect, the cells will not be detected in transplantation assays. However, if these same cells are cultured for a short time such that they re-enter a next G1 phase, they regain an ability to engraft and be detected as HSCs.\textsuperscript{63} Similarly, if recipients are treated with SDF-1 antagonists, S/G2/M cells read out as HSCs. This begs the question: during the time that these cells are unable to engraft, does this mean they are temporarily no longer HSCs? Strictly defining HSC activity using the \textit{in vivo} assay suggests that these cells temporarily cease to be so. An interesting variation on this theme has been proposed by Quesenberry, suggesting the existence of a reversible continuum of hematopoietic states that vary with cell cycle progression.\textsuperscript{64}

History shows that care must be taken when interpreting results from \textit{in vivo} assays. These assays are, by definition, simply surrogate methods to allow the functional identification of a conceptually determined cell. It should be kept in mind that the assay as defined might not overlap perfectly with the cell’s actual properties, or that the concepts used to define that cell might be flawed. For example, the spleen colony assay was thought for many years to be the definitive assay for HSCs, since CFU-S satisfied the then-current conceptual requirements of HSCs. Furthermore, the functional difference
between the primitive cells that gave rise to spleen colonies 7-9 days after transplantation and those that did so after 12-14 days was not initially appreciated, and so no distinction between these two cell types was made. As a result, experiments were designed and results were interpreted on the basis of flawed conclusions.

Similar issues exist for other in vivo assays. One of the original endpoints used in transplantation assays was survival. While it is true that HSCs are ultimately responsible for long-term survival in lethally irradiated recipients, less primitive progenitor types are responsible for radioprotection at early stages. Therefore, for example, if a small number of highly purified HSCs were injected into lethally irradiated mice, they would likely not survive. Therefore, while survival assays may have some relevance for experiments related to clinical engraftment studies, in many cases they have limited usefulness as a stem cell assay.

Another issue is that measurements of overall competitive ability of populations of cells cannot distinguish between variations in LTRC number and variations in competitive ability per individual HSCs. Similarly, limiting dilution assays measure only quantity and not “quality” of HSCs. A possible solution proposed by Nakauchi is to combine the two strategies and determine the activity per HSC

In limiting dilution or purified single cell transplantation experiments, the precise definitions used to identify HSCs are of particular importance for the interpretation of results. In general, if long-term and multilineage donor-derived repopulation is seen, it is determined retrospectively that an HSC was present in the test cells. As technology has developed, this definition and the way it is measured have changed over the years (see table 1.1). Nevertheless, certain issues still remain unresolved. For example, with respect
to the measurement of HSC self-renewal, it is usually assumed that "long-term" repopulation equates with self-renewal. However, the extent of self-renewal that must be observed before the HSC definition is met is still debated. In other words, how "long-term" is long enough? It could be argued that the only true test for self-renewal in vivo is to test the regenerated cells using the same assay (i.e. secondary transplantation), but this is a time- and labour-intensive procedure. It is therefore of utmost importance to interpret each conclusion in light of the HSC definition used.

Another set of issues concerns multipotentiality, the second hallmark feature of HSC activity. Since differentiated cells are identified primarily via FACS, false positives are a possibility. A recent example involves the use of the Gr-1 antibody, which has been found to co-stain a subpopulation of T-cells, resulting in the possibility of classifying recipients as having all their lineages repopulated when in fact no myeloid cells were present. This is of particular importance when comparing HSCs to multipotent progenitors with limited self-renewal, since some lymphoid cells produced early post-transplant have a relatively long half-life and might give a false positive.

In addition, it is commonly assumed that if a few cell types representative of myeloid and lymphoid lineages are produced, that the test cells are fully pluripotent. However, the recent identification of a primitive lymphomyeloid repopulating cell without erythroid or megakaryocytic activity raises interesting questions regarding pluripotency and reminds us that in theory, a cell cannot be classified as pluripotent unless all differentiated lineages are identified.
1.2 Regulation of hematopoiesis

The bone marrow is a complex environment including many hematopoietic and non-hematopoietic cell types and a host of extrinsic molecules produced locally and systemically. Collectively, these maintain a tight regulation of blood cell production and allow rapid response to altered requirements to maintain homeostasis. Most often, external regulation of hematopoiesis involves intercellular communication via growth factors. Growth factor activated signalling events are interpreted by other intracellular signalling molecules and ultimately with the transcription factor repertoire present and the chromatin status in each target cell the gene expression status of the cell is determined. Thus, hematopoiesis is regulated by a combination of intrinsic and extrinsic factors such that intrinsically unique cells may respond differently to stimulation by the same growth factor(s). Over the years, many cytokines have been identified and characterized, including positive or negative regulators of survival, proliferation, self-renewal, and (lineage-specific) differentiation. While many aspects of these processes are intertwined, available evidence suggests that they are often regulated by distinct mechanisms.

1.2.1 Survival and Apoptosis

A well characterized system in which the regulation of survival is paramount is that of the final stages of T-cell development in the thymus. Of the 20-40 million new T-cells produced per day in a young adult mouse, only 2-3% eventually exit the thymus into
the blood. This is due to the double selection process that ensures that the newly
produced T-cells have rearranged their TCR genes in a productive manner yet are not
self-reactive. In the first instance (positive selection), T-cells are programmed to die
unless their T-cell receptors can be stimulated by self-MHC-peptide complexes expressed
by thymic cortical epithelial cells. In the second (negative selection), T-cells whose
receptors bind strongly to self antigens expressed by antigen presenting dendritic cells
receive death signals and undergo apoptosis (reviewed in 68).

Myeloid and erythroid progenitors are constantly being produced in the bone
marrow, but are programmed to die prior to producing their differentiated progeny.
However, in the presence of sufficient levels of the positively regulating growth factors
erythropoietin, GM-CSF, and/or G-CSF, and thrombopoietin, apoptosis is suppressed and
differentiated cells of appropriate types are finally produced and released into the
bloodstream 69, 70. In this way, the body is able to maintain appropriate levels of blood
cells in a timely fashion.

HSCs, on the other hand, appear to be primarily regulated through control of
proliferation, mainly through interactions with the HSC niche (see section 1.2.4.2).
However, the control of apoptosis has been shown to play a role as well. That HSCs are
susceptible to apoptosis has been demonstrated by studies in which the overexpression
of bcl-2 was shown to decrease the death of HSCs in response to a variety of apoptosis
inducing stimuli, including irradiation 71, exposure to chemotherapeutic agents 72, and
growth factor deprivation 73. Conversely, the inducible deletion of a related family
member, mcl-1, resulted in the loss of primitive hematopoietic cells, including HSCs 74. It
was also shown that the overexpression of bcl-2 in combination with Steel factor
signalling was sufficient to prevent apoptosis and allow proliferation in serum-free cultures of HSCs\textsuperscript{75}. Notably, this did not alter the likelihood of self-renewal, indicating that the control of survival, proliferation, self-renewal, and differentiation are distinct processes. However, some growth factors can affect multiple processes. Thrombopoietin, for example, can promote survival\textsuperscript{76}, but also the proliferation of HSCs and differentiation of multipotent progenitors\textsuperscript{77}.

### 1.2.2 Proliferation and Quiescence

Cell cycle progression of hematopoietic cells is directed by the external binding of multiple types of growth factor molecules to specific receptors on the cell surface, which then activate the appropriate intracellular machinery. Well-defined examples include GM-CSF, G-CSF, M-CSF, and IL-3, responsible for the proliferation of myeloid progenitors (reviewed in \textsuperscript{78}). Other growth factors, including thrombopoietin, Steel factor, IL-11, IL-6, and Flt-3 ligand have been shown to be important for the stimulation of proliferation in more primitive hematopoietic cells (reviewed in \textsuperscript{79}). In certain combinations, these growth factors have been shown to have synergistic (or sometimes antagonistic) effects\textsuperscript{80-83}. Cell cycle progression of the most primitive hematopoietic cells can also be negatively regulated by cytokines that inhibit proliferation, such as TGF-\(\beta\)\textsuperscript{84-86}. Quiescence of adult HSCs is thought to be controlled primarily through interactions with the HSC niche (see section 1.2.4.2).

The intracellular machinery involved in cell cycle control includes the highly conserved cyclin-dependent kinases (CDKs), cyclins, and CDK inhibitors (reviewed in
Interestingly, certain components of this machinery are variously expressed in different stages of hematopoiesis (reviewed in \(^8\)). Examples of proteins involved in the regulation of proliferation in primitive hematopoietic cells include the transcription factors MEF/ELF4\(^8\) and Gfi1\(^90,91\), as well as the CDK inhibitors p21\(^{cip1/waf1}\)\(^92\), p16\(^{INK4a}\)\(^93\), and p27\(^{kip1}\)\(^94\), shown to work in cooperation with the MYC antagonist MAD1\(^95\).

A recent study by Yamazaki et al\(^96\) reported that stimulation by thrombopoietin or steel factor induced lipid raft clustering in primitive hematopoietic cells and that this clustering was essential for proliferation to occur. Quiescent cells whose lipid raft clustering was inhibited stayed in G0 and did not divide, regardless of stimulation with mitogenic growth factors. Of note, some of these cells remained viable and functional, and could respond to the growth factor stimulation upon removal of the inhibitor. This suggests that one of the ways that HSC proliferation could be negatively controlled might be through intrinsic or extrinsic modulation of lipid raft reorganization.

1.2.3. Lineage restriction

Interestingly, it has been found that primitive hematopoietic cells express a host of genes normally associated with differentiating or differentiated cell types, albeit at low levels (reviewed in \(^97\)). This has led to the hypothesis that chromatin is generally maintained in an open state in primitive cells, and is thus accessible to transcriptional complexes\(^98\). Progressive alterations in chromatin structure then occur during differentiation, resulting in altered transcription factor expression and the subsequent up-
regulation and down-regulation of appropriate lineage-specific genes in a step-wise fashion. Such alterations in chromatin structure are examples of epigenetic changes, which refer to gene expression alterations that are heritable but not caused by changes in the DNA sequence itself. Epigenetic changes can be initiated and maintained by altering the methylation status of DNA sequences, which recruit repressive chromatin modification complexes, or via the methylation, phosphorylation, or acetylation of specific amino acids of histone tails, which can induce or prevent chromatin condensation.

However, it has been shown that these changes during hematopoietic differentiation are not absolutely irreversible, since cells of one lineage can switch to another lineage in response to specific extrinsic or intrinsic manipulations that result in changes in the transcription factors active in commitment and maintenance of lineage-specific gene expression programs. Some lineage-specific transcription factors have a dual functional role, in that they not only promote expression of genes of one lineage, they repress expression of other lineage-specific genes.

Recently characterized examples include the master transcriptional regulators GATA-1, which is essential for erythroid development, and PU.1, which is essential for the development of B- and T- cells, granulocytes, and monocytes. Interestingly, a functional antagonism exists between GATA-1 and PU.1 that is important for determining erythroid versus myeloid lineage commitment. For example, GATA-1 overexpression causes the reprogramming of myeloid precursors into erythroid cells, while forced PU.1 expression blocks erythroid differentiation. This block can be overcome by co-expression of GATA-1. Direct evidence of functional antagonism was shown in
experiments in which \textit{GATA-1} and \textit{PU.1} binding sites were placed in front of a reporter gene, revealing that each of the two proteins repressed transcriptional activation by the other\textsuperscript{110,111}. Furthermore, it was found that \textit{PU.1} inhibits \textit{GATA-1} target genes expression by recruiting chromatin-modifying proteins to genes to which \textit{GATA-1} is bound\textsuperscript{112}. The repressed chromatin structure thus created has been shown to be released during erythroid development via the down-regulation of \textit{PU.1}\textsuperscript{113}.

More extensive models of how lineage restriction occurs and how cell fate decisions are initially made in HSCs are still debated. A number of studies measuring the lineage outputs of clonally derived colony-forming cells suggest that this can be best described using stochastic mechanisms (see section 3, diversity of HSCs, for more details). In this model, extrinsic factors serve only to regulate proliferation and survival, not lineage choice. This concept is reinforced by observations that the overexpression of various cytokine receptors does not alter lineage fate decisions\textsuperscript{114,115}. However, strong evidence of cytokine-directed lineage choice has been observed in some systems\textsuperscript{116-118}, demonstrating that lineage restriction may be a multifaceted process.

\textbf{1.2.4 Regulation of HSC self-renewal and differentiation}

In order to self-renew, HSCs require functional proliferation pathways along with repression of apoptosis and differentiation pathways. Therefore, many of the regulators discussed in sections 1.2.1 and 1.2.2 could also impact the likelihood of HSC self-renewal. The decision of HSCs to self-renew or to differentiate is governed by a complex
interplay between autonomous signals (intrinsic regulation) and stimuli from the surrounding microenvironment (extrinsic regulation).

1.2.4.1 Intrinsic regulators

The molecular pathways involved in HSC self-renewal are intricate networks of receptors, signalling transducers, transcription factors, and other intracellular proteins. These networks have begun to be unravelled using knockout mice, conditional inactivation strategies, constitutively active or dominant negative proteins, and other techniques. Thus far, relatively few have been identified and characterized, but it is clear that self-renewal is a complex process. The following are a few examples.

The transcription factor Tel/Etv6 is required for the maintenance of HSCs, but not less primitive cells. Conditional inactivation of the Tel/Etv6 gene in B-cell, T-cell, or erythroid progenitors did not affect their differentiated cell output. However, when inactivation was induced in HSCs, a complete depletion of the entire bone marrow was seen within several weeks. As was expected, Tel/Etv6 inactivated bone marrow could not compete effectively against wild-type bone marrow cells when co-transplanted.

Gfi1 is a transcriptional repressor that controls HSC self-renewal by limiting their proliferation. Upon deletion, HSCs proliferate much more rapidly and lose radioprotective ability and competitive reconstitution ability when transplanted. Its mechanism of action is not completely clear but may work via regulation of the CDKi $p21^{CIP1/WAF1}$, which is downregulated in Gfi1-deficient HSCs.
Pten is a tumour suppressor that is a negative regulator of the AKT pathway, which is involved in many cellular processes, including proliferation, survival, and lineage choice. Besides increasing the likelihood of leukemic transformation, the conditional inactivation of Pten in bone marrow HSCs negatively affected HSC self-renewal. Upon transplantation, Pten null HSCs initially engrafted normally but were unable to sustain long-term multilineage WBC production. A transient expansion of Pten\(^{-/-}\) HSCs was seen at early times post-transplant, but over time the HSC pool was exhausted\(^{120}\). Cell cycle analysis revealed that Pten\(^{-/-}\) HSCs were rarely in G0, suggesting a role of Pten in blocking cell cycle progression. Interestingly, Pten deficiency resulted in an alteration of WBC production, with severely reduced B-cell output and a corresponding increase in T- and myeloid cells\(^{121}\).

The JAK-STAT pathway is a common downstream pathway of cytokine-induced signalling. Constitutively activated Stat5 in HSCs promoted in vitro self-renewal of HSCs and a dramatic amplification of downstream progenitors\(^{122}\). Conversely, its deletion resulted in decreased bone marrow and blood cellularity and a loss of competitive ability when transplanted\(^{123}\). Similar results were seen with Stat3. When its activity was decreased in HSCs through expression of a dominant negative STAT3, the competitive reconstituting ability of the transduced HSCs was reduced. Conversely, up-regulation by the expression of a constitutively activated form of Stat3 enhanced the self-renewal and regeneration activity of transplanted HSCs during the initial period of hematologic recovery, and this could be recapitulated upon tertiary transplantation\(^{124}\). Collectively, these studies suggest that the JAK-STAT pathway plays an important role in regulating HSC self-renewal.
The homeobox gene *HOXB4* encodes a transcription factor that is expressed in primitive hematopoietic cells and is rapidly downregulated in more differentiated populations\(^{125,126}\). When *HOXB4* was retrovirally overexpressed in HSCs, a remarkable expansion of HSCs was observed *in vivo*\(^{127-129}\) and *in vitro*\(^{130}\), suggesting that it could act as a positive regulator of HSC self-renewal. Interestingly, *Hoxb4*-deficient mice had no major hematopoietic abnormalities and only a mild proliferation defect\(^{131}\), suggesting that its loss of function could be compensated by a different mechanism, possibly *Hoxa4* and/or *Hoxc4*. In addition, the effects of *HOXB4* overexpression could be enhanced by downregulation of *p21*, suggesting the simultaneous modulation of two independent self-renewal pathways\(^{132}\).

The Polycomb group (PcG) of developmental regulatory genes were originally described in *Drosophila*. The PcG gene *Bmil* is a transcriptional regulator of various homeobox family genes in the mouse\(^{133}\) and is a member of PRC1 (polycomb repression complex 1). The PcG genes encoding members of this complex, including *Bmil*, *Mell8*, and *Mphl/Rae28*, are expressed in the CD34-KSL BM population (which is highly purified for HSCs) and are down-regulated in more differentiated BM populations\(^{134}\). All three PRC1 members have also been shown to play a role in HSC self-renewal.

*Mphl/Rae28* deficient HSCs were able to fully reconstitute transplanted recipients with all lineages for several months and *Mphl/Rae28* overexpression did not result in expansion of primitive hematopoietic cells *in vitro*\(^{134}\). However, *Mphl/Rae28* deficient HSCs were reduced in their ability to expand stem cell numbers, as quantified by limit dilution transplants into secondary recipients\(^{135}\). In addition to a quantitative decrease,
the regenerated *Mph1/Rae28* deficient HSCs were qualitatively deficient, as measured by a lower activity per stem cell\textsuperscript{136}.

*Mel18* null HSCs were shown to express elevated levels of *Hoxb4* and the frequency of HSCs, measured as CRU, was elevated. At the same time, however, there was also a qualitative decrease in repopulating activity per CRU\textsuperscript{137}. However, it should be noted that both of these measures may be skewed due to the previously described defects in T- and B- lymphocyte development in *Mel18*\textsuperscript{-/-} mice\textsuperscript{138}, which results in a lower BM cellularity (potentially affecting HSC frequency) and presumably a reduction in lymphoid cell production in the PB (potentially affecting the WBC output of *Mel18*\textsuperscript{-/-} HSCs). When *Mel18* was overexpressed, the opposite was found, including lower levels of *HoxB4* expression, a lower HSC frequency, and a higher repopulation activity per HSC\textsuperscript{137}.

*Bmil* null HSCs were able to generate a normal spectrum of differentiated blood cells, but only temporarily, due to a marked reduction in self-renewal ability\textsuperscript{139,140}. The lack of self-renewal ability of *Bmil*\textsuperscript{-/-} HSCs was confirmed by their inability to repopulate secondary recipients just six weeks after the primary transplant\textsuperscript{139,140} and explains why *Bmil* null mice die of bone marrow exhaustion within two months of birth\textsuperscript{141}.

Conversely, when *Bmil* was overexpressed in HSCs, a marked increase in self-renewal was seen. HSCs overexpressing *Bmil* were dramatically expanded *in vitro*, and following 10 days of culture, contained 35-fold greater repopulating ability than mock transduced cells\textsuperscript{134}. Recently, *E4F1* was identified to physically and functionally interact with BMII to mediate HSC self-renewal, and its deletion could partially overcome the negative effects of *Bmil* knockout\textsuperscript{142}. Similarly, the deletion of *p16\textsuperscript{INK4A}* and *p19\textsuperscript{ARF}* restored
considerable self-renewal ability in Bmi1 null HSCs\textsuperscript{143}. Interestingly, overexpression of HOXB4 failed to rescue the defective self-renewal of Bmi1\textsuperscript{+/} HSCs, suggesting that Hoxb4 acts upstream of Bmi1\textsuperscript{134}.

The retinoic acid receptor (RAR) family are nuclear receptors that are members of the steroid/thyroid hormone superfamily of transcription factors. Two members of this family, RAR\textsubscript{\alpha} and RAR\textsubscript{\gamma}, are expressed in HSCs, and RAR\textsubscript{\gamma}, but not RAR\textsubscript{\alpha}, has been shown to be a key regulator of HSC self-renewal\textsuperscript{144}. The loss of RAR\textsubscript{\gamma} results in reduced numbers of HSCs and increased numbers of downstream progenitors, while the loss of RAR\textsubscript{\alpha} did not result in any hematopoietic defects. Activation of RAR\textsubscript{\gamma} by stimulation with its natural ligand, all-trans retinoic acid (ATRA), resulted in increased Notch1 and Hoxb4 expression, accompanied by enhanced self-renewal. Therefore, loss of RAR\textsubscript{\gamma} results in reduced numbers of HSCs due to increased HSC differentiation, while RAR\textsubscript{\gamma} activation results in increased HSC self-renewal\textsuperscript{144}.

1.2.4.2 Extrinsic regulators – HSC niche

In recent years, much has been learned about the specific environments within the bone marrow that are of particular importance for the survival and self-renewal of HSCs. This so-called stem cell “niche” contains a unique combination of stromal supporting cells, osteoblasts, and extracellular matrix, and is thought to provide a unique environment that protects HSCs from stimuli, including those that would induce apoptosis, differentiation, or excessive mitogenesis\textsuperscript{145}. It is thought that HSCs in this
environment are usually quiescent with periodic activation in order to produce daughter HSCs (thus increasing HSC numbers) or multipotent progenitors capable of transient amplification and differentiation in order to replenish functional blood cells as needed (Figure 1.1).

It has been hypothesized for decades that the bone medulla surface lining, or endosteum, was important for the maintenance and self-renewal of primitive hematopoietic cells. For example, experiments studying the spatial distributions of primitive hematopoietic cells within the bone marrow showed that the density of granulocytic progenitors increased with distance from the surface of the mouse femur. Conversely, the density of CFU-S decreased towards the bone marrow axis\textsuperscript{146}. Interestingly, the peak density of CFU-S was approximately 100 microns from the bone surface, which might be expected since CFU-S are likely produced by the HSCs more closely associated with the osteoblasts at the endosteal surface. Indeed, the relationship between HSCs and bone may go even “deeper”, as many HSCs can be found within dissociated skeletal bone (Brenton Short, personal communication).

Mouse bone-forming cells, called osteoblasts, are located at the endosteum and are an essential component of the HSC niche. When genetic strategies were employed to increase osteoblast number, increases of HSC numbers were also seen. This increase did not extend to downstream progenitors, suggesting that the supportive role of the osteoblast is specific to HSCs\textsuperscript{147,148}. Conversely, it has also been shown that in a mouse model with decreased osteoblast numbers, hematopoiesis is severely altered\textsuperscript{149}.

Combining recently developed HSC markers with immunohistocytochemical localization techniques have confirmed that HSCs are often localized at the endosteal
surface, associated with osteoblasts. Suzuki et al also monitored this interaction in vivo using Gata2-directed GFP fluorescence, and found that individual quiescent GFP-positive cells were in intimate contact with osteoblasts at the endosteum. Following 5-FU treatment, time-lapse fluorescent imaging revealed that the few remaining GFP+ cells residing at the bone marrow edge did not move, whereas the surrounding GFP− cells were very active. Osteoblasts have also been demonstrated to secrete factors known to modulate stem cell function, including G-CSF, M-CSF, GM-CSF, IL-1, IL-6, as well as cell cycle inhibitory factors such as TGF-β, LIF, TNF-α, and TNF-β (reviewed in ).

Osteopontin, a glycoprotein secreted by osteoblasts, has been shown to have an important role in the modulation of HSC numbers in the niche. It has also been suggested that Wnt proteins secreted by niche stromal cells contribute to HSC self-renewal by activating the Wnt signalling pathway. Direct cell-cell interactions between HSCs and osteoblasts are also important. N-cadherin and beta-catenin, which play roles in adherens junctions, were asymmetrically localized between HSCs and osteoblasts. It has also been shown that the interaction of Tie2, expressed by HSCs, and Ang-1, expressed on the osteoblasts, promotes HSC quiescence. In addition, interaction between the Jagged ligand, expressed on osteoblasts, and Notch family receptors on HSCs, has been shown to promote self-renewal of HSCs.

