ANALYSIS OF DEVELOPMENTALLY PROGRAMMED CHANGES IN
HEMATOPOIETIC STEM CELLS

by

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ABSTRACT

To characterize the extent and timing of changes in hematopoietic stem cell (HSC) properties during ontogeny, experimental strategies were developed to allow quantitative assessment of their proliferative activity, self-renewal potential and differentiation behaviour in vivo. All HSCs in the fetal liver were found to be cycling and following their transplantation into irradiated adult hosts, they rapidly generated daughter HSCs and produced large numbers of granulopoietic progeny. In contrast, adult HSCs, which are predominantly quiescent, regenerated new HSCs more slowly and produced fewer granulopoietic progeny. They also showed a coordinated change in expression of several transcription factors that regulate HSC functions. Interestingly, HSCs retained a fetal phenotype with respect to all these features until 3 weeks after birth and then, within one week, acquired an adult HSC phenotype. Additional studies of serially transplanted HSCs indicated that this switch also took place within the same time frame in adult mice reconstituted with fetal or 3-week post-natal HSCs, suggesting the switch is intrinsically programmed.

To further investigate the mechanism of this switch, an in vitro model suitable for monitoring the survival, proliferation and self-renewal activity of highly purified fetal liver HSCs was developed. Using this model, I found that the cell cycle transit time of optimally stimulated fetal HSCs and adult HSCs is the same, but with lower Steel factor requirements for fetal HSCs. This suggested that the fetal-to-adult switch involves a decreased response to c-Kit activation. Interestingly, the self-renewal behaviour of fetal HSCs expressing a defective form of c-Kit mimicked adult +/+ HSCs, both in vitro and in vivo, but showed no difference in cycling activity, suggesting that Steel factor responsiveness specifically regulates HSC self-renewal responsiveness in vivo. Future studies of changes in gene expression during
the switch, including analyses of c-Kit-defective HSCs as well as normal HSCs, may help to
link the observed changes in Steel factor responsiveness to the molecular mechanisms that
control changes in HSC self-renewal and cycling control during ontogeny.
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<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
</tr>
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<tbody>
<tr>
<td>3H-Tdr</td>
<td>tritiated-thymidine</td>
</tr>
<tr>
<td>5-FU</td>
<td>5-fluorouracil</td>
</tr>
<tr>
<td>α4int</td>
<td>α4-integrin</td>
</tr>
<tr>
<td>AGM</td>
<td>aorta-gonado-mesonephros</td>
</tr>
<tr>
<td>AML</td>
<td>acute myeloid leukemia</td>
</tr>
<tr>
<td>Ang</td>
<td>Angiopoietin</td>
</tr>
<tr>
<td>APC</td>
<td>allophycocyanin</td>
</tr>
<tr>
<td>ATF</td>
<td>activating transcription factor</td>
</tr>
<tr>
<td>ATM</td>
<td>ataxia-telangiectasia mutated</td>
</tr>
<tr>
<td>BIT</td>
<td>bovine serum albumin, insulin and transferrin</td>
</tr>
<tr>
<td>BFU-E</td>
<td>burst forming unit-erythroid</td>
</tr>
<tr>
<td>BM</td>
<td>bone marrow</td>
</tr>
<tr>
<td>Bmi</td>
<td>B cell-specific Moloney murine leukemia virus integration site</td>
</tr>
<tr>
<td>CAFC</td>
<td>cobblestone area forming cell</td>
</tr>
<tr>
<td>Cbf</td>
<td>core-binding factor</td>
</tr>
<tr>
<td>CDK</td>
<td>cyclin-dependent kinase</td>
</tr>
<tr>
<td>CLP</td>
<td>common lymphoid progenitor</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>Cgy</td>
<td>centigray</td>
</tr>
<tr>
<td>CMP</td>
<td>common myeloid progenitor</td>
</tr>
<tr>
<td>CRU</td>
<td>competitive repopulating unit</td>
</tr>
<tr>
<td>CSF</td>
<td>colony stimulating factor</td>
</tr>
<tr>
<td>DNA</td>
<td>deoxyribonucleic acid</td>
</tr>
<tr>
<td>E</td>
<td>embryonic day</td>
</tr>
<tr>
<td>EB</td>
<td>embryoid bodies</td>
</tr>
<tr>
<td>ES</td>
<td>embryonic stem</td>
</tr>
<tr>
<td>FACS</td>
<td>fluorescence activated cell sorter</td>
</tr>
<tr>
<td>FITC</td>
<td>fluorescein isothiocyanate</td>
</tr>
<tr>
<td>FL</td>
<td>fetal liver</td>
</tr>
<tr>
<td>G</td>
<td>gap</td>
</tr>
<tr>
<td>Gapdh</td>
<td>glyceraldehyde-3-phosphate dehydrogenase</td>
</tr>
<tr>
<td>GEMM</td>
<td>granulocytes, erythroblasts, megakaryocytes and macrophages</td>
</tr>
<tr>
<td>Gfi</td>
<td>growth factor independent</td>
</tr>
<tr>
<td>GMP</td>
<td>granulocytes and monocytes progenitors</td>
</tr>
<tr>
<td>HA</td>
<td>hyaluronic acid</td>
</tr>
<tr>
<td>HSC</td>
<td>hematopoietic stem cell</td>
</tr>
<tr>
<td>HF/2</td>
<td>Hanks balanced salt solution containing 2% FCS</td>
</tr>
</tbody>
</table>
HSC – hematopoietic stem cell
Hst - Hoechst 33342
IGF-2 – insulin-like growth factor 2
IL - interleukin
LIF – leukemia inhibitory factor
LTC-IC – long-term culture-initiating cell
M – mitosis
MEM – minimum essential medium
MPD – myeloproliferative disorders
mRNA – messenger ribonucleic acid
NOD/SCID – nonobese diabetic/severe combined immunodeficient
NK – natural killer
OPN – osteopontin
PBS – phosphate buffered saline
PE – phycoerythrin
p.c. - post coitus
PcG – polycomb group
PI – propidium iodide
PI3K – phosphatidylinositol 3’-kinase
Pias – protein inhibitor of activated Stat3
PLC – phospholipase C
Prc – polycomb repressive complex
P-Sp – paraaortic splanchnopleure
Py - pyronin Y
Rho – Rhodamine 123
RNA – ribonucleic acid
RTK – receptor tyrosine kinase
Runx – runt-related transcription factor
S – synthesis
SCF – stem cell factor
SDF-1 – stromal cell-derived factor-1
SF - Steel factor
SFM – serum-free medium
SP – side population
STAT – signal transducer and activator of transcription
TF – transcription factor
TPO – thrombopoietin
WBC – white blood cell
WGA – wheat germ agglutinin
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CHAPTER 1
INTRODUCTION

1.1 General overview of hematopoiesis

Hematopoiesis refers to the process of blood cell production, which occurs throughout life and in the mouse involves the regulated output every day of millions of cells of many different types. These include neutrophils, eosinophils, basophils/mast cells, monocytes/macrophages, erythrocytes, megakaryocytes, platelets, B-lineage cells, T-lineage cells and natural killer (NK) cells for long periods of time (see Figure 1.1). The production of these cells involves the execution of a complex series of molecular changes within more primitive hematopoietic precursors. In addition, these changes have to be co-ordinated with mechanisms that regulate cell proliferation and survival to ensure that the final numbers of new blood cells produced meet physiological demands both during and after normal growth and in response to injury. Understanding the process of hematopoiesis thus requires a description of the complex cellular and molecular mechanisms that allow hematopoietic cells to differentiate into different blood cell types, the regulation of the changes that occur, as well as the elucidation of how these mechanisms interface with signals from the environment.

1.1.1 Definition and identification of HSCs

Blood cell production is sustained throughout life by the proliferative activity of a small population of pluripotent cells with long-term self-renewal potential, referred to as hematopoietic stem cells (HSCs). Initial evidence for the existence in adult hematopoietic tissue of cells with these two properties; namely, pluripotency and self-renewal ability (i.e. the ability to divide without restriction or activation of this pluripotentiality) was provided by
morphological studies of the cells present in the bone marrow of patients with myeloproliferative disorders (MPD). These revealed that, although only a single type of mature blood cell was elevated in the circulation, precursors of the other lineages were also often found at high levels in the bone marrow which led to the suggestion that these disorders all originate in a common pluripotent precursor (1). The first direct support for this concept came from experiments that showed individual transplant-derived spleen colonies containing multiple lineages of myeloid cells carried the same unique cytogenetic marker, inferring the derivation of each from a single pluripotent cell, the so-called colony-forming unit-spleen (CFU-S) (2). Shortly thereafter, clonal marking of longer term transplants in mice provided definitive evidence of a single adult bone marrow cell with lympho-myeloid differentiation potential that could be serially transplanted (3-6).

With the discovery in the 1980s that most CFU-S have, at best, limited self-renewal activity (7-9) and that most CFU-S can be separated prospectively from cells with long-term repopulating activity (7), quantitative transplant-based assays were devised with greater specificity for murine HSCs defined by their long-term lympho-myeloid repopulating activity (10). These assays now typically make use of congenic donor-recipient pairs of mice, that express distinct CD45 epitopes referred to as Ly5.1 and Ly5.2 to enable definitive identification of donor-derived cells among the lymphoid and myeloid cells regenerated in individual irradiated recipients. To allow the frequency of HSCs to be determined in a given “test” cell suspension, the test cells are injected in varying numbers until a limiting dose is achieved – i.e., a transplant dose that causes only a fraction of the mice injected to show continued test-cell derived contributions to their lymphoid and myeloid blood cells long term, i.e., for at least 4 months (10). To enable the “negative” mice to survive, all recipients must be independently protected by a minimal dose of HSCs. These can either be co-transplanted
from a donor of the same genotype as the recipient or can be provided endogenously; for example, using sublethally irradiated recipients with genetically compromised HSCs (11).

HSCs detected using this methodology are called competitive repopulating units (CRUs) (Figure 1.2) and their frequencies are derived by limiting dilution analysis of the proportion of negative recipients obtained in a given set of experiments (Figure 1.3) (10). CRUs thus defined constitute a very small percentage (~.01%) of the cells in the bone marrow of normal adult mice (12;13).

The validity of this approach is based on the assumption that the donor-derived lymphoid and myeloid progeny originated from a single common pluripotent donor cell that was likely to have undergone extensive self-renewal, as indicated by early retroviral marking experiments demonstrating the generation of clonal populations of multilineage cells containing the same common inserts (3-5). Additional evidence that CRU constitute a biologically discrete population have come from more recent experiments showing that CRUs can be purified to near homogeneity using several phenotype-based cell strategies. These have allowed both their pluripotent and self-renewal properties to be demonstrated by tracking the progeny produced in recipients of single cell transplants (14-19). It has also been possible to use cell separation approaches to demonstrate that CRU can be prospectively separated from transplantable pluripotent cells with less durable repopulating ability (17;20-22).

1.1.2. The hierarchical organization of hematopoiesis

The process by which HSC eventually give rise to mature blood cells proceeds as an extensive series of progressive changes spanning multiple cell generations. This is the basis of the creation of a developmental hierarchy in which different intermediate cell types can now be distinguished by a variety of robust, quantitative assays that detect differences in the
proliferation and differentiation capabilities of these cells in vitro or in vivo. Two important features of these assays is their ability to measure the number (rather than just the activity) of the input cell type being evaluated and the fact that there is a range of cell doses over which the output is linearly related to input. Nevertheless they all have the disadvantage that they detect cells retrospectively by virtue of the number and type of progeny produced under defined (optimized) conditions and after a specified length of time. As a result, they are slow, labour-intensive and are generally less precise than direct detection methods.

The long-term culture-initiating cell (LTC-IC) assay and the related cobblestone area forming cell (CAFC) assay are in vitro methods that can detect a cell population that overlaps with CRUs (15;23). These exploit the ability of HSCs and their immediate progeny to be maintained and to proliferate to varying degrees exclusively when cocultured in contact with stromal feeder layers. The principle of these assays is to extend the time of culture to the point that it can be safely assumed that the cells being produced must have derived from HSCs in the original input. This assumption has not been rigorously examined, but correlative studies indicate that the detection of myeloid colony-forming cell (CFC) progenitors (or CA's) beyond 4-5 weeks depends on initiating the cultures with cells that share features with HSCs and that can support their expansion (15;24;25).

Many types of more differentiated cells can be characterized by their ability to form colonies of mature progeny in semi-solid medium containing specific growth factors, hence the term CFC. This type of methodology can detect pluripotent cells that generate colonies containing granulocytes, erythroblasts, megakaryocytes and macrophages (CFC-GEMM) (26;27) as well as different types of lineage-restricted CFCs. Cells that generate only granulocytes and macrophages are termed CFC-GM (28;29) and similar assays and nomenclature exist for cells that generate small or large colonies containing erythroid
progeny (CFC-E and BFU-E, respectively) (30) or megakaryocytes (CFC-Mk and BFU-Mk, respectively) (31).

More recently, the expanded availability of monoclonal antibodies and improved machines for multi-parameter sorting of viable cells in a sterile fashion has led to the identification of surface profile phenotypes that can be used to directly identify cells at different stages of hematopoietic differentiation in defined tissues. However, it should be noted that most of the markers used to establish these profiles do not show fidelity in their expression when the cells are stimulated. The use of phenotypic markers is desirable because of its speed and immediacy, but also has greater limitations of sensitivity.

The first differentiation steps that HSCs undergo usually lead to a loss of either lymphoid or myeloid potential resulting in the generation of either a common lymphoid progenitor (CLP) (32) or a common myeloid progenitor (CMP) (33). CLPs are characterized as Lin−IL-7R+Thy-1−Sca-1lo-c-Kitlo cells in adult mouse bone marrow and these markers identify cells that can produce T, B and NK cells, but not myeloid cells. Conversely, CMPs are FcRγloCD34+ cells and can generate megakaryocytes, erythrocytes, granulocytes and macrophages, but not lymphoid cells. CMPs probably overlap with CFU-S and CFC-GEMM. Later cell types that are restricted to the production of megakaryocytes and erythrocytes only (MEPs; functionally identified as BFU-E) or to the production of granulocytes and monocytes progenitors (GMPs; functionally as CFU-GMs) can also be prospectively isolated by cell surface phenotype from both the bone marrow and the fetal liver (34). However, while this described hematopoietic hierarchy is applicable to the majority, it is not necessarily always the case (35). Examples of such rare cell types were first seen when single cells were plated in culture; the colonies generated contained diverse combinations of cell types not predicted from a rigid hierarchy (36). There are now a number of cells that do not follow the standard
hematopoietic hierarchy briefly outlined in Figure 1.1, such as lympho-myeloid cells that lack erythro-megakaryocytic potential (37) or a mature cell type with the potential to generate both B cells and macrophages (38).

1.2 Development of hematopoiesis

1.2.1. Generation of hematopoietic cells in the mouse embryo

The first cells with transplantable HSC activity appear in the mouse embryo in the yolk-sac (39;40) and aorta-gonado-mesonephrous (AGM) regions (41) on embryonic days (E) 9 and 10 of gestation, respectively. Since these cells have direct access to the circulation, it is thought that they are the origin of the HSCs that appear in the fetal liver on E11. The frequency of HSCs (CRUs) reaches a peak in the fetal liver on E14.5 with maintenance of their numbers until day 16 (42) after which these numbers steadily decline, possibly due to an exodus of HSC to the spleen and bone marrow (43). This redistribution of HSCs into the spleen and bone marrow occurs over a period of several days. HSCs are first detected in the fetal spleen on E13 (44), which then increases over the next 2-3 days (E15.5-16.5), and on E17.5, HSCs are first detected in the fetal bone marrow (45). After birth, HSC numbers continue to decline in the liver and spleen but increase in the bone marrow where HSCs then persist for the lifetime of the mouse. This shift in the tissue location of hematopoiesis appears to be an evolutionarily conserved process and, while the tissues involved differ, it can be seen in organisms as primitive as drosophila and as complex as humans (46).

The murine yolk-sac is a splanchnopleure, which means that it is composed of 2 layers of tissue, extraembryonic mesodermal cells and visceral endoderm cells. This structure develops between E7 and E7.5 (47;48) and is the first site of blood cell production. The inner layer appears to be the origin of primitive macrophages and erythroblasts and endothelial cells
can be seen to arise from the outer layer (49). The simultaneous appearance of erythroblasts and endothelial cells that share expression of Flk-1, CD34, Tie-2, GATA-2, LMO-2 and SCL has suggested that these 2 lineages arise from a common hemangioblast precursor within the developing yolk-sac (50).

The E8 para-aortic splanchnopleure (P-Sp)/aorta-gonad-mesonephros (AGM) region is another important and independent site of hematopoietic cell genesis in mammalian embryos. In mice, this is the first intra-embryonic site in which CFU-S can be detected between from E10 until E11. After this time, the fetal liver takes over as the major hematopoietic tissue (41). It appears that the AGM is not conducive to the generation of HSCs that can be detected by transplantation assays into adult recipients and only after cells from the AGM have further developed in the fetal liver can transplantable HSCs be detected (44).

The fetal liver begins to form on E9 and, at that time, the only hematopoietic cells it contains appear to be committed myeloid precursors (51-53). However, by E11, the fetal liver contains both CFU-S and CRUs (13;54;55). These cells are thought to be derived from colonizing cells that originated in the yolk-sac and/or AGM regions rather than representing the progeny of mesenchymal precursors that are also present in the fetal liver (56). The fetal liver remains the major site of hematopoiesis from E12 until birth (57).

The development of bone marrow begins in the fetus with the formation of a cellular matrix in the bone cavities, followed by their population by an influx of HSCs, most likely emanating from the fetal liver and released into the circulation, starting around E17. Bones that are hematopoietically active are characterized by a particular type of cellular matrix, produced by mesenchymal precursors that migrate into the marrow cavity to generate the stroma of the bone marrow and induce the migration of HSC into these sites (58). This model is supported by the finding that primitive hematopoietic cells (CFU-S) that are genetically
unable to produce stromal derived factor-1 (SDF-1) or its receptor (CXCR4) fail to colonize the bone marrow of fetal mice in spite of apparently normal hematopoiesis in the fetal liver. However, it is important to note that most of these data are limited to studies of CFU-S (45) and comprehensive quantitative data on the frequency and rate of expansion of HSCs in the fetal bone marrow have not been reported.

Recently, the murine placenta has also been found to contain HSC activity, with a peak of activity between E12 and E14, followed by a rapid drop in stem cell frequency up to E15.5, as the liver expands (59). The large number of HSCs found in the placenta raises the question as to whether this is a site of independent stem cell generation or if, similar to the fetal liver, it is a transient niche supporting HSC expansion.

A significant feature of the development of the hematopoietic system in the embryo is the apparent delayed appearance of cells with the properties of HSCs. In fact, the first recognizable hematopoietic cells that arise directly from mesenchymal derivatives are terminally differentiated cells which do not appear to be derived from cells with extensive pluripotent or proliferative ability. This stands in marked contrast to the conventionally recognized hierarchy of hematopoietic cell types present in the adult (described above). These findings have led to the concept that different molecular programs may exist to regulate the generation of hematopoietic cells at different stages of ontogeny (60). It could therefore be informative to characterize more fully the differences that exist between fetal and adult HSCs in order to elucidate the mechanisms by which one evolves from the other.

Interestingly, a recent study provided evidence suggesting that all HSCs in the adult are derived from cells detectable as HSCs in the embryo by using the stem cell leukemia (SCL) locus to direct the expression of a tamoxifen inducible Cre recombinase in hematopoietic and endothelial cells (61). They showed that the progeny of SCL expressing
cells in the E10-11 embryo contribute to the bulk of HSCs in both the fetal liver and adult bone marrow. However, as not all of the cells in the fetus and adult were marked, some de novo generation of HSCs in these tissues can not be ruled out (62).

1.2.2. **Generation of hematopoietic cells from murine ES cells in vitro**

Embryonic stem (ES) cells are derived from the inner cell mass of the embryonic blastocysts and are capable of differentiating into all three primary germ layers of the embryo (endoderm, mesoderm and ectoderm) and therefore to all cell types found in an adult (63). These cells propagate extensively and can be maintained indefinitely ex vivo in an undifferentiated state in the presence of LIF (64). These cells can be genetically modified and reintroduced into blastocysts to allow the identification of critical roles played by genes in all tissue types. To assess the role of a gene at a later stage of development or in a particular tissue specifically, conditional knock-outs can be generated, where the gene is essentially shut-down under the control of a tissue-specific promoter or upon the addition of an exogenous signal at a chosen time during development (65;66).

