Feedback-Mediated Regulation of Human Hematopoietic Stem Cell Fate

by

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A thesis submitted in conformity with the requirements for the degree of Doctorate of Philosophy

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Abstract

Umbilical cord blood (UCB) cells fill a clinical need for hematopoietic stem and progenitor cell (HSPC) transplantation, and the ex vivo expansion of these cells provides an approach to increase their clinical relevance. Ex vivo HSPC expansion has been limited by a poor understanding of the interplay between stem cell autonomous and feedback mediated regulators. We hypothesized that developing strategies to modulate the HSPC microenvironment would enable enhanced expansion of these cells and a greater insight into the underlying biologic mechanisms. We developed a system for the optimized delivery of the transcription factor HOXB4 to human hematopoietic culture and revealed new insights into the context-dependent potentials and limitations of HOXB4. We investigated approaches for the global reduction of inhibitory feedback signaling and developed a fed-batch system that minimizes endogenously produced factors to create a more supportive environment for HSPC self-renewal. This strategy led to an 11-fold expansion of long-term repopulating HSCs in a clinically relevant bioreactor,
producing a novel system for ex vivo expansion and generating a platform to assess HSPC enhancing factors. By combining the fed-batch system with the Notch Delta-1 ligand (DL1), we identified a mechanism whereby DL1 initiated a conversion from IL-6 cis-signaling to trans-signaling, resulting in the modulation of mature cell population production. This demonstrated the impact of cell lineage skewing on microenvironment regulation and the expansion of HSPCs. This work demonstrates how cell-cell interactions and feedback mediated signaling are critical regulators of HSPCs and the manipulation of these regulators can be used both to engineer HSPC expansion processes and to identify novel mechanisms within the hematopoietic system. This provides an important step towards the development of robust methods for the ex vivo expansion of UCB-derived cells and the ultimate goal of achieving cures in hematologic disease.
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1 INTRODUCTION

Portions of this chapter have been published in *Bioessays* (Csaszar et al., 2013). Co-authors include Sandra Cohen and Peter W. Zandstra.
1.1 Clinical Motivation for Hematopoietic Stem Cell Expansion

Hematopoietic stem and progenitor cell (HSPC) transplants have been performed since the 1960s and remain the only widespread clinically curative stem cell therapy. Over 50,000 HSPC transplants are performed annually, of which over 20,000 are allogeneic (Gratwohl et al., 2010). Historically, bone marrow (BM) has been the primary cell source of HSPCs; however, more recently, mobilized peripheral blood (mPB) and umbilical cord blood (UCB) have been established as alternate sources. In the early 2000s, mPB surpassed BM as the most frequently used source of cells for allogeneic HSPC transplants (Bensinger, 2012).

UCB provides a particularly attractive source of HSPCs. Its benefits include non-invasive and rapid availability, reduced chronic graft-versus-host-disease (GVHD) in recipients while preserving favourable graft vs. malignancy effects, decreased risk of infection transmission, and a higher tolerance for human leukocyte antigen (HLA) mismatches (Broxmeyer, 2011; Gluckman, 2009). Additionally, UCB provides a transplantation option to patients for whom a suitable matched unrelated BM or mPB donor cannot be found. This has been reported to be the case in 47% of patients with European ancestry and 79% of patients with non-European ancestry (Barker et al., 2010a). The first successful UCB transplant (UCBT) was performed in 1988 and, today, there are reported to be over 400,000 UCB units banked worldwide and over 20,000 UCBTs have been performed (Gluckman and Rocha, 2009). The ability to collect, prospectively assess, cryopreserve, and bank samples provides flexibility in the use of UCB.

Despite these successes, the use of UCB for HSPC transplants still faces major challenges. Increasing cell dose has been shown to be strongly correlated with more successful UCBT outcomes. A minimum cell dose of $2.5 \times 10^7$ cells/kg, or $2.0 \times 10^5$ CD34$^+$ cells/kg has been recommended, and higher doses are recommended where possible to compensate for HLA mismatches and promote more rapid engraftment (Barker et al., 2010b; Boo et al., 2011; Gluckman, 2009; Liao et al., 2011). Lower numbers of HSPCs per UCB unit (compared to typical BM or mPB isolates) have limited the use of UCB in adult patients. Currently, approximately 40% of unrelated allogeneic transplants in children are performed with UCB, while in adults, UCB accounts for only 7% of unrelated transplants (Liao et al., 2011).
A successful transplant requires a sufficient number of progenitors to participate in rapid short-term neutrophil and platelet recovery, and a sufficient number of long-term repopulating stem cells (LTR-HSCs) to sustain hematopoiesis for the lifetime of the individual. Double UCBTs have provided some advances towards the goal of improving accessibility to UCBTs in adults. Double UCBTs, now performed more frequently than single UCBTs in adults (Rocha et al., 2010), are associated with a decreased relapse rate, although only one unit typically contributes to long-term engraftment (Brunstein et al., 2010; Liao et al., 2011; Sideri et al., 2011). However, double UCBTs do not alleviate concerns regarding delay to neutrophil and platelet recovery. Average times to neutrophil and platelet engraftment are 26 and 53 days respectively for double UCBTs vs. 16 and 20 days respectively for matched related donor transplants from a BM or mPB cell source (Brunstein et al., 2010). This suggests that double UCBTs may not be providing adequate numbers of progenitor cells. Moreover, double UCBTs raise concerns related to higher risk of grade II skin acute GVHD (Sideri et al., 2011), as well as higher material costs.