In studies of hematopoietic regeneration following the ablation of non-quiescent hematopoietic progenitors using 5-fluorouracil treatment, Heissig et al. observed a shift of clusters of proliferating hematopoietic cells from the osteoblastic niche to the “vascular niche”, proximal to the sinusoidal blood vessels in the BM. That putative
HSCs are located at both the osteoblastic and vascular niches has been confirmed using immunohistocytochemical localization\textsuperscript{27}.

1.2.4.3 Studies of extrinsic control of HSC self-renewal \textit{in vitro}

Stringently defined assays are of particular importance when interpreting results of ex vivo manipulated hematopoietic cells. While huge expansions of restricted progenitors and considerable expansions of multipotent progenitors have been described, increases in the numbers of these cells do not necessarily correlate with increases in rigorously defined HSCs\textsuperscript{157};\textsuperscript{158}. The first studies to demonstrate rigorously defined HSC self-renewal \textit{in vitro} used retrovirally marked bone marrow cells grown in stromal cultures for 4 weeks and transplanted into multiple recipients. Expansion of a subset of the input HSCs was revealed by clonal integration patterns shared between the WBCs of multiple recipients up to 7 months post-transplant. Despite the demonstration of occasional expansion, there was an overall net decline of HSC numbers in these cultures\textsuperscript{159};\textsuperscript{160}.

Subsequent studies of \textit{in vitro} self-renewal, primarily utilizing HSC-enriched cell populations cultured in defined serum-free and feeder-free conditions plus recombinant cytokines, have yielded direct evidence of growth factor determined modulation of HSC self-renewal versus differentiation decisions. Using various concentrations of the cytokines Steel factor (SF) and/or Flt3-ligand (Flt3L) plus IL-6 and/or IL-11, maintenance of HSC activity in 7-21 day feeder-free cultures initiated by HSC-enriched
populations of mouse BM has been reported in several studies. Conversely, if IL-1, IL-3, or TNF-α were added to similar cultures, a decrease in HSC activity was seen. However, another report suggests that IL-3, albeit in combination with slightly different cytokines, did not have a negative effect on HSC activity over 10 days in culture. Using various combinations of SF, Flt3L, IL-6, and IL-11, net increases of HSC activity have been documented with the use of 100 ng/ml IL-11 plus 18 ng/ml SF, 100 ng/ml IL-11 plus 50 ng/ml SF plus 100 ng/ml Flt3L, and 50 ng/ml SF plus 20 ng/ml IL-6 plus 100 ng/ml Flt3L. A subsequent multifactorial design analysis using varying combinations and concentrations of SF, IL-11, and Flt3L was performed. This study revealed that while Flt3L or SF alone can stimulate proliferation, maintenance of HSC activity requires activation of the gp130 pathway via IL-6 or IL-11 stimulation. It was also found that the exact concentration of cytokines was very important for HSC amplification. A negative interaction was found between SF and Flt3L when both were used at high concentrations, and stimulation of HSC amplification by IL-11 was observed at a narrow concentration range, with high levels having a negative effect.

Experiments using thrombopoietin (TPO)-null mice have demonstrated that TPO is an important component of in vivo regeneration of HSCs following transplantation. A subsequent study reported that these effects of TPO may be due to an increase in Hoxb4 mediated by an increase in its upstream regulator USF-1. As a single agent, TPO has been suggested to maintain HSC activity over 7 days in serum-containing, feeder-free culture without stimulating their proliferation. This lack of mitogenic activity was confirmed in serum-free, feeder-free cultures in single cell cultures of CD34+ KSL cells cultured with TPO alone. Combining 100 ng/ml TPO with 10 ng/ml of SF
was highly mitogenic yet promoted HSC self-renewal (maintenance) over 3 or 6 days of culture\textsuperscript{65}. Interestingly, similar experiments performed using 100 ng/ml each of Flt3L, SF, and IL-11 showed a dramatic decrease in HSC self-renewal compared to 100 ng/ml TPO plus 10 ng/ml SF\textsuperscript{82}. This data seemed to conflict with the previous findings of Miller et al.\textsuperscript{62}, but this might be explained by the 2-fold difference in SF concentration used. In addition, the Miller et al. experiments used cultures initiated with 15 Lin-Sca+ cells and measured CRU activity after 10 days, while Nakauchi et al. initiated the cultures with single CD34-KSL cells and measured after 3 days. Thus, at the time of transplant, the latter contained only 1 or 2 cells, while the former would have grown exponentially to tens of thousands of cells, raising the possibility of indirect mechanisms being partly responsible for the difference in effect.

A dramatic expansion of HSC activity was observed when unfractionated bone marrow cells were cultured for 3-4 weeks in serum-free media and fibroblast growth factor (FGF)\textsuperscript{170,171}. Although FGF receptors were shown to be expressed on HSCs, the HSC expansion requires the co-culture of non-HSCs for its effect, since the culture of purified KSL cells did not lead to HSC expansion\textsuperscript{170,172}. Yet, the culture of purified KSL cells along with genetically distinct bone marrow cells led to HSC expansion at the same rate\textsuperscript{172}. Interestingly, the addition of SF+IL11+Flt3L to the FGF culture system induced a strong proliferative response and all HSC activity was rapidly lost\textsuperscript{172}. This suggests that the mechanisms of self-renewal induced in the FGF culture system are distinct from those induced by SF, IL11 and Flt3L.

Purified and lipid-modified Wnt3a, a ligand for the Frizzled family of proteins, appears to induce significant HSC expansion over 6 days in cultures containing serum
and SF, although the long-term repopulation ability of the expanded HSCs was not rigorously examined\textsuperscript{173}. Complementary experiments in Bcl-2 transgenic mice where the Wnt signaling pathway was activated using the constitutively active form of $\beta$-catenin demonstrated a similar effect\textsuperscript{174}, possibly in conjunction with Notch signaling\textsuperscript{175}. However, very recent experiments have cast doubt on the applicability of these findings in a normal genetic background\textsuperscript{176}.

All-trans-retinoic acid is a hydrophobic vitamin A analogue that is an agonist for the nuclear retinoic acid receptors (RARs). In 7-day and 14-day cultures containing fetal bovine serum, SF, Flt3L, IL-6, IL-11, and ATRA, maintenance of HSC activity was observed relative to the starting cells. In the same conditions without added ATRA, a slight decrease of HSC activity was seen. Conversely, the addition of a RAR antagonist abrogated all HSC activity within 7 days of culture\textsuperscript{177}. It has since been shown that ATRA's effects are mediated via the nuclear receptor RAR$\gamma$, as RAR$\gamma^{-/-}$ HSCs did not repopulate recipients after being cultured in the presence of ATRA, while RAR$\alpha^{-/-}$ HSCs responded similarly to WT HSCs\textsuperscript{144}.

Zhang and Lodish characterized a minor population of day-15 murine fetal liver, the CD3\textsuperscript{+} fraction (\textasciitilde 2\%), that could support expansion of HSCs in culture. Using Affymetrix microarrays, they then compared the transcriptional profile of these cells with day-15 Gr1\textsuperscript{+} cells and with adult splenic CD3\textsuperscript{+} cells, neither of which could support HSCs in culture, and identified candidate secreted and membrane proteins that were relatively abundant in the fetal liver CD3\textsuperscript{+} cells\textsuperscript{178}. One protein thus identified was insulin-like growth factor 2 (IGF-2). In cultures containing fetal bovine serum, SF, Flt3L, and IL-6, maintenance of HSC numbers was seen over 3 days. However, when a high
concentration of insulin-like growth factor 2 (IGF-2) was added to cultures, a 2-fold expansion of HSCs was observed\textsuperscript{178}. Two additional candidates identified in the original screen, angiopoietin-like proteins 2 and 3, were subsequently shown to support a significant (24 and 30-fold) net expansion of HSCs over 10 days in culture with SF, TPO, IGF-2, and FGF-1\textsuperscript{179}.

1.3 Heterogeneity of HSCs

Heterogeneity within the HSC compartment has been observed and discussed since the field was first established. In general, any observable diversity within the HSC compartment can be attributed to alternate self-renewal and differentiation decisions. For example, a HSC that undergoes limited self-renewal divisions would likely exhaust faster than one that self-renews indefinitely. Or, a HSC whose progeny tend to differentiate down the lymphoid pathways would likely contribute less to the erythrocyte pool. The mechanisms that generate diversity in the HSC compartment have been and continue to be much debated.

Diversity in CFU-S size and considerable size-independent variability in CFU-S regeneration were observed in day 10, 12, and 14 spleen colonies\textsuperscript{16}. Subsequently, lineage output per colony was also found to be heterogeneous\textsuperscript{11}. Because the frequency distributions of secondary colonies formed per primary colony did not fit a Poisson distribution, it was determined that the diversity observed fell far outside what could be expected due to experimental variation. Therefore, it was hypothesized that the variability
was due to either heritable biological heterogeneity, or that the heterogeneity developed during the growth of the colony in a random fashion. This latter hypothesis was described in detail as a stochastic model of stem cell proliferation. This model was subsequently expanded to include the apparently random choice of lineage restriction during differentiation.

The heterogeneity of colony size and self-renewal ability observed within the CFU-S compartment was paralleled by similar (clone size-independent) variability observed in the secondary colony-forming ability of primitive in vitro colony forming cells. Again, the frequency distributions of secondary colonies formed per primary colony did not fit a Poisson distribution, and were thus interpreted to support the stochastic model of stem cell self-renewal and commitment proposed earlier.

Experiments involving the sequential replating of progenitors derived from primitive in vitro colonies demonstrated that these primitive cells could produce diverse combinations of cell lineages. When these progenitors were allowed to divide once and separated, comparison of the progenitor pairs revealed a progressive restriction in lineage potentials, but the lineage choices were made in an apparently stochastic fashion. Similar in vitro paired-daughter cell experiments performed more than a decade later using FACS isolated HSCs also showed dissimilar combinations of lineages and sizes and was also interpreted to support the stochastic model of stem cell commitment.

The advent of retroviral marking of transplantable primitive hematopoietic cells allowed clonal tracking of differentiated cell output over time following transplantation. Heterogeneity and fluctuations in total output, lineage distribution, and longevity were observed within retrovirally marked clones. Attempts to explain
the observed clonal variations included hypotheses of stochastic mechanisms\textsuperscript{1;2;193}, intrinsic differences in the primitive cells themselves\textsuperscript{2;190;194}, or the successive output of variable but relatively short-lived clones\textsuperscript{1;191;195}.

A distinction between long-term and short-term pluripotent repopulating cells was made clear using limiting dilution transplants of bulk bone marrow\textsuperscript{196} or enriched populations\textsuperscript{197;198}. Further studies showing that these two types could be phenotypically separated\textsuperscript{158;199;200} provided compelling evidence that intrinsic differences exist between LT-HSCs and ST-HSCs. Subsequently, multiple additional purification strategies have been found able to phenotypically separate the two cell types (see section 1.1.2 for details).

More recently, additional evidence of heterogeneity within the HSC compartment has been obtained from analyses of single highly purified cells, including variations in transcriptional profiles\textsuperscript{201}, time to first division upon cytokine stimulation in single cell cultures\textsuperscript{65;117;187}, variation in WBC reconstitution levels after single cell transplants\textsuperscript{4;31;65;202-204}, repopulation kinetics post-transplant\textsuperscript{3}, in vivo daughter HSC regeneration\textsuperscript{4;65}, and lineage distribution of cell output in vitro\textsuperscript{187} and in vivo\textsuperscript{4}.

Some of the most direct evidence supporting the intrinsic nature of heterogeneity within the HSC compartment has been generated by the Muller-Sieburg group. They generated clonally repopulated mice by initiating stromal cell-containing cultures with limiting dilutions of unseparated BM, harvested positive cultures 4 weeks later, and transplanted entire cultures into irradiated recipients\textsuperscript{205}. These clonally repopulated mice were analyzed for donor repopulation levels and lineage distributions over time and found to be heterogeneous in overall repopulation kinetics and the WBC types produced.
When HSCs regenerated in the primary recipients were transplanted into secondary and tertiary recipients and similarly analyzed, inter-clonal heterogeneity was still seen, but daughter HSCs derived from any given clone behaved similar to each other in lineage distribution and overall repopulation pattern, and tended to exhibit behaviour similar to the HSC(s) originally injected\textsuperscript{206}. These same mice were then analyzed together with a larger group of recipients clonally repopulated with limiting numbers of fresh BM or single purified cells, for the shape of their repopulation kinetics post-transplant. Only a subset of all the possible repopulation curves was observed, suggesting that the hematopoietic stem cell compartment consists of a limited number of distinct HSC subsets\textsuperscript{207}. Collectively, these results suggest that HSC heterogeneity is due to the existence of epigenetically predetermined HSC subtypes\textsuperscript{208}.

Significant evidence has accumulated to affirm the concept of a HSC-supporting niches within the bone marrow (see section 2.3.2). The existence of multiple niche locations (i.e. osteoblastic and vascular) provides a possible mechanism for alternative extrinsic regulation of HSC behaviour. For example, it has been hypothesized that the osteoblastic niche provides signals to maintain the quiescence of HSCs, while activated HSCs have been associated with the vascular niche\textsuperscript{209}. This is supported by observations of the migration of actively dividing hematopoietic clusters from the osteoblastic to the vascular niche following the ablation of cycling progenitors with 5-fluorouracil treatment\textsuperscript{156}. It has thus been speculated that there may be multiple distinct niches within the bone marrow, and that the differences in these environments might induce alterations in HSC fate decisions\textsuperscript{210}, although this is still controversial\textsuperscript{211}. 

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Functional differences in the behaviour of HSCs from different stages of development have also been demonstrated. In mid-gestation (~14.5 days post-conception), the primary hematopoietic organ is the fetal liver. Thereafter, HSCs gradually migrate from the liver to the bone marrow until shortly after birth, at which point the role of the liver in hematopoiesis is greatly diminished. When compared with adult BM HSCs, fetal liver (FL) HSCs exhibited faster regeneration rates of daughter HSCs\textsuperscript{212,213}, spleen colony forming cells\textsuperscript{214}, and more differentiated cells\textsuperscript{214,215} upon transplantation into irradiated recipients. Another distinct functional difference between fetal and adult HSCs is their cycling status. FL HSCs are exclusively cycling, while most steady-state adult HSCs are quiescent\textsuperscript{63}. Parallel alterations in differentiation properties have also been described, in which FL HSCs produce elevated proportions of myeloid\textsuperscript{45} or lymphoid\textsuperscript{216} cells compared with their adult BM counterparts. Interestingly, it has recently been suggested that an intrinsic switch from these fetal-like to adult-like HSC properties occurs rapidly, between 3 and 4 weeks post-birth\textsuperscript{45,63}.

Several studies have also implicated differences in bone marrow HSCs from young adult versus older adult mice. In the commonly used C57Bl/6J strain, it was observed that absolute numbers of BM HSCs increased with age\textsuperscript{216-221} yet have poorer homing efficiency\textsuperscript{216,221}. Another well-documented observation is that BM HSCs from older mice tend to produce higher proportions of myeloid cells and lower proportions of lymphoid cells (particularly B-cells) than their counterparts from younger mice\textsuperscript{218-222}. Comparison of HSC numbers and other HSC properties between C57Bl/6J and other mouse strains has revealed that these characteristics must be largely genetically determined\textsuperscript{217}.
1.4. Thesis Objectives

The overall aim of my thesis work was to further characterize the properties of HSCs and the extent to which intrinsic and extrinsic mechanisms may alter their subsequent self-renewal or differentiation behaviour. This goal was based on the hypothesis that the well documented heterogeneity in HSC behaviour might be attributable to a previously unrecognized substructure within the HSC compartment. However, because most of the studies to date have made use either of enriched populations of HSCs, or HSCs present at limiting dilutions in a mixture of other cells, it has not been possible to determine whether these were masking an additional level of HSC heterogeneity. I was therefore interested in developing an experimental approach where I could accurately and repeatedly track the clonal behaviours of HSCs in vitro (by studying cultures initiated with single HSCs) or in vivo (by tracking reconstitution obtained in mice injected with a single HSC). To accomplish this I initially helped to develop and validate a strategy to isolate HSCs at very high purities (>30%) and to use these efficiently to initiate short-term clonal cultures and transplant irradiated mice with single cells or in vitro clones. This is presented in the first part of Chapter 2.

I then used these powerful tools to explore the extent to which exogenous growth factors can differentially affect HSC self-renewal decisions in vitro, and whether these decisions were necessarily tied to alterations in cell cycle duration. Extrinsic control of cell differentiation programs is well established, and a link between differentiation and the duration of G1 has been described (reviewed in [87]). In addition, a number of studies of primitive hematopoietic cells have suggested that their proliferative potential may be
inversely correlated with their rate of cell cycle entry\textsuperscript{187,223-225}. However, the link between control of cycling and differentiation in HSCs is complex, since the time taken for highly purified quiescent HSCs to complete a first cell division can be influenced by the combination of growth factors to which they are exposed\textsuperscript{65,82}. I explored this further by comparing initial cell division kinetics and HSC self-renewal in short-term cultures initiated with single purified cells cultured in two different growth factor combinations. The results, also presented in Chapter 2, indicated that combination growth factor signals can differentially alter HSC self-renewal decisions independently of alterations in cell cycle duration.

By analyzing clonal cultures in conditions that support HSC self-renewal, I then asked whether I could identify new behavioural traits of cultured HSC with functionally validated long term multi-lineage repopulating activity \textit{in vivo}. As discussed in section 1.1.2.1, most of the markers used to isolate HSC-enriched populations from steady-state mouse BM are not directly associated with HSC functional potential, since these phenotypes are altered when HSC are activated or stimulated to divide. To search for new identifying properties of HSCs self-renewing \textit{in vitro}, I used a novel microwell array video imaging system to visualize clones derived from individual HSCs over a 4-day period under the conditions I had just confirmed to support HSC self-renewal divisions. Each clone was then recovered and assayed for the presence of HSCs with long term multi-lineage \textit{in vivo} repopulating activity. Time-lapse video images of these assayed clones were then used to correlate visible characteristics of the cultured cells with those that had produced functionally defined daughter HSCs. The results of these studies are described in Chapter 3.
Traditionally, HSCs with rigorously defined long term repopulating activity have been envisaged to comprise a relatively homogeneous population. However, significant variation in the outputs of individual HSCs transplanted \textit{in vivo} is a well recognized hallmark of their behaviour. Nevertheless, the extent to which this heterogeneity in HSC behaviour reflects a predetermined intrinsic diversity among these cells\textsuperscript{208}, or their chance exposure to different environments\textsuperscript{145,226,227}, or stochastic events affecting intrinsic pathways that regulate their behaviour\textsuperscript{180,228-230} remains unresolved. Therefore, I set out to characterize the spectrum of \textit{in vivo} output activity within the HSC compartment, and the extent that this diversity is intrinsically, extrinsically, or randomly regulated. To address this, I performed single cell transplants to examine longitudinally the clonal WBC output of more than a hundred single HSCs (or their \textit{in vitro} generated HSC progeny), by monitoring the peripheral blood of primary recipients over a 6-month period and, in some cases, also of secondary and tertiary recipients. The results, presented in \textbf{Chapter 4}, provide convincing evidence of intrinsic programming of HSCs and challenge the prevailing linear-branching model of HSC differentiation.
Table 1.1 – Definitions of long-term multilineage donor repopulation in various studies

<table>
<thead>
<tr>
<th>Publication</th>
<th>Host</th>
<th>Competitor Cells</th>
<th>min % repopulation</th>
<th>Lineages</th>
<th>Weeks PT</th>
</tr>
</thead>
<tbody>
<tr>
<td>Szilvassy/Eaves PNAS 1990(^5)</td>
<td>8.5 Gy female B6/C3</td>
<td>1%-2\times10^5(5) compromised</td>
<td>detectable by Southern, ~5%</td>
<td>male DNA in BM and thymus</td>
<td>5 to 10</td>
</tr>
<tr>
<td>Morrison/Weissman Immunity 1994(^1)</td>
<td>split 9-9.2 Gy B6</td>
<td>2\times10^5(5)</td>
<td>0.3% of each lineage</td>
<td>B220, Mac1, CD3, Gr1</td>
<td>16+</td>
</tr>
<tr>
<td>Rebel/Lansdorp Blood 1994(^15)</td>
<td>9-9.5 Gy B6</td>
<td>2\times10^5(5)</td>
<td>compromised or 4\times10^5(5) Sca-</td>
<td>20%</td>
<td>None – Ly5.1 only</td>
</tr>
<tr>
<td>Trevisan/Iscove Blood 1996(^5)</td>
<td>3 Gy W41</td>
<td>None</td>
<td>“detectable”, ~0.1-1%</td>
<td>erythroid (via gpi shift assay)</td>
<td>32 to 52</td>
</tr>
<tr>
<td>Osawa/Nakauchi Science 1996(^3)</td>
<td>9.5 Gy B6</td>
<td>500 CD3(^{low}) KSL</td>
<td>“detectable”</td>
<td>Mac1/Gr1, Thy1/B220 (not required)</td>
<td>12 +survival</td>
</tr>
<tr>
<td>Miller/Eaves PNAS 1997(^5)</td>
<td>9 Gy B6 or 4 Gy W41</td>
<td>10(5) or nothing</td>
<td>1%</td>
<td>Gr-1+, SSClow</td>
<td>16+</td>
</tr>
<tr>
<td>Sudo/Nakauchi JEM 2000(^21)</td>
<td>9.5 Gy B6</td>
<td>2\times10^5(5)</td>
<td>1%</td>
<td>Gr1/Mac1, CD4/CD8, B220</td>
<td>12</td>
</tr>
<tr>
<td>Ema/Nakauchi JEM 2000(^6)</td>
<td>9.5 Gy B6</td>
<td>2\times10^5(5)</td>
<td>1%</td>
<td>Gr1/Mac1, CD4/CD8, B220</td>
<td>12</td>
</tr>
<tr>
<td>Adolfsson/Jacobsen Immunity 2001(^24)</td>
<td>9.5 Gy B6</td>
<td>1.5-2\times10^5(5)</td>
<td>0.5% of total, 0.1% myeloid</td>
<td>Gr1/Mac1</td>
<td>16</td>
</tr>
<tr>
<td>Szilvassy/Eaves ExpHem 2003(^23)</td>
<td>split 9 Gy B6</td>
<td>2\times10^5(5)</td>
<td>5%</td>
<td>Gr1/Mac1, Thy1, B220</td>
<td>5,10,17,26</td>
</tr>
<tr>
<td>Uchida/Eaves ExpHem 2003(^36)</td>
<td>4 Gy W41</td>
<td>None</td>
<td>0.05% of each lineage</td>
<td>B220, CD5, Gr1/Mac1</td>
<td>16+</td>
</tr>
<tr>
<td>de Haan/Miller Dev Cell 2003(^17)</td>
<td>10 Gy B6</td>
<td>2\times10^5(5)</td>
<td>2% of each lineage</td>
<td>Gr1/Mac1, Thy1, B220</td>
<td>8</td>
</tr>
<tr>
<td>Matsuzaki/Okano Immunity 2004(^17)</td>
<td>10.5 Gy B6</td>
<td>2\times10^5(5)</td>
<td>1%</td>
<td>Gr1/Mac1, B220/CD3</td>
<td>12</td>
</tr>
<tr>
<td>Kiel/Morrisson Cell 2005(^27)</td>
<td>split 11 Gy B6</td>
<td>2\times10^5(5)</td>
<td>above background (0.1-0.3%)</td>
<td>Gr1/Mac1, B220, CD3</td>
<td>16</td>
</tr>
<tr>
<td>Ema/Nakauchi Dev Cell 2005(^4)</td>
<td>9.5 Gy B6</td>
<td>2\times10^5(5)</td>
<td>1%</td>
<td>Gr1/Mac1, CD4/CD8, B220</td>
<td>12 to 16</td>
</tr>
<tr>
<td>Zhang/Lodish Blood 2005(^40)</td>
<td>split 10 Gy B6</td>
<td>1-2\times10^5(5)</td>
<td>1%</td>
<td>Thy1, B220, Mac1, Gr1, Ter119</td>
<td>16</td>
</tr>
<tr>
<td>Rossi/Weissman PNAS 2005(^20)</td>
<td>split 3.2 Gy B6</td>
<td>3\times10^5(5)</td>
<td>“detectable”</td>
<td>Mac1, B220, TCR8+</td>
<td>28</td>
</tr>
<tr>
<td>Dykstra/Jervis PNAS 2006(^22)</td>
<td>4 Gy W41</td>
<td>None</td>
<td>1% of total @ 15wk, 1% of each lineage at any time</td>
<td>Gr1/Mac1, B220/CD5</td>
<td>4,8,12,16</td>
</tr>
</tbody>
</table>
Figure 1.1 – Hierarchical model of hematopoiesis (adapted from Bryder et al\textsuperscript{210}).
HSCs are placed at the top of this hierarchy and are the only cells with extensive self-renewal ability. Cells of all mature blood cell types are continually produced through a series of successive differentiation and amplification steps. Cells defined LTRC, STRC, and CFU-S (Sections 1.1.1 and 1.1.2) are thought to represent cells in the top levels of the hierarchy, as shown.
Figure 1.2 – Functional detection of HSC by transplantation in vivo.
HSCs can be retrospectively identified by their ability to produce multiple WBC types for an extended period of time following transplantation into an irradiated recipient. In this way, the HSC’s hallmark properties of self-renewal and multipotentiality can be functionally assessed. Test cell(s) congenic for a single cell surface marker (CD45) are injected into irradiated recipients. After an extended period of time (usually 16 weeks), a blood sample is collected and the tested by FACS for the presence of donor-derived WBC. If WBC of both myeloid and lymphoid lineages are present at this time, it is assumed that the original test cells must have contained a HSC (with self-renewal and multipotent abilities).
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Chapter 2