The generation of the hematopoietic lineage from ES cells shows similar kinetics in vitro to that seen during normal embryonic development and proceeds through similar intermediates, from hemangioblasts to the long-term and short-term repopulating hematopoietic cells and to committed progenitors of the myeloid and lymphoid lineages, which has allowed ES cells to be used to derive various hematopoietic lineages (67-73).
1.3 Differences between fetal and adult hematopoiesis

1.3.1 Mature cells

Many features of terminally differentiating myeloid cells remain the same throughout ontogeny and, as noted above, appear to be produced by cells undergoing a similar sequence of changes at the molecular level. Nevertheless, some differences are known, the most notable examples being the types of haemoglobins produced by erythroblasts in fetal and adult mice (74) and the speed with which these cells transit the cell cycle (75;76). Human fetal progenitors also demonstrate a faster growth rate than their adult bone marrow counterparts, as demonstrated by the speed and size of colonies they generate in vitro (76). In addition, cell tracking studies have shown directly that human fetal liver-derived CD34+ cells divide in vitro with a shorter cell cycle time than do those derived from cord blood (77).

In the case of lymphoid cells, differences in the programs of maturing T-cells, B-cells and NK cells have also been described. These result in differences in the T-cell receptor gene rearrangements exhibited by fetal and adult T-cells (78), differences in the surface marker profiles of fetal and adult B-lineage cells (79) and differences in the receptors expressed by fetal and adult NK cells. In addition, there appears to be marked changes in the growth factor requirements of fetal and adult lymphoid cells (80). For example, interleukin-7 is critical to T- and B-cell development, as demonstrated by mice with a homozygous null mutation of the IL-7 gene (81). However, these same mice have detectable B lymphopoiesis in the fetus that is not seen in the adult, indicating the operation of compensatory mechanisms that can support fetal B-cell differentiation that are either absent or not effective in the adult (82).
1.3.2 HSCs

A number of differences in the way more primitive hematopoietic cells may differentiate are also well documented. Summarized below are the differences in kinetic, phenotypic, homing, self-renewal and growth factor responsiveness properties of fetal and adult HSCs.

1.3.2.1 Kinetic properties

Transplants of murine fetal liver cells (E14-18) repopulate the bone marrow and spleen of irradiated recipients faster than do transplants of adult bone marrow cells (54) with a 16-hour vs. a 25-hour doubling time of the cell populations produced in the bone marrow in the first 3 weeks post-transplant (75). These classical experiments clearly point to differences in parameters that regulate the output of cells from hematopoietic cells with repopulating activity but do not discriminate between differences in HSC self-renewal potential, cell cycle transit times and or possible differences in rates of cell loss due to cell death.

Early studies also showed that primitive cells in the fetal liver and adult bone marrow differ in their proliferative activities, as indicated by the proportion that these cells are in cycle. For example, comparison of the cycling status of CFU-S from fetal liver and adult bone marrow revealed that those from fetal liver are mostly in cycle, whereas those from adult bone marrow are mostly quiescent (54). Subsequent studies confirmed that these differences extend to the HSC compartment with >80% of adult bone marrow HSCs being resistant to in vivo treatment with 5-fluorouracil (5-FU), a cytotoxic agent, and 40% of the cells in a subset of fetal liver cells that contains the HSCs being found to be in the S/G2/M phase of the cell cycle (83;84).

A number of genes alter the cycling characteristics of HSC, including p21, p27, p18, cyclinD (85-90) and Gfi-1 (91). Various genes appear to regulate the proliferation of fetal
liver and adult bone marrow HSC differently. As discussed below in detail, these include genes for Steel factor (SF), c-Kit (92-94) and Tie-2 (95) that control HSC interactions with their microenvironment, as well as a number of intrinsic signaling molecules and transcription factors that appear to be involved in regulating HSC function (see Section 1.4).

1.3.2.2 Phenotypic properties

Fetal and adult HSCs have also been found to display a different spectrum of markers on their cell surface and to exhibit differences in their dye efflux and adhesion properties (Table 1.1). Notable examples include a poor ability of fetal liver HSCs to adhere to fibronectin and a positive expression of C1qRp (96), a C-type lectin-like type I transmembrane protein recognized by the monoclonal antibody AA4.1 (97). Fetal liver HSCs also express readily detectable levels of CD11b (Mac1), an integrin component that is commonly found on mature macrophages from all developmental stages, but not on adult bone marrow HSCs (43;98). The demonstration that Mac-1 is expressed on fetal liver HSCs was one of the first indications of the lack of differentiation stage-specificity of many phenotypic markers. This highlights their inherent unreliability as surrogate direct indicators of HSCs and, in particular, the problems caused by the indiscriminate use of standard lineage-negative antibody cocktails to obtain enriched populations of HSCs from manipulated cell suspensions (96;99;100).

Rebel et al. (1996) avoided this, and carefully characterized the phenotypic similarities between fetal and adult stem cells as being the expression of Sca-1, c-Kit, MHC class I and CD43 antigens, as well as high binding to wheat germ agglutinin (WGA+) and the lack of expression of B220, Gr-1, Ly-1, Thy-1, CD71 and Fall-3. As differences, they found that fetal liver HSCs stain positively with monoclonal antibodies against AA4.1, Mac-1 and CD45RB and fetal liver also retain Rhodamine 123 (are Rho123+) whereas adult stem cells do
not (98). In mice, CD34 and CD38 are also developmentally regulated HSC cell surface markers. Murine fetal liver HSCs are CD34⁺CD38⁻ whereas adult bone marrow HSCs are CD34⁻CD38⁺ (101-104). The receptor tyrosine kinase Tie-2 may also be differentially expressed by HSCs during ontogeny, with reports suggesting that murine fetal liver HSCs express Tie-2 but adult HSCs do not (105-107). However, the Tie-2 antibody is not yet a reliable material and may prove to label both adult and fetal HSCs. HSCs from the fetal liver also do not efflux Hoechst 33342 and are thus not detectable within the gate used to identify adult HSCs as side population (SP) cells using fluorescent activated cell sorting (FACS) (108).

With a few exceptions (e.g. c-Kit (92)), most developmentally regulated cell surface markers expressed on HSCs change in adult HSCs as a function of an alteration in their proliferative activity and/or cytokine activation. Therefore, it seems likely that a similar explanation might apply to the phenotypic differences exhibited by fetal liver HSCs. For example, 5-FU treatment of mice leading to activation of their HSCs in vivo (109) and/or direct activation of adult HSCs in vitro with cytokines causes the CD34⁻, CD38⁺, Mac-1⁻, SP, Rho⁻ phenotype of quiescent HSCs to acquire a CD34⁺, CD38⁻, Mac-1⁺, non-SP, Rho⁺ phenotype, identical to that characteristic of fetal liver HSC (102;104;108;110-112). Also noteworthy is the lack of consistency in the phenotypic profiles of murine and human HSCs. For example, human fetal liver HSCs are CD34⁺ (113), sharing the murine CD34 expression pattern of fetal liver HSCs, but bearing the SP phenotype of adult bone marrow HSCs.

1.3.2.3 Homing and mobilization

Another important property of HSCs is their motility and ability to migrate in response to specific chemoattractants. HSCs migrate from one site to another throughout life; i.e., from the fetal liver to the fetal spleen and bone marrow in the embryo and later a small percentage
of HSCs are constantly found in the peripheral circulation (114). This property underlies the ability of HSCs from both mice and humans to be transplanted intravenously. The mechanisms that attract and retain HSCs in the bone marrow, as well as those that allow their release, are still not fully understood although a number of molecular ligand-receptor pairs controlling HSC adhesion and migration have been identified.

VLA-4 (α4β1 integrin) expressed on HSCs and its receptor VCAM-1 on bone marrow stromal cells are critical mediators of adhesion and mobilization, as shown by the fact that treatment of mice with antibodies to VLA-4 and VCAM-1 results in HSC mobilization. This appears to involve signaling through the c-Kit pathway, with both c-Kit and VLA-4 being downregulated on HSCs when they are mobilized (115-125). Most recently, the α4 integrin component of VLA-4 has been shown to be required for the competitive function and self-renewal of HSCs in vivo due to their reduced retention within the niche in its absence (126).

C-Kit is also thought to play a direct role in HSC adhesion to the endosteal bone, but not necessarily in their migration (127).

CD44 is another adhesion molecule expressed by HSCs (128). CD44 has many ligands one of which is hyaluronic acid (HA), the major component of the ECM in the bone marrow (129) and E-selectin, which is expressed by endothelial cells (130). Some reports have suggested that antibodies to CD44 could affect the homing of HSCs (131;132), yet CD44⁻/⁻ mice are healthy and fertile with normal numbers of in vivo-homing HSCs (133). Nevertheless, in vitro, the SDF-1-mediated induction of human progenitor cells to migrate on HA was associated with their assumption of a polarized morphology and the formation of pseudopodia at the leading edges of which CD44 was concentrated (132). Interestingly, high levels of CD44 expression on tumour cells mediates their metastatic spread (134).
Matrix-bound cytokines, such as Steel Factor (SF) and Flt3-ligand (135-137) may also serve as adhesion molecules for their respective receptors (c-Kit, the receptor for SF, and Flt-3, the receptor for Flt3-ligand), both of which are expressed on the surface of HSCs. Certainly membrane-bound SF expressed on stromal cells can cause the adhesion of c-Kit-expressing HSCs to them (138) and thus play a role in HSC retention in the bone marrow, that when reversed leads to their mobilization (139).

SDF-1 may be a major regulator of HSC homing and is produced in large amounts by bone marrow stromal cells (140). Conversely, administration of antagonists of SDF-1 into normal adult mice (or humans) causes the HSCs in the bone marrow to detach from their niche and enter the circulation – a process referred to as mobilization (141). SDF-1 is a member of the chemokine family of molecules but is unique in its ability to bind to a single receptor, CXCR4, a member of the G-coupled receptor family. CXCR4 is also the only chemokine receptor found to be expressed on HSCs thus conferring its response specificity to SDF-1 (142). However, CXCR4 is not restricted in its expression to primitive hematopoietic cells and is highly expressed on B-lineage cells and many other cell types including numerous kinds of metastatic tumor cells (143-145). In vitro, the chemoattractant properties of SDF-1 on HSCs (and derivative progenitor types) have been well documented using transwell assays (140). Activation of CXCR4 leads to the activation of phosphoinositol-3 kinase which in turn activates PKC-zeta, whose downstream targets include Pyk-2 and ERK1/2 (146).

Interestingly, the levels of CXCR4 expression on the cells it chemoattracts can vary widely and this parameter is not predictive of either their in vitro chemoattractant responsiveness (147-149) or their in vivo homing activity (150). Mice in which either the SDF-1 or CXCR4 genes have been inactivated die at a late stage of embryogenesis with gross bone marrow failure in spite of apparently normal hematopoiesis in the fetal liver, likely due
to an impaired ability of the fetal liver HSC to migrate into or be retained within in the bone marrow (147;151-154). SDF-1 elicits multiple responses in CXCR4-expressing cells including the induction of a chemoattractant migratory response by HSCs (142;155), inhibition of HSC cycling (156), and HSC mobilization (155;157). Additional mediators of HSC chemotaxis are likely to be found, such as the recent discovery of the P2Y-like receptor, GPR105, (158).

Migration of HSCs across the endothelium occurs preferentially in the G₀/G₁ phase of the cell cycle and fetal HSCs are more efficient than adult mobilized PB HSCs at this process (159). Similarly, the mobilized HSCs found in the circulation are predominantly G₀/G₁ cells (160;161) even when the method of mobilization is shown to initiate their prior entry into division within the bone marrow (162). Interestingly, adult HSCs stimulated to proliferate in vitro or in vivo display an engraftment defect during their period of transit through S/G₂/M (84;163;164), although this defect could not be demonstrated for human fetal liver HSCs (165). Some differences in the expression of adhesion and homing molecules were found when G₁/G₀ vs S/G₂/M cells enriched in their content of HSCs were compared, such as a higher expression of VLA-4 and a lower expression of CD44 in the G₁/G₀ cells (163).

Adhesive interactions between HSCs and their microenvironment also play a role in the regulation of HSC survival and proliferation as well as in their homing and mobilization.

1.3.2.4 Self-renewal

As noted above, after the first appearance of HSC on the 9th and 10th days of embryogenesis, it is thought that the later expansion of this compartment occurs by the execution of symmetric self-renewal divisions; i.e., cell cycles in which competency for multi-lineage differentiation is maintained but not activated in both daughter cells. Self-renewal divisions are the hallmark
attribute of all stem cells and much effort has been devoted to elucidating this process with a view of devising ways to manipulate it. Controlled enhancement of HSC self-renewal ex vivo offers the potential both to develop strategies for obtaining amplified HSC populations and their mature progeny for a variety of therapeutic applications and to create models for investigating mechanisms of leukemogenesis (166;167). Fetal liver HSCs have garnered interest as a population with seemingly greater intrinsic self-renewal potential than bone marrow HSCs, based on the sustained larger PB cell outputs by fetal liver HSCs as compared to bone marrow HSCs (99;168;169). In addition, fetal liver-derived cells reconstitute more daughter CRUs after a given period (168;169) and can typically be serially transplanted more times than adult bone marrow cells. However, it appears that HSC numbers are not fully regenerated in irradiated transplanted mice, even though mature blood cell output is fully restored (170;171) and when the transplant dose is corrected for the actual content of CRU, huge expansions of adult bone marrow CRU can be documented (172). As yet, similar experiments have not been performed with HSCs regenerated in primary recipients of fetal liver-derived HSCs. Thus, it has been difficult to discern the separate contributions of increased self-renewal activity and greater survival or shorter cell cycle times of fetal liver HSCs on the greater regenerative activity they have been found to display in experiments that do not measure these responses independently. Notably, only a single paper has been published describing the kinetics of normal HSC expansion in mice transplanted with adult bone marrow cells (172) and similar data does not exist for fetal liver HSCs. As a result, it is not known when recipients of primary transplants should be assessed for secondary CRU content in order to provide a quantitative index of the self-renewal activity of the cells transplanted.
1.3.2.5 Growth factor responsiveness

There is now definitive evidence that the self-renewal of HSCs can be modulated by exposure to different extrinsic factors, both in vivo and in vitro. In vivo evidence is seen following virtually every HSC transplant, where the number of HSCs initially injected can be shown to increase at least 10-20 fold (169; 172), compared to their maintenance at a constant level in normal adult mice. The same shifts seen in HSC self-renewal also occur when normal (+/+) HSCs are transplanted into W-deficient recipients (defective in normal c-Kit signaling) and are stimulated to expand their numbers (94; 173).

Significant efforts have been made to identify factors that influence HSC self-renewal in vitro, although these have been focused primarily on HSCs from young adults (174; 175) or from samples of human cord blood (176) and relatively little is known about self-renewal control in fetal liver HSCs. The difficulty in finding one or more growth factors that will promote an expansion of HSC is not due to an inability to identify factors that can optimize the entry of HSCs into division (77), but rather because of the relative inability of any growth factors identified to date to effectively sustain the stem cell potential of the stimulated HSC. Interestingly, certain GFs have been shown to stimulate HSC expansion but only over a narrow range (i.e., IL-6), while others (e.g. Steel factor, SF) attain their maximal effects only at high doses (>300ng/mL) (175). In the case of fetal liver HSCs, even less is known about factors that may promote HSC self-renewal but some additional candidates have been identified (e.g., Fibroblast growth factor-1 (177), thrombopoietin (174), Wnt proteins (178), Sonic hedgehog (179) and TAT-HoxB4 (180).
1.4 Genes that regulate HSCs

1.4.1 Genes critical to both fetal and adult HSCs

Studies of the generation and activity after transplantation of HSCs from mice in which specific genes have been modified or completely inactivated, or are overexpressed, have provided a significant body of information about specific intrinsically expressed receptors, signaling intermediates and transcription factors that appear to be important in controlling HSC proliferation and self-renewal. Many transcription factors regulate the self-renewal, proliferation, and/or the early differentiation of HSCs. Those shown to play a critical role (as demonstrated by inactivation studies) include: Activating transcription factor (ATF) 4, required for high-level proliferation in the fetal liver (181), Core-binding factor β, required for fetal liver HSC emergence and normal maturation (182;183), c-Myb and p300, required for self-renewal and differentiation, respectively (184-186), c-Myc, required for differentiation (187), Gata-2, for normal proliferation, especially in adult bone marrow HSCs after cytotoxic treatment (188;189), Ikaros, required for normal numbers of HSC (190-192), Lmo-2, required for emergence of HSCs (193;194), meis1, required for the proliferation/self-renewal of HSCs (195;196), MEF, required for normal maintenance of adult HSC quiescence (197), mel-18, required to negatively regulate proliferation, by binding to cyclinD2 (198-200), mixed-lineage leukemia, required for emergence of fetal liver HSCs (201), and those shown to play an important role (as demonstrated by overexpression studies): Hox genes (202-204), and rae-28 (205), both resulting in enhanced self-renewal. There are also non-transcription factors that play critical roles in stem cells, such as signaling intermediates STAT3, required for self-renewal (206;207), STAT5, required for normal competitive repopulating ability (208-212), and the cyclin-dependent kinase inhibitors described above p21, p27, p18, required to maintain quiescence, progenitor proliferation and to inhibit self-renewal, respectively (85-
87;90); receptors, such as the retinoic acid receptor \( \gamma \), that mediates cell cycle arrest (213) and the receptor for thrombopoietin, c-mpl, required for normal numbers of HSCs (214); as are secreted factors such as transforming growth factor-\( \beta \), a positive regulator of competitive repopulation in adult HSCs (215) and wnt, a positive regulator of proliferation and expansion (167;178;216;217).

Through studies of chimeras and using conditional gene knock-out strategies, it has also become possible to determine whether identified candidates act in a cell autonomous fashion and whether they may differentially affect fetal and adult HSCs, particularly where marked deleterious effects on fetal cells are obtained. In the last decade, with the advent of methods to obtain highly purified populations of murine HSCs from both fetal and adult sources, it has also been possible to determine whether candidate regulators are differentially expressed in these populations.

It is interesting to note that many of the genes found to have important effects on HSC were first identified because of their involvement in fusion proteins created by translocations associated with different types of acute myeloid leukemia (AML) in humans (218). This is not surprising, given that such diseases are thought to originate after sufficient accumulation of genetic deregulation in HSCs.

1.4.2 Genes more critical to fetal than adult HSCs

1.4.2.1 Runx1

Runx1 (runt-related transcription factor1) is the murine homolog of a human gene originally called \( AML-1 \) to reflect its discovery on chromosome 21 from the cloning of a common breakpoint in human AML cells that carry a 8;21 translocation (219). Runx1 encodes the DNA-binding subunit of a member of the core-binding factor (Cbf) family of transcription
factor complexes and has primarily activating functions in myeloid cells (220-222). It has, therefore, also been called Chfa2. Target genes identified include CyclinD3, IL-3, GM-CSF and RAG1 and TCRα (223-227).

A critical role for Runxl in early normal hematopoietic development was first revealed by the generation of two Runxl-deficient mouse lines, both of which died mid-gestation as a result of bleeding in the central nervous system and soft tissues, with complete loss of fetal liver hematopoiesis (228;229). All hematopoietic cell lineages were found to be affected by the loss of Runxl, with the exception of primitive erythropoiesis in the yolk-sac, which was minimally affected. Similarly, in vitro, Runxl-ES cells could generate normal numbers of primitive erythroid precursors but were impaired in their ability to generate blast colony-forming cells (cells that have properties of hemangioblasts) and failed to produce definitive hematopoietic CFCs (230). In the fetal liver of Runxl heterozygotes, hematopoietic progenitor numbers were found to be reduced by comparison to wild-type controls, suggesting that Runxl has a dose effect on mechanisms that regulate the production of primitive hematopoietic cells in the fetus. Nevertheless, in adult haploinsufficient animals, no phenotype was detected, implying the operation of different control mechanisms in the adult or an ability of other genes to substitute for, or compensate for, a lack of Runxl (231).

An inducible gene-targeting strategy for Runxl was then pursued in order to assess directly the effect of an absence of Runxl in adult cells that had been generated from cells that produced Runxl at normal levels (232-234). A complete absence of Runxl in the adult did not impair continued production of hematopoietic cells suggesting that it is dispensible for the maintenance of adult HSC activity although enhanced granulopoiesis and defective megakaryocytopoiesis and early lymphopoiesis was noted. Competitive repopulation assays confirmed that Runxl- adult bone marrow cells contain elevated short-term myeloid
repopulating activity (<2 months) but are similar to +/+ bone marrow cells in their ability to
repopulate irradiated mice with mature neutrophils, monocytes and megakaryocytes for more
prolonged periods.

Using a "knock-in" marker strategy, Runx1 was shown to be expressed in
mesenchymal and endothelial cells at all sites in the yolk-sac and embryo proper where the
first definitive hematopoietic cells first appear, as well as in all of the HSCs that emerge
during this process. However, when expression of Runx1, itself, was deleted, a complete
block was seen in the generation of hematopoietic cells at these locations (235;236). Taken
together, these findings indicate an essential role of Runx1 in the generation of fetal HSCs
from hemangioblasts in the embryo but not for their later self-renewal. However, it is
interesting to note that forced expression of RUNX1-ETO (a fusion protein shown to act as a
potent dominant negative inhibitor of Runx1 during development (228;229) in CD34+ human
cord blood cells enhanced the long term retention of lympho-myeloid differentiation potential
as well as an ability to repopulate irradiated NOD/SCID mice (237). Thus, although Runx1
may be dispensible for the maintenance and initial differentiation of adult HSCs, these cells
appear to remain sensitive to pathways that can be modulated by changes in Runx1 activity.

1.4.2.2 Notch

Notch genes were discovered almost 80 years ago in Drosophila and found to encode type 1
transmembrane glycoprotein receptors that are cleaved after activation by binding their
specific ligands, either Jagged1 or 2, or Delta (238). Cleavage results in the translocation of
intracellular Notch to the nucleus where it can act as a transcriptional activator, for example
for the c-myc promoter (239). Of the 4 mammalian notch homologs, Notch-1 has been most
extensively studied and its ability to inhibit HSC differentiation (promote HSC expansion)
documented (240-242). Homozygous inactivation of Notch-1 causes embryonic lethality by day 9.5 (243;244) and at this time HSCs could not be detected when they were assayed for their ability to repopulate conditioned newborn mice (245). Further studies showed that in vitro, Notch-1−/− ES cells proliferated normally and were not impaired in their ability to generate either EBs or EB-derived definitive erythroid and myeloid CFCs. Following injection into wild-type blastocysts, they contributed initially to the CFC and CFU-S populations produced up to E10.5 of gestation. However, after that time, these cells rapidly disappeared and were not replaced even though a high degree of chimerism persisted in other organs (246).

In contrast, elimination of either Jagged1 or Notch1 or both in adult HSCs using an inducible Cre-loxP-mediated inactivation strategy revealed no effects on the ability of the affected HSC to exhibit their self-renewal or multipotentiality either postransplant or endogenously after treatment of the mice with 5-FU (247). These findings strongly suggest that Notch1 plays a differential role in regulating the ability of fetal and adult HSCs to execute self-renewal divisions when stimulated to proliferate.

1.4.2.3  

Scl

The stem cell leukemia (Scl) gene encodes a basic helix-loop-helix family member transcription factor, that can either activate or repress transcription, depending on the composition of the multicomponent complexes it forms and plays an important role at multiple stages of hematopoietic cell differentiation, as reviewed in (248). The Scl protein would thus be expected to be able to play different roles through interactions with different partners present in varying levels in specific cell types (248). Some of these are known to be critical for hematopoiesis, such as E2A, and the bridge protein LMO-2, which also binds GATA-1
(see below). This type of complex can activate the c-Kit promoter, through functional interaction with Sp1 (249). Although studies of cells from Scl\(^{+/+}\) heterozygotes with a lacZ/w reporter gene knocked into one of the Scl genes have now shown that Scl is expressed in a number of tissues, it has also been possible to establish that, within the hematopoietic system, Scl is expressed at the highest levels in the most primitive cells, including HSCs (61;250;251).

The Scl gene was first targeted for disruption in 1995 by 2 groups, who both found that this resulted in embryonic lethality by E9.5, with complete loss of blood formation in the yolk-sac (252;253). Scl\(^{-/}^-\) ES cells are also unable to contribute to hematopoiesis in mouse chimeras, demonstrating the cell-autonomous requirement for Scl in the generation of both primitive and definitive hematopoiesis in the fetus (254;255). In vitro, Scl\(^{-/}^-\) ES cells were able to generate flk-1\(^+\) blast-CFC but the colonies then produced contained only vascular smooth muscle cells and no detectable endothelial or hematopoietic cells, suggesting that Scl may be required for the first steps in the transition from a mesodermal precursor to a hemangioblast (250;256). Recent mRNA knockdown studies in zebrafish suggest that Scl may also play important roles in the generation of endothelial and hematopoietic cells in this organism but not until after the hemangioblast stage when each of these lineages has become distinct (257).

To assess the role of Scl in adult hematopoiesis, conditional knockout mice have been generated and studied. In one such study, analysis of bone marrow cells from targeted adult mice revealed that Scl, like Runx1 and Notch-1, is dispensable for continued HSC function. However, in the case of the Scl\(^{-/}^-\) HSCs, the ability to generate differentiated myeloid and lymphoid progeny was preserved, but the generation of mature erythroid and megakaryocytic precursors was compromised (258). Similar results were reported by the second group who
found that *Scl* was required for the short-term repopulation of irradiated recipients but did not appear necessary for HSC self-renewal (259;260).

Interestingly, transduction of HSCs with vectors encoding either a wild-type *Scl* c-DNA or a dominant-negative (dn) form also had downstream effects on hematopoiesis without altering the long-term repopulating capacity of the transduced HSCs. The specific effects were the promotion of short-term myeloid repopulation by the *Scl*-transduced cells and promotion of lymphoid contribution by the *dnScl*-transduced cells (261).

### 1.4.3 Genes more critical to adult than fetal HSCs

Genes critical to adult bone marrow HSCs but not fetal liver HSCs may be considered much easier to study as knock-outs of these might be anticipated to allow the organism to initially develop normally with the consequences of the mutant phenotype occurring later after birth. However, it is not clear when this point would be expected, since very little is known about the timing or relevance of extrinsic versus intrinsic factors in regulating changes in the HSC compartment between fetal life and adulthood. Nevertheless, some gross differences have become apparent from single generation transplants suggesting that genes more critical to adult than fetal hematopoiesis can be identified.

#### 1.4.3.1 Bmi-1

B cell-specific Moloney murine leukemia virus integration site-1 (*Bmi-1*) is a gene encoding a member of the Polycomb group (PcG) of transcription factors. Two general types of multimeric PcG complexes are recognized. Both are involved in silencing genes through directing the modification of chromatin structure, one in the initiation of this process (called polycomb repressive complex 2 or Prc2) and the other in the stable maintenance of gene
repression (called polycomb repressive complex 1 or Prcl), and as a result, both are considered strong candidates for playing important roles in orchestrating the epigenetic changes in gene regulation that must occur during the self-renewal and initiation of lineage restrictive events that occur in stem cells (262;263). Bmi-1 is a member of the Prcl complex and other members of the Prcl complex are Mel-18, Rae28, Ringl and M33. Bmi-1 is expressed at elevated levels in highly purified HSC populations isolated from both fetal liver and adult bone marrow and acts to repress the CDK inhibitor p16^{ink4a} and p19^{Arf} (263;264).

As predicted for a gene that differentially targets the self-renewal of adult HSCs, homozygous deletion of Bmi-1 allows the affected mice to survive until about 1-2 months after birth at which point they die with hypocellular marrows. Assessment of the cellular composition of the fetal liver of Bmi^{-/-} mice showed these have a normal cellularity with unaltered numbers of c-Kit^{+} Sca1^{+} Thy1.1^{lo} lin^{-} cells, a population that is normally enriched in the fetal liver HSCs. However, when Bmi-1^{-/-} fetal liver cells were transplanted into adult irradiated mice, no B-lineage cells were produced and overall multi-lineage repopulating activity was reduced initially and declined irreversibly to undetectable levels within 8 weeks (265) unless Bmi-1 expression was restored, in which case HSC at near normal frequencies could be demonstrated (264;265). This demonstrates that the initial generation and expansion of fetal liver HSCs is not impaired by a lack of Bmi-1, but later, this transcription factor becomes increasingly critical for maintenance of the HSC compartment both when the cells are allowed to develop endogenously or when transplanted directly into an adult irradiated environment. Consistent with this interpretation was the finding that cells with the c-Kit^{+} Sca1^{+} Thy1.1^{lo} lin^{-} HSC phenotype were still detectable in the bone marrow of 4-5 week-old Bmi-1^{-/-} mice but at 10-fold reduced numbers and these were even more defective in their ability to reconstitute primary recipients (264;265). Interestingly, the greater biological effect
of a Bmi-1 deficiency in adult bone marrow cells was mirrored by similar differences in the extent of activation obtained on Bmi-1 target genes including p16\textsuperscript{ink4a} and p19\textsuperscript{Arf} (265), although later experiments showed that these did not translate into detectable effects on proliferative activity or HSC apoptosis (199).

The latter investigation also showed that forced over-expression of Bmi-1 could enhance the frequency of symmetrical divisions by primitive pluripotent hematopoietic cells stimulated by cytokines to proliferate in vitro, corroborating the hypothesis that Bmi-1 acts to maintain the competency of HSCs (199).

1.4.3.2 \textit{c-Kit}

\textit{C-Kit} is a proto-oncogene that encodes a type 3 cell-surface tyrosine kinase receptor also referred to CD117 (266). The ligand that binds to and activates c-Kit has been variably designated as c-Kit-ligand, Stem cell factor (SCF) and Steel factor (SF) because it is expressed on HSCs and is the product of the Steel gene. Both c-Kit and its ligand are produced as transmembrane proteins that can be variably cleaved proteolytically to yield an additional soluble form with full binding activity (267;268). SF binding induces homodimerization and tyrosine phosphorylation of the receptor, forming docking sites for SH2 domain-containing signaling intermediates (269). These include phosphatidylinositol 3'-kinase (PI3 K) and phospholipase C\textgamma-1 (270), the Src family kinases Src, Lyn, Fyn (271), the GTPase activating protein GAP (272), proteins of the p21Ras-MAPK pathway (269) and STAT1, 3 and 5 (273-275). Additional signaling molecules that couple to c-Kit, including Vav (276), Jak2 (277), Dok (278), Tec (279) and the tyrosine phosphatase SHP-1 (246). C-Kit is expressed on all HSCs and hematopoietic progenitors throughout embryonic and adult life and signaling downstream of the c-Kit receptor is required for many aspects of normal hematopoiesis.
The W (encoding c-Kit) and Steel (encoding Steel factor) loci were first identified from early genetic studies of spontaneously arising mutant white spotted mice that shared the same profile of pleiotropic phenotypes affecting coat colour, sterility and red blood cell production (280). These phenotypes are caused by abnormalities in the generation of melanocytes, germ cells and erythroid cells. Severe W mutations conferred a cell-intrinsic defect on primitive hematopoietic cells normally detected as CFU-S, whereas severe Steel mutations conferred a defect that was not transplantable and restricted to the environment of hematopoietic tissues (281). The W^{41}/W^{41} mouse has a single nucleotide substitution in the coding region of the c-Kit gene that results in partial impairment of the tyrosine kinase activity of the SF receptor. The fetal liver of these mice contains a normal number of transplantable HSCs but their longterm competitive activity is slightly reduced (94). Moreover, if they are simply allowed to expand without being transplanted, the numbers attained in the bone marrow by early adulthood is ~10-fold lower than that of wild-type mice and the repopulating activity per W^{41}/W^{41} HSC is also impaired (93). Similarly, the fetal liver of Sl/Sl mice, which do not express a functional SF protein, have been found to contain ~40% of the normal number of cells with a Sca1^+ Thy1.1^{lo} lin^- phenotype on E13 and these then increased at the same rate as their counterparts in wild-type embryos until E15 when these embryos all die from anemia (92).

1.4.3.3 \textit{Gfi-1}

The growth factor independent-1 (Gfi-1) gene encodes a zinc-finger transcription factor with repressor activity and was identified as a common site of Moloney murine leukemia virus integration in lymphoma cell lines that acquire IL-2 independence (282). Gfi-1 is expressed in HSCs and CLPs and granulopoietic progenitors but not in CMPs or megakaryocyte-erythroid...
progenitors (283). The Gfi-1 protein interacts with protein inhibitor of activated Stat3 (Pias) to mediate proliferative responses to cytokines and can enhance Stat3 signaling in primary T cells (284). There have not yet been any reports of experiments to measure HSC numbers or activity in the fetal liver of Gfi-1<sup>-/-</sup> mice, but the fact that young Gfi-1<sup>-/-</sup> mice are not anemic and have sustained myelopoiesis suggests that Gfi-1 is not critical for the initial appearance and early expansion of HSCs (285). However, adult Gfi-1<sup>-/-</sup> bone marrow cells showed a greatly reduced ability to repopulate primary irradiated recipients and an almost complete failure to generate cells that will repopulate secondary recipients (91;283).

Interestingly this late appearance of defective HSC self-renewal differed from that elicited by deletion of Bmi-1 where the HSCs conversion to a quiescent population was not altered (199). In contrast, in the case of Gfi-1<sup>-/-</sup> mice, both Hoechst 33342/PyroninY staining of the lin<sup>-</sup>Sca1<sup>+</sup>c-Kit<sup>+</sup> population isolated from the bone marrow of adult Gfi-1<sup>-/-</sup> mice and +/+ controls (164;165) and BrdU labelling studies (286;287) of these cells tracked over time showed an increased proportion of the HSCs from the adult Gfi-1<sup>-/-</sup> mice to be proliferating (91;283). In these cells GATA-2 was slightly upregulated and the cell cycle inhibitor p21<sub>ciP<sub>1</sub>Wafl</sub> was 10-fold down-regulated (91). This is a significant observation since adult p21<sub>ciP<sub>1</sub>Wafl</sub>-null mice have a similar HSC defect (85).

1.4.3.4. **Tie2**

Tie2 is a tyrosine kinase receptor expressed on the surface of endothelial cells and both fetal liver and adult HSCs (105;106;288;289). Tie2 and its ligands, the angiopoietins, have been suggested to play a role in the recruitment and mobilization of adult HSCs from the bone marrow (290). Initial observations indicated that Tie-2<sup>/-</sup> embryos display reduced hematopoietic differentiation as cultured explants from early stages of development (from the
P-Sp) in Tie2−/− embryos did not generate expected numbers of differentiated cells (291). Nevertheless, fetal liver hematopoiesis was not significantly affected by inactivation of the Tie2 gene whereas marked failure of Tie2−/− hematopoiesis was seen in the bone marrow of adult mice (95). The reason for the adult dependence of HSC on Tie-2 signaling may be related to the nature of the niche occupied by HSCs in the adult. HSCs are known to be concentrated at the periphery of the marrow cavity adjacent to osteoblasts that secrete Angiopoietin-1 (Ang-1), a ligand for Tie-2, that can maintain adult HSCs in vitro (292) and promotes adult HSC quiescence (293).

1.5. Experimental objectives

As reviewed above, strong evidence of differences in the proliferative status and regenerative potential of fetal liver and adult bone marrow HSCs has been found, although neither of these putative differences have been rigorously documented or quantified. Surprisingly, attempts to maintain fetal liver HSCs in vitro have shown that these HSCs are even more difficult to sustain than adult bone marrow HSCs (294;295) in spite of the fact that both the proliferative activity and the regenerative ability of fetal liver HSCs in vivo is thought to be greater. These latter findings suggested that effects of specific growth factors on the ability of fetal liver HSCs to maintain their undifferentiated status might be different from the results obtained for adult bone marrow HSCs (175) and predicted by studies of human cord blood and adult bone marrow LTC-IC expansion in vitro (296).

Based on this information, I set out to define more precisely how the cycling and self-renewal activity of the HSCs present at different stages of development change over time, to determine how these might relate to changes in other potential indicators of HSC function (i.e., output of different types of differentiated cells), and to examine whether any changes
observed were intrinsically or environmentally regulated and to try to determine how they might be regulated at a molecular level.

My overall working hypothesis was that the reported greater in vivo regenerative activity of transplanted fetal liver HSCs is due to intrinsic differences in the self-renewal potential of these cells as compared to their adult counterparts. This required designing experiments that would allow their self-renewal activity to be measured independently of their cycling activity.

Accordingly, I first executed a series of experiments to quantify the proportion of functionally defined HSCs that are in G0 (quiescent) vs G1/S/G2/M (actively proliferating) using a variety of existing methodologies that could be directly coupled to assessing CRU frequencies. These methods were then applied to HSCs from different sources and stages of development of the mouse starting with E14.5 fetal liver and finishing with bone marrow cells from 10 week-old young adult mice. The results of these experiments are presented in Chapter 2.

I then designed experiments to determine whether the self renewal properties of fetal liver HSCs are truly different from adult bone marrow HSCs, and, if so, to identify when they change. The first step was to delineate the precise kinetics of HSC regeneration in vivo from a fixed number of transplanted HSCs. These data were used to define a time when differences in HSC regenerative activity could best be compared and then applied to transplants of HSCs from the same sources assessed for changes in their proliferative activity in Chapter 2. To test whether HSC from fetal liver and adult bone marrow can transit the cell cycle at different rates (i.e. have different cell cycle transit times) a method for purifying fetal liver HSCs to near homogeneity was developed and HSC proliferation was then monitored by direct visualization
of these cells in single cell cultures. The results of these experiments are presented in Chapter 3.

A final series of experiments was undertaken to explore a possible mechanism for the changes in HSC behaviour documented in Chapters 2 and 3. Given the importance of SF-stimulation of adult HSCs to maximize the self-renewal of adult HSCs in vitro (175) in contrast to an apparent weak effect of deficient SF signaling on fetal HSCs (92;94) a change in SF signaling sensitivity might underlie the changes observed in HSC cycling control and self-renewal potential. To test this hypothesis, I first undertook experiments to compare the dependence of fetal liver and adult HSCs on SF stimulation in vitro and then asked how this dependence might be altered in HSCs from W^{41}/W^{41} mice that have defective c-Kit signaling and whether any differences observed might be predictive of their self-renewal behaviour as assessed using the secondary transplant endpoint developed in Chapter 2. The results of these experiments are presented in Chapter 4.
Figure 1.1: Hematopoiesis

A simplified overview of the concept of hematopoiesis that progresses from stem cells to mature blood cells. Subsets of lineages are shown.
**Figure 1.2:** Competitive repopulating unit (CRU) assay.

Schematic of the principle components of a CRU assay: recipients are irradiated and transplanted with graded doses of congenic cells; 16 weeks must elapse before the presence of multilineage donor-type cells detected in the peripheral blood of the recipient can indicate whether a CRU was present in the test cell population or not. A low dose of irradiation (400 cGy instead of 900 Cgy) eliminates the need to ensure recipient survival by co-injection of helper cells with the test population, but does not eliminate the competing endogenous stem cell pool. W41 recipients have compromised HSCs (attenuated c-Kit signaling), and can therefore be used in combination with a low dose of irradiation.
**Figure 1.3**: Limiting dilution analysis (LDA) to determine the frequency of CRU within a test population.