Different In Vivo Repopulating Activities of Purified Hematopoietic Stem Cells Before And After Being Stimulated To Divide In Vitro With The Same Kinetics†

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2.1. Introduction

Life-long blood cell production is dependent on the continuous activation of a small population of multi-potent hematopoietic stem cells (HSCs) found primarily in the bone marrow (BM)\(^1\):\(^2\). This population is maintained by the ability of HSCs to divide and generate daughter cells that, at least half of the time, retain the same proliferative and differentiation potentialities as the original parent HSCs. Murine HSCs can be quantitated by limiting dilution assays that detect their longterm (>4 months) multi-lineage differentiation activity in competitively repopulated hosts\(^3\):\(^5\). The self-renewal activity of these cells in vivo has been well documented and, when individual HSCs are evaluated, remarkable heterogeneity in their sustained self-renewal responses is seen\(^6\). At a molecular level, very little is known about the regulation of the HSC self-renewal process. One approach has been to look for effects of various growth factor conditions on the preservation or loss in vitro of HSC function. However, this requires systems for tracking highly purified HSCs and their progeny in order to distinguish between changes in HSC numbers caused by their differentiation rather than their death. At the time the present studies were being pursued, several protocols for isolating murine HSCs at purities of 15-20% had been reported\(^7\):\(^{11}\) and recent studies using HSC-enriched cell populations had already yielded some evidence of growth factor-determined modulation of HSC self-renewal versus differentiation decisions in vitro\(^12\):\(^{14}\). Interestingly, these findings are paralleled by a lack of evidence for growth factor control of lineage choice in "normal" hematopoietic cells\(^15\):\(^{18}\). Taken together, these observations suggest a model of HSC regulation in which the stem cell state is maintained by intracellular regulators that
block the activation of multiple latent lineage-specific differentiation programs. Such regulators may be distinct from transcription factors that progressively restrict which differentiation program will ultimately be activated. Candidate genes regulating the maintenance of HSCs in an undifferentiated state would be Stat3 and HoxB4, whereas candidate genes regulating differentiation would be Gata-1, PU.1 and Pax5.

In other systems, extrinsic control of cell differentiation decisions is well established. In some of these, alterations in the duration of G1 have been implicated (reviewed in 24). Interestingly, a number of studies of primitive hematopoietic cells have suggested that their proliferative potential may be inversely correlated with their rate of cell cycle entry. However, the link between control of cycling and differentiation in HSCs is complex, since the time taken for highly purified quiescent HSCs to complete a first cell division can be influenced by the combination of growth factors to which they are exposed. In addition, the downstream checkpoint regulators involved appear to differ between HSCs and the early differentiated progenitor cells they generate.

During an initial series of experiments designed to analyze the properties of different fractions of side population (SP) cells isolated from adult mouse BM on the basis of their ability to efflux Hoechst 33342 (Ho), we noted that greater than 40% of the lineage marker-negative (lin-) Rhodamine-123-negative (Rho-) subset of SP cells have longterm multi-lineage in vivo repopulating ability when assessed using single cell transplants. We then used these highly purified HSCs to investigate further the relationship between the kinetics of HSC mitogenesis in vitro and the maintenance of HSC function. Our results show that 2 growth factor cocktails with the same potent
mitogenic effects on HSCs elicit marked differences in their execution of a self-renewal division.

2.2. Results

2.2.1. Characterisation of the in vivo repopulating activity of lin⁻Rho⁻ SP cells from adult mouse BM

Figure 2.1A shows a typical FACS profile of the SP cells in suspensions of normal adult mouse BM (Pep3b-Ly5.1) after co-staining with Rho and antibodies against various lin markers. In this experiment, 0.2% of the starting BM population was contained within the SP fraction defined by a verapamil-sensitive ability to efflux Ho. Approximately 50% of the SP BM cells were lin⁻ but only 2 ± 1 % proved to be both lin⁻ and Rho⁻ (ie ~0.004% of the starting BM cells, as shown in the lower left gate in Fig. 2.1B). In a preliminary experiment to assess the content of HSCs in the lin⁻ Rho⁻ subset of SP BM cells, 10 W41 mice were each injected with ~10 cells (10 FACS-sorted lin⁻ Rho⁻ SP events) from congenic Pep3b-Ly5.1 donors. Stable donor-derived repopulation (4-68%), including all major WBC lineages (B-, T-, and G/M cells) was seen in 9 of the recipients for at least 6 months post-transplant. The 10th mouse did not show any evidence of donor-derived (Ly5.1⁺) WBCs throughout this period. When these numbers (one negative mouse out of 10 injected with ~10 test cells each) were used to derive a preliminary estimate of the HSC frequency in the lin⁻ Rho⁻ SP fraction using Poisson statistics and the method of
maximum likelihood, a value of 1 HSC per 4 lin⁻ Rho⁻ SP cells was obtained (67% CI = 1/3 to 1/7).

A more definitive quantification of HSC frequency within the lin⁻ Rho⁻ SP fraction was then obtained directly by an analysis of 105 mice injected with single cells. As summarised in Table 2.1, 42 (40%) of these mice showed detectable levels of repopulation (37/94 W⁴¹ and 5/11 B6 recipients). Forty of the 42 clones produced were multi-lineage (B + T + G/M) and 35 of these were maintained in vivo for at least 4 months. Two additional clones were classified as ‘lineage restricted’ because no T-cells were detected in them at any time point; both of these also had low levels of total repopulation (0.3 and 0.7%). An example of a long-lived multi-lineage clone is shown in Fig. 2.1C. As also shown in Table 2.1, retrospective analysis revealed a significantly reduced (2.4-fold, p=0.005, chi-square test) frequency of repopulation of the sublethally irradiated female W⁴¹ recipients of single male cells in comparison to all other donor-recipient sex combinations (for which no host anti-graft effect would have been anticipated³⁴). In the latter (sex antigen-compatible) recipients, 28/66 (42%) of the lin⁻ Rho⁻ SP cells met the most stringent criteria of longterm multi-lineage repopulation and 34/66 (52%) had some in vivo repopulating activity.

The level of repopulation attained by individual multi-lineage clones at 4 months post-transplant varied widely, i.e., from 0.2% to 75% of the total number of circulating WBCs (Fig. 2.2A, left panel), independent of the genotype (W⁴¹ or B6) of the recipient (data not shown). Interestingly, unlike the frequency of mice that became repopulated, the level of repopulation subsequently achieved was independent of the donor-host sex
Further follow-up on 9 of the in vivo clones for another year revealed 2 general patterns of clone development (Fig. 2.3A). Approximately half (4/9) showed an initial large output of WBCs at 8 weeks that rapidly subsided to a detectable, but much lower output of WBCs over the next year. The other 5 clones were small or not yet detectable at 8 weeks and did not achieve maximum WBC outputs until at least 6 months post-transplant although they also showed a subsequent decline over the latter 8 months of follow-up. Interestingly, the distribution of the different lineages of mature WBCs contained within each of these clones was highly variable regardless of the initial kinetics of clonal expansion, and in some clones, marked fluctuations in these distributions were also seen over time. Three examples are shown in Fig. 2.3B.

Femoral BM cells obtained by aspiration 6 months post-transplant from 3 highly repopulated (>50% Ly5.1+ WBCs) mice that had each received a single lin− Rho− SP cell were transplanted into pairs of secondary recipients. All 6 of these secondary recipients showed sustained (>8 months) multi-lineage repopulation by the progeny of the initial cells injected into the 3 primary mice (Table 2.2). BM cells were aspirated from 4 of the secondary recipients (again at the 6-month post-transplant time point) and injected into 8 tertiary recipients. Six months later, 4 of these tertiary recipients showed high levels of multi-lineage repopulation by cells derived from the same original lin− Rho− SP cell (Fig. 2.1D). Another 2 of these tertiary mice died prior to analysis and the remaining 2 were negative.
2.2.2. Most of the HSCs are in the Rho\(^-\) subset of the lin\(^-\) SP fraction of adult BM

To determine whether the properties of the HSCs present in the lin\(^-\) Rho\(^-\) SP fraction were representative of the majority of the HSCs in adult mouse BM, the remaining lin\(^-\) SP cells were subdivided into a Rho\(^+\) fraction (lower middle gate in Fig. 2.1B) and a Rho\(^-\) fraction (lower right gate in Fig. 2.1B) as defined in the Methods. The frequency and hence total number of HSCs in each of these fractions was then determined by limiting dilution analysis. As shown in Table 2.3, no HSC activity was detected in the Rho\(^+\) subset of lin\(^-\) SP cells and the frequency of HSCs in the Rho\(^+\) fraction was \(-0.3\%\). Thus, although the lin\(^-\) Rho\(^-\) fraction contained only 2\% of the SP cells, it was calculated to contain 88\% of all HSCs present in the SP fraction (assuming all HSCs are lin\(^-\)), with 11\% of the HSCs being found in the lin\(^-\) Rho\(^+\) fraction and <1\% in the lin\(^-\) Rho\(^-\) fraction. Calculation of the average size of the clones produced by the minor population of Rho\(^\pm\) HSCs and their content of B, T and GM cells showed no differences in any of these parameters with those obtained for the more prevalent Rho\(^-\) HSCs: the average clone size at 4 months for the Rho\(^\pm\) HSCs was 24\% of the total WBC value versus 22 ± 4\% for the Rho\(^-\) HSCs and the average % B, T and GM cells within each clone was 33\%, 49\%, and 18\% for the Rho\(^\pm\) HSCs versus 37 ± 3\%, 46 ± 4\%, and 17 ± 3\% for the Rho\(^-\) HSCs.

2.2.3. A minor fraction of lin\(^-\) Rho\(^-\) SP cells are LTC-ICs

We next sought to determine the frequency of lin\(^-\) Rho\(^-\) SP cells that could be detected in vitro as LTC-ICs. In one experiment (A in Table 2.4), single lin\(^-\) Rho\(^-\) SP BM cells were sorted directly into a 96 well plate pre-loaded with irradiated feeder cells. In a second
experiment (B in Table 2.4), single cells of this phenotype were first sorted into a plate
pre-loaded with medium only and then visually confirmed cells were transferred to wells
with feeders. In both experiments, the frequency of LTC-ICs was less than the frequency
ascertained for HSCs using the in vivo assay and the average LTC-IC frequency from
analyses of a total of 123 single lin' Rho' SP cells was 24%.

2.2.4. Lin' Rho' SP cells cultured in SF and TPO or SF and IL-11 (± FL)

demonstrate the same initial division kinetics but their progeny exhibit different in
vivo repopulating activities

We then took advantage of the high purity of HSCs in the lin' Rho' SP fraction to
compare the mitogenic and self-renewal responses of HSCs to different growth factor
cocktails. Single lin' Rho' SP cells were deposited using the FACS into separate wells of
a 96-well plate containing either 10 ng/ml of SF and 100 ng/ml of TPO or 300 ng/ml of
SF, 20 ng/ml of IL-11 ± 1 ng/ml of FL. Each well was first examined 4-16 hours later
using an inverted microscope to identify those containing a single viable (refractile) cell.
These cells were monitored every 4-8 hours thereafter to determine their kinetics of entry
into a first division, and in some cases (with the SF+IL-11+FL and SF+TPO cocktails
only), a second division. The first growth factor cocktail was chosen because the initial
kinetics of activation and self-renewal behavior of HSCs purified by a different method
had already been reported\(^\text{13}\). The other 2 cocktails were chosen because we had
previously found that they stimulate optimal and equivalent HSC expansion in 10-day
cultures of Sca-1\(^+\) c-kit\(^+\) lin' cells\(^\text{35}\). Fig. 2.4 shows the combined results of 6 experiments
in which the proliferation kinetics of a total of 424 single lin⁻ Rho⁻ SP cells cultured with either SF+IL-11+FL or SF+TPO were determined. No cell division (appearance of doublets) was seen in any of the cultures before 24 hours of incubation. However, over the next 24 hours ~90% of the cells cultured in SF+IL-11+FL and ~75% of those cultured in SF+TPO had completed a first division with slight increases in both of these values over the following 12 to 16 hours. Both the first and second divisions were highly synchronous and occurred with similar kinetics in the 2 cocktails used for these studies. Thus ~70% of the cells that divided within 60 hours did so between 29 and 41 hours after initiating the cultures and the second division followed ~14 hours later. In 2 additional experiments, a total of 249 cells were cultured either in SF+IL-11+FL or in SF+IL-11 only. Similar assessment of the rate of doublet formation showed the results in these 2 conditions to be the same as those indicated in Fig. 2.4 (data not shown).

Doublets that had been observed to appear from single cells in the previous 6 hours (candidate G₁ cells) were injected as unseparated pairs of cells into irradiated recipient mice to determine whether at least one daughter cell had retained HSC activity. The timing of this selection was chosen to avoid the anticipated complication of a loss of in vivo engraftment ability that reversibly affects HSCs as they transit S/G2/M.36,37 Intriguingly, 14 (48%) of 29 recipients (11 of 20 W41 and 3 of 7 B6 mice) injected with doublets generated in cultures containing SF+IL-11+FL showed longterm multi-lineage repopulation from these cells and an additional W41 recipient showed longterm lineage-restricted (G/M plus B but no T cells) repopulation (Table 2.1). Moreover, the overall levels of repopulation (i.e. 0.6-55%, Fig. 2.2, center panel) spanned the same range seen
in recipients of the original lin−Rho−SP cells (Fig. 2.2, left panel). In marked contrast, only 4 (11%) of the 36 doublets generated in cultures containing SF+TPO showed HSC activity in injected mice (all W41) and the WBC outputs that were obtained from these were all relatively low (i.e. 0.4-2.1%, Fig. 2.2, right panel). However, almost half (16/36) of the recipients in this second group were female and had been injected with male cells whereas there were no sex-incompatible combinations in the assays of doublets generated in response to SF+IL-11+FL. Therefore, the differential effect of these 2 growth factor cocktails would likely be overestimated unless the comparison was restricted to sex-matched combinations in the SF+TPO group. Nonetheless, when this was done, a significant difference was still seen (14/29 = 48% of mice injected with doublets generated in SF+IL-11+FL vs 3/20 = 15% of mice injected with doublets generated in SF+TPO, p = 0.008, chi-square test).

2.3. Discussion

In this study we demonstrate the ease with which an essentially pure population of HSCs (>40% with longterm multi-lineage in vivo repopulating activity, ~25% with LTC-IC activity) can be reproducibly obtained from normal adult mouse BM in a single sorting step. This is achieved by the selection of cells that actively efflux both Rho and Ho using the powerful double wave-length emission analysis of Ho-effluxing cells31 after stringent removal of all cells expressing B220, Ly1, Mac1, Gr1 and the antigen recognized by Ter119. These are all properties assigned to HSCs more than a decade ago7,32 and used since by many investigators but not previously combined in this particular strategy. A
very similar approach involving the additional use of Sca-1 and pyroninY staining was recently reported\textsuperscript{38} but with a reported 3-fold lower HSC frequency in the recovered population. Goodell et al reported that the majority of HSCs in normal adult B6 BM have an SP phenotype\textsuperscript{31} and here we show that 88\% of these have a Rho\(^{-}\) phenotype as previously shown for HSCs that express Sca-1\textsuperscript{33}. Therefore HSCs isolated from adult mouse BM on the basis of their lin\(^{-}\) Rho\(^{-}\) Sp characteristics would be assumed to be representative of the majority of HSCs and display the same CD34\(^{+}\), Thy-1\(^{0}\), Sca-1\(^{+}\), c-kit\(^{+}\) phenotype used variably by others to also obtain highly enriched HSC populations\textsuperscript{8-11}. This assumption is further supported by the finding that the frequency of lin\(^{-}\) Rho\(^{-}\) SP cells in unfractionated adult mouse BM (0.004\%, of which half appear to be HSCs) corresponds closely to the reported frequency of HSCs (0.001\%) measured functionally using the same transplantation procedure as that employed here\textsuperscript{39}. P-glycoprotein, the product of the \textit{mdr-1a/1b} genes in the mouse, can efflux both Rho and Ho\textsuperscript{40} and is exclusively responsible for the Rho\(^{-}\) phenotype of HSCs as shown by studies of \textit{mdr-1a/1b}\(^{-}\) mice\textsuperscript{41,42}. \textit{Bcrp/Abcg2} is another verapamil-sensitive ABC transporter that can also efflux Ho and appears to be responsible for the SP (Ho\(^{-}\)) phenotype of HSCs\textsuperscript{41,43}. Thus the differential expression of only 7 cell surface molecules appears sufficient to allow a functionally defined homogeneous population of HSCs to be isolated from normal adult mouse BM.

Analysis of other subsets of the lin\(^{-}\) SP population showed that \textasciitilde90\% of HSCs were contained in the Rho\(^{-}\) fraction with the remaining 10\% displaying a reduced ability to efflux Rho. Thus, the expression (or function) of p-glycoprotein appears to be less
stable than that of Bcrp/Abcg2 within the HSC population present in adult mouse BM. Furthermore, we could not discern any differences between the Rho⁻ and Rho⁺ HSCs in terms of the types or numbers of progeny they produced over a period of at least 4 months. These findings are consistent with the possibility that changes in the ability of HSCs to efflux Rho may be reversible and not necessarily indicative of a change in HSC potential. Reversible changes in other phenotypic properties of HSCs including expression of Mac1, CD34 and CD38 have also been reported. Evidence of biologic heterogeneity even within the lin⁻ Rho⁻ SP population of HSCs was seen when the kinetics of their output of mature WBCs was compared in single cell reconstituted mice monitored over a period of greater than 1 year. As noted by others in a related study of mice repopulated with limiting numbers of HSCs, we observed 2 patterns of reconstitution. These patterns were distinguished by a rapid versus a delayed onset of clonal expansion and accompanying differences in the stability of the clone size initially attained. Interestingly, stability of the overall clone size did not extend to specific WBC lineages (B, T and G/M) whose numbers fluctuated in a highly clone-specific fashion. Thus, clonal stability can be a deceiving parameter when documented at the level of total mature WBCs. Further experiments will be required to determine if and how clone size may be related to HSC self-renewal activities post-transplant. Additional evidence of heterogeneity within the lin⁻ Rho⁻ SP fraction of BM cells is provided by the finding that HSCs were twice as numerous as cells detectable as LTC-ICs. Thus, although previous studies have formally demonstrated that some LTC-ICs can generate HSCs, indicating a degree of overlap between cells detectable by both assays, this
overlap is clearly not complete. Unfortunately, since neither of these functionally defined cell types accounted for greater than 50% of the lin' Rho' SP cells, the present data do not clarify whether all lin' Rho' SP LTC-ICs are also HSCs or whether they represent only a partially overlapping population.

An unexpected finding that emerged from a retrospective analysis of sublethally irradiated female W^41 mice transplanted with single male lin' Rho' SP cells was a 2.4-fold decrease in the HSC frequency measured as compared to experiments in which myeloablated and/or sex-antigen-compatible hosts were used. A role of the weak Y-antigen has been described^34 but not previously shown to affect HSC frequency determinations. The effect seen here may be attributable to the combined use of single cell transplants and sublethally irradiated W^41 recipients whose residual immunocompetence is likely to have been greater than in +/- mice given a myeloablative dose of radiation plus a radioprotective graft of 10^5 normal BM cells. It is interesting to note that the distribution of clone sizes generated in the female mice repopulated with single male HSCs was not altered suggesting that the effect on HSC detection was restricted to an early period post-transplant.

The finding that in sex antigen-compatible donor-host combinations, more than 50% of the lin' Rho' SP cells could repopulate transplanted mice and more than 40% produced durable clones is remarkable in terms of the extraordinary HSC detection efficiency these numbers imply. This is of particular interest in view of the value of 10% recently documented as the 24-hour BM homing efficiency of unpurified HSCs measured using a second transplant to detect the fraction of HSCs that can be recovered from the
BM of primary recipients 24 hours after being transplanted. One possible explanation of the 4-fold discrepancy between the 2 values is that many BM-derived HSCs that re-home to the BM within 24 hours of being injected into the bloodstream are transiently compromised in their ability to do so again. Alternatively, some of the original HSCs injected may home initially to other tissue sites and only later return to the BM to proliferate and differentiate. Evidence that longterm repopulating HSCs have a high detection efficiency (at least 40%) in irradiated mice has also been recently reported using different methods of HSC purification and detection, providing additional support for a significant disparity between the proportion of injected HSCs detectable in the BM 24 hours later and the proportion that can produce a detectable clone over the subsequent 4 months. This finding also indicates that freshly isolated populations in which only 10-20% of the cells demonstrate longterm in vivo HSC activity are not likely to be more than 20-40% pure.

The actual biologic purity of phenotypically defined cell isolates has important implications for their use in gene expression analyses to identify candidate gene products responsible for maintaining the stem cell state of HSCs. These analyses will be confounded by the significant contamination of such populations with cells that do not possess HSC activity, particularly since the contamination is likely to consist of downstream, but closely related, cell types. Another comparative approach is now suggested by the observation here that HSC function appears to remain unaltered for periods of up to 16 hours in vitro in the presence of potently mitogenic growth factors. Given the likelihood that many changes in gene expression would be initiated during this
time, comparative analyses of such briefly cultured HSCs may provide a strategy to select for "stem cell" genes whose continued expression would not be perturbed.

Finally, our studies provide evidence of a dissociation between the growth factor regulation of HSC mitogenesis and their self-renewal behavior since differences in the latter could be demonstrated in spite of identical cell division kinetics predicted by and confirmatory of those described by others\textsuperscript{13,55}. Specifically, we found the frequency of first division doublets with HSC activity to be the same as the frequency of HSCs in the original lin- Rho- SP cells when they had been stimulated to divide in the presence of SF+IL-11+FL. Thus every HSC in mouse BM that has a lin\textsuperscript{-} Rho\textsuperscript{-} SP phenotype can be stimulated to execute a self-renewal division in the first cell cycle it completes in vitro when stimulated with this growth factor cocktail. In contrast, the second growth factor cocktail, consisting of a lower concentration of SF and a relatively high concentration of TPO produced first division doublets that showed a pronounced loss of HSC activity in spite of a similar HSC mitogenic response. This loss of HSC activity cannot be simply attributed to a decreased proportion of HSCs stimulated to divide since it was already shown by Ema et al\textsuperscript{13} that only 25\% of the cells that remain quiescent for the first 3 days (<15\% of the initial total) retain HSC activity when cultured under these conditions. Therefore, it can be concluded that a significant proportion (>40\%) of the lin\textsuperscript{-} Rho\textsuperscript{-} SP cells that divide in the first 48 hours in SF+TPO were HSCs prior to culture and that more than half of them lose HSC activity by the time they complete a first division.

Taken together, these findings confirm the ability of growth factor receptor-mediated signals to alter HSC self-renewal decisions\textsuperscript{12,14,56-58} and demonstrate that these
can be differently enacted within a single cell cycle without affecting the rate at which the cells progress through that cycle. Additional exploitation of this experimental model should allow further dissection of the mechanisms involved in regulating the stem cell state of primitive hematopoietic cells.

2.4. Materials and methods

2.4.1. Mice

C57Bl/6J:Pep3b-Ly5.1 (Pep3b) mice were used as BM donors and C57Bl/6J $W^41/W^41$-$Ly5.2$ ($W^41$) or occasionally C57Bl/6J-Ly5.2 (B6) mice as recipients. All mice were bred and maintained in the animal facility at the BC Cancer Research Centre in microisolators and were provided with sterilized food and water. $W^41$ mice were irradiated with 400 cGy and B6 mice with 900 cGy of $^{137}$Cs $\gamma$-rays prior to being injected intravenously with one or more test cells as indicated. All B6 recipients were also injected with a radioprotective transplant of $10^5$ normal B6 BM cells. Both types of recipients were then given water acidified with hydrochloric acid for the following 8 weeks. It has been previously shown that the frequency of repopulating cells detected in sublethally irradiated (400 cGy) $W^41$ mice and radioprotected B6 mice given 900 cGy is the same, as are the repopulation levels and lineage ratios obtained$^{5,14,59}$. Therefore, we pooled the results from these two recipient types. To obtain BM cells for transfer to secondary and tertiary recipients, femoral BM aspirates were performed on anaesthetized mice as described$^{60}$ and 3-10 x $10^6$ cells were then injected per recipient.
2.4.2. Cell preparation and flow cytometry

BM cells were suspended in Hank’s balanced salt solution (HBSS, StemCell Technologies, Vancouver, BC, Canada) containing 2% fetal bovine serum (HF, StemCell Technologies) and, in most cases, the majority of the lin+ cells were then removed immunomagnetically on a column (StemSep, StemCell Technologies) as described by the supplier. Cells were then suspended at 10^6 cells/ml in pre-warmed Iscove's modified Dulbecco’s medium (IMDM) supplemented with 10 mg/ml bovine serum albumin, 10 μg/ml insulin, and 200 μg/ml transferrin (BIT, StemCell Technologies), 100 units/mL penicillin, 100 μg/mL streptomycin (both from StemCell Technologies), 10^{-4} M 2-β-mercaptoethanol (2-ME, Sigma) and 0.1 μg/mL of Rho (Molecular Probes Inc., Eugene, OR). After 30 minutes incubation at 37°C, the cells were washed with HF and resuspended at 10^6 cells/ml in the same medium without Rho. The cells were then immediately incubated with 0.1 μg/ml of Ho (Molecular Probes) for 90 minutes at 37°C, while a parallel aliquot was also incubated with 50 μM verapamil (Sigma Chemicals, St.Louis, MO). Cells were then washed, resuspended at 10^7 cells/ml in ice-cold HF plus 5% rat serum (Sigma) and 3 μg/ml of a Fc receptor blocking antibody (2.4G2) followed by monoclonal antibody staining for 30 minutes on ice. Antibodies used for analysis or sorting were biotinylated anti-Mac1 (M1/70: monocytes), anti-Gr1 (RB6-8C5: granulocytes), anti-B220 (RA3-6B2: B-lymphocytes), anti-Ly1 (53-7.3: T-lymphocytes), and fluorescein isothiocyanate (FITC)-conjugated anti-CD45.1 (anti-Ly5.1, from clone A20, originally obtained from Dr. G. Spangrude, University of Utah and subsequently maintained in the Terry Fox Laboratory) and biotin-conjugated TER-119 and
allophycocyanin (APC)-conjugated anti-CD45 obtained from Becton Dickinson (BD, San Jose, CA). The lineage cocktail consisted of B220, Gr1, Mac1, Ly1, and TER119 antibodies. Cells stained with biotinylated antibodies were washed and incubated for 20 minutes on ice with Streptavidin-phycoerythrin (SA-PE, BD), then washed once with HF and once with HF plus 2 μl/ml propidium iodide (PI, Sigma). Cells were analysed using a FACSsort or FACSCalibur (BD) with CellQuest software (BD) and sorted on a triple laser FACS Vantage equipped with UV, argon, and helium-neon lasers (BD). Within the lin’ SP fraction, Rho- cells were defined as those showing less fluorescence in the FL-1 channel than that exhibited by >99.9 % of unstained cells, Rho+ as those comprising ~60% of the next most Rho+ cells and Rho+ cells as the remaining lin’ SP cells. In 2 experiments, CD45- cells were excluded using anti-CD45-APC staining to increase the clarity of the SP profile (data not shown).

2.4.3. Single cell liquid cultures

Single lin’ Rho- SP cells were sorted using the single cell deposition unit of the sorter into the individual wells of round-bottom 96 well plates preloaded with 200 μl of IMDM containing BIT, antibiotics, 2-ME and 40 μg/ml human low density lipoproteins (LDLs, Sigma). Just prior to transfer to 37°C either 300 ng/ml murine Steel factor (SF, StemCell Technologies) plus 20 ng/ml human interleukin (IL)-11 (Genetics Institute, Cambridge, MA) with or without 1 ng/ml human Flt3-ligand (FL, Immunex, Seattle, WA) as indicated, or 10 ng/ml SF plus 100 ng/ml human thrombopoietin (TPO, Genentech, Inc.,
South San Francisco, CA) were added. After 4-16 hours incubation, each well was checked using an inverted microscope and all those that contained one and only one refractile (viable) cell were identified. These wells were subsequently re-examined at 4-8 hour intervals to determine the kinetics of a first and, in some experiments, also a second division (i.e., by tracking the appearance first of a doublet and then of 3 or 4 cells).

2.4.4. Assays for hematopoietic activity

Long-term culture-initiating cells (LTC-ICs) were measured by assessing the in vitro colony forming cell (CFC) content of 4-week co-cultures of single sorted cells seeded onto previously established, irradiated mouse marrow feeder layers\textsuperscript{62}. To assess single cells (or doublets) for their in vivo repopulating activity, single lin\textsuperscript{−} Rho\textsuperscript{−} SP cells were deposited using the single cell deposition unit of the sorter directly into the individual wells of a 96-well plate and then 2-16 hours later, the presence of a single cell in each well (and later doublets) was verified using an inverted microscope. In initial experiments, cells were harvested after the first 2-4 hours of incubation in wells pre-loaded with IMDM plus BIT plus LDLs to allow those containing viable single cells to be identified. Subsequent results obtained with cells incubated for up to 16 hours with added growth factors showed the same frequency of repopulating cells as detected after 2 hours (data not shown) and hence all data for single cells incubated for 2-16 hours were pooled. After cells to be injected were identified, the entire plate was placed on ice until injection to prevent any cell cycle progression during that interval. For cells assayed in W\textsuperscript{41} recipients, the entire volume of each well was harvested into a 1 ml syringe pre-
loaded with 200 µl of saline and then taken up and down several times into the syringe before being injected intravenously. For cells to be assayed in B6 recipients, a 1 ml syringe was first loaded with 200 µl of medium containing $10^5$ freshly isolated B6 BM cells. These were then combined with the contents of the well prior to being injected. To assess donor-derived repopulation, peripheral blood samples were collected 2 and 4 months post-transplant, and in some cases at later intervals up to 15 months post-transplant. Following ammonium chloride (NH₄Cl) treatment to lyse the red blood cells (RBCs), the white blood cells (WBCs) were stained with FITC-conjugated anti-CD45.1 for the detection of donor-derived (Pep3b) cells, together with biotinylated anti-B220, anti-Ly1, and anti-Gr1 plus anti-Mac1, to detect B-cells, T-cells, and granulocytic/monocytic (G/M) cells, respectively. SA-PE was used as a secondary reagent to detect the biotin-labelled populations. A minimum of 5,000 viable cells were analyzed on a FACSCalibur (BD). Unless otherwise indicated, recipients were considered to be multi-lineage repopulated if their WBCs contained greater than 0.05% CD45.1⁺ events of each lineage (B220, Ly-1, and Gr1/Mac1). The gates that were used excluded >99.99% of the events obtained when the WBCs from unmanipulated W41 or B6 mice were stained with the anti-CD45.1, B220, Ly-1 and Gr1/Mac1, antibodies. The frequencies of HSCs in the limiting dilution experiments were calculated with L-calc™ software (StemCell Technologies) which uses Poisson statistics and the method of maximum likelihood. The frequency of HSCs in the single-cell transplant experiments were calculated directly from the proportion of positive mice. In both cases, only mice
showing long-term (≥4 months) multi-lineage repopulation were defined as positive and all other recipients were classified as negative.
Table 2.1. Frequency and type of repopulation in irradiated recipients of single lin' Rho' SP BM cells or their immediate progeny

<table>
<thead>
<tr>
<th>Cells injected</th>
<th>Sex of donors And recipients</th>
<th>No. of repopulated mice*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Total no. of mice injected</td>
<td>Multi-lineage, longterm</td>
</tr>
<tr>
<td>Single lin'Rho' SP cells</td>
<td>M to M, F to F, F to M</td>
<td>66</td>
</tr>
<tr>
<td></td>
<td>M to F</td>
<td>39</td>
</tr>
<tr>
<td>SF + IL-11 + FL doublets</td>
<td>M to M, F to F, F to M</td>
<td>29</td>
</tr>
<tr>
<td></td>
<td>M to F</td>
<td>0</td>
</tr>
<tr>
<td>SF + TPO doublets</td>
<td>M to M, F to F, F to M</td>
<td>20</td>
</tr>
<tr>
<td></td>
<td>M to F</td>
<td>16</td>
</tr>
</tbody>
</table>

M = male, female = female

*Multi-lineage longterm repopulation = 3 lineages (B220⁺, Gr1/Mac1⁺ and Ly1⁺) of donor-derived WBCs at ≥6 weeks.
Lin-restricted longterm repopulation = <3 lineages of donor-derived WBCs at ≥6 weeks.
Multi-lineage short term repopulation = 3 lineages of donor-derived WBCs at 8 but not 16 weeks.
Table 2.2. Single Lin- Rho- SP cells can generate sufficient daughter HSCs to repopulate secondary and tertiary recipients

<table>
<thead>
<tr>
<th>Recipient</th>
<th>% Ly5.1+ donor repopulation in PB, 6 mo post-transplant</th>
<th>Lineage distribution of Ly5.1+ cells, %</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>B220</td>
</tr>
<tr>
<td>Lin-Rho-SP cell A:</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Primary recipient A</td>
<td>62</td>
<td>43</td>
</tr>
<tr>
<td>Secondary recipient A1</td>
<td>83</td>
<td>20</td>
</tr>
<tr>
<td>Tertiary recipient A1a</td>
<td>21</td>
<td>58</td>
</tr>
<tr>
<td>Tertiary recipient A1b</td>
<td>47</td>
<td>42</td>
</tr>
<tr>
<td>Secondary recipient A2</td>
<td>81</td>
<td>18</td>
</tr>
<tr>
<td>Tertiary recipient A2a</td>
<td>30</td>
<td>34</td>
</tr>
<tr>
<td>Tertiary recipient A2b*</td>
<td>16</td>
<td>43</td>
</tr>
<tr>
<td>Lin-Rho-SP cell B:</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Primary recipient B</td>
<td>54</td>
<td>17</td>
</tr>
<tr>
<td>Secondary recipient B1</td>
<td>3</td>
<td>47</td>
</tr>
<tr>
<td>Tertiary recipient B1a</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Tertiary recipient B1b</td>
<td>died</td>
<td>--</td>
</tr>
<tr>
<td>Secondary recipient B2</td>
<td>10</td>
<td>42</td>
</tr>
<tr>
<td>Tertiary recipient B2a</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Tertiary recipient B2b</td>
<td>died</td>
<td>--</td>
</tr>
<tr>
<td>Lin-Rho-SP cell C:</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Primary recipient C</td>
<td>68</td>
<td>48</td>
</tr>
<tr>
<td>Secondary recipient C1</td>
<td>1</td>
<td>17</td>
</tr>
<tr>
<td>Secondary recipient C2</td>
<td>1</td>
<td>23</td>
</tr>
</tbody>
</table>

Secondary recipients A1 and A2 were transplanted with BM cells from primary recipient A, B1 and B2 from B, C1 and C2 from C. Tertiary recipients A1a and A1b were transplanted with BM cells from secondary recipient A1, A2a and A2b from A2, B1a and B1b from B1, B2a and B2b from B2.

*Mouse died 5.5 months post-transplant, thus data shown for A2b is 5 months post-transplant.
Table 2.3. Frequency of HSCs in the Rho± and Rho+ subsets of lin-SP adult mouse BM cells

<table>
<thead>
<tr>
<th>Lin-SP Subset*</th>
<th>% of SP†</th>
<th>Cells/mouse</th>
<th>Positive mice/total</th>
<th>HSC frequency‡</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rho+/-</td>
<td>29±9</td>
<td>1000</td>
<td>4/4</td>
<td>1/270 (1/200-1/380)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>300</td>
<td>4/6</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>100</td>
<td>2/10</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>30</td>
<td>2/8</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>10</td>
<td>0/8</td>
<td></td>
</tr>
<tr>
<td>Rho+</td>
<td>15±5</td>
<td>300</td>
<td>0/5</td>
<td>&lt;1/2000 (&lt;1/740-&lt;1/5400) §</td>
</tr>
<tr>
<td></td>
<td></td>
<td>100</td>
<td>0/6</td>
<td></td>
</tr>
</tbody>
</table>

* Defined by the gates shown in Figure 2.1B

† Values represent ± standard error of the mean (SEM) from 3 experiments.

‡ HSC frequencies were calculated from the proportion of repopulated mice measured at 6 months post-transplant. Values shown in brackets are the upper and lower limits defined by ± SEM (67% CI).

§ The value shown was calculated assuming one mouse given the maximum number of cells tested had been positive and serves to indicate the sensitivity of the assay in this instance.
Table 2.4. LTC-IC assay of single Lin- Rho- SP cells

<table>
<thead>
<tr>
<th>Experiment</th>
<th>LTC-IC Frequency</th>
<th>Colonies per LTC-IC*</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>20/66 (30%)</td>
<td>44 ± 15</td>
</tr>
<tr>
<td>B</td>
<td>10/57 (18%)</td>
<td>29 ± 20</td>
</tr>
<tr>
<td>A+B</td>
<td>30/123 (24%)</td>
<td>39 ± 12</td>
</tr>
</tbody>
</table>

* Values represent the mean ± SEM from 3 replicates for each of exp'ts A and B.
Figure 2.1. FACS profiles of the lin' Rho subsets of freshly isolated mouse BM SP cells and their first and third generation clonal progeny produced in serially transplanted mice. (A) FACS profile of unseparated adult mouse BM cells after staining with Ho and analysis of the fluorescence at two wavelengths as described. The gate indicates the SP fraction of cells whose high Ho efflux ability is blocked by verapamil (0.2% of total). (B) FACS profile of adult mouse BM SP cells concurrently stained with Rho and lin-PE. The gates indicate Rho' (2%, left gate) Rho(+) (~60%, middle gate), and Rho(+) (~38%, right gate) populations of lin' SP cells defined as described in the Methods. (C) FACS profiles of a highly repopulated recipient of a single lin' Rho' SP cell. (D) FACS profiles of a tertiary recipient of ~10^7 BM cells from a secondary recipient of ~10^7 BM cells aspirated from the mouse shown in (C).
Figure 2.2. Repopulation levels in mice transplanted with single lin⁻ Rho⁻ SP BM cells and doublets derived from them in vitro. This figure shows the proportions of donor-derived WBCs measured 4-6 months post-transplant in multilineage repopulated recipient mice injected with single lin⁻ Rho⁻ SP BM cells (triangles), or doublets originating from single lin⁻ Rho⁻ SP BM cells after being cultured in 300 ng/ml SF, 20 ng/ml IL-11, and 1 ng/ml FL (squares) or 10 ng/ml SF and 100 ng/ml TPO (circles). Open symbols represent the repopulation levels seen in female W⁴¹ recipients of male cells; solid symbols are all other donor-recipient combinations.
Figure 2.3. Long-term repopulation kinetics of single cell-transplanted mice. (A) Total donor-derived (Ly5.1\(^+\)) WBCs detected in 9 mice transplanted with a single lin\(^-\) Rho\(^-\) SP BM cell and followed for 15 months. On the left are shown the clones that exhibited a pattern of initial rapid expansion (peak size at 8 weeks post-transplant) followed by a decline to low but detectable levels throughout the subsequent period of observation. On the right are shown the remaining clones whose size did not reach a peak until \(\geq 24\) weeks post-transplant. (B) Distribution of B220\(^+\) (circles), Gr1/Mac1\(^+\) (squares), and Ly1\(^+\) (triangles) WBCs in 3 randomly selected clones (indicated in panel A).
Figure 2.4. Cell division kinetics of lin' Rho' SP BM cells cultured in 2 different cytokine cocktails. Single lin' Rho' SP BM cells were deposited into the individual wells of 96 well plates (using the cell sorter) in a total of 6 experiments and then the cells were cultured in serum-free medium containing either 300 ng/ml SF plus 20 ng/ml IL-11 and 1 ng/ml FL (circles: 4 experiments, 27 to 154 cells per experiment, 248 cells total), or 10 ng/ml SF and 100 ng/ml TPO (triangles: 4 experiments, 21 to 71 cells per experiment, 176 cells total). At 4-8 hour intervals, each well was examined and cells scored as having completed a first division (solid symbols) when 2 or more cells were first seen, and a second division (open symbols) when 3 or 4 cells were first seen. Each symbol shows the proportion of single viable cells initially identified in a given experiment that had divided (once or twice) by the time point shown. All data from all 6 experiments are shown.
2.5. References


Chapter 3

High Resolution Video Monitoring of Hematopoietic Stem Cells Cultured in Single-Cell Arrays Identifies New Features of Self-renewal†

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†A version of this chapter has been published:

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3.1. Introduction

Time-lapse video imaging offers unique opportunities to determine how specific physical properties of individual living cells change with respect to one another over time and under different conditions. It has been used for more than half a century\textsuperscript{1-4} to study cell morphology during attachment and migration\textsuperscript{5,6}, cell lifetimes\textsuperscript{7,8}, growth\textsuperscript{9}, death\textsuperscript{10,11}, contact inhibition\textsuperscript{12}, clonal heterogeneity\textsuperscript{13}, and mitosis\textsuperscript{14}. Software for extracting and analyzing cell lineage\textsuperscript{15} and morphology\textsuperscript{16} data from videos of cells also has an extensive history. Time-lapse studies of primitive hematopoietic cells have provided information about their cell membrane dynamics when co-cultured with stromal cells\textsuperscript{17,18} or fibronectin\textsuperscript{19}, their kinetics of division\textsuperscript{20}, their morphology and migration\textsuperscript{21}, their localization \textit{in vivo}\textsuperscript{22} and their simultaneous expression of different fluorescent proteins\textsuperscript{23}.

Here we asked whether time-lapse video imaging could be used to identify new behavioral traits of hematopoietic stem cells (HSC) with functionally validated long term multi-lineage repopulating activity \textit{in vivo}. A number of groups have reported methods for obtaining highly purified (>20% pure) populations of HSCs from normal adult mouse bone marrow\textsuperscript{24-29}. One of these methods involves isolating cells lacking surface markers characteristic of mature blood cells; (i.e., lineage marker-negative, or lin\textsuperscript{-} cells) that efficiently efflux the fluorescent dyes, Rhodamine-123 (Rho\textsuperscript{-} cells) and Hoechst 33342\textsuperscript{26}. Efflux of Hoechst 33342 results in the appearance of a side population of cells (SP cells) in 2 dimensional plots of fluorescent events\textsuperscript{30}. In mouse bone marrow (BM), the subset of lin\textsuperscript{-}Rho\textsuperscript{-}SP cells represents \(~0.004\%\) of all the cells. Assessment of the blood cells generated in mice following injection of single lin\textsuperscript{-}Rho\textsuperscript{-}SP cells has shown that 40\% of these cells can produce all blood cell types for many (>4) months\textsuperscript{26}. Interestingly, most of the markers used to isolate HSC-enriched populations from steady-state mouse BM are not directly associated with HSC functional potential, since these phenotypes are
altered when HSC are activated or stimulated to divide\textsuperscript{31-35}. In fact, very few stable properties of HSCs, apart from their defining developmental potential, have been identified. To search for new identifying properties of HSCs that are useful when they are proliferating, we developed a novel microwell array imaging system to visualize clones derived from individual HSCs over a 4-day period under conditions that support HSC self-renewal divisions\textsuperscript{26;36;37}. Each clone was then recovered and assayed for the presence of HSCs with long term multi-lineage \emph{in vivo} repopulating activity. Video images of these assayed clones were then used to correlate visible characteristics of the cultured cells with those that had produced functionally defined daughter HSCs.

3.2. Results

3.2.2. Cell division kinetics of CD45\textsuperscript{mid}lin\textsuperscript{Rho}\textsuperscript{SP} cells determined by high resolution video tracking.

In vivo transplantation assays of 83 freshly isolated CD45\textsuperscript{mid}lin\textsuperscript{Rho}\textsuperscript{SP} cells showed that 31\% of these were functionally detectable HSCs (Figures 3.1A and 3.1C). Additional cells of this phenotype were shipped overnight from Vancouver, BC to Waterloo, ON and then 67 of these were loaded into the individual wells of 3 silicone microwell array chambers containing serum-free medium (SFM) and 300 ng/ml murine Steel factor (SF) plus 20 ng/ml human interleukin-11 (IL-11) and 1 ng/ml human flt3-ligand (FL). The arrays were incubated at 37\(^\circ\)C for 4 days and imaged using a 5x objective at 3-minute intervals throughout this period to allow the morphology and behavior of each cell and its progeny to be recorded and tracked (Figures 3.2B and 3.2C). This sequence of images provided precise information about the timing of every cell division that occurred (n=679) and hence the duration of each intervening cell cycle. From these data we constructed pedigree diagrams for each of the 67 clones generated (Figure 3.2D). The average
times to the first, second and third division determined for all cells that completed these cycles were 39.5±7.6, 18.2±5.2 and 15.8±3.8 hours, respectively (Figure 3.2E). Sister cells (i.e., paired progeny derived from the same parental cell) divided with remarkable synchrony throughout the culture period (Figure 3.2F). Transplantation data were obtained on 61 individually harvested clones and the results of these showed that 17 of the 61 clones (28%) contained HSCs. This finding demonstrated that a high proportion of the input HSCs had executed at least one self-renewal division during imaging, in spite of the overnight transit of the cells before and after the 4-day culture period (Figures 3.1B and 3.1D).