Poisson statistics describe the probability distribution of random counts and state that the frequency of negative outcomes (recipients that are not repopulated in all lineages long-term) is equal to $e^{-nf}$ (where $n$ equals the number of cells tested, and $f$ equals the frequency of stem cells (1 in $f$)). The log linear relationship of the frequency of negatives $= -n/f$. so when $n=f$, the frequency of negatives $= e^{-1} = 0.37$

CRU assay results can thus be plotted based on the above to determine at which test cell dose the proportion of negative outcomes is equal to 37%.
Table 1.1: Phenotypic similarities and differences between fetal liver and adult bone marrow HSCs

<table>
<thead>
<tr>
<th>Fetal Liver</th>
<th>Adult Bone Marrow HSCs</th>
</tr>
</thead>
<tbody>
<tr>
<td>B220^ Gr1^- Ly1^- Thy-1^- CD71^- Fall-3^- Ter119^- Nk1.1^- CD4^-</td>
<td></td>
</tr>
<tr>
<td>Sca-1^c-Kit^ MHC class I^ CD43^ WGA^</td>
<td></td>
</tr>
<tr>
<td><strong>C1qRp</strong></td>
<td><strong>C1qRp</strong></td>
</tr>
<tr>
<td><strong>AA4.1</strong></td>
<td><strong>AA4.1</strong></td>
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<tr>
<td><strong>Mac1</strong></td>
<td><strong>Mac1</strong></td>
</tr>
<tr>
<td><strong>CD45RB</strong></td>
<td><strong>CD45RB</strong></td>
</tr>
<tr>
<td><strong>Tie-2</strong></td>
<td><strong>Tie-2</strong></td>
</tr>
<tr>
<td>Rhodamine-bright</td>
<td>Rhodamine-dull</td>
</tr>
<tr>
<td>Hoechst-bright (non-side population)</td>
<td>Hoechst-dull or side population (SP)</td>
</tr>
<tr>
<td><strong>CD34</strong></td>
<td><strong>CD34</strong></td>
</tr>
<tr>
<td><strong>CD38</strong></td>
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CHAPTER 2

HEMATOPOIETIC STEM CELLS PROLIFERATE UNTIL AFTER BIRTH AND SHOW A REVERSIBLE PHASE-SPECIFIC ENGRAFTMENT DEFECT

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Kristen McKnight isolated E18.5 fetal bones, David Kent isolated RNA and generated cDNA from my samples, and Lindsay McCaffrey assisted with CRU analysis.
INTRODUCTION

Hematopoietic stem cells (HSCs) are defined as cells with multi-lineage hematopoietic differentiation potential and sustained self-renewal activity. Operationally, HSCs are detected by their ability to regenerate longterm multi-lineage hematopoiesis in myeloablated recipients. HSC numbers can be quantified by endpoints that measure this regenerative activity in genetically distinguishable, radio-protected hosts transplanted with limiting numbers of HSCs (1). HSCs are also characterized by extensive heterogeneity. Variability in many HSC properties is dictated by changes in their state of activation and the consequent changes in these properties are thus reversible. For example, most of the HSCs present in normal adult mice are in a deeply quiescent (G_0) state (2-4) and, in association with this status, they express CD38 but not CD34 or Mac1 (5;6). These G_0 HSCs also actively exclude certain fluorescent dyes, such as rhodamine-123 (Rho) (7;8) and Hoechst 33342 (Hst) (9). The latter property underlies the detection of adult mouse HSCs as "side population" (SP) cells (10). However, when HSCs are activated, they rapidly down-regulate expression of CD38 (6;11), increase expression of CD34 (12) and Mac1 (13;14) and acquire a Rho-bright, non-SP phenotype (15). In association with these changes, some of the HSCs begin to differentiate and hence permanently lose their longterm repopulating activity, but many do not, in spite of their transiently altered phenotype (16). Another property of HSCs that appears to vary reversibly is their ability to exit from the circulation into the bone marrow and re-initiate hematopoiesis. Quiescent adult mouse HSCs can execute this process at near unit efficiency in suitably myelosuppressed hosts, as shown by their ability to be detected at purities of ≥20% following intravenous injection (15;17;18). However, notable changes in HSC engrafting potential have also been found to accompany the progression of HSCs through the cell cycle both in vitro and in vivo (3;19-21). Specifically, HSC activity was not detectable in suspensions of adult or
neonatal hematopoietic cells in S/G2/M, even when substantial HSC activity could be found in the corresponding G1 cells. The transient nature of the silencing of HSC homing activity during the progression of these cells through S/G2/M is inferred from the fact that the populations studied did not contain G0 HSCs, or were expanding their HSC content, although formal documentation of the re-acquisition of repopulating activity by incapacitated S/G2/M HSCs was not documented. The molecular mechanisms that control the bone marrow-homing activity of HSCs are not fully elucidated, although a number of cell surface ligand-receptor interactions with known involvement in cell adhesion and chemotaxis have been implicated. These include stromal cell-derived factor-1 (SDF-1)/CXCR4, Steel factor (SF)/c-kit, CD44/Hyaluronic acid (HA) or osteopontin (OPN), VLA-4/V-CAM1 and P2Y-like receptor and an unknown ligand (22-25). The expression and activity of some of these appear to be variably affected by cytokine exposure (26;27); however, their specific involvement in the engraftment defect of HSCs in S/G2/M has remained unclear.

In the mouse embryo, pluripotent hematopoietic cells with longterm repopulating ability first appear in the aorta-gonado-mesonephros (AGM) region on the 9th day of gestation (28). These cells then migrate to the fetal liver and later to the bone marrow with continuing expansion of their numbers until young adulthood is reached (29). Most of the HSCs in the embryonic day (E) 14.5 liver have phenotypic characteristics of activated adult HSCs (CD38+, Mac1+, CD34+, Rho+, non-SP) (13;14), as might be expected for an expanding HSC population. The proportion of HSCs in the E14.5 fetal liver that are proliferating has been previously estimated from phenotyping studies to be ~40% (3), although a more direct measurement of this fraction as not been reported.

In this study, we sought to investigate the possibility that the HSC S/G2/M engraftment defect is constant through ontogeny, while the cycling status of HSCs is not.
As a first step towards elucidating the mechanisms that regulate changes in HSC turnover and engraftment properties during ontogeny, we designed experiments to assess their proliferative status in mice at different stages of development. Our results show that the entire HSC population remains in cycle until the 3rd week after birth regardless of the tissue in which the HSCs are located. Then within a week, the majority of the HSCs switch abruptly from an actively dividing to a quiescent state. Until this switch occurs, those HSCs that are in S/G2/M show the same engrafting defect previously demonstrated for adult HSCs that have been stimulated to divide. Interestingly, prior to the establishment of a quiescent HSC population, the HSCs in S/G2/M were found to express higher levels of SDF-1 than those in G1 and their defective engrafting activity could be completely reversed, either by holding them ex vivo for a few hours until they re-entered G1, or by pre-treating the host with a specific antagonist of stromal cell-derived factor-1 (SDF-1).

RESULTS

All HSCs in the E14.5 fetal liver are rapidly proliferating

To measure the proportion of HSCs that are in cycle in the E14.5 fetal liver, we used 3 complementary strategies. In the first, we injected pregnant mice on E13.5 with 100 mg/kg of 5-fluorouracil (5-FU) and then removed the fetuses 16 hours later, prepared cell suspensions from the fetal livers and measured the number of HSCs present using a limiting dilution transplantation assay for longterm (16-week) competitive repopulating units (CRUs) (1). In these experiments, we detected very few CRUs in the fetal livers of the 5-FU-treated embryos (Figure 2.1A, left panel, 3 experiments). A comparison of the yields of CRUs from the 5-FU-treated fetal livers with the control fetal livers from pregnant mice injected on E13.5 with
phosphate buffered saline (PBS) indicated that the 5-FU treatment had reduced the expected CRU population in vivo by more than 1000-fold.

We then assessed the cycling status of HSCs in the E14.5 fetal liver by measuring the proportion of CRUs that survived a 16-hour exposure to high-specific activity $^3$H-thymidine ($^3$H-Tdr) (30). Sixteen hours was anticipated to be sufficient to allow all cycling HSCs to enter S-phase, as confirmed later (see below), with minimal exit of any quiescent cells from G0 (31), as demonstrated for adult bone marrow HSCs, most of which are in G0 (Figure 2.1A, right panel). For these experiments, the Ter119$^+$ (erythroid) cells were first removed from the fetal liver cells to give a 10-fold enrichment in HSC content and the cells were then incubated in a serum-free medium supplemented with 50 ng/ml SF only. This growth factor condition was chosen based on other data demonstrating that freshly isolated E14.5 fetal liver CRUs are maintained at input numbers for 16 hours under these conditions (Chapter 4). The results of the $^3$H-Tdr suicide experiments showed that this treatment reduced the number of CRUs in the suspensions of E14.5 fetal liver cells more than 100-fold (P<.001, Figure 2.1A, middle panel), whereas the same treatment had no significant effect on the recovery of CRUs in similarly treated lineage-marker-negative (lin$^-$) bone marrow cells from young adult (10 week-old) mice by comparison to either control cells (incubated without $^3$H-Tdr, Figure 2.1A, right panel, P=0.17) or the starting values (data not shown).

We then assessed the distribution of CRUs between the G0 and G1/S/G2/M fractions of E14.5 Ter119$^+$ fetal liver cells. These subsets were isolated by fluorescence activated cell sorting (FACS) on the basis of their staining with Hst and pyronin Y (Py) (32). A representative FACS profile of the Hst and Py-stained cells is shown in Figure 2.2A. The combined results of in vivo assays of the sorted cells from 4 independent experiments are shown in the left part of Figure 2.3. These indicate that all of the transplantable CRU activity
was confined to the G1/S/G2/M fraction. Based on the total number of G0 cells assayed, the proportion of quiescent HSCs could be estimated to be less than 0.02%.

HSCs undergo a complete and abrupt change in cycling activity between 3 and 4 weeks after birth

Since HSCs are known to be present in the bone marrow of mice at later times of gestation, it was of interest to investigate whether HSCs first become quiescent in the fetus at that site. To address this question, we used the 16-hour \(^3\)H-Tdr suicide assay to determine the cycling status of the CRUs present in the bone marrow of mice at E18.5. For comparison, we also evaluated the cycling status of CRUs in the E18.5 fetal liver. The frequency of CRUs in these 2 tissues was 1 per \(10^5\) and 1 per \(7 \times 10^4\) total nucleated cells (Supplementary Table 1); i.e., \(-5\) and 3.5-fold lower than in adult bone marrow (1 per \(2 \times 10^4\) total nucleated cells (33)), and 6 and 4-fold lower than in the E14.5 fetal liver (1 per \(1.7 \times 10^4\) total nucleated cells (33)). After overnight exposure to high-specific activity \(^3\)H-Tdr, no CRUs could be detected in the suspensions of either the E18.5 fetal bone marrow cells or the E18.5 fetal liver cells, in contrast to the control cells incubated in the same medium without \(^3\)H-Tdr (Figure 2.1B). Thus all HSCs in the fetus, irrespective of their location, appear to be rapidly proliferating.

To further investigate the pace and timing of the transition of HSCs into a largely quiescent population, we analyzed the cycling status of CRUs in lin\(^-\) bone marrow cell suspensions from 3 and 4 week-old (weanling) mice. In initial experiments, the frequencies of CRUs in the lin\(^-\) bone marrow cells obtained from the 3 and 4 week-old mice were found to be the same (1 per \(6.5 \times 10^3\) and 1 per \(6.3 \times 10^3\) lin\(^-\) cells; Supplementary Table 2) and \(-2\)-fold lower than in the lin\(^-\) bone marrow cells from 10 week-old (young adult) mice (1 per \(2.9 \times 10^3\) lin\(^-\) cells). Bone marrow cells from 3 and 4 week-old mice were then fractionated by FACS
into their component $G_0$ and $G_1/S/G_2/M$ subsets based on the gates shown in the left panels of Figure 2.2B and 2.2C, and the sorted $G_0$ and $G_1/S/G_2/M$ cells were assayed separately for CRU activity. Re-analysis of the sorted $G_0$ and $G_1/S/G_2/M$ fractions after staining for Ki67 confirmed that the cells expressing this proliferation marker were confined to those we had designated as $G_1/S/G_2/M$ (see representative profiles in the right panels of Figure 2.2B).

Remarkably, the results of the in vivo assays showed that all of the CRUs detected in the bone marrow of 3 week-old mice were also confined to the $G_1/S/G_2/M$ fraction, whereas $>98\%$ of the CRUs in the bone marrow of 4 week-old mice were found in the $G_0$ fraction (Figure 2.3). Thus, there is a rapid down-regulation of CRU proliferative activity in the bone marrow of mice between 3 and 4 weeks of age with little change in CRU numbers.

**HSCs in S/G2/M show a specific and reversible engraftment defect regardless of their developmental origin or route of injection into assay recipients**

Given the previously reported engraftment defect of adult HSCs stimulated to enter S/G2/M (19), it was of interest to determine whether the number of proliferating HSCs present early in development might be routinely underestimated due an inability of those in S/G2/M to be detected. To investigate this possibility, the $G_1/S/G_2/M$ population of E14.5 Ter119° fetal liver cells was subdivided into its component $G_1$ and S/G2/M fractions and then each of these 2 subsets was assayed separately for CRUs. In this case, the gate settings chosen to separate the $G_1$ (2n DNA) and S-phase cells (>2n DNA) were validated by the profiles obtained when the sorted cells were stained with propidium iodide (PI) and reanalyzed by FACS (Figure 2.4A, left panel).

All CRU activity detectable in the $G_1/S/G_2/M$ fraction of Ter119° E14.5 fetal liver cells was confined to the $G_1$ subset (left bars in Figure 2.4B and control values in the left side of
Figure 2.5A). Similar experiments performed with lin\(^{-}\) bone marrow cells from 3 week-old mice showed that the CRUs in the G\(_1\)/S/G\(_2\)/M population from this source were likewise confined to the G\(_1\) fraction (control values in the right side of Figure 2.5A and data not shown). It is of interest to note that the S/G\(_2\)/M defect was specific to repopulating cells able to produce progeny in all lineages for at least 16 weeks. In contrast, cells with short term repopulating activity (8 weeks) were readily detected in the S/G\(_2\)/M fraction as well as in the G\(_1\) fraction, thus confirming the restriction of this cell-cycle-dependent engrafting defect to cells with sustained multi-lineage repopulating activity (34;35).

To determine whether the apparent engraftment defect of proliferating CRUs was reversible, we first assayed the CRU content of aliquots of the same isolated G\(_1\) and S/G\(_2\)/M cells after they had been incubated for 6 hours at 37\(^\circ\)C in serum-free medium containing 50 ng/ml of SF. During this time, many of the G\(_1\) cells progressed into S/G\(_2\)/M and many of the S/G\(_2\)/M cells moved into G\(_1\), as seen by their altered PI (Figure 2.4A, right panel) or Hst (data not shown) staining profiles. In vivo assays showed that CRU activity reappeared when the S/G\(_2\)/M cells were cultured for 6 hours, whereas the CRU activity originally present in the G\(_1\) cells was partially lost (middle and left bars of Figure 2.4B).

We next asked whether the inability of intravenously transplanted CRUs in S/G\(_2\)/M to engraft recipient mice might be overcome by injecting the cells directly into the femoral bone marrow space. However, intrafemoral injection did not enable any CRUs in this subset of Ter119\(^{+}\) El4.5 fetal liver cells to be detected (Figure 2.4B) even though the frequency of CRUs measured in the corresponding G\(_1\) El4.5 fetal liver cells after intrafemoral injection was the same as when the latter were transplanted intravenously (1 per 3.6 x 10\(^3\) cells versus 1 per 3.8 x 10\(^3\) cells).
The $S/G_2/M$ engraftment defect of HSCs can be overcome by pretreatment of the host with a SDF-1 antagonist

Previous reports have shown that SDF-1 can promote both the mobilization (36) and the homing (37-39) of HSCs. However, the mobilization of primitive hematopoietic cells can also be stimulated by blocking SDF-1/CXCR4 signaling, as achieved by in vivo administration of AMD3100, a SDF-1 antagonist (40). In addition, it has recently been shown that in vivo administration of AMD3100 can increase the competitive engrafting ability of transplanted marrow cells in unirradiated hosts (41). These suggested that targeting the SDF-1/CXCR4 pathway might also influence the variable engraftment properties of cycling HSCs, either by influencing the HSCs themselves or the transplanted host. To investigate these alternatives, we asked whether either pretreating the hosts or the cycling HSCs to be transplanted with a specific antagonist of SDF-1 might alter the level of repopulation obtained 16 weeks later.

The SDF-1 antagonist used in these experiments was SDF-1G2 (also called P2G because it is identical to SDF-1 except that the proline at position 2 has been converted to glycine (42)). SDF-1G2 is thus structurally quite different from AMD3100 but similar in its ability to block SDF-1 from binding to CXCR4 without activating CXCR4 (42;43). SDF-1G2 also shares with AMD3100 an ability to elicit effects on primitive hematopoietic cells both in vitro and in vivo (44). When mice were injected with 10 µg of SDF-1G2 (or PBS) 2 hours prior to the transplantation of FACS-sorted $G_1$ or $S/G_2/M$ cells and then analyzed for the presence of donor-derived blood cells 16 weeks later, the results for E14.5 fetal liver and 3-week mouse bone marrow cells were similar (Figure 2.5A). Treatment of recipients with SDF-1G2 had no effect on the repopulating activity of CRUs in $G_1$. In contrast, SDF-1G2 pretreatment of recipients of $S/G_2/M$ cells enabled long-term multi-lineage repopulation to be readily detected (7 and 4 of 10 mice transplanted with fetal liver and 3-week bone marrow $S/G_2/M$ cells,
respectively, vs. 0 of 10 in both sets of controls injected with PBS, in a total of 3 experiments). Moreover, the SDF-1G2 pretreated hosts showed levels of repopulation by both sources of S/G2/M cells that were indistinguishable from those seen in mice transplanted with G1 cells (Figure 2.6). On the other hand, if the SDF-1G2 treatment was applied directly to the cells to be transplanted for 30 minutes before they were injected, no difference in the engrafting activity of the transplanted G1 or S/G2/M cells was seen by comparison to untreated controls over a wide range of SDF-1G2 and SDF-1 concentrations tested, either with or without added SF (Figure 2.5B).

**HSCs in S/G2/M express higher levels of SDF-1 transcripts than HSCs in G1**

To begin to understand the mechanism behind the observed HSC S/G2/M engraftment defect and how it might be overcome by SDF-1G2 pre-treatment of the host, we isolated highly purified populations of HSCs from E14.5 fetal livers and from the bone marrow of 3 week-old mice (lin^- Sca-1^+ CD43^+ Mac1^+ cells representing ~20% pure HSCs, Bowie, MB and Eaves, CJ, manuscript in preparation) and sorted these into their corresponding G0/G1 and S/G2/M fractions as revealed by Hst staining. Aliquots of from ~200 to 800 cells were collected from each fraction in 3 independent sorting experiments and transcript levels for Gapdh, c-Kit, c-mpl, CD44, a4-integrin (a4int), VCAM-1, CXCR4 and SDF-1 were measured by quantitative real-time analysis of the cDNAs prepared from the isolated RNA extracts, as described in the Methods. Transcripts for all of these genes were consistently detected in both the G0/G1 and S/G2/M fractions of the highly purified suspensions of HSCs cell populations from fetal liver and 3 week bone marrow, including SDF-1, which had not previously been shown to be expressed by HSCs (Figure 2.7). Interestingly, SDF-1 was also the only one of the genes
assessed that was found to be expressed at significantly different levels in G_0/G_1 and S/G_2/M HSCs (9-fold higher in the latter, P<.05).

DISCUSSION
This study presents 2 new and clinically relevant features of HSC regulation. The first is the unanticipated sudden and complete change in HSC proliferative activity that occurs in juvenile mice between 3 and 4 weeks of age. Both the abruptness and the reproducibility of this change suggest an underlying mechanism that is tightly controlled and broadly active. It is notable that this change was not linked to the migration of HSCs during late embryogenesis from the microenvironment of the fetal liver to that of the bone marrow, but rather, was strictly associated with the developmental status of the donor. Thus, although differences between bone marrow and fetal liver niches and stromal cells have been sought and described (45–48), these differences do not appear to directly determine the cycling activity of the HSCs they are thought to regulate. The present data are more consistent with a model in which the mechanism of HSC cycling control in vivo is indirectly controlled by external cues, perhaps via changing stimulation of the stromal cells that then alter the signals they deliver, as suggested by studies of the longterm marrow culture system (44;49) and of elements of the bone marrow microenvironment in vivo (25). However, internally programmed changes in HSC responsiveness to external factors could also contribute to a developmentally controlled alteration in HSC cycling activity.

In humans, an abrupt change in HSC proliferative activity at an analogous point in development (2–4 years) has been inferred from measurements of the rate of decline in telomere length of circulating leukocytes (50). This suggests that the mechanisms involved in regulating HSC proliferative activity during ontogeny may be preserved across these species.
and the mouse will be a relevant model for their future elucidation. It is interesting to note that, in the mouse, a number of other changes in hematopoietic cell properties or output parameters have already been found to change during ontogeny in concert with this transition of the HSC compartment from a predominantly cycling to a predominantly quiescent population. These changes include the initial acquisition of an SP and Rho<sup>dim</sup> phenotype by HSCs (15), and the completion of appearance and rapid cycling of adult-type (Ly49<sup>+</sup>) natural killer cells and peripheral T-cells (51;52).

Many other differences in the properties of fetal and adult HSCs and the programs they dictate have also been described (13;53;54). It will clearly be of interest to determine the extent to which these may be programmatically linked to the mechanisms that precipitate the change in HSC cycling that occurs in mice between 3 and 4 weeks of age. Several genes have been implicated in the differential control of HSC behavior at different stages of development. These include genes encoding various transcription factors, i.e., Runx1 (55), Notch (56), Scl (57), bmi1 (58;59) and Gfi-1 (60;61), as well as the growth factor receptors, c-Kit (62;63) and Tie2 (64). Further delineation of the molecular basis of the unique programs operative in fetal HSCs and how these regulate fetal HSCs cycling are of major interest as this information could provide new strategies for expanding HSCs and offer potential insights into mechanisms of leukemogenesis.

The second significant set of findings emanating from our studies are the universality and pronounced extent of the engraftment defect found to characterize cycling HSCs in the S/G<sub>2</sub>/M phases of the cell cycle, the specificity of this effect for hematopoietic cells with prolonged versus short term repopulating activity, and the reversibility of this defect either following their progression into G<sub>1</sub>, or by pre-treating the host with a specific antagonist of SDF-1. Interestingly, the corrective effect of in vivo administered SDF-1G2 could not be
replicated by treatment of the cells with this agent prior to injection. The in vivo effect of SDF-1G2 could also not be mimicked by intrafemoral injection of the test cells. The inability of intrafemoral injection to overcome the defective engraftment of HSCs in S/G2/M suggests that this defect is likely mediated by events that affect the transplanted HSCs after they have entered the bone marrow environment.