3.2.3. Association of smaller clone sizes and longer cell cycle times with retention of HSC activity.

Retrospective analysis showed that the 4-day clones containing HSCs were significantly smaller than those in which HSCs were not detected (log₂ average size 8.8±1.1 cells, n=17 versus 17.6±1.2 cells, n=44, p<.005, Figure 3.3C). This difference in clone size corresponds to an average difference of one fewer cell generation (3.1 versus 4.1) in the clones in which HSC self-renewal divisions were subsequently shown to have occurred. The average cell cycle time of cells that completed 1, 2, and 3 divisions was also significantly longer for all 3 cycles (p<.005) in the HSC-containing clones as compared to those without detectable HSCs (Figure 3.3A). The best discrimination between these 2 types of clones was obtained by combining all 3 cell cycle times (Figure 3.3B). Note that for these calculations, we excluded clones in which 3 divisions or more did not occur although there were only 7 such clones in all. Interestingly, neither of the 2 cells that remained viable, but did not divide during the 4-day imaging period, displayed repopulating activity when subsequently injected into mice. Also, clones containing HSCs had significantly (P<0.05, one tailed t-test) greater asymmetry between the cell cycle times of the
daughters of the clone founder than clones in which HSCs were not detected (see Supporting Information for details).

### 3.2.4. Association of a late prevalence of cells with uropodia with loss of HSC activity.

We also looked for other features of cell behavior in clones that might be associated with their retention (or loss) of HSC activity including the acquisition and loss of different types of cellular projections. During the first 14-18 hours only 6 of the 67 wells (~9%) contained cells with lagging posterior projections (uropodia) although 45 (~67%) contained cells with other cytoplasmic extensions. At later times, uropodia became more prevalent, particularly in some clones (Figures 3.4A and 3.4B). Filopodia (long, thin projections) were observed with high resolution imaging (20X and 40X objectives) on most cells at the start and end of the period of monitoring, but these were not consistently visible in the lower resolution images collected every 3 minutes (using the 5X objective) and were therefore not included in the present analysis.

When cells were scored for the presence or absence of uropodia during the final 12 hours of the 4-day culture period, the majority of the cells in 25 of the clones (~37%) contained uropodia. A significant association (p<0.05) with the presence or absence of HSC activity was only found in the latter case, where none of the clones with a late predominance of cells with uropodia were found to contain HSCs.

### 3.2.5. Identification of a combination of monitored parameters that are predictive of HSC self-renewal divisions.

We then asked whether combining 2 different parameters of cell behavior in the clones (time to 3rd division and lack of uropodia on the fourth day of culture) would identify HSC-containing clones more efficiently than either of these parameters on its own. To apply the first parameter,
we chose a minimal cell cycle time that included all HSC-containing clones and excluded a maximum number of non-HSC-containing clones. To define such a cutoff in a way that could be applied to other data sets, we set it equal to the mean time to the 3rd-division measured on the entire data set minus 0.5 standard deviations (SD). For the data set shown in Figure 3, this value was 67.23 hours. This value was then used as a gate to subdivide clones into 2 groups; those clones in which the first cell to reach a 3rd mitosis did so in less than 67.23 hours, and those in which the first cell to reach a 3rd mitosis took longer than this threshold period (see Methods and Table 3.2 for additional information). We then further subdivided the clones into 2 groups based on whether or not the majority of the cells within the clone displayed uropodia at any point during the final 12 hours of culture. Selection of clones in which the time to the 3rd division was >67.23 hours and ≤0% of the cells exhibited uropodia in the last 12 hours of culture identified clones that contained HSCs at a 2.26-fold higher frequency than in the original 61 clones analyzed (Figure 3.4D, Table 3.1).

The robustness of these criteria to identify HSC-containing clones was then tested by applying them to similar data acquired from 2 independently executed experiments of the same design. As in the first experiment, maintenance of HSC activity was evident in the clones analyzed after culturing single CD45midlinRhoSP cells for 4 days (Figure 3.2E and 3.2F). Importantly, application of the same criteria identified in the first experiment to the data obtained from the 2 later experiments allowed the HSC-containing clones to again be predicted with a 2 to 3-fold increased efficiency (Table 3.1).

3.3. Discussion

Here we describe a novel time-lapse video monitoring system that allows high-resolution real-time tracking of cells in multiple expanding clones in vitro to be coupled with functional
assays of the individually harvested clones at the end of the monitoring period. These unique features have made it possible to address new questions about the biology of HSCs that previously have not been amenable to investigation. The objective of our study was to identify new parameters that might be associated with HSC self-renewal divisions in vitro. From a survey of numerous cell features (see Supporting Information for details of other features considered), we identified 2 that each showed a significant association with clones containing HSCs after 4 days of culture: a prolonged cell cycle time measured over 3 divisions and a reduced proportion of progeny with uropodia at any time between 84 and 96 hours of culture. In combination, these parameters identified all of the HSC-containing clones in each of the 3 experiments performed and consistently enhanced the identification of HSC-containing clones 2- to 3-fold independent of the starting purities of the HSCs tested (see controls in Table 3.1) or other inter-experimental variations likely to have occurred. This suggests that these biomarkers are indeed robust features of mouse BM HSCs.

These findings extend the results of previous studies that correlated longer cell cycle times of primitive hematopoietic cells of both mouse \(^{38}\) and human \(^{39,40}\) origin with the retention of their primitive cell properties. The experiments described here have taken this line of investigation a step further through the use of a more highly purified HSC starting population, a higher spatial-temporal resolution monitoring system, and functional assessment of the HSC activity retained (or not) by each tracked clone. In this way, a link between HSC cell cycle time and their self-maintenance in culture could, for the first time, be definitively established. Schroeder \(^{41}\) has recently described a complementary computer-aided culture and time-lapse imaging system that he has used to describe the generation of HSC-derived clones on stromal cell feeder layers but without data for HSC activity in the clones produced. We anticipate that
further use of both systems will provide valuable new insights into how primitive hematopoietic cells interact with external cues to regulate their self-renewal and differentiation potential.

Multiple studies have previously associated a variety of cell projections with primitive hematopoietic cells\textsuperscript{42-46}. In particular, Frimberger et al.\textsuperscript{47} observed several types of projections on the leading edge and periphery of cells in HSC-enriched populations using high-speed optical-sectioning microscopy and inverted fluorescent video microscopy. Giebel and colleagues\textsuperscript{48} have described the appearance of uropodia at the rear pole of human CD34\textsuperscript{+} cells. Here, we found that the late presence of uropodia was negatively associated with retained HSC activity. Clearly, attention to the criteria used to define different categories of projections as well as the particular culture conditions used and the time in culture at which they are assessed will be important to future investigations of whether these projections play a role in HSC biology.

Although our approach is potentially applicable to any HSC-containing population, all candidate biomarkers would need to be screened again if a different isolation strategy were used, because the non-HSC component of such populations would likely be different. A strength of the approach used here is that it can be adapted to any source of HSCs or HSC isolation strategy because it makes no assumptions about the biological homogeneity of the cells being monitored. The most useful biomarkers are, however, those that can be directly linked to the defining developmental properties of HSCs. The technology and experimental design described here thus represent an important advance in the definitive identification of such features. In addition, the system we have described has the flexibility of allowing specific cells with tracked histories to be removed by micromanipulation at any time point and then assayed or analyzed. Cells containing reporter genes or labeled surface or internal components will further broaden the scope of cellular events that can be monitored. We thus anticipate increasing application of this powerful technology to many areas of cell biology.
3.4. Materials and Methods

3.4.1. Mice

Bone marrow donors were 8-12 week-old C57Bl/6J-Ly5.1 or -Ly5.2 mice. Transplant recipients were Ly5-congenic C57Bl/6J-W^d/W^d mice sublethally irradiated with 360 cGy X-rays at ~350 cGy per minute. Peripheral blood (PB) was collected at 4, 8, 12, and 16 weeks post transplant and the leukocytes were then stained with antibodies for donor and recipient CD45 allotypes plus lymphoid and myeloid specific markers. Long term repopulation was defined as the detection of donor-derived leukocytes at >1% levels in the PB for at least 16 weeks. Multi-lineage repopulation was defined as the detection of >1% of both donor type lymphoid and myeloid cells at 4, 8, 12, and/or 16 weeks after transplantation. Evidence of both long term and multi-lineage repopulation in the same recipient was used to infer that ≥ HSC had been injected. For further details, see Supporting Information.

3.4.2. Purification, culture and shipment of CD45^mid^lin^-Rho^-SP cells

Cell purification was performed as previously described\textsuperscript{26}, with minor modifications. See Supporting Information for details. For controls, single CD45^mid^lin^-Rho^-SP cells were sorted into the individual wells of a round-bottom 96-well plate containing 100-200 µl of serum-free media (SFM), visually confirmed, and were then injected individually directly into sublethally irradiated C57Bl/6J-W^d/W^d recipients. The, CD45^mid^lin^-Rho^-SP cells to be imaged were sorted and collected into a 1.4 ml Eppendorf tube pre-filled with SFM in Vancouver, BC, and then shipped via overnight courier (18-22 hours) at 4°C to the University of Waterloo in Ontario. On arrival, the cells were then warmed to 25°C and 300 ng/ml murine SF (StemCell Technologies, Vancouver, BC), 20 ng/ml human IL-11 (Genetics Institute, Cambridge, MA), and 1 ng/ml human FL (Immunex, Seattle, WA) added to the medium. Single CD45^mid^lin^-Rho^-SP cells were
then micromanipulated into the individual microwells of an array chamber (Figure 3.2A, prepared as described below) which was then placed at 37ºC in a humidified, 5% CO₂ atmosphere and imaged every 3 minutes using phase contrast optics. The time of cytokine addition was set as zero hours of culture time for all experiments. At the end of the 4 days of culture, the clones in the arrays were harvested individually, placed into separate 0.65 ml microcentrifuge tubes and shipped via overnight courier at 4ºC to Vancouver, where the cells in each tube were then resuspended and injected into individual sublethally-irradiated C57Bl/6J-W^{a1}/W^{a1} recipients.

### 3.4.3. Videotracking system

Cells were cultured in custom-designed microwell chambers. Briefly, these were constructed by applying silicone gel to a glass coverslip to form a film approximately 20 μm thick and a 100-μm wide glass scraper was then used to machine 2 sets of perpendicular rows to form the array wells before the gel set (Figure 3.2A). A glass tube was then affixed around the array to form a reservoir to contain the culture medium. To deposit the cells within the array, the entire reservoir was filled with 1 ml of medium containing approximately 50 cells which were then allowed to settle. Each of the 40 microwells was then loaded with a single cell by repositioning the settled cells using a glass micropipet guided by a 3-axis motorized micromanipulator. The micropipets were made from capillary tubes (Drummond, 3-000-203-G/X) using a vertical pipet puller (Kopf, Model 720) and cut with a single-crystal diamond-tipped glass etcher to give an opening 15-30 μm wide. Images were obtained on a Zeiss Axiovert 200 microscope equipped with phase contrast optics and a Sony XCD-SX900 digital camera. Cells were exposed to light only during imaging. Each cell in each image of the approximately 1850-image time courses was scored for morphological characteristics, location, and parentage using human-assisted custom cell tracking.
software that generated pedigree diagrams with other data superimposed upon them for visualization. Data from these diagrams were then imported into standard analysis programs (Excel, MATLAB, Prism) to test correlations between candidate biomarkers and HSC activity.

3.4.4. Purification of CD45$^{mid}$Lin$^-\text{Rho}^-\text{SP}$ cells

Bone marrow cells were collected by flushing femurs and tibias with Hank’s balanced salt solution containing 2% fetal bovine serum (HF, StemCell Technologies, Inc, Vancouver BC, Canada). Following lysis of the red blood cells with a buffered ammonium chloride solution (StemCell), the majority of the cells expressing CD5, CD11b, CD45R, 7-4, Ly-6G, and glycophorin A were removed immunomagnetically (using the EasySep™ negative selection protocol and the biotinylated Mouse Hematopoietic Progenitor Cell Enrichment Cocktail from StemCell as described by the supplier). Cells were then washed, spun, and re-suspended at 10$^6$ cells/ml in pre-warmed Iscove's modified Dulbecco’s medium (IMDM) supplemented with 10 mg/ml bovine serum albumin, 10 μg/ml insulin, and 200 μg/ml transferrin (BIT, StemCell) and 0.1 μM 2-mercaptoethanol (Sigma) (collectively called serum-free medium, SFM) as well as 0.1 μg/ml of Rho (Molecular Probes Inc., Eugene, OR). After 30 minutes incubation at 37°C, the cells were washed with HF, resuspended at 10$^6$ cells/ml in the same medium minus Rho, and immediately incubated with 5 μg/ml of Hoechst 33342 (Sigma) for 90 minutes at 37°C. Cells were then washed with ice-cold HF and resuspended at 10$^7$ cells/ml in ice-cold HF followed by staining for 30 minutes on ice with additional biotinylated EasySep™ Mouse Hematopoietic Progenitor Cell Enrichment Cocktail (StemCell) and anti-CD45-allophycocyanin (APC, BD Biosciences). After washing the cells once with ice-cold HF, they were incubated on ice with phycoerythrin (PE)-conjugated streptavidin (BD Biosciences) for visualization of residual Lin$^+$ cells. The cells were then washed once with ice-cold HF and resuspended in ice-cold HF plus 1
μl/ml propidium iodide (PI, Sigma). Cells were sorted in Vancouver using a FACSVantage equipped with UV, argon, and helium-neon lasers (BD Biosciences). Using gates set to exclude PI events, cells with a CD45midlinRhoSP phenotype were selected (Figure 3.5).

3.4.5. In vivo repopulation assay

For in vivo assays of freshly isolated single (control) cells, the entire volume of each well containing a visually confirmed single cell was harvested into a 1 ml syringe preloaded with 300 μl of saline and then taken up and down several times into the syringe before being injected intravenously. For in vivo assays of 4-day clones shipped in microcentrifuge tubes, the entire volume was loaded into a 1 ml syringe and injected intravenously. PB samples were collected from the tail vein of injected mice 4, 8, 12, and 16 weeks later. Following lysis of the red blood cells with ammonium chloride (StemCell), the leukocytes were resuspended in HF plus 3 μg/ml of an anti–mouse IgG receptor antibody for 20 minutes to minimize nonspecific staining. Cells were then stained with antibodies for donor and recipient CD45 allotypes (anti-CD45.1-APC and anti-CD45.2–fluorescein isothiocyanate [FITC]) plus anti-Ly6g-PE/anti-Mac1-PE for myeloid cells, or anti-B220-PE for B-cells, or anti-CD5-PE for T-cells (CD45.2–FITC purified and conjugated in the Terry Fox Laboratory, Vancouver BC; CD45.1–APC from eBiosciences, San Diego CA; all others from BD Biosciences, San Jose CA). To calculate levels of donor-derived leukocytes in the PB of the recipient mice, events negative for CD45.1 and CD45.2 or positive for both CD45.1 and CD45.2 were excluded, and the % of cells co-staining with each lineage marker and the donor CD45 allotype was determined (Figure 3.6). Long term repopulation was defined as the persistence of donor leukocytes at >1% for at least 16 weeks post-transplantation. Multi-lineage repopulation was defined as the detection of both donor type lymphoid and myeloid cells at levels >1% of all lymphoid or myeloid cells at any point after transplantation.
Evidence of both long term and multi-lineage repopulation in the same recipient was used to infer that at least one HSC had been injected.

3.4.6. Calculation of the time to a 3rd division exclusion parameter

We chose a cutoff time that in the first experiment included all HSC-containing clones and excluded a maximum number of non-HSC-containing clones. To make this cutoff time applicable to future data sets we defined it as 0.5 SD less than the mean time to 3rd division for the entire data set. Application of this criterion to the clones in the first experiment allowed all HSC-containing clones to be selected (Figure 3.7). For clones in which one or more cells did not complete a 3rd division, estimation of the missing values was performed as follows: In each clone where all 4 possible 3rd divisions occurred, the ratio of the average 3rd cycle time to the average 2nd cycle time was calculated. The average of these ratios was then multiplied by the observed 2nd cycle time to obtain estimated 3rd cycle times for clones where all 4 3rd divisions did not occur. For these clones, estimated times to 3rd division were then calculated by adding the estimated time to 3rd division to the observed times to 1st and 2nd division. In clones that completed a 2nd division but not a 3rd division, either the estimated time to 3rd division or the total culture time, whichever was greater, was assigned to each cell. Note that this estimation procedure cannot be applied to clones containing less than 3 cells. For 2-cell clones, the estimated time to 3rd division was calculated as follows. In each clone where all 4 possible 3rd divisions occurred, the ratio of the average 2nd plus 3rd cycle time to the 1st cycle time was calculated. The average of these ratios was then multiplied by the observed 1st cycle time and added to the observed 1st cycle time to obtain an estimate of the time to 3rd division for the 2-cell clones.
3.4.7. Image tracking methodology and candidate biomarker testing

The location, morphological characteristics, and parentage of each cell in each image of the ~1850-image time course sequences were documented and tracked using custom software (available from the authors on a collaborative basis). This software presents a table for each image in the time course that allows multiple characteristics to be assigned a coded score. Cells were scored as having a projection if one was visible for at least 3 consecutive images (spanning at least 6 minutes) in the video. The software can then derive values such as cell speed and cell-cell distance from the scored characteristics and superimpose the results on pedigree diagrams. These data were used to identify candidate biomarkers (see Table 3.2 for examples of biomarkers considered). Additional biomarkers are still being investigated. Data were imported from the custom software into standard analysis programs (Excel, MATLAB, Prism) to test correlations between candidate biomarkers and clones identified from the in vivo assays as HSC-containing or not. Candidate biomarkers with significantly different scores between HSC- and non-HSC-containing clones were used to develop criteria that would exclude as many non-HSC clones as possible (high selectivity) while retaining most or all HSC-containing clones (high sensitivity). Each criterion had a parameter that could be adjusted to achieve these goals. In the case of the uropod criterion, the maximum % of cells with uropodia that a clone at late times contained was the most discriminating criterion. In the case of the time to a 3rd division criterion, it was the factor by which we multiplied the SD before subtracting it from the mean that proved most useful. A 3D surface plot in which the fold-enrichment was plotted as a function of both criteria was then generated and the peak in this plot used to identify the optimal parameter values for the two criteria (>50% of cells with uropodia, and a factor of 0.5, respectively). Regions of the plot in which not all HSC wells passed were excluded.
Table 3.1 – Application of selection criteria developed from the ‘training set’ of data to results from 2 additional experiments.

<table>
<thead>
<tr>
<th>TRAINING SET</th>
<th>Fresh (control)</th>
<th>Cultured</th>
<th>Non-gated</th>
<th>Gate 1</th>
<th>Gate 2</th>
<th>Gates 1,2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number of HSC containing clones</td>
<td>6/11</td>
<td>17/61</td>
<td>17/36</td>
<td>17/40</td>
<td>17/27</td>
<td></td>
</tr>
<tr>
<td>Percentage</td>
<td>55</td>
<td>28</td>
<td>47</td>
<td>43</td>
<td>63</td>
<td></td>
</tr>
<tr>
<td>Fold Enrichment</td>
<td>1.00</td>
<td>1.69</td>
<td>1.53</td>
<td>2.26</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>TEST SET #1</th>
<th>Fresh (control)</th>
<th>Cultured</th>
<th>Non-gated</th>
<th>Gate 1</th>
<th>Gate 2</th>
<th>Gates 1,2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number of HSC containing clones</td>
<td>3/16</td>
<td>4/24</td>
<td>4/13</td>
<td>4/18</td>
<td>4/12</td>
<td></td>
</tr>
<tr>
<td>Percentage</td>
<td>19</td>
<td>17</td>
<td>31</td>
<td>22</td>
<td>33</td>
<td></td>
</tr>
<tr>
<td>Fold Enrichment</td>
<td>1.00</td>
<td>1.85</td>
<td>1.33</td>
<td>2.00</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>TEST SET #2</th>
<th>Fresh (control)</th>
<th>Cultured</th>
<th>Non-gated</th>
<th>Gate 1</th>
<th>Gate 2</th>
<th>Gates 1,2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number of HSC containing clones</td>
<td>4/18</td>
<td>5/73</td>
<td>5/42</td>
<td>5/35</td>
<td>5/27</td>
<td></td>
</tr>
<tr>
<td>Percentage</td>
<td>24</td>
<td>6.8</td>
<td>12</td>
<td>14</td>
<td>19</td>
<td></td>
</tr>
<tr>
<td>Fold Enrichment</td>
<td>1.00</td>
<td>1.74</td>
<td>2.09</td>
<td>2.72</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* Excluding clones containing one or more cell with a cumulative time to a 3rd division faster than the mean minus 0.5 SD.
† Excluding clones containing >50% of cells with uropodia during the final 12 hours of culture.
### Table 3.2: Candidate biomarkers considered in the search for those with the most power to distinguish HSC-containing from non HSC-containing clones.

<table>
<thead>
<tr>
<th>Criterion (per well unless noted)</th>
<th>Sub-criterion</th>
<th>Score</th>
<th>% that pass gate</th>
<th>% that pass gate</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean length of cell cycle (hours)</td>
<td>2nd</td>
<td>HSC wells 21.1 +/- 1.4</td>
<td>Non-HSC wells 16.9 +/- 0.6</td>
<td>Yes</td>
</tr>
<tr>
<td>Mean cumulative time to division (hours)</td>
<td>1st</td>
<td>44.0 +/- 1.9</td>
<td>37.9 +/- 1.0</td>
<td>Yes</td>
</tr>
<tr>
<td></td>
<td>2nd</td>
<td>65.1 +/- 2.4</td>
<td>53.3 +/- 1.9</td>
<td>Yes</td>
</tr>
<tr>
<td></td>
<td>3rd</td>
<td>82.7 +/- 2.2</td>
<td>70.4 +/- 1.7</td>
<td>Yes</td>
</tr>
<tr>
<td>Clone size (cells)</td>
<td>48 h</td>
<td>1.6 +/- 0.2</td>
<td>2.2 +/- 0.2</td>
<td>Yes</td>
</tr>
<tr>
<td></td>
<td>72 h</td>
<td>4.1 +/- 0.3</td>
<td>7.7 +/- 0.7</td>
<td>Yes</td>
</tr>
<tr>
<td></td>
<td>96 h</td>
<td>9.5 +/- 1.0</td>
<td>24.9 +/- 2.9</td>
<td>Yes</td>
</tr>
<tr>
<td>Founder divided</td>
<td>-</td>
<td>17/17 (100%)</td>
<td>42/44 (95%)</td>
<td>No</td>
</tr>
<tr>
<td>50% or more cells with uropodia</td>
<td>First 12 h</td>
<td>0/17 (0%)</td>
<td>6/44 (14%)</td>
<td>No</td>
</tr>
<tr>
<td></td>
<td>Last 12 h</td>
<td>0/17 (0%)</td>
<td>30/44 (68%)</td>
<td>Yes</td>
</tr>
<tr>
<td>Any pseudopodia on &gt; 50% of cells in first 12 h</td>
<td>-</td>
<td>11/17 (65%)</td>
<td>31/44 (70%)</td>
<td>No</td>
</tr>
<tr>
<td>1 or more deaths</td>
<td>-</td>
<td>1/17 (6%)</td>
<td>1/44 (2%)</td>
<td>No</td>
</tr>
<tr>
<td>Difference between daughters of founder (i.e. asymmetry in first generation)</td>
<td>Cycle time (hours)</td>
<td>2.4 +/- 0.6</td>
<td>1.2 +/- 0.2</td>
<td>Yes</td>
</tr>
<tr>
<td></td>
<td>Mean migration speed (microns/hour)</td>
<td>2.8 +/- 0.9</td>
<td>2.8 +/- 0.8</td>
<td>No</td>
</tr>
<tr>
<td>Mean migration speed of founder in first 12 h (microns/hour)</td>
<td>-</td>
<td>11.0 +/- 2.4</td>
<td>11.6 +/- 1.1</td>
<td>No</td>
</tr>
</tbody>
</table>

1 The gates were designed to pass all HSC wells while excluding as many non-HSC wells as possible, except in the case of the difference between cell cycle times of the daughters of the founder, in which case this was not possible due to a few very symmetric sisters in the HSC well population. In this case the gate was designed to maximize the fold-enrichment.