Quantitative analysis of the level of expression of 7 candidate genes in the G1 and S/G2/M subsets of purified cycling HSCs from both fetal liver and 3 week bone marrow sources confirmed the expected expression of c-Kit, c-mpl, CD44, α4int, VCAM-1 and CXCR4 and further revealed that these cells also all contain SDF-1 transcripts. Moreover, although the transcript levels were not different between the G1 and S/G2/M fractions for c-Kit, c-mpl, CD44, α4int, VCAM-1 and CXCR4, a 9-fold increase in SDF-1 expression was noted in the S/G2/M HSCs. Previous work has suggested that the ability of transplanted HSCs to reach a niche within the bone marrow that can support their self-maintenance may depend on the strength of the SDF-1 gradient they encounter within the bone marrow space causing them to migrate towards the osteoblasts that line the bone (22). According to such a model, the ability of HSCs to express varying levels of SDF-1 in the absence of changes in their expression of CXCR4, might be anticipated to regulate their ability to respond to other more distal sources of SDF-1. Up-regulated expression of SDF-1 during the progression of HSCs through S/G2/M, as demonstrated here, might then be sufficient to interfere with an appropriate intra-bone marrow migratory response resulting in the rapid differentiation, death or irreversible sequestration of these cells in a site where they could not be stimulated to divide. Timed blockade of CXCR4 on cells within the bone marrow by injected SDF-1G2 might then be envisaged to increase in the level of intra-marrow SDF-1 to a point that transiently restores an effective chemoattractant gradient for the otherwise insensitive HSCs in S/G2/M. Such a
possibility has, in fact, recently been modeled in the zebrafish, where overexpression of SDF-1 in the germ cells was found to prevent the normal migration of these cells towards endogenous SDF-1 signals (65).

In the hematopoietic system, it is interesting to note that longterm repopulating SDF-1$^{-}$ HSCs could engraft irradiated hosts whereas only short term repopulation was obtained from CXCR4$^{-}$ cells (66;67). In addition, as would be predicted from the explanation we have advanced, forced overexpression of CXCR4 in retrovirally-transduced (i.e. proliferating) human HSCs was able to enhance the in vivo engrafting activity of these cells (68) and, conversely, treatment with antibodies to CXCR4 had the opposite effect (69). However, SDF-1 levels in the bone marrow are also subject to regulation, for example, as occurs following the administration of granulocyte colony-stimulating factor (G-CSF) (70).

Recently, Chen et al. (41) found that administration of AMD3100 pre-transplant can produce a modest improvement in the engraftment of quiescent adult bone marrow HSCs transplanted into non-irradiated hosts. The mechanism proposed was that the injected AMD3100 initiated the mobilization of endogenous HSCs within the marrow thereby improving the ability of the incoming transplanted HSCs to compete for niche occupancy. The studies of Chen et al. thus differed in several respects from those described here where we have observed an enhanced engraftment by HSCs that was more marked and exclusive to HSCs that were in S/G2/M at the time of injection. Therefore, it seems unlikely that the mechanisms responsible for the enhanced engraftment seen in both experimental models are similar, in spite of the fact that they are both mediated by treatment of the host with an SDF-1 antagonist.

The fact that proliferating human HSCs show the same engraftment defect when they transit S/G2/M is noteworthy (20) and underscores the clinical implications of these findings.
For example, our results predict that intrafemoral injection of transplants is unlikely to be a useful strategy for improving the therapeutic effectiveness of HSCs induced to expand in vitro. To date, interference of SDF-1 action by specific CXCR4 inhibitors has been used primarily for enhancing the yield of HSCs from donors for transplantation into myeloablated patients (71;72). Another application of such inhibitors suggested by the findings reported here could be to treat recipients of transplants of cycling cells. Thus significant benefit might also be derived by pretreatment of the host, particularly when transplants of genetically modified or cultured cells are to be administered since half of the HSCs in an asynchronously dividing population would be expected to be in S/G2/M.

MATERIALS AND METHODS

Mice. Ly5-congenic strains of C57Bl/6 mice were used as donors and recipients. All recipients were also homozygous for the $W^{41}$ allele. Mice were bred and maintained in microisolators with sterile food, water and bedding at the BC Cancer Research Centre according to protocols approved by the University of British Columbia Animal Care Committee.

Cells. Single cell suspensions were prepared in Hank’s balanced salt solution containing 2% fetal calf serum (FCS) (HF/2, StemCell Technologies). Enriched populations of HSCs were obtained by immunomagnetic removal of Ter119$^+$ or lin$^+$ cells from fetal liver and bone marrow cell suspensions, respectively (using EasySep™, StemCell Technologies). Antibodies used for isolation of lin$^-$ cells between 4 and to 10 weeks of age were anti-B220, Ter119, anti-Gr1, anti-Ly1 and anti-Mac1 (StemCell Technologies). To isolate lin$^-$ cells from 3 week-old
mice, the Macl antibody was omitted because Macl was known to be expressed on fetal and cycling HSCs (13;14;73).

*Tritiated* $^3$H-Tdr suicide assay. Cells were suspended at $10^6$/ml in Iscove's medium containing $5 \times 10^{-5}$ mol/l 2-mercaptoethanol, a serum substitute (BIT™, StemCell Technologies) and 50ng/ml murine SF (StemCell Technologies). Equal volumes were then incubated at 37°C, in 5% CO$_2$ in air for 16 hours in 35 mm petri dishes in the presence or absence of 20 µCi/ml of $^3$H-Tdr (25 µCi/mmol; Amersham). The cells were then harvested, washed twice with Iscove's medium containing 2% FCS and limiting dilution CRU assays performed.

*FACS isolation and analysis of cells in different cell cycle phases.* Cells were suspended in HF/2 containing 1 µg/ml Hst (Molecular Probes/Invitrogen) either 10 µmol/l fumitremorgin C (a gift from Dr. Susan Bates, NIH, Bethesda, MD) or 50 µmol/l reserpine (Sigma Chemicals) and then incubated at 37°C for 45 minutes. Pyronin Y (Py; Sigma) was added at 1 µg/ml and the cells incubated for another 45 minutes at 37°C. Cells were washed twice in HF/2 with 1 µg/ml PI (Sigma) in the second wash and were finally resuspended in HF/2 with PI and kept on ice in the dark until being sorted (PI cells only) on a 3 laser FACS Vantage (Becton Dickinson). For analysis of DNA content, cells were either re-stained with Hst only using the same protocol, or with PI at least 1 hour after storage at 4°C of cells that had been washed twice in ice-cold PBS with 0.1% glucose and fixed in 1 ml of ice-cold 70% ethanol. To stain the cells with PI, cells were washed twice with 2% PBS and resuspended in PBS with 0.1% glucose, 5 µg/ml PI and 200 µg/mL RNAse A. Cells were then incubated for at least 1 hour at 4°C and then analyzed directly on a FACSCalibur (Becton Dickinson). To stain sorted cells
for Ki67, the cells were washed and resuspended in 50 μl of ice-cold 80% ethanol and then incubated at -20° C for at least 2 hours. The fixed cells were washed twice in 300 μl of PBS with 1% FCS and 0.09% NaN3 (pH=7.2). Fluorescein isothiocyanate (FITC)-conjugated anti-human Ki67 antibody (Becton Dickinson) was then added and the cells incubated for 30 minutes at room temperature in the dark. Cells were then analyzed by FACS, using cell stained with a FITC-conjugated mouse IgG1 (Becton Dickinson) as a control.

In vitro treatment of S/G2/M HSCs. Sorted cells were incubated at 37°C in 5% CO2 in air in the wells of a round-bottom 96-well plate in serum-free media (as for the 3H-Tdr suicide assays) with one of the following 6 additions: 100 ng/ml SDF-1 (a gift from I. Clark-Lewis, Biomedical Research Centre, University of British Columbia, Vancouver, BC, Canada) or 300 ng/ml SDF-1G2 but no SF, or 50 ng/ml SF alone, or 50 ng/ml SF plus 100 ng/ml SDF-1 or 300 ng/ml SDF-1 or 300 ng/ml SDF-1G2. Cells were then harvested from the wells and equal aliquots injected into recipient mice such that each mouse received the equivalent of either 4 x 10^3 starting G1 cells or 1.2 x 10^4 starting S/G2/M cells.

CRU assay. Recipient (W^d1/W^d1) mice were sublethally irradiated with either 400 cGy of 137Cs γ-rays or 360 cGy of 250 kVp X-rays and then injected intravenously with the test cells except when injected intrafemorally as indicated. Intrafemoral injections were performed as described (74). CRUs were identified by their ability to have generated ≥1% donor Ly5-type blood cells including Ly1+ (T-cell), B220+ (B-cell) and Gr1/Mac1+ (granulocyte/monocyte) subsets that could be detected ≥16 weeks after transplantation (75). CRU frequencies were calculated using the L-calc program (StemCell Technologies) from the proportions of mice that were negative for this endpoint. Recipients treated with SDF-1G2 (a gift from I. Clark-
Lewis) were injected intravenously with 10 μg per mouse of SDF-1G2 dissolved in PBS 2 hours after being irradiated and were then transplanted another 2 hours later. This schedule was used in an attempt to minimize direct interaction of the injected HSCs with SDF-1G2 in the circulation (based on the likely rapid clearance of SDF-1G2) and maximize any potential effect on the host by keeping the interval between injecting the SDF-1G2 and the transplant as short as possible. Controls were injected with PBS instead of the SDF-1G2.

**Real-time PCR.** Cells were sorted into 1 ml HF/10 and RNA was isolated using the PicoPure™ RNA Isolation Kit (Arcturus Biosciences Inc.) as recommended by the supplier including a 15 minute DNAsel treatment (Qiagen) on the column at room temperature. RNA was eluted into an 11 μl volume and stored at -80°C. A cDNA preparation was then generated using the SuperScript™ III First-Strand Synthesis System for RT-PCR (18080093, Invitrogen) again as recommended by the manufacturer, with the reaction scaled up to use 25μl.

Quantitative real-time PCR was performed using the following primer pairs (5' to 3'): a4int (NM_010576.2) forward primer AGGACACACCAGGCATTCAT, reverse primer CCTCAGTGTTTCGTTTGGTG; CD44 (NM_009851.1) forward primer CTTTATCCGGAGCACCTTGCCACC, reverse primer GTCACAGTGCGGGAACTCC; c-Kit (NM_021099.2) forward primer ACAAGAGGAGATCCGCAAGA, reverse primer GAAGCTCAGCAAATCATCCAG; c-mpl (NM_010823.1) forward primer AGTGGCAGCACCAGTCATCT, reverse primer GAGATGGCTCCAGCACCTT; CXCR4 (NM_009911.2) forward primer CGGAGTCAGAAATGCCAGCAAGA, reverse primer CTGGTCAGTCTTTATATCTGGAAAA; Gapdh (NM_008084) forward primer AACTTTGGCATTTGTCAAAGG, reverse primer ATGCAGGGATGATGTTCTGG; SDF-1 (NM_001012477) forward primer GAGCCAACGTCAGCATCTG, reverse primer
CGGGTCAATGCACACTTGTC; **VCAM-1** (NM_011693.2) forward primer
TGATTGGGAGAGACAAAGCA, reverse primer AACAACCGAATCCCCAACTT. The
relative expression changes were determined with the $2^{-\Delta CT}$ method (76), and the
housekeeping glyceraldehyde-3-phosphate dehydrogenase (**Gapdh**) gene transcript was used
to normalize the results.

*Statistical analyses.* Comparisons were made using the Wald test.
Figure 2.1: All fetal HSCs are sensitive to cell cycle-specific drugs.

Cells from different mouse embryonic tissues were analyzed for CRU content either 16 hours after injection of the pregnant mother with 100 mg/kg 5-FU (or PBS), or after in vitro incubation of the cells for 16 hours with high-specific activity $^3$H-Tdr (or not). A. The left panel shows the effects of the 5-FU injection on E14.5 fetal liver (FL) CRUs, data pooled from 3 independent experiments. The middle panel shows the effects of $^3$H-Tdr on E14.5 FL CRUs and the right panel shows the (lack of) effect of $^3$H-Tdr on CRUs from adult (10-week-old) mice assessed in parallel (data pooled from 6 independent experiments). B. The left and right panels show the effects of $^3$H-Tdr on E18.5 fetal bone marrow and FL CRUs (data pooled from 4 and 4 independent experiments, respectively). The middle panel shows the complete data set from the limiting dilution analysis of the E18.5 fetal bone marrow cells.
Figure 2.2: FACS profiles of the distribution of different lin² populations in G₀, G₁ and S/G₂/M.

A. The left panel shows a representative FACS contour plot for E14.5 Ter119² fetal liver (FL) cells after staining with Hst and Py. Right panel shows the profile for the same cells after staining for Ki67. B. The left panel shows a representative FACS contour plot for lin² bone marrow (BM) cells from 3 week-old mice after staining the cells with Hst and Py. The middle panel shows the profile for the sorted G₀ cells after staining for Ki67 (>90% of the G₀ cells showed no Ki67 expression). The right panel shows the profile for the sorted G₁/S/G₂/M cells after staining for Ki67 (>99% of the G₁/S/G₂/M cells expressed Ki67). C. and D. Representative FACS contour plots for lin² BM cells from 4 week-old and 10 week-old mice after staining the cells with Hst and Py.
Figure 2.3: The cycling activity of CRUs is down-regulated between 3 and 4 weeks of age.

Results shown are the number of CRUs per $10^5$ initial total viable cells. For each tissue source, the difference in the yields of CRUs in the 2 subsets compared was significantly different ($P < 0.001$). For fetal liver (FL), these were depleted of Ter119$^+$ cells; for the 3 and 4 week-old bone marrow (BM) cells, all lin$^+$ cells except Mac1$^+$ cells had been removed and for, the 10 week-old BM cells, all lin$^+$ cells including Mac1$^+$ cells were removed. Values shown are the mean ± SEM from data pooled from at least 3 experiments per tissue.
Figure 2.4: Hoechst/Pyronin-sorted HSCs display an absolute but transient S/G2/M engraftment defect.

A. E14.5 Ter119⁺ fetal liver (FL) cells in G1/S/G2/M were fractionated into their component G1 and S/G2/M subsets, leaving a slight separation between them. Aliquots of the sorted subsets were then stained with PI (or Hst/Py, data not shown). The sorted cells were cultured for 6 hours and then were stained again with PI. This showed that, during this 6 hour culture period, approximately a third of the cells originally in G1 had progressed into S/G2/M and a similar proportion of the cells originally in S/G2/M had progressed into G1. B. CRUs per 10⁵ initial Ter119⁺ FL cells for G1 and S/G2/M fractions before and after 6 hours in culture. There was a 3.5-fold loss of CRUs when G1 cells were cultured for 6 hours (P<.01), but no loss when the cultured cells were re-sorted for G1 cells (P=.36). Conversely, we detected a >65-fold increase (P<.001) in the number of CRUs detected when CRUs in S/G2/M were cultured and a >128-fold increase (P<.001) when the cultured cells were sorted for G1 cells. Values shown are the mean ± SEM of results from at least 3 experiments.
Figure 2.5: The engraftment defect of HSCs in S/G2/M is corrected by treatment of the host, but not the cells, with SDF-1G2.

A. Effect of injecting prospective recipients, 2 hours post-irradiation and 2 hours prior to transplant with 10 ng/ml SDF-1G2(+) or PBS (-). Starting equivalents of 4,000 G1 cells per recipient mouse or 12,000 S/G2/M cells per recipient mouse were similarly tested. Results show a new ability of fetal liver (FL) HSCs in S/G2/M and 3 week bone marrow (BM) HSCs in S/G2/M to engraft only when they are transplanted into SDF-1G2 treated recipients, whereas treated recipients were no more likely to be engrafted long term by HSCs in G1 than were untreated recipients. Results are combined from 3 independent experiments.

B. Effect of in vitro treatment of sorted Ter119' FL cells in G1 or S/G2/M for 30 minutes at 37°C in serum-free medium plus various additives, as shown, on CRU detection. When present, SF was used at a concentration of 50 ng/mL, SDF-1 at either 100 ng/ml or 300ng/m and SDF-1G2 at 300 ng/ml. In vitro treatment had no significant effect on the number of mice that subsequently showed multi-lineage repopulation from starting cells in either G1 or S/G2/M. Results are combined from 3 independent experiments.
Figure 2.6: Donor-derived repopulation of SDF-1G2-treated mice.

Shown are representative FACS profiles of donor-specific cells detected after dual staining for the donor-type Ly5 allotype and various lineage-specific markers. 

A. Example of a positively engrafted PBS-treated recipient of fetal liver (FL) cells in G1. 

B. Example of a positively engrafted SDF-1G2-treated recipient of FL cells in S/G2/M. 

C. Example of a PBS-treated recipient of FL cells in S/G2/M that does not show donor-derived hematopoiesis.
Figure 2.7: Gene expression analysis of the G1 and S/G2/M subsets of highly purified lin- Sca1+CD43+Mac1+ HSCs from fetal liver and 3-week bone marrow.

Gene expression in G1 was set equal to 1 and the fold change in transcript levels in the corresponding S/G2/M fraction is shown. Results shown are the mean ± SEM of data from 2-3 biological replicates measured in triplicate. The difference between the level of SDF-1 transcripts between the 2 pairs of G1 and S/G2/M samples is significant, P<.05)
Table 2.1: Limiting dilution data for CRU frequency determinations for E18.5 fetal liver and bone marrow cells (data pooled from 5 experiments).

<table>
<thead>
<tr>
<th>No. of cells injected per mouse (x10^3)</th>
<th>No. of negative mice per total mice injected</th>
<th>No. of cells injected per mouse (x10^2)</th>
<th>No. of negative mice per total mice injected</th>
</tr>
</thead>
<tbody>
<tr>
<td>2800</td>
<td>1 / 9</td>
<td>4725</td>
<td>0 / 4</td>
</tr>
<tr>
<td>250</td>
<td>0 / 6</td>
<td>2800</td>
<td>1 / 9</td>
</tr>
<tr>
<td>80</td>
<td>5 / 20</td>
<td>2500</td>
<td>0 / 3</td>
</tr>
<tr>
<td>20</td>
<td>11 / 14</td>
<td>1575</td>
<td>1 / 4</td>
</tr>
<tr>
<td></td>
<td></td>
<td>800</td>
<td>6 / 12</td>
</tr>
<tr>
<td></td>
<td></td>
<td>200</td>
<td>9 / 15</td>
</tr>
</tbody>
</table>

CRU frequency (range defined by ± SEM): fetal liver 1 / 73,000 ± 1 / 59,000, bone marrow 1 / 95,000 ± 1 / 75,400.
Table 2.2. Limiting dilution data for CRU frequency determinations for lin⁻ bone marrow cells from 3 and 4 week-old mice (pooled data from 2 experiments).

<table>
<thead>
<tr>
<th>3-week bone marrow</th>
<th>4-week bone marrow</th>
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<tbody>
<tr>
<td>No. of cells injected per mouse (x10³)</td>
<td>No. of negative mice per total mice injected</td>
</tr>
<tr>
<td>12</td>
<td>0 / 3</td>
</tr>
<tr>
<td>4</td>
<td>4 / 6</td>
</tr>
<tr>
<td>CRU frequency (range defined by ± SEM)</td>
<td>1 / 6,500</td>
</tr>
<tr>
<td>(range defined by ± SEM)</td>
<td>+ 1 / 4,000</td>
</tr>
<tr>
<td>by ± SEM)</td>
<td>- 1 / 10,000</td>
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CHAPTER 3

HEMATOPOIETIC STEM CELLS TRANSITION ABRUPTLY AND
PROGRAMMATICALLY FROM A FETAL TO AN ADULT STATE.

The work presented in this Chapter was submitted for publication

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Pamela A. Hoodless and Connie J. Eaves

David Kent isolated the RNA and generated the cDNA preparations, Brad Dykstra assisted
with the analysis of stage-specific GM-contribution, Kristen McKnight isolated E18.5 fetal
bones and Lindsay McCaffrey assisted with CRU analysis.
INTRODUCTION

The identification in the 1950's of cells in adult mouse bone marrow that can individually regenerate all lineages of the blood and lymphoid systems provided the first direct evidence of a pluripotent hematopoietic stem cell (HSC) (1;2). Since that time much effort has been devoted to the development of quantitative assays for enumerating, characterizing and purifying HSCs. Currently there is general agreement that HSCs can be most specifically identified by endpoints that detect competitively and permanently (>4 months) repopulated immunologically compatible recipients whose endogenous blood-forming system has been seriously compromised (e.g., by irradiation and/or genetic modification). HSC quantitation can then be achieved using a limiting dilution approach coupled with a strategy for keeping the recipients alive with a minimally competitive source and dose of HSCs (3). This has allowed many shared biological properties and associated molecular features of HSCs from different stages of ontogeny to be identified (4). However, in spite of these common properties, extensive heterogeneity in their individual behaviour is a hallmark of every source of HSC thus far studied (5-8).

Changes in the behaviour of HSCs from different stages of development have also been recognized for many years. Of particular note is the finding that HSCs from midgestation mouse fetal livers (embryonic day (E)14.5) regenerate HSCs in irradiated recipients at a faster rate than is seen with transplants of adult bone marrow HSCs (9-11). This, in turn, results in an increased rate of regeneration of derivative myeloid progenitor cell types detected by short term colony assays both in vivo (colony-forming unit-spleen, CFU-S) and in vitro (colony-forming cells, CFCs) and their more mature progeny (6;11;12). The size, content and longevity of clones produced in vivo by individual HSCs likely reflects their immediate self-renewal history; that is, the frequency with which they have generated one or
more daughter HSC(s) during their expansion post-transplant. However, changes in other HSC properties during development could also affect their clonal outputs post-transplantation: for example, changes that control their proliferative activity, cell cycle time, survival, or lineage-restriction.

Comparisons of the responses of genetically-altered HSCs and their wild type counterparts stimulated in identical environments have led to a growing list of intrinsic determinants of HSC self-renewal activity (13-20), and a few of these have been reported to affect fetal and adult HSCs differentially (13;20). Definitive evidence that the self-renewal of HSCs can be modulated by the types and concentrations of growth factors to which the HSCs are exposed both in vivo (21) and in vitro (14;22-24) has also been demonstrated. Some of these latter effects differ on fetal and adult HSCs, such as in vitro conditions that support adult but not fetal HSC self-renewal (25). Interestingly, the timing of changes in the influence of specific determinants of HSC regenerative properties during development has not been previously explored. Recently, we showed that fetal mouse HSCs are maintained as a wholly cycling population until 3 weeks after birth, at which point they switch within one week to a largely quiescent population (as shown in Chapter 2). Here we show that this rapid change in HSC proliferative activity is accompanied by a similarly abrupt switch in the regenerative and differentiation properties these cells display after transplantation into irradiated recipients. In addition, we show that this switch occurs independently of the age of the host in which the HSCs are amplifying and is accompanied by pronounced changes in their transcriptional control.
RESULTS

Kinetic analysis of the different rates of HSC production by transplants from fetal and adult sources

Previous reports have provided some limited, albeit key information about the different kinetics of HSC regeneration in irradiated recipients of fetal and adult HSCs (6; 18), including effects of the dose as well as the source of the HSCs (CRUs) initially transplanted (26). Therefore, to maximize the differences displayed by HSCs from different sources, while also restricting other possible contributing variables, the number of CRUs transplanted into each primary W41 recipient was set at 10, regardless of the tissue from which they had been obtained (i.e., the contents of either $10^5$ Ter119<sup>+</sup> E14.5 fetal liver cells (27) or $10^5$ lin<sup>-</sup> adult bone marrow cells (28)). Groups of recipients were then sacrificed from one to 24 weeks later and the changing bone marrow content of in vivo amplified donor-derived HSCs determined by performing limiting dilution transplantation assays in secondary W41 recipients. This general experimental design is shown schematically in Figure 3.1A.

The pooled results from 4 such experiments revealed 2 distinct and highly consistent patterns of CRU amplification in primary recipients of fetal liver and adult bone marrow CRUs (Figure 3.1B). Initially (during the first week), there was no net change in the number of donor-derived HSCs detected in the bone marrow of the transplanted mice. Then, between the first and second week, the number of fetal liver-derived CRUs expanded rapidly (8-fold), whereas during the same interval, bone marrow-derived CRUs increased their numbers very little (1.5-fold). Thereafter, the rates of expansion of the 2 populations of CRUs in vivo became similar, doubling approximately every week, until saturation of the regenerated CRU content.
To determine whether the different kinetics of CRU regeneration obtained from transplants of fetal liver and adult bone marrow cells might be due to the co-transplantation of accessory cells unique to one of these tissues, the rates of regeneration of the progeny of each was re-examined in mice injected with both (i.e., transplants of Ly5.1 fetal liver cells containing 10 CRUs were co-injected with Ly5.2 adult bone marrow cells, also containing 10 CRUs, into both Ly5.1 and Ly5.2 W41 recipients) and the CRU output from each donor CRU type was then measured 2, 4 and 8 weeks later in secondary recipients. The results of this experiment with mixed primary transplants showed that the kinetics of CRU regeneration from the co-injected fetal liver and adult bone marrow CRUs were the same as when these cells were transplanted separately (data not shown).

Taken together, these results show that fetal and adult HSCs expand at markedly different rates in irradiated mice during the second week after transplantation and that these differing rates of expansion are cell autonomous.

Identification of an abrupt and programmed switch in the regenerative properties of HSCs between 3 and 4 weeks after birth

We next sought to investigate the timing and pace of change in HSC (CRU) regenerative properties from those typical of the CRUs present in the E14.5 fetal liver to those typical of the CRUs present in adult bone marrow. We therefore repeated the experiment shown in Figure 3.1A using bone marrow cells from mice at intermediate stages of development (i.e. E18.5 fetal bone marrow, and 3 and 4-week post-natal bone marrow) as the cells initially transplanted. In each case, preliminary experiments were undertaken to define the number of cells that contained 10 CRUs. This dose was then injected into each primary recipient and secondary limiting dilution transplants were performed from one to 4 weeks later to determine
the kinetics of expansion of HSCs derived from the injected cells initially transplanted into the primary recipients. These experiments focused on the initial 4-week period post-transplant because the greatest difference between the rate of expansion of fetal liver and adult bone marrow CRUs in vivo had been seen at this time (Figure 3.1B). As shown in Figure 3.2A and B, the results of the experiments with CRUs from donors at intermediate stages of development revealed that the E18.5 fetal bone marrow and week 3 post-natal bone marrow CRUs expanded with the same kinetics as the E14.5 fetal liver CRUs, whereas the week 4 post-natal bone marrow CRUs behaved just like the transplants of adult bone marrow CRUs.

We next asked whether this abrupt transition in the regenerative properties of CRUs was unique to the weanling mouse entering puberty or whether a similarly timed switch might also be seen during the expansion of fetal liver-derived CRUs in adult recipients. To discriminate between these possibilities, we transplanted secondary mice with 10 CRUs that had been regenerated in primary recipients of 10 fetal liver CRUs transplanted 6 weeks previously. We then assessed the kinetics of expansion of the progeny of these in vivo expanded fetal liver CRUs during their further expansion in the secondary recipients by performing limiting dilution CRU transplants in tertiary recipients (Figure 3.1A). This experiment showed that within 6 weeks, the CRUs derived from fetal liver CRUs had acquired the regenerative properties of adult bone marrow CRUs (Figure 3.2C). Thus, regardless of the age or physiological conditions of the host in which fetal liver CRUs proliferate, they generate a cohort of CRUs with “adult” CRU regeneration properties within the same time frame.
Identification of an abrupt and programmed switch in the differentiation behaviour of HSCs between 3 and 4 weeks after birth

To investigate whether the changes in HSC (CRU) regenerative activity were accompanied by parallel changes in their differentiation properties, we examined the different types of WBCs they produced at 16 weeks (Figure 3.3). The percentage of all donor WBCs that expressed Ly6G and/or Mac1 (GM) was more than 2-fold higher in recipients of fetal liver CRUs than in recipients of adult bone marrow CRUs. A fetal liver-like GM output was obtained in primary recipients of E18.5 bone marrow and 3 week-old bone marrow cells, as well as in secondary recipients of CRUs from mice transplanted with fetal liver CRUs 1-2 weeks earlier. In contrast, an adult-like output change of GM was seen in secondary recipients of CRUs from mice transplanted with 3 week-old bone marrow cells, 2 weeks earlier. Similarly, this adult-like output was seen in primary recipients of 4 week-old bone marrow cells, secondary recipients of adult bone marrow cells, and tertiary recipients of CRUs from secondary mice that had been transplanted 1-2 weeks earlier with bone marrow from primary recipients of fetal liver CRUs transplanted 6 weeks previously (refer to Figure 3.1A schematic – indicated by asterisks on the 3° line). Interestingly, a GM output intermediate between that exhibited by fetal liver and that by adult bone marrow CRUs was seen in secondary recipients of CRUs from primary mice that had been transplanted with 3 week-old bone marrow cells for just 1 week (chequered bar), suggesting that the CRUs ultimately tested were comprised of a mixture of fetal-like and adult-like CRUs. Collectively, these findings point to the operation of an intrinsically controlled switch that alters the differentiation properties of CRU between 4.5 and 5.5 weeks after E14.5 (i.e. approximately 3-4 weeks after birth), regardless of whether the fetal liver CRUs are left in situ in their endogenous developmental setting or are transplanted directly into a myeloablated adult recipient.
The cell cycle transit time of self-renewing HSC does not change during ontogeny

The different rates of HSC (CRU) expansion measured in recipients of fetal and adult CRUs could be influenced by several parameters: differences in the frequency of symmetric self-renewal divisions undertaken, differences in HSC cell cycle transit times and differences in HSC death. These are difficult parameters to measure in vivo but can be assessed in single cell cultures of highly purified CRU populations maintained under conditions that optimize each of these parameters. We have previously reported the kinetics of division of approximately 40% pure populations of adult bone marrow CRUs cultured under conditions that allow a 2- to 4-fold net expansion of their numbers (14;24). Experiments showed that El4.5 fetal liver CRUs could be purified to purity of approximately 10% by isolation of the lin’Sca-1‘Mac1’CD43’ subset as shown by both limiting dilution transplants and transplants of single purified cells (Figure 3.4A). In preliminary experiments (for a detailed description please refer to Chapter 4), we found that 50 ng/ml SF gave a full net maintenance of fetal liver CRU numbers after 48 hours in culture and this growth factor condition was superior in this regard to any of a variety of other growth factor cocktails tested, including that found to be optimal for adult bone marrow CRUs (i.e., 300 ng/ml SF + 20 ng/ml IL-11 ± 1 ng/ml Flt3-ligand (14;24)). The kinetics of cell division of fetal liver HSCs were then monitored visually in single cell cultures containing 50 ng/ml SF and the results compared to previously published data for adult bone marrow HSCs (14;24).

As shown in Figure 3.4B, under these conditions, fetal liver HSCs divided with the same 14-hour cell cycle transit time as adult bone marrow HSCs, but without any initial delay, due to the fact that all of the fetal liver HSCs are in cycle whereas all of the purified adult bone marrow HSCs are quiescent (see Chapter 2). In addition, it can be seen that there was essentially no cell death in these cultures as previously shown for optimally stimulated adult
bone marrow HSCs (24). Thus, under optimally mitogenic conditions that also preserve HSC activity, all of the fetal liver HSCs completed a first division within 28 hours whereas adult bone marrow HSCs would be just starting to divide at this time. More prolonged monitoring of the fetal liver HSC cultures showed that the fetal liver cells continued to proliferate in the same semi-synchronous fashion as previously shown for adult bone marrow HSCs and were thus completing their second and third divisions at the same times as adult bone marrow cells were found to be completing a first and second division, respectively (Figure 3.4C). Based on these findings, it seems likely that the expansion of fetal liver and adult bone marrow HSCs stimulated in the bone marrow of myeloablated mice also occurs with minimal HSC death, with the same cell cycle transit times for both sources of HSCs. Accordingly, differences in the frequency of symmetric self-renewal divisions would be inferred. Interestingly, the same kinetics of division were seen when these highly purified fetal liver cells were cultured in the cocktail used for adult bone marrow HSCs (i.e., 300 ng/ml SF + 20 ng/ml IL-11), even though this was found to lead to a rapid loss of HSC activity (see Chapter 3), suggesting that the cell cycle transit times of HSCs undergoing an initial differentiation step is the same as that of HSCs maintaining self.

Identification of differences in gene expression between purified fetal liver and adult bone marrow HSCs that also distinguish the same phenotypes of cells in the bone marrow of 3 and 4 week-old mice

Identification of an abrupt transition between 3 and 4 weeks after birth of a number of properties in HSCs that distinguish these cells in the fetal liver and adult bone marrow (see Figure 3.5) suggested that changes in these properties might be accompanied by corresponding changes in gene expression. To test this hypothesis, highly enriched E14.5 fetal liver (lin'Sca-
(lin−Rho<sup>dull</sup>SP) (24) HSCs populations were isolated (~20% and 40% pure, respectively), RNA extracts obtained and cDNA preparations made. Similar isolates were obtained from the lin−Sca-1<sup>+</sup>Mac1<sup>+</sup>CD43<sup>+</sup> and lin−Rho<sup>dull</sup>SP cells isolated from 3 and 4-week old bone marrow cells, respectively, based on the assumption that the phenotype of their HSCs, like their cycling (Chapter 2), self-renewal and differentiation properties (Chapter 3) would be similar to the phenotype of fetal liver HSCs (Chapter 3) and adult bone marrow HSCs (24), respectively. We then looked for changes in the levels of transcripts for a number of candidate genes. Several genes previously reported to play a role in HSC expansion (cyclinD2, Ikaros, rae-28 and MEF) were found to be more highly expressed (P<.05) in fetal liver and 3-week bone marrow HSCs as compared to HSCs in 4 and 10 week bone marrow (Figure 3.6). Bmi-1, c-Kit, Gfi-1, Notch1 and Ship showed no difference in expression in any of the HSC-enriched samples. However, a marked and permanent upregulation of expression of ATM, Ezh2, Gata-2 and Runx1 was noted in the purified HSCs of 4 and 10 week-old bone marrow as compared to their earlier counterparts (P<.05). Increased expression of Scl in adult vs. fetal HSCs was also noted, but expression of this gene was not tested in the 3 and 4-week bone marrow HSCs.

**DISCUSSION**

Here we have identified a unique time point in the expansion of HSCs when these cells undergo an abrupt and tightly regulated change in properties that control their ability to execute symmetric self-renewal divisions. After this point, the HSCs show a decreased ability to rapidly generate large outputs of progeny in long-term in vivo reconstitution assays. Concomitant with this change in self-renewal control is an alteration in the relative proportions of terminally differentiated cells they generate, as evidenced by a changed average
output of granulocytes and monocytes. Of note, the timing and unexpectedly abrupt kinetics of these co-ordinated changes in HSC properties mirror precisely the change in cycling status of HSCs in vivo (Chapter 2). Until 3 weeks after birth, the HSCs remain a wholly cycling population, but between 3 and 4 weeks after birth they are converted to a largely quiescent population. Taken together, these findings point to the existence of a novel and rapidly executed master switch that controls a spectrum of key HSC properties. Interestingly, although there is no previous report of such a switch at this time, several studies have described changes in other HSC properties around this time (expression of CD34, CD38, and podocalyxin and Hoechst33342 and Rhodamine-123 efflux properties) (29-35).

We also show here that when optimally stimulated, fetal liver HSCs (E14.5) and adult bone marrow HSCs have negligible apoptotic frequencies and identical cell cycle times (14 hours). In this latter respect, they differ from the more mature progeny that are generated from these cells which, in the fetal liver, clearly cycle more rapidly (36,37). Thus the enhanced rate of expansion of fetal liver HSCs after transplantation into irradiated adult hosts is likely due to differences in the intrinsic mechanisms that control their self-renewal ability, as opposed to differences in regulators of their cell cycle transit times. The concept of an intrinsically determined switch regulating HSC functions was further supported by experiments indicating that the rate of HSC regeneration post-transplant changes after a fixed period of HSC expansion, independent of the particular in vivo environment in which the expansion took place (Figure 3.2C).

To gain further insight into genes that might be involved in these developmentally programmed changes in HSC behaviour, we looked for correlated patterns of change in the expression of a number of candidates previously implicated in HSC control (reviewed in Chapter 1). This survey included the following specific genes: ATM, which has been shown to
play a role in mediating the stress-response of HSCs (38); *Bmi-1, mel-18, rae-28*, and *Ezh2*, PcG proteins thought to be important in the initiation (*Ezh2*) and maintenance (*Bmi-1, mel-18, rae-28*) of gene repression and involved in the regulation of HSC self-renewal (39-41); *cyclinD2*, a gene that is upregulated in proliferating cells and may contribute to HSC expansion when overexpressed (42); 2 genes that play critical roles in adult but not fetal HSCs; i.e. *c-Kit*, the receptor for SF, a ligand that promotes self-renewal signalling (13) and *Gfi-1*, a zinc finger repressor thought to restrict the proliferation of HSCs (43); *Gata-2*, whose expression in fetal and adult HSCs is driven by different promoters (44;45); *Ikaros*, a DNA-binding subunit critical for HSCs expansion (46); *MEF*, a gene whose absence in HSCs results in their increased quiescence (47); *Notch1* (48-50), *Runx1* (51-53) and *Scl* (54-57) 3 genes critical to the generation of HSCs in the fetus but not in the adult (Chapter 1); and *Ship*; of interest due to a possible role in HSCs (58) and of major interest to the lab of Dr. Gerry Krystal, also in the Terry Fox Laboratories.

These studies revealed a remarkably consistent pattern of gene expression in the 4 populations analyzed (E14.5 fetal liver HSCs and 3, 4 and 10 week-old bone marrow HSCs). Although changes in the expression of *Gata-2* were consistent with the fact that the promoter used by this gene changes during development (45), none of the results obtained for other categories of genes were predicted. For example: within the PcG genes: expression of *Bmi-1* was unchanged whereas expression of *rae-28* was down-regulated after 3 weeks and the opposite was true for *Ezh2* and *mel-18*. Previous evidence of differential roles in fetal liver and adult bone marrow HSCs (e.g., *Runx1, Scl* and *Notch1*) was similarly unpredictive. Nevertheless, for those genes whose expression did change, a remarkably consistent pattern was identified, as might be expected for a major switch in programming, in which genes involved in the expansion of HSCs through promotion of cell cycle or self-renewal (*CyclinD2*,
Ikaros, MEF and rae-28) were more highly expressed in HSCs with fetal liver-like properties and genes involved in HSC maintenance (ATM, Ezh2, Gata-2, and mel-18) were more highly expressed in HSCs with adult properties.

It is interesting to note that the levels of expression of ATM, Ezh2, and Gata-2 were particularly elevated in the HSCs from 4-week bone marrow. It is thus conceivable that the products of these genes, or the regulators of their expression, may play an important role in regulating the actual transition that takes place in these cells between 3 and 4 weeks of age. It will also be important to confirm the HSC purities in the 3 and 4-week bone marrow phenotypes analyzed and to investigate their potential functional effects on influencing the developmental switch herein described.

MATERIALS AND METHODS

Mice. C57Bl/6-Pep3B-Ly5.1 (Pep3B) and congenic C57Bl/6-W41/W41-Ly5.2 (W41) mice were bred, maintained and used in experiments as donors and recipients, respectively, in the Animal Resource Centre of the BC Cancer Agency according to protocols approved by the University of British Columbia in accordance with Canadian Council of Animal Care guidelines. All mice were kept under microisolation conditions and supplied with sterile food and water.

Cell preparation. Livers were removed from E14.5 Pep3b fetuses and fetal femurs from E18.5 Pep3b fetuses. Both tissues were placed in Hank's balanced salt solution (HF: StemCell Technologies) containing 2% fetal bovine serum (FBS) (HF/2), and a cell suspension obtained by forcing the tissue through a sieve using the plunger of a 3 ml syringe. Cell aggregates were removed using a 70 µm filter and, where indicated, Ter119+ (erythroid) cells were removed.
immunomagnetically (using EasySep reagents and equipment; StemCell Technologies) as recommended by the supplier. Bone marrow cells were harvested from 3 week, 4 week and 3-4 month-old Pep3B mice by flushing excised femurs and tibiae with Dulbecco’s Minimum Essential medium (DMEM) containing 2% FBS. Where indicated, Lin⁺ (B220⁺, Ter119⁺, Ly6G⁺, Mac1⁺, Ly1⁺) cells were removed immunomagnetically (using EasySep).