2 The data suggest a correlation but n may not be sufficiently large.

3 Includes lamellipodia, uropodia, and processes that generally radiated from the cell bodies in directions that were not consistently from the leading nor trailing edges. Resolution at 5x magnification was too low to detect filopodia and so they were not scored, but were visible at higher magnification.

4 Similar results were obtained by taking the ratio of the cell cycle times instead of the difference.

Mean values are ± SEM, n=61.
Figure 3.1 - *In vivo* repopulation characteristics of single CD45\textsuperscript{mid}lin\textsuperscript{Rho}SP cells or their clonal progeny. A. Representative FACS profiles from a mouse repopulated with a single CD45\textsuperscript{mid}lin Rho\textsuperscript{SP} cell. B. Representative FACS profiles from a mouse repopulated with the *in vitro* progeny of a single CD45\textsuperscript{mid}lin Rho\textsuperscript{SP} cell cultured for 4 days in an array chamber. C. Proportion of PB leukocytes produced from a single freshly isolated CD45\textsuperscript{mid}lin Rho\textsuperscript{SP} cell transplanted 16 weeks previously. Filled circles identify mice in which the level of donor-type leukocytes indicated that at least one HSC was present in the clone injected (>1% donor-type leukocytes at 16 weeks and >1% of both lymphoid and myeloid cells present at some point during the period the mice were serially monitored). Open circles represent mice in which some donor leukocytes could be detected at 16 weeks (>0.1%) but these were either <1% of the total and/or had not shown all lineages to have been included in the cells produced. Mice showing no (<0.1%) repopulation by donor-type cells are not shown. Horizontal bars show the geometric mean size of the clones produced *in vivo* from the injected HSCs of HSC-containing clones. D, E, and F. Proportion of donor-type leukocytes seen in the PB of mice injected 16 weeks previously with a 4-day clone derived from a single CD45\textsuperscript{mid}lin Rho\textsuperscript{SP} cell in the first imaging experiment (D) and in the second 2 experiments (E and F).
Figure 3.2 - Description of the high resolution time lapse array system and representative culture results.  

A. A digital image of an array showing 40 silicone microwells each capable of holding up to ~150 cells that can be tracked simultaneously. 

B. Higher power view of a representative well containing one CD45<sup>mid</sup>lin<sup>Rho</sup>SP cell suspended in SFM plus 300 ng/ml SF, 20 ng/ml IL-11 and 1 ng/ml FL. 

C. Close-up of the well shown in B after 4 days at 37°C. 

D. The pedigree diagram of the clone that developed in the well shown in C, illustrating the precision with which sequential cell divisions could be timed. 

E. Cell cycle time histogram of 67 individually cultured CD45<sup>mid</sup>lin<sup>Rho</sup>SP cells. A delayed initial cell cycle was observed followed by synchronously maintained subsequent divisions. Cells that did not complete the corresponding cell cycle were excluded from this histogram. 

F. Comparison of the cell cycle times of individual progeny pairs demonstrating the pronounced synchrony retained between such ‘sister’ cells in spite of the wide range of cycle times observed. Cells whose sisters did not complete the corresponding cell cycle were not included in the plot. 

G. Example of part of a clone in which many cells have large trailing projections (uropodia). Arrows indicate cells with uropodia. 

H. Example of part of a clone in which very few cells have uropodia.
Figure 3.3 - HSC activity is associated with smaller clone sizes and longer cell cycle times.

A. Duration of the 1st, 2nd, and 3rd cell cycles was significantly longer in clones containing HSC than in clones without HSCs. Cells that did not complete a 1st, 2nd, or 3rd cell cycle were excluded from this analysis. B. The cumulative time to a 3rd division of cells in HSC-containing clones was significantly longer than the corresponding value for clones without HSCs. Clones in which there were <3 cell divisions but the cells remained viable until the end of culture were assigned a time to 3rd division equal to the total culture time. Error bars represent SEM, n=67. C. Comparison of 4-day clone size distributions for those that contained HSCs and those that did not. Horizontal bars indicate the geometric mean values which are significantly different (p<.005). On average, clones with HSCs executed one less division over the 4 days than clones that did not contain HSCs.
Figure 3.4 – Use of behavioral parameters defined by cell tracking to predict HSC-containing clones. Circles indicate the mean time to a 3rd division in each clone. Bars indicate the ranges of these times. Arrows indicate that one or more of the cells did not complete a 3rd division by the end of the culture period. Asterisks indicate wells in which the original cell had not yet divided at 96 hours when the cultures were terminated. Filled circles represent clones that contained a detectable HSC, open circles represent clones that did not. Grey symbols represent clones that were excluded by one or both of the 2 criteria applied (i.e., the average time to a 3rd division was <67.23 hours and/or >50% of cells within the clone displayed uropodia during the final 12 hours of culture). Black symbols identify the 34 clones that were not excluded by either criteria (i.e., the fastest time to a 3rd division was >67.23 hours and ≤0% of cells within the clone displayed uropodia during the final 12 hours of culture). The latter allowed the frequency of HSC-containing clones in the remainder to be increased from 28% to 63%, a 2.26-fold increase.
Figure 3.5. Purification of CD45$^{mid}$lin'Rho'SP cells. Stained bone marrow cells were first gated using FSC/SSC (A) and PI (B) to exclude debris, erythrocytes, dead cells, and cell clumps. Gates were then set around the CD45$^{mid}$ (C), SP (D), and lin'Rho' (E) populations. (F) All 5 gates in combination resulted in the isolation of $\sim$0.004% of the original bone marrow cells.
Figure 3.6. Sample calculation of the donor-derived leukocyte level in the PB of a transplanted mouse. As a control, a non-transplanted C57Bl/6J-\(W_{d}^{d}/W_{d}^{d}\)-Ly5.1 recipient stained with both CD45.1 and CD45.2 showed no events in the CD45.2 single positive (donor) gate (A). To calculate the level of donor-derived leukocytes present in the PB of a transplanted mouse, events negative for CD45.1 and CD45.2 or positive for both CD45.1 and CD45.2 were first excluded (B), and the overall level of donor-derived leukocytes was then calculated within the single positive events (C). Mice whose PB contained >1% donor cells at 16 weeks were considered to be repopulated long term. To calculate the donor contribution to the myeloid lineages, the donor contribution of the Ly6g/Mac1 positive compartment was calculated (D). Similarly, to calculate the donor contribution to the lymphoid lineages, the donor contribution of the B cells (E) and T cells (F) was calculated. If >1% of the myeloid and >1% of the lymphoid cells were of donor origin, mice were considered to have shown multi-lineage repopulation by the injected cell(s). Evidence of both long term and multi-lineage repopulation in the same recipient was used to infer ≥ HSC had been injected.
Figure 3.7 – Calculation of time to a 3rd division exclusion parameter. Construction of the time to a 3rd division exclusion gate as the average of the mean time to a 3rd division for all clones minus one half the SD, in this case 67.23 hours. Dotted symbols indicate an estimated time to a 3rd division.
References:


33. Uchida N, Dykstra B, Lyons K et al. ABC transporter activities of murine hematopoietic stem cells vary according to their developmental and activation status. Blood 2004;103:4487-4495.


Chapter 4

Longterm symmetric propagation in vivo of functionally distinct subtypes of hematopoietic stem cells†

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†A version of this chapter has been submitted for publication
4.1. Introduction

The mammalian blood-forming system is a complex dynamic cellular system designed to sustain the required levels of multiple lineages of short-lived mature blood cells throughout life. It is now well established that all mature blood cell types are continuously generated from a relatively small population of undifferentiated self-sustaining pluripotent hematopoietic stem cells (HSCs). In the adult, this process usually spans many cell divisions during which lineage choices are first progressively restricted and then executed in a well co-ordinated sequence (reviewed in 1). The properties of pluripotent hematopoietic cells and the intrinsic and extrinsic molecular mechanisms that regulate their initial specification and subsequent maintenance or differentiation are amongst the most intriguing, but still poorly understood aspects of hematopoiesis. The investigation of these questions has been confounded by the biological heterogeneity now recognized among hematopoietic cells with the same apparent breadth of pluri potency. One aspect of this heterogeneity is manifested as differences in the durability of mature blood cell production displayed both in vivo2-6 and in vitro7. The fact that differences in the types of white blood cells (WBCs) produced and the longevity of their production can be associated with phenotypically distinct and prospectively separable subsets of hematopoietic cells has led to the widely held view that the earliest stages of hematopoiesis can be modelled as a linear branching hierarchy8. The demonstration of parallel changes in HSC gene expression programs has provided further support for this model9-11. These investigations have been greatly facilitated by the introduction of in vivo repopulation assays that use prolonged outputs of mature blood cell types as endpoints to allow input HSCs with putative lifelong self-sustaining ability to be specifically discriminated from cells that display more limited growth or differentiation activity4;12-14.
Adult HSCs with long term reconstituting activity (>1% contribution to the circulating WBCs at 4-6 months post-transplant\(^4\,5\,15\,17\)) have been conceptualized as a relatively homogeneous population distinct from multi-lineage but short term reconstituting cells (STRCs, also sometimes referred to as ST-HSCs, and MPPs\(^18\,19\)) or more restricted myeloid or lymphoid differentiation potential (CMPs and CLPs\(^20\)). Nevertheless, even amongst HSCs with long term reconstituting activity, significant variation in progeny outputs is a well recognized hallmark of their behaviour when examined at a clonal level. Historically, this was first noted at the level of HSCs in experiments that tracked the different numbers and types of cells produced in mice transplanted with retrovirally-marked HSCs\(^21\,25\). Later, when limiting dilution HSC assays were established, the functional heterogeneity of individual HSCs was confirmed\(^26\,31\). More recently, additional evidence of heterogeneous HSC behavior has been obtained from analyses of mice repopulated with purified populations of HSCs\(^32\,34\). Nevertheless, the extent to which this heterogeneity in HSC behavior may reflect a predetermined intrinsic diversity\(^35\), or chance exposure to different environments\(^36\,38\), or stochastic events affecting intrinsic pathways that regulate HSC behavior\(^39\,42\) remains unresolved.

To address these possibilities, we have taken advantage of the recent development of a robust method for isolating HSCs at a high purity (~30%)\(^17\,33\). This made it practical to transplant a large number of mice with single HSCs (or their immediate \textit{in vitro} generated HSC progeny). Their individual clonal WBC outputs were then tracked \textit{in vivo} over a 6-month period and their individual self-renewal activities evaluated in secondary and tertiary recipients (Figure 4.1). Analysis of these data revealed 4 distinct patterns of WBC output in the primary recipients, 2 of which were robustly and faithfully transmitted to secondary and tertiary recipients. However, when the initial cells were first stimulated to divide \textit{in vitro}, the reconstitution pattern
of their progeny rapidly changed, although with remarkable symmetry within clones. These findings suggest the existence of adult HSC subsets that have intrinsically determined differentiation programs that are stably perpetuated through many self-renewal divisions in vivo, but are also subject to rapid alteration in vitro.

4.2. Results

4.2.1. Recipients of a single HSC (or HSC-derived in vitro clone) display one of 4 distinct patterns of WBC reconstitution

The power of single cell transplants is that all progeny subsequently detected at any time can be ascribed to the same original starting cell. Here we used multi-parameter fluorescence-activated cell sorting (FACS) to isolate the CD45\textsuperscript{mid}, lineage marker-negative, Rhodamine-123\textsuperscript{dull}, Hoechst 33342-excluding side population (CD45\textsuperscript{mid}lin Rho SP) fraction of normal adult mouse bone marrow (BM) cells, that we have previously shown to contain ~30% HSCs\textsuperscript{17,33}. We then injected 352 sublethally irradiated Ly5-congenic $W^{41}/W^{41}$ mice with visually confirmed, single cells of this phenotype, or with in vitro clones generated from such cells after 4 days in serum-free medium containing a growth factor cocktail that supports a modest expansion of HSCs\textsuperscript{43}. Sublethally irradiated $W^{41}/W^{41}$ mice were used as recipients because they provide an environment for the detection of individual HSCs that is equivalent to lethally irradiated mice in conventional limiting dilution assays while avoiding the complication of having to transplant additional cells to ensure survival of the recipients\textsuperscript{14,15}. The number and proportion of myeloid (granulocytes/monocytes, GM) and lymphoid (B-cell and T-cell) WBCs of the donor Ly5 genotype present in the blood of each of the 352 mice transplanted was then determined 4, 8, 12,
16 and 24 weeks later (Figure 4.1 and Figure 4.8). Significant longterm reconstitution (i.e., >1% of the total circulating WBCs present 16-24 weeks post-transplant) from the injected cell or clone was obtained in 93 of the 352 mice (26%). Another 26 of the 352 recipients (7%) showed a detectable but unsustained transplant-derived contribution to the circulating WBCs (>0.5% prior to 16 weeks, but <1% at 16-24 weeks). The small fraction of the CD45midlin-Rho~SP cells responsible for this latter activity may be viewed as contaminating STRCs according to current functional definitions. Accordingly, we eliminated these latter mice from all subsequent analyses in order to ensure the exclusion of STRCs as well as later cell types with more restricted differentiation potentials (e.g., CMPs and CLPs) that are not present in the SP fraction. In the remaining 233 primary recipients (66%), donor-derived WBCs were not detected in the blood at any time (<0.5% at all time points).

The specific levels of donor-derived WBCs in each of the 93 mice that showed significant longterm repopulation varied considerably between mice throughout the 6 month period of follow-up (Figure 4.2A), as expected. We then calculated the separate contribution of donor cells at 16 weeks post-transplant to each lineage (GM, B or T). When these 16-week lineage-specific contributions were plotted as normalized ratios for each mouse on a ternary graph (see Figure 4.8 for details), the ratios segregated into 4 distinct clusters (Figure 4.3). We have designated these as α, β, γ and δ. The α and β clusters were defined, respectively, by donor-derived GM:(B + T) ratios of ≥2 and 0.25 to 2. Mice that had donor-derived GM:(B + T) ratios of <0.25 were further subdivided into 2 clusters according to whether there was a continuing ≥1% donor contribution to the myeloid lineage as well as to both lineages of lymphoid cells at 16 weeks (γ-type), or whether they contributed exclusively to the lymphoid lineages at this time (δ-type). Values for transplants of freshly isolated single cells (n=49) and 4-
day clones (n=44) clustered in the same ways (Figure 4.9, panels A and B) and were therefore pooled for subsequent analyses.

Interestingly, the contribution of donor-derived cells to the total number of WBCs 16 weeks post-transplant was not a highly discriminating feature of each cluster (Figure 4.2B), although each cluster did display a distinct kinetic pattern of reconstitution when the average values were plotted as a function of time post-transplant (Figure 4.2C). Specific examples of the 4 reconstitution patterns are shown in Figures 2D and 2E. In the upper panels of these figures, the data are displayed as additive contributions of the donor-derived GM, B and T cell contributions to the total WBC pool as a function of time after the initial cells were injected. In the lower panels of these figures, the results are displayed as separate specific contributions to each of the 3 types of WBCs monitored. The second method of data presentation is useful because it is not confounded by variations in total GM, B or T values that may change during the course of the experiment. Note that when the average lineage-specific contributions were plotted over time, 4 distinct patterns were again evident (Figure 4.2F). Based on these findings, we defined the cells responsible for each WBC reconstitution pattern operationally as α-, β-, γ- and δ- longterm reconstituting cells (LTRCs).

4.2.2. α- and β-LTRCs display extensive self-renewal activity in vivo with long term preservation of the original pattern of WBC reconstitution

Given the different patterns of WBC reconstitution observed in primary recipients of single LTRCs (or their immediate LTRC progeny generated in vitro), it was of interest to determine whether these would be equally or differentially associated with long-term in vivo self-renewal activity. To test this, we transplanted pairs of sublethally irradiated Ly5-congenic
mice with the regenerated BM cells harvested individually from 46 of the 93 clonally repopulated mice 6-7 months after the initial cell(s) had been injected. The pattern of WBC reconstitution in each of the secondary recipients was then monitored and analyzed as in the primary mice. The results for the 16-week time point showed that cells from 21 of the 46 primary clones contained detectable secondary LTRCs in one or both secondary recipients (i.e., >1% of the circulating WBCs were of the same Ly5 genotype as the initially transplanted cell(s)). Strikingly, all 21 of the primary clones containing secondary LTRCs originated from α- or β-LTRCs, and this included almost every α- or β-LTRC tested (10/11 α-LTRCs and 11/12 β-LTRCs) (Figure 4.4A and Figure 4.10A). In contrast, no γ- (0/6) or δ-LTRCs (0/17) demonstrated in vivo self-renewal in these experiments. Interestingly, the LTRC subtype appeared more predictive of secondary reconstituting activity than either the total donor-derived WBC levels in the primary recipients or the extent of the individual donor contribution to the GM, B or T lineages (Figure 4.10B). Paired tertiary transplants were also performed on BM cells harvested from 21 of the successfully repopulated secondary mice and the reconstituted GM, B and T cells were then monitored in the tertiary recipients for up to 24 weeks post-transplant. These assays showed that most of the α- and β-LTRCs had undergone further expansion in the secondary mice (Figure 4.4A).

Interestingly, the lineage-specific WBC contributions seen in the secondary and tertiary recipients at 16 weeks (see examples in Figure 4.4B), as well as the overall reconstitution patterns obtained (see examples in Figure 4.5A), were both highly reminiscent of the 4 patterns identified in the primary mice. Moreover, comparison of each cohort of serial transplants revealed a striking preservation of each particular reconstitution pattern obtained in multiple recipients of cells from the same original LTRC (see examples 1-3 in Figure 4.4B for α- and β-
LTRCs, respectively). The similarity in the reconstitution patterns displayed by the progeny of individual α- or β-LTRCs is even more obvious when the paired recipients of cells from the same primary or secondary donors are compared (Figure 4.5A). Taken together, these findings point to an extensive self-renewal ability of α- and β-LTRCs and very high stability of their WBC reconstituting properties over many divisions (given that in most cases, only a 10% sampling of the progeny of 1 cell repopulated 2 of 2 secondary recipients and 10% of their progeny usually repopulated further pairs of tertiary recipients).

Evidence of a change from one reconstitution pattern to another was, however, noted in approximately half of the secondary recipients of cells from α- and β-LTRC-repopulated primary mice (Figure 4.4A). Specifically, in 5 of the 10 α-LTRC-repopulated primary mice tested, one or both of the secondary recipients showed a more balanced donor-derived GM:B + T ratio at 16 weeks, typical of β-LTRCs (examples 4 and 5 in Figure 4.4B for α-LTRCs). Similarly, in 5 of the 11 β-LTRC-repopulated primary mice tested, one or both of the secondary recipients showed a low donor-derived GM:B + T ratio at 16 weeks, typical of γ- or δ-LTRCs (examples 4 and 5 in Figure 4.4B for β-LTRCs). These findings indicate that some α-LTRCs can also produce β-LTRCs (and/or γ- and δ-LTRCs) and some β-LTRCs can also produce γ- and δ-LTRCs. However, these pattern switches are clearly not common events in vivo since many α- and β-LTRCs sustained the production of the same type of daughter LTRCs for over a year.

It is important to note that 11 of the 16 α-LTRCs identified in the original set of transplants would not have been classified as HSCs by conventional endpoints (i.e., >1% donor contribution to the total WBCs and >1% donor contribution to the GM, B and T lineages at 16 weeks) due to their failure to produce mature B- and/or T-cells until after 16 weeks post-transplant. However, of the 6 whose progeny were analyzed in secondary transplants, 3
produced lymphoid cells in numbers consistent with traditionally defined HSCs (examples 1 and 4 in Figure 4.4B for α-LTRCs). Thus α-LTRCs can be precursors of conventionally defined HSCs even though they might not, themselves, be recognized as HSCs in a primary recipient followed for up to 6 months.

4.2.3. LTRC subtypes are rapidly but symmetrically altered in vitro

We next asked whether HSCs stimulated to proliferate in vitro generate the same or a different distribution of LTRC subtypes. As noted above, the transplanted 4-day in vitro clones produced the same 4 patterns of WBC reconstitution at 16 weeks post-transplant as seen in recipients of freshly isolated cells (Figure 4.9). However, after 4 days in vitro, during which time >95% of the input cells divide at least once\textsuperscript{17,33}, the cells harvested produced a different ratio of WBC reconstitution patterns. The proportion of primary mice displaying γ and δ patterns increased from 17/49 (35% for freshly isolated cells) to 36/44 (82% for the 4-day clones) and the proportion of those with α and β patterns decreased correspondingly from 32/49 (65%) to 8/44 (18%) (Figure 4.6). Since the cloning efficiency in vitro under these conditions is so high\textsuperscript{17,33}, the increased number of γ and δ patterns seen must be attributable either to the generation of clones of γ- and/or δ-LTRCs that arise either from non-LTRCs or to cells that initially were α- or β-LTRCs. After 10 days in culture, no α- or β-LTRCs were detected and all mice repopulated with any LTRCs remaining in the 10-day clones appeared to have been transplanted with γ- and/or δ-LTRCs (Figure 4.6 and Figure 4.9C).

To determine whether the altered reconstitution patterns obtained by the cultured cells reflected the properties of a single persisting LTRC in each clone or whether they might be shared by multiple LTRCs present in the same clone, we transplanted equal portions of 18
different 10-day clones into 2-3 mice each and then again followed the WBC output patterns obtained in each mouse (Figure 4.1). From these analyses, 8 subdivided 10-day \textit{in vitro} clones produced >1% of the circulating WBCs at 16 weeks later in 2 or more recipients. This result demonstrates that multiple LTRCs had been generated in at least 8 of the 18 clones tested (44%). Moreover, recipients repopulated with portions of the same clone displayed remarkably similar kinetics of WBC reconstitution and lineage-specific contributions from the injected cells (see examples in Figure 4.5B). Thus, marked symmetry was also evident in the generation of LTRCs \textit{in vitro}, in spite of a rapid change in the types of LTRCs being produced.

4.3. Discussion

For more than 50 years it has been appreciated that the BM of mice contains cells with an impressive ability to produce all types of mature blood cells for many months. The subsequent development of methods to quantify such cells in limiting dilution transplantation assays and prospectively isolate them as unique populations separate from cells with more restricted proliferative or differentiation potential has led to the widespread adoption of a linear branching model of hematopoiesis\textsuperscript{47}. Recent studies of various gene knockout mice have provided further support for the concept that different stages of hematopoietic cell development in this complex hierarchy are differentially dependent on the functional contributions of specific key genes, and that the intrinsic regulation of short and long term renewal of pluripotentiality may involve mechanistically distinct processes\textsuperscript{48-50}. Historically, data suggesting that the vast majority of HSCs in the adult were quiescent and, once activated, could only be propagated for 2 or 3 serial transplants were used to infer a model in which lifelong blood production had to be sustained by the sequential recruitment and removal of HSCs from an ultimate reservoir of cells with similar
finite self-renewal potentialities\textsuperscript{22,40}. More recent experiments have altered this view with the
demonstration that all adult mouse HSCs are likely to divide approximately once a month\textsuperscript{51,52}
and that, when serially transplanted, can expand their numbers at least 8000-fold\textsuperscript{53}. Similarly,
analyses of the average telomere length in WBCs from humans of different ages suggests that
these WBCs derive from a pool of HSCs that are slowly but continuously entering the cell cycle
throughout adult life\textsuperscript{54}. Nevertheless, key questions about the durability and intrinsic control of
self-renewal potential of individual HSCs \textit{in vivo} remain largely unanswered.