**Purification of HSCs.** E14.5 Ter119⁺ fetal liver and 3 week-old bone marrow cells were first incubated with biotinylated anti-Gr1 (RB6-8C5, granulocytes), anti-B220 (RA3-6B2, B lymphocytes), and anti-Ly1 (53-7.3, T lymphocytes), all of which were prepared in the Terry Fox Laboratory, TER-119, anti-CD4 and anti-NK1.1 (all from Becton Dickenson [BD]), and phycoerythrin (PE)-labelled anti-Sca-1 (BD) and various combinations of the following: fluorescein isothiocyanate (FITC)-conjugated anti-CD43, or anti-CD34 and allophycocyanin (APC)-conjugated anti-Mac1 or c-Kit (all from BD). 4 week-old bone marrow or 10 week-old bone marrow cells were similarly stained for lineage-antibodies, including Mac1, selecting those that are negative for these, and enriched for cells that did not retain the dyes Rhodamine and Hoechst 33342, as previously described (24). Staining was carried out with cells in ice-cold HF plus 5% rat serum (Sigma Chemicals) and 3 μg/ml Fc receptor blocking antibody (2.4G2) at 10⁷ cells/ml for at least 30 minutes in the dark. Cells were then washed in HF and incubated for an additional 30 minutes on ice with streptavidin-PE-TexasRed. Cells were washed again in HF/2 and then resuspended in HF/2 plus 2 μg/ml propidium iodide (PI, Sigma). Cells were kept cold and protected from light during the analysis and sorting on a FACS Vantage or FACS Aria (BD).
Transplantation and HSC quantification. W41 mice were irradiated with 360 cGy of 250 kVp X-rays and then varying numbers of Pep3b cells injected as indicated. HSCs were identified retrospectively by their ability to produce B, T and granulopoietic WBCs in the W41 mice for ≥16 weeks after staining peripheral blood (PB) samples with antibodies for donor (Ly5.1) and recipient (Ly5.2) CD45 allotypes plus lymphoid (B220 and Ly1) and myeloid (Gr1 and Mac1) cell surface markers, as previously described (Chapter 2). Mice were considered to be repopulated by ≥HSC when >1% of the total PB WBCs were Ly5.1+ and these included a contribution of ≥1% Ly5.1+ cells to the Ly1+, (Ly6G/Mac1)+ and B220+ populations. Donor-type HSC frequencies in the injected test populations were then determined from the proportions of recipients scored using Poisson statistics and the method of maximum likelihood (L-Calc software, StemCell Technologies) (28). To evaluate the frequency of HSCs from transplants of single purified cells, the cells were sorted using the FACS into the individual wells of a 96-well round-bottom plate containing 200 μl of serum-free medium (SFM) and visually confirmed to contain only one cell per well. SFM consisted of Iscove's MEM supplemented with a serum substitute (BIT™, from StemCell Technologies) and 10⁻⁴ M 2-mercaptoethanol. The entire contents of each well (1 cell + SFM) were then taken up into a 1ml syringe and injected intravenously into individual irradiated W41 recipients. In this case, the frequency of HSCs was calculated directly from the proportion of injected mice that showed multi-lineage Ly5.1 repopulation for 16 weeks. Values for total HSCs regenerated per transplanted mouse were calculated by multiplying HSC frequencies by 4x the number of cells recovered from 2 femurs and 2 tibias, assuming 2 femurs and 2 tibias contain ~25% of the total bone marrow cells in an adult mouse (59).
Short term cultures. Single lin⁻Sca-1⁺CD43⁺Mac1⁺ E14.5 fetal liver cells were deposited using the automatic cell deposition unit of a FACS Vantage directly into the individual round bottom wells of a 96-well plate containing SFM with either 300 ng/ml Steel factor (SF) and 20 ng/ml IL-11 or 50 ng/ml SF only. Wells were then maintained at 37°C for up to 50 hours and monitored by direct visualization at 4-hour intervals using an inverted microscope to determine the timing of the first and second cell divisions (the interval during which first 2, and later 3 or 4 cells were first seen in each well).

Real-time PCR. Cells were sorted into 1 ml HF/10 and RNA was isolated using the PicoPure™ RNA Isolation Kit (Arcturus Biosciences Inc.) as recommended by the supplier including a 15 minute DNAsel treatment (Qiagen). RNA was eluted into an 11 μl volume and stored at -80°C. A cDNA preparation was then generated using the SuperScript™ III First-Strand Synthesis System for RT-PCR (1808093, Invitrogen) again as recommended by the manufacturer, with the reaction scaled up to use 25μl. Quantitative real-time PCR was performed using the following primer pairs (5' to 3'): ATM (NM_007499.1) forward GCAGAGTGCTGAGGGTTTGT and reverse AACTTCCAGCAACCTTCACC; Bmi-1 (NM_007552.3) forward AAACCAGACCACCTCTGAACA and reverse TCTTCTTCTCTCATCTCATTTTTGA; c-Kit (NM_021099.2) forward GATCTGCTCTGCGTCCTGTT and reverse CTGATTGTGCTGGATGGATG; CyclinD2 (NM_009829.2) forward GGCCAAGATCACCCACACT and reverse ATGCTGCTCTGGAGGTGGATT; Ezh2 (NM_007971.1) forward CATCGAAGGCAGTGGAGTC and reverse GTCTGGCCCATGATTATTCTTC; Gata-2 (NM_008090.3) forward TGACTATGGGACGAGTCTC and reverse ACACACTCCCCCGCCTTCT; Gfi-1 (NM_010278.1) forward
CTGCTCATTCACTCGGACAC and reverse ATTTGTGGGGCTTCTCACCT; *Ikaros* (NM_001025597) forward CCTGAGGACCTGTCCACTACC and reverse ACGCCCATTCTCTTCATCAC; *MEF* (NM_019680) forward TCTGTGGATGAGGAGGTTCC and reverse GGGTGCTGGAGAAGAACTCA; *mel-18* (NM_009545.1) forward TTCCCCCTCTTAACGATTTG and reverse GATCCTGGAGGCTGTTCCT; *Notch-1* (NM_008714.2) forward GCACAA CTCCACTGATCCTG and reverse GCAAGGCCGACTTGCCTA; *rae-28* (NM_007905.1) forward GTCCAGGCCAGATGTAT and reverse CCCCAT TAGGCATCAGGA; *Scl* (NM_011527.1) forward TGAGATGGAGATT TCTGATGGTC and reverse CAAATGCCCCATTCACATT; *Gapdh* (NM_008084) was used as an endogenous control: forward AACTTTGGCATTGTGGAAGG and reverse ATGCAGGGATGATGTTCTGG.

*Statistical Analyses.* Comparisons were made using the Wald test except in Figure 3.3, where the students T-test was used.
Figure 3.1: Fetal liver HSCs self-renew to a greater extent than bone marrow HSCs in a transplant.

A. Schematic of the experimental design followed to measure the rate of self-renewal of 10 HSCs in vivo or 10 HSCs previously expanded for 6 weeks in an adult recipient from 10 starting fetal liver (FL) HSCs. B. Results shown are the number of donor CRU per recipient, as determined by secondary CRU assays, from the transplant of E14.5 FL (black circles) or 10 week-old bone marrow (BM) (open squares) after x weeks in a primary recipient. Values shown are the mean ± SEM of results from 4 experiments.
Figure 3.2: HSCs switch from fetal liver-like SR to adult bone marrow-like SR abruptly and intrinsically.

Results shown are the number of donor CRU per recipient, as determined by secondary CRU assays, from the transplant of 10 donor HSCs, assayed after weeks 1, 2, 3 and 4. For comparison, the similar results from 10 fetal liver (FL) (grey circles) and 10 bone marrow (BM) (grey squares) are shown. Values shown are the mean ± SEM of results from 2 experiments. A. E18.5 fetal bone marrow HSCs self-renew at the same rate as E14.5 fetal liver CRU. Open circles denote the E18.5 fetal bone marrow (FBM)-generated CRU per recipient. B. A clear transition in self-renewal potential from fetal liver-like to bone marrow (BM)-like is revealed between 3 and 4 week-old BM HSCs. Open diamonds denote the 3-week-old BM-generated CRU per recipient and closed diamonds denote the 4-week-old BM-generated CRU per recipient. C. After 6 weeks in a transplant, FL-derived HSCs self-renew at the same rate as BM CRU. Closed triangles denote the donor CRU. The experimental design is shown in Figure 3.1B.
Figure 3.3: Fetal liver-like and adult bone marrow-like HSCs generate distinct PB lineage contribution patterns when transplanted into irradiated recipients.

Bars indicate the mean %GM of donor WBC at 16 weeks post-transplant of 4 to 24 recipients of multiple (~3-6) HSCs of the source indicated. Error bars indicate the standard error of the mean. The percentage of all donor WBCs that express Ly6G and/or Mac1 (GM) is more than two-fold higher in recipients of fetal liver (FL) HSCs than recipients of adult bone marrow (ABM) HSCs (leftmost bars). With one exception, HSCs from other developmental stages and/or transplanted into secondary or tertiary recipients show distinctly FL-like or ABM-like repopulation patterns (remaining bars). Black bars indicate a significantly (p<0.05) greater GM contribution than primary recipients of ABM. White bars indicate a significantly lower (p<0.05) GM contribution than primary recipients of FL. Checkered bar indicates both a significantly greater % GM than ABM recipients and a significantly lower % GM than FL recipients.
Figure 3.4: Purification and culture of fetal liver HSCs reveals a similar cell cycle length as adult bone marrow HSCs.

A. Lin−Sca-1+CD43+Mac1+E14.5 fetal liver (FL) cells are enriched for HSC activity. Cells were isolated based on the indicated phenotypes and assayed for CRU content by either limiting dilution, or where appropriate, by single-cell injection. Values shown are the mean ± SEM of results from at least 2 experiments. LSCD43Mac1+ cells were assayed in 4 different experiments by limiting dilution and 5 different experiments by single cell injection. B. Schematic of the experimental set-up followed to measure the in vitro division kinetics of purified HSCs. C. In vitro division kinetics of single, pure (20%) FL HSCs compared to single, pure (40%) bone marrow (BM) HSCs reveals a same cell cycle transit time. The percentage of cumulative divisions is shown with respect to time in culture. FL HSCs are shown as open circles when cultured in self-renewal conditions of 50 ng/ml SF and closed circles when cultured in differentiating-conditions of 300 ng/mL SF and 20 ng/mL IL-11.
A distinct and abrupt transition in HSC behaviour occurs both endogenously and post-transplantation, after the same length of time. HSCs switch from fetal liver-like to adult bone marrow-like behavioural characteristics. Pre-switch HSCs are characterized by actively cycling, high rates self-renewal in transplants and high GM-regeneration per donor-derived WBCs. Post-switch HSCs are characterized by a large percentage in quiescence, low rates of self-renewal in transplants and low GM-regeneration per donor-derived WBCs.
Figure 3.6: Gene expression analysis of purified fetal liver, 3-wk, 4-wk and 10-wk-old bone marrow HSCs.

Gene expression in the adult bone marrow (ABM) or 4 week bone marrow (BM) HSCs was calculated as a fold-change relative to gene expression in the fetal liver (FL) or 3 week BM HSCs, respectively, with values within each comparison normalized to levels of Gapdh expression from each. A. Shown are the results for changes in gene expression between FL and ABM HSCs, with level of gene expression from FL HSCs set equal to 1. B. Shown are the results for changes in gene expression between 3 week BM HSCs (set to 1) and 4 week BM HSCs. Results shown are the mean of the biological duplicates of the mean of the individually measured triplicates ± SEM of the duplicates.
**Table 3.1:** CRU frequency determinations for purified (1/10) single fetal liver HSC-derived clones under two conditions (pooled data from 2 experiments).

<table>
<thead>
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<th>Condition 1: 50 ng/ml SF</th>
<th>Condition 2: 300 ng/ml SF + 20 ng/ml IL-11</th>
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<tr>
<td>No. positively-engrafted mice per total tested</td>
<td>No. positively-engrafted mice per total tested</td>
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<tr>
<td>Single clones</td>
<td>Pooled clones (2)</td>
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<td>2 / 18</td>
<td>4 / 10</td>
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<td>CRU : 1 / 10</td>
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REFERENCES


CHAPTER 4

STEEL FACTOR RESPONSIVENESS REGULATES THE HIGH SELF-RENEWAL PHENOTYPE OF FETAL HEMATOPOIETIC STEM CELLS

The work presented in this Chapter was submitted for publication

Michelle B. Bowie and Connie J. Eaves

David Kent isolated the RNA and generated cDNA.
INTRODUCTION

In the studies described in Chapters 2 and 3, a developmental switch that appears to simultaneously alter two key HSC properties - proliferative status and self-renewal potential - was identified. To investigate the molecular basis of this switch, with respect to the change in self-renewal potential, an in vitro model that supports fetal HSC self-renewal divisions was sought. Much effort has been directed to analyzing the growth factor requirements for maintaining bone marrow HSCs in culture and cocktails that support moderate expansion of these cells have been identified (1-11). Similar efforts with fetal liver HSCs are more limited and have shown that their growth factor responses are quite different from those of adult HSCs (12). The disparity between the high self-renewal capacity of fetal liver HSCs in vivo (13-15) and the inability to achieve their maintenance in vitro has led to a series of experiments testing the potential of various cell types to substitute for soluble growth factors in this regard (1;16;17), followed by the report that IGF-2, a product of some supportive cell types, might be effective (17).

The W41 mouse has a single point mutation in the tyrosine kinase domain of the SF receptor, c-Kit, resulting in attenuated signalling following ligand binding as compared to the wild-type (+/+) receptor (18). The importance of c-Kit activation for stimulating adult HSC self-renewal has been well documented (4;9;19;20). The effect of the W41 mutation on HSC generation in vivo is much less severe in the fetus than in the adult (19). Evidence of a minimal requirement of fetal liver cells with a HSC phenotype to be stimulated by SF in order to expand their numbers has also been reported (21). These findings led us to hypothesize that the SF responsiveness of HSCs might also be programmed to switch; to a decreased state. This decrease in SF responsiveness in HSCs might thereby reduce both their ability to execute symmetric self-renewal divisions and their ability to be mitogenically activated and therefore
be an important regulator of the switch in HSC properties described in Chapters 2 and 3. The studies described in this Chapter were designed to test this possibility that a change in SF responsiveness can account for the change in HSC cycling status and/or self-renewal activity as shown in Chapters 2 and 3.

RESULTS

Fetal liver CRUs are more sensitive to SF than adult bone marrow HSCs

In a first series of experiments, 11 cocktails of 5 different growth factors were assessed in 12 experiments for their ability to maintain fetal liver CRUs in culture for at least 48 hours. The results are shown in Figure 4.1. Consistent with previous reports, most cocktails failed to sustain a significant level of CRU activity over a 48 hour period in culture. However, an important exception to this result was obtained when only SF was present at a concentration of 50 ng/ml. Under this condition, all the fetal liver HSCs divided (see Figure 3.4C), but the number of CRUs present after 2 days was maintained. Intriguingly, 50 ng/ml of SF in the presence of 20 ng/ml IL-11 was not able to maintain fetal liver CRUs for 48 hours in culture. This suggests that 20 ng/mL IL-11 is inhibitory to fetal liver CRU self-renewal, in the presence of 50 ng/ml SF alone. To more fully define the SF sensitivity of fetal liver HSCs, a full dose response curve was then generated using the same experimental design. As shown in Figure 4.2, the generation of daughter CRUs in these cultures was optimal when SF was present at a concentration of 50 ng/ml SF, with significantly reduced CRU outputs at 2x higher or 5-fold lower SF concentrations. By comparison, optimal self-renewal of cultured CRUs from adult bone marrow requires 300 ng/ml SF (4). Thus the maintenance of in vivo repopulating activity by fetal liver and adult bone marrow CRUs stimulated to divide is highly
dependent on SF activation but, in this regard, fetal liver CRUs are 6-fold more sensitive to SF than their adult counterparts.

**Fetal liver and adult bone marrow HSCs express the same levels of c-Kit.**

Next, a series of experiments were undertaken to determine whether the different responses exhibited by fetal liver and adult bone marrow HSCs could be attributed to differences in their expression of the SF receptor, c-Kit. Accordingly, the lin$^{Sca1^+CD43^+Mac1^+}$ and lin$^{Rho^dull SP}$ subpopulations of fetal liver and adult bone marrow cells, respectively, from Pep3b (+/+ ) mice (~20% and ~30% pure HSCs, as described in Chapter 3 and (22), respectively) were isolated and the levels of c-Kit mRNA and cell surface protein then determined (Figure 4.3). Quantitative RT-PCR measurements of c-Kit transcript showed no difference between the 2 highly enriched HSC populations compared. Flow cytometric analysis of the level of c-Kit protein expressed on the cell surface in the same highly purified CRU populations yielded similar results to the mRNA data, as expected from historical data obtained from less purified populations (Figure 4.3 and (21)).

**W41 fetal liver CRUs mimic +/+ adult bone marrow CRUs in their SF requirement for self-maintenance in vitro**

The W41 mutation encodes a form of c-Kit that confers a reduced signalling capacity of this receptor following SF binding; W41 CRUs would thus be characterized by a reduced SF sensitivity than their +/+ counterparts. Given that W41 mice have normal numbers of HSCs in the fetus but not in the adult, this reduced SF sensitivity might only affect HSC expansion once these mice age past 3 weeks, the same time point at which self-renewal potential, cycling activity and multilineage potential decrease. To directly assess the SF responsiveness of W41
CRU self-renewal at different stages of development, we used the same type of dose-response analysis of 2-day cultured cells performed on +/- CRUs (Figure 4.2). These experiments showed that the E14.5 fetal liver CRUs from W41 mice have a SF sensitivity very similar to adult bone marrow CRUs from +/- mice, with a peak response in terms of W41 CRU self-maintenance in cultures that contained 500 ng/ml SF. When adult bone marrow CRUs from W41 mice were tested in the same way, their SF sensitivity was found to be at least 10-fold further reduced. Thus, in spite of a greatly reduced ability to activate normal pathways downstream of c-Kit, the CRUs in W41 mice appear to undergo the same switch in SF responsiveness that characterizes the development of adult CRUs from +/- mice. To determine whether the decreased SF responsiveness of CRUs in the W41 fetal liver relative to +/- fetal liver CRU might nevertheless be sufficient to maintain these cells in cycle in vivo, lin^-Scal^+CD43^+Mac1^+ fetal liver cells were isolated from W41 E14.5 fetal livers and then stained with Hst to determine the distribution of G0/G1 and S/G2/M cells within this fraction of +/-CRU-phenotype cells. Although the CRU content of this phenotype in the W41 fetal liver was not assessed, the frequency of these cells among the Ter119-depleted cells was the same as in the corresponding +/- Ter119-depleted cells (1/4115 ± 913 W41 fetal liver cells, n=2 vs. 1/4944 ± 834 +/- fetal liver cells, respectively), consistent with a similar frequency of CRUs (19). The Hst staining revealed an approximately equal proportion of HSCs between the G0/G1 (2N DNA) and S/G2/M (>2N DNA) fractions (Figure 4.4), indicating that the W41 fetal liver HSCs are a cycling population, despite lower SF responsiveness.
The proliferative activity of W41 CRUs is not impaired in the fetus but the self-renewal in vivo mimics the characteristic reduced activity of adult +/+ CRUs

W41 HSCs are hyposensitive to soluble SF in vitro, like +/+ adult bone marrow HSCs, but their sensitivity to the more potent, membrane-bound SF (23) under transplantation conditions in vivo might be similar to +/+ fetal liver. We therefore examined the self-renewal activity of W41 fetal liver CRUs in vivo, using the same serial transplant design outlined in Chapter 3. Accordingly, the 10^4 fetal liver cells (estimated to contain 10 W41 fetal liver CRUs (19)) were transplanted into lethally irradiated Pep3b mice and then at weekly intervals up to 4 weeks later, the number of W41 CRUs regenerated in the bone marrow of these hosts was measured by performing limiting dilution CRU assays in secondary Pep3b recipients. The results, shown in Figure 4.5, indicate that the rate of W41 fetal liver CRU self-renewal in vivo was the same as previously characterized for CRUs from +/+ adult bone marrow (Chapter 3), not +/+ fetal liver.

DISCUSSION

Based on observations in Chapter 3, suggesting that fetal liver and adult bone marrow HSCs self-renew using different mechanisms, an initial objective was to identify critical signalling pathways for fetal liver HSC self-renewal. In vitro analysis of potentially supportive growth factor conditions revealed that 50 ng/mL of SF, in the absence of other growth factors, can maintain fetal liver HSCs for 48 hours. This condition obviously does not recapitulate the HSC-supportive environment of the E14.5 fetal liver and suggests that a variety of other parameters may be required for optimal regenerative potential, such as cell-cell interactions. It did, however, draw attention to the fact that 50 ng/mL is a lower dose than that previously described as optimal for adult HSCs (4).
The objective of the experiments was then to determine whether a change in SF signalling might explain the reduced proliferative activity and self-renewal potential acquired by HSCs during development. Here we demonstrate that a change in SF signalling alone is not sufficient to alter the proliferative activity of HSCs, as W41 E14.5 fetal liver HSCs are a cycling population, but we provide evidence that the reduced self-renewal potential of HSCs, both in vitro and in vivo, can be caused by a reduced sensitivity to SF. Dose response curves for SF-dependent HSC self-renewal in vitro revealed that the sensitivity of fetal liver and adult HSCs differed by a factor of approximately 6 (50 ng/mL vs. 300 ng/mL), occurred independently of the amount of c-Kit expressed by these cells and correlated with their self-renewal behaviour in vivo as assessed quantitatively in a transplant-based regeneration assay. As predicted by earlier studies, this change in SF sensitivity was not mediated by a change in c-Kit expression. Therefore, it seems more likely that these differential effects of SF are due either to a developmentally regulated change in the surface organization of c-Kit, or in the coordinated integration of c-Kit activation within additional signalling pathways, or in downstream events that mediate effects of c-Kit activation on self-renewal decisions. For example, in the latter case, a downstream effector of c-Kit might be envisaged to become limiting as a result of the fetal-to-adult switch. Previous studies have shown that the concentration of SF required to maximize the mitogenesis of adult HSCs is much lower than what is required to maximize their self-renewal in vitro (4). This differential requirement for c-Kit activation might explain why the reduced signalling capacity of W41 fetal liver HSCs would be sufficient to sustain their proliferation at a high rate in the fetus, in spite of an impaired self-renewal activity when stimulated to grow in the microenvironment of the irradiated adult bone marrow. The findings of Iscove and Nawa that the self-renewal of adult
bone marrow HSCs from +/+ mice could be enhanced by in vivo administration of SF (and IL-11) is consistent with this hypothesis (20).

It is important to acknowledge that soluble SF is not as potent as membrane-bound SF in c-Kit signalling and in vivo, membrane-bound SF is critical to adequately sustain hematopoiesis (23). Therefore, the SF concentration requirements documented here and in previous studies (4) presumably mirror those required to match the signalling achievable from cell-bound SF in vivo.

Although not pinpointed here, we propose that the change in SF responsiveness is likely to occur abruptly between 3 week and 4 weeks after birth, similarly to the observed change in self-renewal potential at this time point (Chapter 3). This could easily be determined by future comparison of the SF dependence of HSC self-renewal in vitro from 3 and 4 week-old mice.

MATERIALS AND METHODS

Animals. C57Bl/6:Pep3B (Ly5.1) and W41 (Ly5.2) mice were used as donors and recipients, respectively, or vice versa, as indicated. Cell suspensions were prepared from E14.5 fetal liver and the bone marrow of 10 week-old adult mice as described in Chapter 2.

CRU assay. When Pep3B mice were used as recipients, they were irradiated with 2 doses of 400 cGy X-rays, separated by 4 hours. W41 recipients were irradiated once with 360 cGy X-rays. All transplants were injected intravenously. Recipients were analyzed for multi-lineage (Gr1, Mac1, B220, Ly1) donor-derived repopulation after 16 weeks and CRU frequencies calculated as described in Chapter 2.
HSC cultures. Fetal liver cells depleted of Ter119\(^+\) cells and W41 bone marrow cells depleted of lin\(^+\) cells (EasySep, StemCell Technologies) were cultured at a concentration of 10\(^6\) cells/ml, for 48 hours in serum-free medium with various growth factors as indicated. After 48 hours, cells were harvested and CRU assays were performed.

RNA analysis. The lin\(^-\)Sca-1\(^+\)CD43\(^+\)Mac1\(^+\) fraction of E14.5 fetal liver cells and the lin\(^-\)Rho\(^\text{dull}\)SPCD45\(^{\text{mid}}\) fraction of adult bone marrow cells were isolated by FACS as described in Chapter 2 and (22), respectively. RNA was isolated, transcribed into cDNA and quantitative-RT-PCR was then performed (as described in Chapter 2). Primers for c-Kit (NM_021099.2) were as follows: forward ACAAGAGGAGATCCGCAAGA and reverse GAAGCTCAGCAAATCATCCAG, for Gapdh (NM_008084) were as follows: forward primer AACTTTGGCATTGTGGAAGG, reverse primer ATGCAGGGATGATGTTCTGG.

Protein expression. E14.5 fetal liver and week 10 bone marrow cells were stained with lin\(^-\) (no Mac1) Sca1\(^+\)CD43\(^+\) and lin\(^-\) SP Rho\(^\text{dull}\) as described in Chapter 2 and (22), analyzed by FACS and the geometric mean of their c-Kit fluorescence intensities determined and compared.

Cell cycle analysis. The lin\(^-\)Sca1\(^+\)CD43\(^+\)Mac1\(^+\) fraction of E14.5 Ter119\(^+\) fetal liver cells from were co-stained with Hoechst 33342 and their distribution in G\(_0\)/G\(_1\) vs. S/G\(_2\)/M assessed using gates to distinguish cells with 2n vs. >2n DNA, respectively, as described in Chapter 2.

Assays of HSC self-renewal in vivo. 10\(^5\) unfractionated fresh W\(^{df}\)/W\(^{df}\) fetal liver cells, containing an estimated 10 CRUs (19), were injected intravenously into irradiated primary Pep3b hosts and then 1, 2, 3 and 4 weeks later, groups of these were sacrificed and the bone
marrow cells from these primary recipients was harvested from all four leg bones. Single cell suspensions were prepared and tested for regenerated $\mu^d/\mu^d$ CRU content by limiting dilution assays performed in secondary irradiated Pep3b recipients, as described above and in Chapter 3.

Statistical Analysis. Comparisons were made using the Wald test
Figure 4.1: Comparison of the effects of different growth factor cocktails on fetal liver CRU self-maintenance in vitro

The number of CRUs recovered after 48 hours from each culture is expressed as a percent of the input number. CRU numbers were determined by 16-week limiting dilution transplantation assays as described in Chapter 2. Values shown are the mean ± SEM of results pooled from 2 to 5 experiments. Growth factor concentrations are in ng/ml.
Figure 4.2: Steel factor dose response curves for the in vitro self-renewal of +/+ and W41 CRUs from fetal liver and adult bone marrow.

Data shown for each source of CRUs are expressed as a percent of the number of CRUs recovered in the culture that yielded the maximum CRU output: +/+ fetal liver (FL) (open circles); W41 FL (solid circles); W41 bone marrow (BM) (solid squares). Data for +/+ BM is redrawn from results presented in (4).
Figure 4.3: Comparison of c-Kit expression on fetal liver and adult bone marrow HSCs.

A. Representative profile of E14.5 Ter119- fetal liver (FL) viable, lin− cells assessed for Sca1 and CD43 expression, further analyzed for intensity of c-Kit protein expression. B. Shown are the results of fold-change in cKit gene expression relative to gapdh between purified Lin− Sca1+CD43+Mac1+ FL HSCs (as shown in A) and Lin−Rho+ SP bone marrow (BM) HSCs (as shown in C) on the left, and the geometric mean fluorescence intensity (M.F.I) of c-Kit protein expression in each of these same populations. C. Representative profile of adult BM viable cells, selected within the Lin−Rho+ SP fraction, further enriched as SP cells and assessed for intensity of c-Kit protein expression.
Figure 4.4: Cell cycle analysis of W41 fetal liver HSCs.

Shown is a representative staining of viable, lineage$^-$ E14.5 W41 fetal liver cells (n=2). These cells were further enriched for Mac1$^+$ cells and their distribution in G$_0$/G$_1$ vs. S/G$_2$/M was assessed by Hoechst staining.
Figure 4.5: W41 fetal liver HSCs self-renew at the adult bone marrow HSC self-renewal rate.

Results shown are the number of donor CRU per recipient, as determined by secondary CRU assays, from the transplant of 10 donor W41 fetal liver (FL) HSCs, assayed after weeks 1, 2, 3 and 4. For comparison, the similar results from +/+ FL (dotted line) and +/+ bone marrow (BM) (dashed line) are shown, as described in Chapter 3. Values shown are the mean ± SEM of results from 4 experiments.
REFERENCES


CHAPTER 5

CONCLUSIONS AND FUTURE DIRECTIONS

In this thesis, I have presented the results of experiments that, for the first time, define the timing of changes in HSC proliferative activity, self-renewal and differentiation behaviour that occur during development. These have revealed the unexpected discovery of an abrupt and coordinated change in all of these properties between 3 and 4 weeks after birth. For each of these parameters, a quantitative assay/endpoint was devised and then HSCs from fetal, weanling and young adult mice were assessed with respect to each parameter.

The first evidence of this “switch” came from studies of the proliferative activity of the HSCs present at each of these stages of development, as assessed using 3 different methodologies (as described in Chapter 2), and subsequently confirmed by gene expression analyses of highly purified HSCs populations isolated from these different sources. These studies indicated that all HSCs are maintained in a state of constant turnover until 3 weeks after birth regardless of their location (in the liver or bone marrow) but then, within a period of one week >80% have transitioned into a quiescent state without further change in this level of proliferative activity through to adulthood.

Interestingly, when the self-regenerative properties of HSCs from the same stages of development were subsequently examined (Chapter 3), these were also found to transition from a fetal phenotype to an adult phenotype at exactly the same time. Thus, it was possible to show that the HSCs in transplanted bone marrow from 3 week-old mice displayed the rapid HSC-regenerating activity characteristic of fetal HSCs, whereas the results for similarly assessed HSCs from the bone marrow of 4 week-old mice were superimposable on the data for...
HSCs from adult bone marrow, which display a much slower HSC-regenerating activity. In addition, I undertook a series of experiments with highly purified fetal liver HSCs in vitro which demonstrated that, unlike their differentiating progeny (1;2), the cell cycle transit time of optimally stimulated fetal and adult HSCs is identical. These experiments are important as they strongly suggest that the different rates of HSC regeneration obtained by fetal and adult HSCs in the bone marrow of irradiated adult mice is determined by differences in their self-renewal response to the conditions activated in these hosts.

As also shown in Chapter 3, the spectrum of differentiated progeny generated by transplanted HSCs also changes at precisely the same time during development (resulting in a reduced output of circulating granulocytes and monocytes by the HSCs present in mice that are 4 weeks-old or more). Both the distinctive high self-renewal behaviour and higher output of granulocytes and/or monocytes were also found to change after the same duration of fetal HSC proliferation in transplanted adult hosts. Thus the switch itself, as well as its precise timing, does not appear to be dependent on extrinsic conditions that are determined either by the site or the developmental status of the host, favouring the view that they are intrinsically programmed.

The unexpected speed and synchrony of these changes suggests the operation of some master “switch” mechanism. And, if the mechanism that regulates the intrinsic responsiveness of HSCs to factors that control their cycling status proves to be independent of the environment in which the HSCs are located, as found for their self-renewal potential and distribution of mature progeny output, this would imply that this putative switch may, itself be intrinsically regulated and pre-programmed – perhaps determined either by time and/or the number of divisions HSCs are stimulated to execute.
Because of the evidence indicating that all HSCs are cycling up until 3 weeks after birth in the normal mouse, genetic strategies to promote HSC quiescence might be anticipated to cause a delay in the timing of the ‘switch’ seen here to occur between 3 and 4 weeks of age. Recently, the loss of the transcription factor MEF was shown to promote the quiescence of adult HSCs (3). Therefore, it would be interesting in the future to examine if this might also be true of fetal HSCs, and if so, whether the predicted delay in altered self-renewal activity would also be seen. This is of particular interest, given that Chapter 3 also shows that MEF is up-regulated in pre-switch HSCs. The other genes found to be higher in expression, such as Ikaros (4-6) or rae-28 (7), both genes shown to be critical to HSC expansion, would also be candidates of interest for this strategy. MEF−/− bone marrow HSCs have also been found to respond differently when maintained under homeostatic conditions as compared to conditions activated in response to hematologic injury. Conversely, forced overexpression in pre-switch HSCs of genes found to be higher expressed in post-switch HSCs might also facilitate inducing a premature post-switch state. Particularly, ATM, Ezh2 and Gata-2, the genes with the highest fold change in the 4 week versus 3 week bone marrow HSCs would be strong candidates to study. These findings point to the likelihood that a small set of genes, including MEF, rae-28, Ikaros, Ezh2, Gata-2 and ATM, may be part of a program that co-regulates HSC proliferative activity and self-renewal function. Other candidates are the cyclin-dependent kinase inhibitor (CKI) p21Cip1/Waf1 which when deleted, has been shown to increase specifically the proportion of cycling HSCs (but not their derivative progenitors) and under these stress conditions self-renewal activity appeared to be compromised (8), the p18INK4C CKI, shown to limit the potential of adult HSC self-renewal in vivo (9), or genes within the specific clusters on chromosome 11, shown to be disproportionately distinctly expressed in
DBA/2 versus C57BL/6 mice (10), specifically related to their demonstrated differences in HSC proliferation (11).

One can also envisage various mechanisms that might mediate such effects; for example, a very long-lived (or functionally sequestered) protein or mRNA that decayed at a slow rate to finally reach a critical threshold level. This type of cell division-related threshold has been well studied in the case of telomeres, and the regulation of telomere length and telomerase activity is tightly linked to cell cycle regulation (12). Yet telomeres seem unlikely to be playing this type of critical role in murine HSCs as it has been proposed that telomere shortening evolved as a tumor suppressor mechanism not found in short-lived mammals, such as mice (13). In fact, it has been demonstrated that significant numbers of generations of mice must be followed before defects due to telomere shortening become evident, as mouse telomeres are extremely long (14;15). However, this does not preclude that a particular length of telomeres, long as it may be, is a threshold signal for a cascade of ‘switch’ responses to begin. The demonstration that adult bone marrow HSCs have shorter telomeres than those from the fetal liver or cord blood seems to suggest a continued decline in telomere length occurs with age (16).

The rapid switch that takes place in the type of globin expressed in maturing erythroblasts has also been well-studied. There, a role for the chromatin-remodeling complex (PYR complex) and its TF binding partner, Ikaros, has been well demonstrated. Mice null for *Ikaros* not only show a significant loss of HSCs (4), but display a delay in the murine embryonic to adult β-globin switch (17). cDNA array analysis on these mice indicated that several hematopoietic-specific genes across all lineages were changed in the day14 embryos. Therefore *Ikaros*, *PYR* and a number of other chromatin-remodelling genes, such as members
of the polycomb-group (*Bmi-1, mel-18, rae28, Ezh2*) (18-20) continue to be strong candidates to test for playing a role in the HSC switch demonstrated in this Thesis.

It is worth noting that if such a switch was determined to be cell division-dependent, it would not necessarily imply that one final HSC division was required for a transition from the fetal to the adult state. This could instead be quickly and easily regulated without the need of a change-of-state cell division, by changing any number of factors within the same cell: a loss or gain in a transcription factor, or set of transcription factors, again past a given threshold; any epigenetic changes, etc. Complicating the matter further is the heterogeneity of the adult HSC, for example with obvious subtypes related to differences in their patterns of repopulation in vivo (21).

Further investigations of HSC properties that are similarly altered during development may also offer new clues to the mechanisms that regulate the changes observed here. As described in the Introduction to this Thesis (Chapter 1), there are a number of cell surface markers whose expression is different between adult and fetal HSCs and in some cases, evidence of a post-natal change between 3 and 10 weeks of age has been reported (22-28). More carefully timed analyses will be useful to determine how closely these changes track relative to the functional changes described in this thesis. If coordinated, common transcription factor binding sites in the promoters of genes encoding for these cell surface markers, along with those of genes found in Chapter 3 to change in coordination, could then be sought in silico. Particularly, to look for activators of expression of genes found to be up-regulated in post-switch HSCs and repressors of those down-regulated in this population, as playing a role in promoting the switch itself.
A corresponding mechanistic change in HSCs has been described in Chapter 4, which points to a role for c-Kit signalling, responsible, at least, for the change in self-renewal potential. Chapter 4 describes a change in the HSC responsiveness to SF signalling as HSCs progress through development, to explain the reduced self-renewal potential that HSCs acquire during development, as described in Chapter 3, but not their reduced proliferative potential.

A full dose-response curve showed that the sensitivity of fetal liver and adult HSCs differ, but that W41 fetal liver HSCs were similar to +/+ adult HSCs. However, purified fetal liver HSCs were shown to express the same level of c-Kit mRNA as adult bone marrow HSCs and even the same mean fluorescence intensity of c-Kit expressed on the cell surface of pure fetal liver and adult bone marrow HSCs, suggesting that there is likely to be a developmentally regulated change in the surface organization of c-Kit or in a downstream or cooperative signalling component.

W41 fetal liver HSCs were demonstrated to also share the same in vivo self-renewal kinetics as adult bone marrow HSCs, indicating that HSC responsiveness to SF signalling was directly related to the rate of self-renewal. These results suggest that the levels of SF expressed in the bone marrow of an irradiated recipient is limiting to adult bone marrow, but not +/+ fetal liver HSCs, and treatment of recipients of bone marrow HSCs with SF could therefore be a means by which to increase the rate of regeneration following bone marrow transplantation.

These findings raise the question of which effectors of SF signalling, potentially transcription factors, might be regulating the intensity of c-Kit downstream signalling, as these may be critical points of control for the potential 'master switch'. A set of transcription factors acting as negative regulators of expression of key components to c-Kit signalling would be presumed to be up-regulated at a point in time between 3 and 4 weeks post-birth.
Alternatively, complexes involved in the repression of promoter accessibility of genes mediated c-Kit signalling may be up-regulated, either as direct mediators of chromatin condensation or by enhancing rapid methylation of the promoter region; each of which would result in a decreased amount of expression of such key genes in HSCs after 3 weeks post-birth.

In Chapter 2, an examination of the differences in cycling status of HSCs through development highlighted one HSC-cycling property that did not change through development: HSCs in S/G2/M phase of the cell cycle have a perpetual inability to engraft irradiated recipients. This has obvious clinical ramifications, as expanded HSCs are routinely used when there is a need for HSCs in the clinic and therefore a significant proportion of these are useless under current procedures. Chapter 2 provides evidence that this defect can be overcome when the recipient environment specifically (not the test cells) is pre-treated with an antagonist to SDF-1. However, the mechanism by which the host interacts with a cell autonomous property such as the cell cycle status is still unclear, specifically after treatment with the SDF-1 antagonist. We have proposed a logical model based on the additional evidence that SDF-1 is up-regulated in S/G2/M HSCs compared to HSCs in G1, suggesting that the high levels of SDF-1 in these cells saturates the CXCR4 receptors expressed on these same cells, preventing their ability to respond to SDF-1 gradients, unless these gradients were very strong. In Chapter 2, we hypothesized that SDF-1G2 treatment can generate a stronger gradient of SDF-1 in the host, allowing S/G2/M HSCs to now engraft. It is of great interest to determine whether SDF-1G2 treatment can result in increased levels of SDF-1 expressed at niche cells.

Since the SDF-1 antagonist acts on CXCR4 in the niche, but not cell-autonomously on stem cells, the population of cells in the recipient's environment that do express CXCR4 and mediate the observed effects are of great interest to identify and characterize. Various candidates, such as stromal and epithelial cells could be isolated and tested in vitro for their
differential ability to bind to \( S/G_2/M \) and \( G_0/G_1 \) HSCs. As well, the ability of \( S/G_2/M \) and \( G_0/G_1 \) HSCs to migrate through these cells could be tested, both before and after SDF-1G2 treatment of the niche cells under investigation. Such experiments will provide evidence of the mechanism that explains these exciting observations.

There are potential clinical applications of the findings presented in this Thesis. For example, in Chapter 2, the results of experiments on the \( S/G_2/M \) engraftment defect predict that intrafemoral injection of transplants is unlikely to be a useful strategy for improving the therapeutic effectiveness of HSCs induced to expand in vitro. Particularly exciting is the idea that pre-treatment of recipients of cycling cells with an SDF-1 antagonist could potentially improve the therapeutic effectiveness of these cells by as much as 100% (assuming that half of the HSC in a cycling population are in \( S/G_2/M \)). The induction of adult bone marrow HSC cycling is a consequence of culturing to expand these prior to transplantation or to genetically modify these; currently readily used and increasingly attempted strategies, respectively. The information presented in Chapter 4 could also provide new strategies for expanding HSCs, particularly as one growth factor condition, 50 ng/ml SF, has now been shown to maintain fetal liver HSCs in culture. Again, the ability to maintain fetal liver HSCs in culture is a necessity for successful attempts at genetic modification.

The induction, development, and progression of a leukemic disease is thought to arise through deregulation of the cycling, self-renewal and/or differentiation control in HSCs. This thesis has presented new evidence of controlled changes in all of these properties: Chapter 2 reveals programmed changes in the cycling status of HSCs, Chapter 3 reveals programmed changes in the self-renewal and differentiation potential of HSCs and Chapter 4 identifies a role for SF responsiveness in regulating differences in HSC self-renewal potential, but not the cycling status of HSCs. This information thus provides the potential to gain further insights
into leukemogenesis, through continued delineation of the molecular basis of the unique programs operative in pre-switch HSCs and how these regulate the pre-switch HSC properties described.
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