To address these questions, we took advantage of recently developed methods for
obtaining a population of BM that is highly enriched for HSCs (~30% purity) and is also
phenotypically separate from less primitive cell types with more restricted reconstituting and/or
differentiation ability (including ST-HSCs, MPPs, CMPs and CLPs). Measurements of WBC
outputs in the present study showed that ST-HSCs accounted for less than 10% of the total
number of cells/clones assayed. The purity of HSCs in the starting populations tested and their
minimal contamination with less primitive cells thus made it practical to analyze, over long
periods of time (up to 18 months), the progeny of a large number of single initial HSCs. Using
this approach, we showed that the kinetics of WBC reconstitution could be subdivided into 4
distinct patterns. The fact that 2 of the patterns were also consistently associated with robust
self-renewal along with stable perpetuation or symmetric conversion of the reconstituting pattern
in their progeny strongly argues that these 4 reconstituting patterns are intrinsically pre-
determined in HSCs \textit{prior} to transplant. Accordingly, we have assigned them the operational
designations of $\alpha$-, $\beta$, $\gamma$- and $\delta$-LTRCs. Unfortunately, as yet there is no known method for
subdividing the rare CD45\textsuperscript{mid}lin\textsuperscript{Rho}SP BM cells to allow these functionally recognized subsets
to be prospectively isolated. Hence candidate molecular differences remain inaccessible to
definition, although this would clearly be a future interesting avenue of investigation.

The cells defined here as β-LTRCs correspond to what has been traditionally envisaged
as highly competitive HSCs because of their rapid and sustained pluripotent differentiation
activity in primary recipients and extensive self-renewal activity in secondary and tertiary mice.
γ-LTRCs meet the expectation of a class of less competitive HSCs with rapidly apparent and
strong initial pluripotent differentiation activity, but declining outputs of all lineages at 16 weeks
and insufficient self-renewal activity to produce daughter LTRCs detectable in secondary mice
transplanted with cells harvested from primary mice after 6 to 7 months. δ-LTRCs also lack this
degree of self-renewal activity and, although pluripotent and capable of long-term repopulation,
do not produce myeloid cells beyond the first 4 months post-transplant. δ-LTRCs thus appear to
share the functional properties of other cells described as STRCs. α-LTRCs are the most
interesting and novel type of LTRCs identified here because they typically produced detectable
levels of mature WBCs only after many weeks and those eventually detected were largely
myeloid for many months. Nevertheless, α-LTRCs also possessed extensive self-renewal
activity and in vivo could occasionally regenerate LTRCs that were able to produce significant
numbers of lymphoid progeny in secondary recipients.

The presence in adult mouse BM of phenotypically distinct subsets of HSCs that produce
mature WBCs after a long delay has been noted by others, although it was not appreciated
that they might display a unique lineage output program. Evidence of sustained lineage biases in
the repopulation patterns of HSCs has been previously suggested from analyses of the
reconstituting activity of cells present in 4-week stromal cell-containing cultures initiated with
limiting dilutions of unseparated BM cells. Interestingly, in those experiments, a subtype of
self-renewing lymphoid-biased HSCs was also proposed, which was not observed in the current study. This may be explained by the different histories of the cells used in the 2 studies, which we show here to be a critical parameter.

Figure 4.7 summarizes the relationships found between the 4 types of LTRCs we describe. β-, γ- and δ-LTRCs can be readily accommodated within the classical hierarchical scheme but α-LTRCs do not fit readily into this paradigm. Many α-LTRCs generated myeloid cells throughout 3 cycles of hematopoietic reconstitution over a period of 18 months with low or negligible contributions to the lymphoid lineages, thus allowing their distinction and independent maintenance to be convincingly documented. Serial transplants of the in vivo generated progeny of β-LTRCs also suggested stable self-renewal of this program for similarly extended periods, although it should be recognized that the output of multiple LTRC subtypes in secondary and tertiary transplants might be indistinguishable from that of β-LTRCs. Interestingly, occasional production of β-LTRCs (and/or γ- and δ-LTRCs) from α-LTRCs was seen in vivo and may also have occurred in vitro. In vivo production of exclusively γ- and/or δ-LTRCs from β-LTRCs was also sometimes noted. On the other hand, evidence of the generation of α-LTRCs from β-LTRCs was not obtained, although this would not have been detectable if any β-LTRCs (and/or γ- or δ-LTRCs) had also been present since the lymphoid cells they produced would override the pattern characteristic of co-transplanted α-LTRCs. Future experiments utilizing limiting dilution transplants into secondary recipients would circumvent this problem and will thus be of interest to clarify the full spectrum of inter-program conversions that α- and β-LTRCs can undertake.

Perhaps the most striking and unexpected result of the present study is the symmetry of reconstitution behavior sustained by clonally amplified LTRCs both in vivo, where the input program was stably maintained over many self-renewal divisions, and in vitro, where there was a
rapid shift to less competitive repopulation programs. Thus, even when many daughter cells from the same in vivo or in vitro clone were injected and the repopulation patterns were different from those characteristic of the parental cells, the patterns produced by different aliquots of the same clone mimicked one another with extraordinary similarity. Collectively, our findings suggest that the repopulation patterns later displayed by primitive pluripotent hematopoietic cells are intrinsically pre-set, possibly at a much earlier point in their development. This would suggest the possibility that genes involved in setting the pace and ease of activation of particular WBC differentiation lineages may be epigenetically modified in different ways in different HSC subsets. Such a possibility would also fit with current evidence of lineage priming of pluripotent LTRCs and their subsequent differentiation by progressive suppression of lineage options (reviewed in \textsuperscript{59}). However, regardless of the underlying mechanism(s), it now seems clear that self-renewal divisions of HSCs in vivo do not randomly reset the differentiation options of daughter HSCs.

Preliminary repopulation studies suggest that type α-LTRCs may be less prevalent in embryonic day 14.5 fetal liver. Among 10 mice that were each repopulated with single fetal liver HSCs, the numbers repopulated by α-, β-, γ- and δ-LTRCs were 0, 30%, 30% and 40%, respectively (compared to the distribution in adult BM of 27%, 39%, 12% and 22%, see Figure 4.9). Interestingly, several studies suggest that cells with type α characteristics may increase as mice age \textsuperscript{60-63} Such a shift is consistent with our data suggesting a reduced or absent frequency of α-LTRCs in fetal mice and might contribute to the decrease in lymphoid cells characteristic of aging mice (reviewed in \textsuperscript{64}). Understanding the mechanisms that allow α- and β-LTRCs to sustain their characteristic WBC output patterns should give new insights into the pathways
involved in HSC differentiation. The findings reported here may also help to clarify the heterogeneity seen in genetically similar leukemias.

4.4. Materials and Methods

4.4.1. Mice

BM donors were 8-12 week-old C57Bl/6J-Ly5.1 or -Ly5.2 mice. All transplant recipients were Ly5-congenic C57Bl/6J-W41/W41 (W41/W41) mice given a sublethal dose of irradiation (360 cGy X-rays at ~350 cGy per minute).

4.4.2. Purification, culture and transplantation of CD45midlin−Rho−SP cells

Single viable (propidium iodide-negative) CD45^mid^lin^−^Rho^−^SP cells were sorted by FACS as previously described into the individual wells of a round-bottom 96-well plate containing 100-200 μ l of serum-free medium, centrifuged at 700 rpm, and visually confirmed. Some single cells were then injected intravenously into sublethally irradiated W41/W41 recipients, as described. Others were cultured for 4 or 10 days with 300 ng/ml murine Steel factor (StemCell Technologies, Vancouver, BC), 20 ng/ml human IL-11 (Genetics Institute, Cambridge, MA) and 1 ng/ml human Flt3-ligand (Immunex, Seattle, WA). Clones present in 4-day culture plates were then individually harvested and injected intravenously into sublethally irradiated W41/W41 recipients. Clones present in 10-day cultures were subdivided into equal aliquots and then injected intravenously into groups of 2-3 sublethally irradiated W41/W41 recipients. 24 weeks post-transplant, BM was harvested from selected repopulated primary recipients of single
CD45mid lin Rho-SP cells or 4-day clones, and the cell content equivalent of one femur (~10% of the total BM) was injected into each of 2 secondary sublethally irradiated W41/W41 recipients. BM was similarly harvested from selected secondary recipients 24 weeks post-transplant and the equivalent of one femur from each secondary mouse was injected into a pair of tertiary irradiated W41/W41 recipients.

4.4.3. Analysis of in vivo repopulation

Peripheral blood samples were collected from the tail vein of mice 4, 8, 12, 16 and 24 weeks after transplantation. Following lysis of the red blood cells with ammonium chloride (StemCell), the WBCs were stained with antibodies for donor and recipient CD45 allotypes (anti-CD45.1-allophycocyanin [APC] and anti-CD45.2-fluorescein isothiocyanate [FITC]) plus anti-Ly6g-phycoerythrin [PE]/anti-Mac1-PE for myeloid (GM) cells, or anti-B220-PE for B-cells, or anti-CD5-PE for T-cells (CD45.2-FITC purified and conjugated in the Terry Fox Laboratory, Vancouver BC; CD45.1-APC from eBiosciences, San Diego CA; all others from BD Biosciences, San Jose CA). To calculate repopulation levels, events negative for CD45.1 and CD45.2 or positive for both CD45.1 and CD45.2 were excluded, and the contributions of the injected (donor) cells to the populations of circulating GM, B, T, and total WBCs were calculated. Recipients with >1% donor-derived WBCs at 16 and/or 24 weeks post-transplant were considered to be repopulated with LTRCs. Different LTRC subtypes (α, β, γ and δ) were discriminated as described in the Results. Further details are given in Figure 4.8.
Figure 4.1. Schematic Representation of the Overall Experimental Design

158 freshly isolated CD45.1 CD45<sup>mid</sup>lin<sup>-</sup>Rho<sup>-</sup>SP cells and 194 in vitro clones (generated from single CD45<sup>mid</sup>lin<sup>-</sup>Rho<sup>-</sup>SP cells cultured for 4 days in 300 ng/ml Steel factor, 20 ng/ml IL-11 and 1 ng/ml Flt3-ligand) were transplanted into sublethally irradiated W<sup>41</sup>/W<sup>41</sup> recipients. At regular intervals post-transplant, WBC samples were analyzed for the presence of donor cells of B-cell, T-cell and myeloid (GM) lineages. 24 weeks post-transplant, BM was harvested from 46 positive recipients and 1 femur equivalent was injected into each of 2 secondary sublethally irradiated W<sup>41</sup>/W<sup>41</sup> recipients (total of 96, of which 90 survived). Similarly, BM was harvested from 21 selected secondary recipients at 24 weeks post-transplant and injected into pairs of tertiary recipients (total of 42, of which 40 survived). 10-day in vitro clones were also generated from single CD45<sup>mid</sup>lin<sup>-</sup>Rho<sup>-</sup>SP cells, and 18 such clones were injected into groups of 2-4 sublethally irradiated W<sup>41</sup>/W<sup>41</sup> mice each (total of 48 mice).
Figure 4.2. WBC Outputs in Recipients of Single LTRCs or Their Clonal Progeny Generated *In Vitro*

(A) Donor contributions to the total circulating WBCs for each of the 93 reconstituted mice shown individually.
(B) Variations in the donor contributions to the total circulating WBCs at 16 weeks post-transplant of all 4 LTRC subtypes. Each point represents an individual mouse. Horizontal bars indicate mean values.

(C) Distinct patterns of WBC reconstitution by each of the 4 LTRC subtypes. Values are the mean ±SEM of all mice in each LTRC group as defined in the text.

(D) and (E) Examples of individual mice repopulated with freshly isolated LTRCs (D), or LTRCs from 4-day clones generated in vitro (E). Top panels: Colored areas represent donor WBC of GM (red), B-cell (blue) and T-cell (yellow) lineages as a percentage of all WBCs over time post-transplant. Data are stacked such that the sum of each lineage contribution represents the percentage donor contribution to all WBCs. Bottom panels: For each time point, the separate donor contributions to the GM (red), B-cell (blue) and T-cell (yellow) lineages are shown as bars, and the percentage donor contribution to the total WBCs is shown as a grey area.

(F) Distinct patterns of donor contributions to the GM (red), B-cell (blue) and T-cell (black/yellow) lineages over time are shown. Values are the mean ±SEM of data from all mice in each LTRC group.
Figure 4.3. Identification of LTRC Subtypes in Ternary Plots of Their Lineage-specific Contributions at 16 Weeks Post-transplant

(A) Comparison of the ratio of donor clone contributions to the GM (up), B-lineage (lower left) and T-lineage (lower right) WBCs. If a clone were contributing only GM cells, the value would lie at the upper vertex; if only B cells, the value would lie at the left vertex; and if only T-cells, at the right vertex. If a clone were contributing equally towards all 3 lineages, the value would lie in the centre of the triangle. Greater relative contributions to the GM, B or T lineages shift the values to the top, lower left or lower right, respectively. The graph can be divided into 3 sections based on specific myeloid to lymphoid contribution ratios: High GM:(B+T) = ratio > 2:1; low GM:(B+T) = ratio < 1:4; balanced GM:(B+T) = ratio between 1:4 and 2:1.

(B) Subdivision of LTRCs according to the ratio of their contributions to the myeloid and lymphoid lineages at 16 weeks post-transplant. α-LTRCs display a high GM:(B+T) contribution, β-LTRCs a balanced GM:(B+T) contribution, γ-LTRCs a low GM:(B+T) contribution, δ-LTRCs contribute B-cells and/or T-cells but no (<1%) GM cells at 16 weeks.
### Figure 4.4. Clonal Propagation of Repopulation Patterns in Secondary and Tertiary Recipients.

(A) Summary of the repopulation patterns seen in pairs of secondary and tertiary recipients transplanted with the progeny of each of the 4 LTRC subtypes.
(B) Five examples of serial transplants originating from each of the 4 LTRC types are shown. Bars represent the percent donor contribution to the GM (red), B-cell (blue) and T-cell (yellow) lineages at 16 weeks post-transplant in primary, secondary and tertiary recipients (as indicated). Negative recipients (<1% donor WBCs at 16 weeks post-transplant) are indicated with an asterisk.

nd = not done.
† indicates the recipient mouse died before 16 weeks post-transplant.
Figure 4.5. Intra-clonal Comparisons of LTRC Progeny
(A) Results in paired secondary recipients. For each time point, the donor contributions to the GM (red), B-cell (blue) and T-cell (yellow) lineages are shown as bars and the donor contribution to the total WBCs is shown as a grey area.
(B) Results for subdivided 10-day clones. For each time point, the donor contributions to the GM (red), B-cell (blue) and T-cell (yellow) lineages are shown as bars, and the donor contribution to the total WBCs is shown as a grey area.
Figure 4.6. Rapid Alteration of LTRC Distributions In Vitro

(A) The distribution of α-, β-, γ-, or δ-LTRCs identified in freshly isolated CD45midlinRhoSP BM cells.
(B) The distribution of α-, β-, γ-, or δ-LTRCs identified in 4-day clones generated in vitro from freshly isolated CD45midlinRhoSP BM cells.
(C) The distribution of α-, β-, γ-, or δ-LTRCs identified in subdivided 10-day clones generated in vitro from freshly isolated CD45midlinRhoSP BM cells. Portions of 8 clones were injected into a total of 21 recipients.
Figure 4.7. Schematic Diagram of the Relationships Between LTRC Subtypes and the Mature WBC Types They Generate.

β-, γ-, or δ-LTRCs appear to form a hierarchy that corresponds to conventional models of HSC differentiation where the propensity for generating mature myeloid progeny diminishes progressively before pluripotentiality is lost. α-LTRCs represent a novel cell type, in which a very strong propensity for myeloid cell output can be independently and exclusively sustained over multiple cycles of hematopoietic reconstitution, although crossover to the β-, γ-, or δ-LTRC stream is not precluded.
Figure 4.8. Representative Examples of Mice Repopulated with Each LTRC Subtype Illustrating the Gating Strategies and Relevant Calculations.

(A) Viable WBCs are initially identified on the basis of their forward/side light scattering characteristics and lack of staining with PI, followed by the exclusion of events negative for CD45.1 and CD45.2 or positive for both CD45.1 and CD45.2.

% donor of WBC: (3.05+1.41) + (0.54+6.23) + (0.21+6.8) = 7.4%
% donor of GM: 3.05/(3.05+6.23) = 30.6%
% donor of B: 0.54/(0.54+6.23) = 1.24%
% donor of T: 0.21/(0.21+6.8) = 5.6%

Normalized GM:B:T ratio for ternary graph: 36.56 : 1.23 : 0.51 = 0.955 : 0.032 : 0.013

% donor of WBC: 11.1/(11.1+4.52) = 71.1%
% donor of GM: 11.1/(11.1+4.52) = 71.1%
% donor of B: 35.4/(35.4+13.6) = 72.2%
% donor of T: 23.8/(23.8+11.4) = 67.6%

Normalized GM:B:T ratio for ternary graph: 71.1 : 72.2 : 67.6 = 0.337 : 0.342 : 0.321

% donor of WBC: 0.01/(0.01+8.22) + (1.83+6.59) + (6.47+1.8) = 8.31%
% donor of GM: 0.01/(0.01+8.22) + (1.83+6.59) + (6.47+1.8) = 8.31%
% donor of B: 1.83/(1.83+32.4) = 5.3%
% donor of T: 6.47/(6.47+39.4) = 14.4%

Normalized GM:B:T ratio for ternary graph: 0.01 : 5.3 : 14.4 = 0.003 : 0.373 : 0.727

GM : B + T ratio: 0.05/(3.9+14.1) = 0.003
(B) The overall donor repopulation level is then calculated as a proportion of the single positive events.

(C) As a control, a non-transplanted C57Bl/6J-W41/W41-Ly5.1 recipient stained with both anti-CD45.1 and anti-CD45.2 antibodies shows no events in the CD45.2 single positive (donor) gate.

(D) Representative examples of 4 mice injected 16 weeks previously with one of each of the 4 LTRC subtypes are shown plotted on a ternary graph.

(E) Here the corresponding 16-week FACS plots, gating strategy, and relevant calculations are shown for the same 4 examples as in (D) after gating for PI WBCs as described in the first 2 panels.
Figure 4.9. Ternary Plots of the Lineage-Specific Contributions Show the Same LTRC Subtypes in Fresh and Cultured Cells but in Different Proportions

(A) Ternary plot of 16-week lineage-specific contributions for individual mice transplanted with single freshly isolated CD45<sup>mid</sup>Lin<sup>Rho</sup>'SP LTRCs.

(B) Ternary plot of 16-week lineage-specific contributions for individual mice transplanted with entire 4-day LTRC-containing clones generated \textit{in vitro} from single freshly isolated CD45<sup>mid</sup>Lin<sup>Rho</sup>'SP cells.
(C) Ternary plot of 16-week lineage-specific contributions for individual mice transplanted with subdivided 10-day clones that were generated \textit{in vitro} from single freshly isolated CD45^{mid}lin^{-} Rho^{SP} cells and that contained multiple LTRCs.

(D) Ternary plot of 16-week lineage-specific contributions for individual mice transplanted with single freshly isolated lin^{-}Sca-1^{-}CD43^{+}Mac1^{+} LTRCs from E14.5 fetal liver (FL).

(E) Table summarizing the representation of the different LTRC subtypes in the different sources of cells tested. FL = fetal liver.
Figure 4.10. Differential Association of Secondary LTRC Activity with Different Measures of Primary WBC Output

(A) α- and β-LTRCs, but not γ- or δ-LTRCs, demonstrate long-term self-renewal in vivo. Each point represents a primary recipient whose BM cells were transplanted into 2 secondary recipients 6-7 months post-transplant. The black points indicate that one or both secondary recipients were repopulated (>1% donor WBCs at 16 weeks post-transplant). The white points indicate that neither of the 2 secondary recipients was repopulated.

(B) Percent donor contribution to the myeloid lineage predicts for secondary LTRC activity in vivo better than the percent donor contribution to either the total WBCs or the B- or T-cell lineages. Each point represents a primary recipient whose BM cells were transplanted into 2 secondary recipients at 6-7 months post-transplant. The black points indicate that one or both secondary recipients were repopulated (>1% donor WBCs at 16 weeks post-transplant). The white points indicate that neither of the 2 secondary recipients was repopulated.
4.5. References


Chapter 5

Discussion and Future Directions

5.1 Major contributions

In the studies presented in this thesis, we analyzed the functional properties of a large number of individually purified HSCs to obtain new information about the regulation of HSC self-renewal both in vitro and in vivo. This approach allows the clonal outputs of individual HSCs to be unambiguously assessed over time. It thus provides a powerful strategy for dissecting the unique characteristics of individual HSCs within a large and heterogeneous HSC population. In recipients of single HSCs, variable WBC repopulation levels were documented at 16 weeks post-transplant (Ch 2-4). When the contribution to the WBC pool was tracked at multiple time points, distinct reconstitution patterns were discerned (Ch 2 and 4). Differences in cell cycle times (Ch 2 and 3) and clone size in vitro (Ch 3) were also observed. Diverse ratios of differentiated WBC types produced in vivo from single transplanted cells allowed the identification of distinct HSC subtypes (Ch 4), and evidence that these subtypes were intrinsically pre-determined was obtained from the observation that their defining reconstitution patterns were faithfully transmitted through many self-renewal divisions in vivo.

In Chapter 2, I provided definitive evidence that two equally mitogenic growth factor cocktails can have remarkably disparate effects on HSC self-renewal, even within the span of a single cell cycle. Specifically, the frequency of first-division doublets with HSC activity was found to be the same as the frequency of HSCs in the original lin−Rho−
SP cells when they had been stimulated to divide in the presence of 300 ng/ml SF plus 20 ng/ml IL-11 plus 1 ng/ml Flt3L, a combination described by Audet et al.\textsuperscript{1,2} to maximize HSC self-renewal over culture periods of up to 10 days. Thus every HSC in mouse BM that had a lin$^-$/Rho$^-$/ SP phenotype could be stimulated to execute a self-renewal division in the first cell cycle it completed \textit{in vitro} when stimulated with this growth factor cocktail. In contrast, the second growth factor cocktail, consisting of a lower concentration of SF (10 ng/ml) and a relatively high concentration of TPO (100 ng/ml), produced first-division doublets that showed a pronounced loss of HSC activity in spite of a similar HSC mitogenic response. Therefore, it could be concluded that a significant proportion (>40%) of the lin$^-$/Rho$^-$/ SP cells that divide in the first 48 hours in SF + TPO were HSCs prior to culture and that more than half of them lose HSC activity by the time they complete a first division. These findings extend previous results with less purified cells suggesting that growth factor receptor–mediated signals can alter HSC self-renewal decisions (see Chapter 1, section 1.2.4.3 for details) and demonstrate for the first time that these can be differently enacted within a single cell cycle without affecting the rate at which the cells progress through that cycle. These findings further demonstrate that growth factor regulation of HSC mitogenesis and self-renewal are dissociated control mechanisms, inviting interest in the future elucidation of the molecular basis of this dissociation.

In Chapter 3, I describe the use of a novel time-lapse video microscopy system to link data from high-resolution real-time monitoring of expanding HSC-derived clones \textit{in vitro} with the results of \textit{in vivo} reconstituting assays of the same clones evaluated individually. The scale of these experiments made it possible to search for distinct
features of in vitro cell behaviour that are associated with HSC self-renewal divisions. From a survey of numerous candidate features, we identified two that showed a significant association with 4-day HSC-containing clones. One feature was a prolonged cell-cycle time measured over three successive divisions. The second was the presence of a reduced proportion of progeny with uropodia at any time between 84 and 96 hours after initiation of the cultures. In combination, these parameters identified all of the HSC-containing clones in each of the three experiments performed and consistently enhanced the identification of HSC-containing clones 2- to 3-fold independent of the starting purities of the HSCs tested. These findings build on the results of previous studies that correlated longer cell-cycle times of self-renewing pluripotent cells with clonogenic activity in methyl cellulose cultures and extend them to HSCs. Through the use of a highly purified HSC starting population, increased spatial–temporal-resolution of the assessment of cell cycle times, and in vivo assessment of whether or not each tracked clone retained functional HSC activity, a link between HSC cell-cycle time and their self-maintenance in culture was definitively established.

In Chapter 4, I presented the results of longitudinal analyses of WBC outputs performed on a large number of recipients of single purified HSCs (or in vitro clones derived from HSCs). These analyses revealed that the adult HSC compartment can be resolved into 4 subtypes. Two of these stably and autonomously propagate their unique patterns of WBC reconstitution through many self-renewal divisions in vivo. Conversely, when stimulated to proliferate in vitro, HSCs generated within the same clone were shown to rapidly shift to less competitive patterns of WBC output, although remarkable similarity was again seen in the reconstitution patterns displayed by cells within the same
in vitro clone. Thus, even when many daughter cells from the same in vivo or in vitro clone were injected and the repopulation patterns were different from those characteristic of the parental cells, the patterns produced by different aliquots of the same clone mimicked one another with extraordinary similarity. Collectively, these findings suggest that one of a small number of predetermined, but not irrevocable programs of differentiation behavior are imposed on HSCs at an early point in their development and that these are then symmetrically propagated even when they are altered. The most likely mechanism for establishing and maintaining such symmetry during many HSC self-renewal divisions and yet allow rapid alterations in the face of appropriate external cues would seem to be via the epigenetic modification(s) of genes involved in setting the pace and ease of activation of particular WBC differentiation lineages. Regardless of the underlying mechanisms, it now seems clear that self-renewal divisions of particular LTRCs in vivo do not reset the differentiation options of their LTRC progeny.

Together, these results show that individual members of the HSC compartment show previously unappreciated pre-existing heterogeneity, and that external influences on HSC self-renewal and differentiation in vivo remain delimited by these intrinsic properties.
5.2 Implications and Future Directions

5.2.1 Basis of Functional Heterogeneity in HSCs

Over the years there has been a continuing debate regarding the basis of observed heterogeneity in the HSC compartment. While stochastic modelling may provide useful descriptions and hypotheses regarding the potential range of behaviours that HSC may display, the data presented in this thesis indicate that individual HSCs have predetermined self-renewal and subsequent differentiation properties although these can also be modified by exposure to different environmental cues. This concept has been reinforced by observations of inbred mouse strain-specific variation in the self-renewal and aging properties of HSCs\textsuperscript{4-7}. Further studies have identified some of the genetic loci responsible for these differences\textsuperscript{8-11}, and have revealed that these are complex traits that involve multiple genes\textsuperscript{12}. In addition, even in identical genetic backgrounds, intrinsic differences in HSC properties have been described when comparing HSCs from mice of different ages\textsuperscript{13,14} or developmental stages\textsuperscript{15}. Furthermore, using \textit{in vitro} limiting dilution techniques, Muller-Sieburg has described heritable intrinsic heterogeneity between HSCs from mice of the same age\textsuperscript{16}.

The observations described in this thesis have extended these latter findings using single cell transplants to directly examine the functional properties of a large number of individually purified HSCs, including assessments of their self-renewal abilities by secondary and tertiary transplantations. This permitted the identification of four distinct HSC subtypes based on the ratios of differentiated WBC types that they produced \textit{in vivo}. 

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The two subtypes capable of self-renewal in vivo autonomously propagated their unique patterns of WBC production through many self-renewal divisions in vivo. If the observed heterogeneity could be explained by random internal or external events, we would have predicted that a similar degree of heterogeneity would be seen in secondary and tertiary recipients. However, the serial transplant results described in Chapter 4 suggest that this is not the case, and thus provide support for the alternative view; i.e., that this heterogeneity is largely intrinsically determined and not due to microenvironmental influences.

These results also affirm that the control of lineage output and the control of self-renewal are two distinct processes. For example, \( \gamma \)- and \( \delta \)-LTRCs lack extensive self-renewal ability but can produce significant numbers of lymphoid and myeloid lineages. Conversely, \( \alpha \)-LTRCs can self-renew extensively but produce primarily myeloid progeny. Based on our results, we have proposed the model shown in figure 5.1a to explain the relationships between the 4 LTRC subtypes identified. \( \beta \)-, \( \gamma \)-, or \( \delta \)-LTRCs appear to form a hierarchy that corresponds to conventional models of HSC differentiation (see section 1.1.3 for details) where the propensity for generating mature myeloid progeny diminishes progressively before pluripotentiality is lost. \( \alpha \)-LTRCs represent a novel cell type, in which a strong propensity for myeloid cell output can be independently and exclusively sustained over multiple cycles of hematopoietic reconstitution, although direct or sequential transitioning into \( \beta \)-, \( \gamma \)-, and \( \delta \)-LTRCs is not precluded.

An alternate model is shown in figure 5.1b. In this model, the \( \beta \)-LTRC is at the top of the hierarchy. \( \beta \)-LTRC can self-renew to produce additional \( \beta \)-LTRCs. Upon
division, if a progeny LTRC loses multi-lineage capacity, it becomes an \( \alpha \)-LTRC, whereas if it loses extensive self-renewal ability, it becomes a \( \gamma \)-LTRC. In general, these are one-way transitions, but in some cases, partial reversion may be seen. It should be noted that data from Muller-Sieburg et al\(^{16}\) also suggests the existence of a self-renewing LTRC that has lost the majority of its myeloid differentiation potential. However no example of such a cell was observed in the present studies.

While these models describing the relationships between LTRC types are both consistent with the observations described, further experiments are required to verify to what extent these models correct. From the data presented in Chapter 4, it is not possible to determine whether beta-LTRCs can produce alpha-LTRCs and if so, at what frequency. This is because the secondary and tertiary transplants were not performed using limiting LTRC numbers, and thus any alpha-LTRCs produced would be masked if co-transplanted with any other LTRC types due to their production of lymphoid cells. Another unresolved question regards the alpha-LTRCs that gained lymphoid differentiation ability upon serial transplantation. Through the use of serial transplants of limiting numbers of HSCs, it could be determined whether one, some, or all of the regenerated HSCs switched this functional property. By performing an additional round of transplants, it would be possible to determine if this gain of lymphoid differentiation ability was due to the production of beta-, gamma-, or delta-LTRCs. These issues are impossible to resolve with the current data, and would require performing the secondary and tertiary transplants at the single LTRC level. Ideally, this would be accomplished using single re-purified HSCs from the primary recipients. However, the CD45\(^{\text{mid}}\)Lin\(^-\)Rho\(^-\)SP purification strategy is no longer as effective for regenerated HSCs, and so an
alternate purification strategy would be required. The SLAM receptors have recently been identified as promising candidates for the isolation of HSCs from previously transplanted mice\(^\text{17}\). An alternative would be to perform the transplants using limiting numbers of cells such that each recipient would be very likely to have received either one or zero HSCs.

The latter model (Fig. 5.1b) might be explained by invoking both extrinsic and intrinsic control parameters. For example, alternate niches might exist that influence the progeny outcome of the division of \(\beta\)-LTRCs\(^\text{18}\). One niche might promote the production of additional \(\beta\)-LTRCs, another might promote the transition to \(\alpha\)-LTRCs, and yet another might promote the transition to \(\gamma\)-LTRCs. However, to explain the nearly exclusive regeneration of \(\alpha\)-LTRCs over many self-renewal divisions \textit{in vivo}, this model requires that the changes induced by these alternate niches are then fixed and largely irreversible even when cells are transferred to secondary or tertiary hosts.

Changes in the relative sizes of these putative niches might also be invoked to explain why the HSC compartments of fetal liver, young adult BM, and aged adult BM differ with respect to their proportional content of the four LTRC subtypes. For example, during embryogenesis and early development, conditions may encourage the formation of additional \(\beta\)- and \(\gamma\)-LTRCs, but not \(\alpha\)-LTRCs. During adulthood and especially as aging progresses, the conditions that promote the formation of additional \(\alpha\)-LTRCs would thus increase. Alternatively, the formation of \(\alpha\)-LTRCs from \(\beta\)-LTRCs might be a low-frequency stochastic event and these cells simply accumulate with age.

To determine whether the different HSC characteristics of fetal liver, young adult BM, and aged adult BM are due to exclusively intrinsic events or whether the
environment is primarily responsible, it would be appealing to transplant HSCs from
different stages of development into each of these environments and measure the
regeneration of the different LTRC types produced by limiting dilution assays in
secondary recipients. In addition, it would be interesting to alter the expression of genes
shown to be involved in HSC aging, such as $Ezh-2^{19}$ or $p16^{20,21}$, and determine whether
the aging-related functional changes are mitigated. Similarly, if the expression of genes
associated with fetal liver HSCs such as $AML1/Runx1^{22}$ or $scl^{23}$ are manipulated, it would
be interested to determine if the numbers or types of LTRCs are altered.

A close examination of the data in Chapter 4 also reveals "microheterogeneity" within
the 4 LTRC subtypes defined here. When LTRCs were clonally amplified in vivo
or in vitro, the WBC output over time is remarkably similar in "sister" LTRCs. That this
occurs both in vivo and in vitro suggests that there is a degree of intrinsic programming in
each individual LTRC that specifies the behaviour of all of its progeny in response to
external stimuli. This provides further support for the intrinsic nature of HSC
heterogeneity. To investigate the mechanisms involved and possible involvement of
epigenetic changes to specific genes, it would be of particular interest to develop new
methods for isolating enriched populations of different types of LTRCs and compare their
chromatin status.

5.2.2 HSC self-renewal

The HSC compartment is maintained by the process of self-renewal, which refers
to a cell division in which one or both of the daughters of an HSC retains its
differentiation and proliferative potentialities. The decision of HSCs to self-renew or
become more restricted is governed by a complex interplay between intrinsically set properties and stimuli from the surrounding microenvironment (external cues). Several of the known mechanisms regulating self-renewal of HSCs are discussed in detail in section 1.2.4 of this thesis. Experimentally, self-renewal is difficult to define. In fact, it is fundamentally impossible to determine whether a daughter HSC shares the same properties as its parent since once the daughter cell is produced, the parent no longer exists in its original form. In addition, it is difficult to measure every possible key parameter to determine whether two cells are functionally identical. Thus, the criteria used to define functional equivalency are at best crude approximations of molecular events being implicated but not yet clarified. In the case of HSCs, it is generally assumed that long-term differentiated cell output is a useful indicator of self-renewal, based on the assumption that cells without self-renewal ability (i.e., non-HSCs) will exhaust their ability to produce mature progeny prior to the termination of the assay. This is one of the basic premises behind long-term in vitro assays such as CAFCs or LTC-ICs, as well as in vivo long-term repopulation assays. Since HSCs are often defined by their readout in a particular assay, in many situations the most rigorous and relevant definition of self-renewal is to test daughter cells for HSC activity using the same assay as applied to the starting population.

In this thesis, I have explored aspects of HSC self-renewal by analyzing in vitro and in vivo clones initiated with single highly purified HSCs. In Chapter 2, I show that HSC self-renewal in vitro can be altered within a single cell cycle, in response to differential extrinsic factors. In Chapter 3, I have described that in vitro self-renewal (or lack thereof) associates with cell cycle cycle length, clone size and lack of presence of
uropods. In Chapter 4, I show that self-renewal in vivo, as determined by secondary transplantability, does not associate particularly well with total repopulation level, lymphoid cell output, or even the presence or absence of multi-lineage repopulation. Rather, in vivo self-renewal can be best predicted by the absolute contribution to myeloid WBCs produced at 16-24 weeks post-transplant, as well as a moderate to high proportion of myeloid versus lymphoid cell output. However, it should be noted that in the experiments just described, quantification of HSC self-renewal was not performed; rather, a simple “yes or no” answer was obtained in each case. An added layer of information could be obtained by the execution of rigorous limiting dilution transplants to determine the extent of self-renewal in each case. This would contribute a valuable additional parameter with which to examine the functional heterogeneity of HSC.

It is interesting to note that when HSCs are stimulated to divide, the daughter cells produced tend to share similar functional properties with each other, suggesting the existence of intrinsic mechanisms in the original HSCs that delimit the properties of their progeny. Interestingly, in vivo, the progeny HSCs tend to retain the functional characteristics of the parent HSC, even after many self-renewal divisions. In contrast, I have demonstrated that HSC characteristics can be rapidly altered when they are stimulated to divide in vitro, although the daughters tend to be altered in a fashion that is predetermined by the parent HSC. This leads to the interesting question of how and why the in vivo and in vitro environments tested here have such discrepant effects on HSC self-renewal. There are many extrinsic and intrinsic factors that co-operate to allow HSC self-renewal to occur in vivo. Some of the factors involved in this process are discussed in section 1.2.4.1 and 1.2.4.2 of Chapter 1 of this thesis. When HSCs are stimulated in vitro,
However, the conditions are more defined, and there are likely one or more components that are lacking by comparison to the environments in vivo where HSCs self-renew. One component that seems to be of particular importance to HSC self-renewal is Steel factor (SF) as described in section 1.2.4.3 of Chapter 1. Indeed, recent data generated in our lab suggest self-renewal ability can be differentially altered in vivo even prior to a first cell division, in cultures with sufficiently reduced levels of SF but otherwise identical conditions (David Kent, personal communication). This effect was not due to alterations in apoptosis, since apoptosis was not seen in either condition, nor was HSC entry into and rate of progression through the cell cycle altered.

It would also be of interest to modify the culture conditions and determine whether the culture-induced shift of LTRC types described in Chapter 4 can be altered. For example, if conditions previously demonstrated to increase HSC self-renewal were used, might the culture-induced shift be mitigated? Examples of such strategies might include the addition of Tat-HOXB4, activation of the Wnt/Frizzled signalling pathway, the use of stromal feeder layers, or alternative cytokine conditions such as FGF-1. Additionally, transgenic systems could be envisaged with inducible expression of intrinsic regulators known to increase HSC self-renewal, such as Bmi-1 overexpression and/or Pbx knockdown.

It has also become apparent that differences in self-renewal ability exist between HSCs from different stages of development. In particular, HSCs from day 14.5 fetal liver have been shown to exhibit faster HSC regeneration rates in vivo than their adult BM counterparts. It has recently been determined that these different HSC regeneration properties are largely intrinsically determined, and that a transition from fetal-like to

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adult-like occurs rapidly, between 3 and 4 weeks post-birth\textsuperscript{15}. Differences in self-renewal ability between HSCs from young and old mice have also been suggested\textsuperscript{14,35}, but this has not yet been as rigorously addressed.

However, it is also interesting to consider the extent to which different environments may play a role in altering the self-renewal behaviour of HSCs from different developmental stages. In recent studies of fetal liver and young adult HSCs, it has been suggested that several characteristics, including their self-renewal potential, are intrinsically modulated independently of the environment in which the HSCs are expanding\textsuperscript{15}. However, this data is not complete and will require additional experiments to be conclusive. With respect to the differences between young and old HSCs, environment does seem to play a partial role, as determined by experiments where young and old HSCs are transplanted into young and old recipients\textsuperscript{14,36}. An exciting next step would be to transplant fetal liver, young adult BM, and old adult BM HSCs into prenatal, young, and old mice, and thus resolve the extent of intrinsic versus extrinsic determination of behaviours seen in HSCs from different stages of development.

5.2.3 Intrinsic Determination of Lineage Output Patterns by Individual HSCs.

As discussed in Chapter 1, primitive hematopoietic cells express low levels of many genes normally associated with differentiating or differentiated cell types (reviewed in\textsuperscript{37}). It has thus been hypothesized that open chromatin structure is maintained in primitive cells, with progressive epigenetic changes occurring during differentiation\textsuperscript{38}. 

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Changes in transcription factor levels coincide with these epigenetic changes, resulting in the up-regulation and down-regulation of appropriate lineage-specific genes in a step-wise fashion. Along with earlier work by Muller-Sieburg, the data presented in Chapter 4 demonstrate the existence of differences in lineage output in mice repopulated with single HSCs which is transferred stably through many self-renewal divisions. Because these differences are present within (genetically identical) cells from the same individuals and yet can be so stably propagated, an epigenetic mechanism appears likely. HSCs with different regions of active or inactive chromatin could influence the production or differentiation of certain downstream cell types. For example, Muller-Sieburg has shown that lymphoid progenitors produced by a “myeloid-biased” (α-LTRC-like) HSC demonstrate a blunted response to IL-6. Presumably, in the HSC, the self-renewing program and myeloid differentiation program are active, but the lymphoid differentiation program has been dampened.

It would thus be of particular interest to attempt to modify the epigenome of HSCs and determine whether different patterns of reconstitution could be obtained, and if the distribution or symmetry of LTRC types present were altered. One strategy would be to alter the expression in HSCs of one or more of the Polycomb group genes, which have well-known roles in epigenetic regulation (reviewed in). Another approach would be to remodel the chromatin in a more random fashion by treatment with drugs that interfere with histone deacetylation or DNA methylation. Indeed, several examples of such drugs have been suggested to modify HSC properties in culture, including 5-azo2’deoxycytidine, valproic acid, trichostatin A, trapoxin, and chlamydocin.
Interestingly, Sudo et al\textsuperscript{13} and Rossi et al\textsuperscript{14} suggest that self-renewing cells with \(\alpha\)-LTRC characteristics increase with mouse age. A similar phenomenon has also been described by other groups, albeit less rigorously\textsuperscript{36,47,48}. This shift in the HSC compartment with age might partly explain the well-documented decrease in lymphopoiesis with age (reviewed in \textsuperscript{49}). As a result, the \(\alpha\) type is seen as a ‘defective HSC’\textsuperscript{13,40} that has retained self-renewal ability, lost lymphoid ability, and accumulates with age. While I did not examine aged mouse HSCs in this study, I did show using a serial transplantation model that a single \(\alpha\)-LTRC can produce LTRC progeny that have a significant capacity for lymphopoiesis, suggesting that this ‘defect’ is not necessarily a permanent one. Nonetheless, it would be intriguing to test the LTRCs present in old mouse BM and determine whether the same LTRC types are seen but in different proportions, or whether the LTRCs themselves have changed their properties. In particular, it would be interesting to compare the \(\alpha\)-LTRCs from young BM with the similar cells in old BM.

The successful prospective isolation of the LTRC types identified in Chapter 4 would be an important accomplishment to provide additional support for their intrinsic nature, and would enable multiple avenues of further study. A first step towards identifying a phenotype would be to search for markers that subdivide the CD45\textsuperscript{mid}Lin\textsuperscript{-}Rho\textsuperscript{SP} population, followed by functional analysis to determine whether the LTRC types are segregated between the subpopulations. Potential candidates to test include CD34\textsuperscript{50}, CD27\textsuperscript{51}, Flk2/Flt\textsuperscript{32,53}, endoglin/CD105\textsuperscript{54}, SLAM family receptors CD150 and CD244\textsuperscript{55}, EPCR/CD201\textsuperscript{56}, or \(\alpha\)-2 integrin/CD49b\textsuperscript{57}.
If a phenotype could be identified that would allow distinct LTRC types to be separately isolated, this would permit comparative studies of their gene expression profiles to identify genes that are differentially expressed and hence might be considered candidates responsible for the different functional properties of these cells. Indeed, techniques enabling the global transcriptional profiling of single hematopoietic stem cells are now available\textsuperscript{58}. Purified LTRCs of the various types would also enable the analysis of the epigenomic differences between them. In light of the hypothesized role of chromatin remodelling in maintaining their distinctive characteristics, this would be a particularly applicable strategy. Recent advances in chromatin immunoprecipitation technology have allowed this technique to be performed on samples as small as 100 cells\textsuperscript{59}, which might enable the chromatin status of purified LTRCs to be analyzed. In addition, \textit{in vitro} tracking of prospectively isolated cells would allow for further characterization of LTRC properties \textit{in vitro}. Similarly, analysis of dissociated doublets and quadruplets could provide new insights into specific LTRC relationships. A complementary approach would be to attempt to prospectively identify LTRC types by carefully observing their behaviour with the time lapse video microscopy system described in Chapter 3. If certain characteristics could be associated with particular LTRC subtypes, it might allow for a higher throughput screening of candidate markers for prospective isolation. In addition, it might enable more rapid testing for various culture conditions that are better able to maintain LTRCs \textit{in vitro}.

It may also be informative to assay for and compare LTRC types in different mouse strains. If the LTRC proportions are sufficiently different between different mouse
strains, it would demonstrate a distinct genetic component, and further may present an opportunity to single out genes that contribute to the difference between the strains.

5.3. Concluding Comments

For decades, the HSC compartment has been seen as a black box – early experiments clearly revealed that it exists, but what exactly was inside was not fully appreciated. Retroviral marking experiments, along with purification strategies that revealed functionally distinct subsets have provided a glimpse into the variety of cell potentialities within this primitive subset, but the exact details were still not elucidated. The advent of advanced purification strategies has ushered in an era of new possibilities, permitting a range of existing techniques to be modified to directly study single HSCs. Recent examples include global transcription analysis\textsuperscript{58}, lipid raft clustering\textsuperscript{60}, localization \textit{in vivo}\textsuperscript{61}, and the analysis of lineage potential \textit{in vitro}\textsuperscript{62}. To this impressive list, we can now add the examples described in this thesis, including cytokine effects during initial cell cycling \textit{in vitro} (Chapter 2), time-lapse photography of cells in culture (Chapter 3) and the analysis of HSC potentials using single cell transplantations \textit{in vivo} (Chapter 4). Collectively, this work has opened up the black box a little bit, and the mysteries of the HSC compartment have begun to be unravelled.

Certainly, it is now clear that HSCs are not functionally homogeneous. The studies described here have demonstrated that this diversity is primarily intrinsic yet can be influenced by external factors. This is an exciting realization, since it suggests that
different heritable molecular states exist for HSCs that define their functional properties, leading to the possibility of prospective identification of HSCs with different properties and the elucidation of the molecular mechanisms that distinguish them. Undoubtedly, some of the genes that are uniquely regulated in HSCs with different functional properties, and the mechanisms by which they are regulated, would offer insights into the control of the fundamental HSC properties of self-renewal and lineage output. If the pathways affecting these decisions could then be modulated, it would have far-reaching implications for many aspects of hematopoietic stem cell biology. For example, HSCs of particular types could conceivably be amplified ex vivo for transplantation purposes, for treatment specific to a particular blood disorder. Moreover, these pathways might be informative in the development of methods for generating hematopoietic cells from embryonic stem cells, and the subsequent production of differentiated blood cells of specific types. In addition, a greater understanding of these mechanisms could improve our understandings of leukemogenesis, as well as identify possible molecular targets for cancer therapy.
Figure 5.1. Hypothesized relationships between LTRC subtypes

β-, γ-, or δ-LTRCs appear to form a hierarchy that corresponds to conventional models of HSC differentiation where the propensity for generating mature myeloid progeny diminishes progressively before pluripotentiality is lost. α-LTRCs represent a novel cell type, in which a very strong propensity for myeloid cell output can be independently and exclusively sustained over multiple cycles of hematopoietic reconstitution, although crossover to the β-, γ-, or δ-LTRC stream is not precluded. In panel A, α-LTRCs and β-LTRCs are seen as two discrete HSC types with unique lineage output properties, as described in chapter 4. In panel B, an alternate hypothesis is shown. β-LTRCs are seen as the only “true” HSCs, with both multipotentiality and self-renewal ability. Through subsequent intrinsic changes, the β-LTRCs lose either self-renewal ability and become γ-LTRCs, or lose multipotency and become α-LTRCs. In mice repopulated with multiple LTRCs, these changes would not be seen since they would be masked by any remaining β-LTRCs, but their unique characteristics become apparent through single cell transplantations.
5.4. References