The current average cost of UCB graft acquisition has been reported to be $34,200 per unit in the Americas and $24,300 per unit in Europe (Bart, 2010), and ongoing break-even costs to public banks have been estimated to be a minimum of ~$15,000 per unit, and potentially much greater depending on the size of the bank that is being maintained (Meyer et al., 2005; Sirchia et al., 1999). The FDA has recently approved HEMACORD, as the first licensed UCB cell product, to the New York Blood Center. This decision is consistent with the need for standardized metrics for un-manipulated cell populations, and will help with setting a clear baseline to compare expanded products against. The decision may also create additional costs and complexities related to graft acquisition as different UCB banks may need their own product designations (Allison, 2012). In addition to the graft acquisition costs, a recent cost analysis study found that patients receiving a myeloablative double UCBT had an average cost per day survival of $2,082 as compared to $1,016 for a myeloablative matched related donor transplantation, likely due to the longer time to engraftment and thus longer period of hospitalization for UCBT patients (Majhail et al., 2009).

These clinical and economic limitations have motivated the field of ex vivo UCB expansion as an alternate approach to obtain appropriate numbers of HSPCs. This requires the ability to
manufacture an UCB product that has both robust increases in LTR-HSCs, and appropriate composition of committed progenitors.

1.2 The Hematopoietic System

The hematopoietic system is a complex hierarchical system comprised of a heterogeneous population of blood cells. The hematopoietic stem cell (HSC) resides at the top of the hierarchy, with the capability to self-renew as well as to differentiate into all the cells of the hematopoietic lineage (Bryder et al., 2006; Doulatov et al., 2012). Development of mammalian hematopoietic cells is first seen in the extra-embryonic yolk sac of the embryo, as the hemangioblast gives rise to committed endothelial and hematopoietic progenitors. However, definitive HSCs with complete, long-term, multilineage repopulating potential first appear at E10.5 in the AGM region of the embryo body and the vitelline and umbilical arteries (Dzierzak and Speck, 2008). As development progresses, HSCs are found in the placenta, yolk sac, fetal liver, and UCB, as they transition to their adult niche. Murine HSCs first appear in the bone marrow at E15 (Ratajczak, 2008).

In adults, HSCs primarily reside in niches within the bone marrow, where they maintain homeostasis of the blood system by self-renewing to maintain the stem cell supply, and differentiating into committed hematopoietic progenitor cells, which further differentiate to produce mature blood cells. Most mature blood cells have short lifespans, requiring a continuous production of cells on the order of $10^{10}$ red blood cells and $10^9$ white blood cells to be generated each hour (Handin et al., 2002). Apart from maintaining the supply of mature cells in a state of homeostasis, HSCs must also have the regulatory ability to respond to physiological demands, such as infection or blood loss. With each cell fate decision, HSCs may remain quiescent, undergo apoptosis, or divide, depending on the requirements of the hematopoietic system and the resulting environmental signals within the niche. Upon division, the HSC will either self-renew into daughter cells with identical stem cell potential, or it may differentiate along a specific hematopoietic lineage.

The LTR-HSCs have the ability to self-renew for the lifespan of the individual in order to maintain the hematopoietic system. Further down the hematopoietic hierarchy, short-term
repopulating HSCs (STR-HSCs) and multipotent progenitors (MPPs) have a decreased propensity for self-renewal, but still retain the ability to produce all mature hematopoietic cells. Although often denoted as distinct and identifiable cell populations, the distinction among the cells populations at the top of the hematopoietic hierarchy is not static, but is constantly evolving with the development of more sophisticated stem cell characterization assays. For example, the identification of an intermediate-term repopulating HSC population in the mouse system, with repopulating characteristics that place it between the LTR-HSC and the STR-HSC, has led to the addition of a functionally and phenotypically distinct population to the hematopoietic hierarchy (Benveniste et al., 2010). Another study, which demonstrated the ability to separate murine HSCs into four categories with unique self-renewal and lineage contribution characteristics, has provided an example of further segregation of the LTR-HSC population (Dykstra et al., 2007). Moreover, the relative frequencies of these sub-populations change throughout development and with aging (Benz et al., 2012). Thus, HSC populations do not fit neatly into rigid compartments and it may be most relevant to consider the primitive cells of the hematopoietic hierarchy to be lying along a dynamic continuum as opposed to falling into distinct categories (Kirkland, 2004; Quesenberry et al., 2005).

Further down the hematopoietic hierarchy, MPPs undergo differentiation into lineage committed cells. With each differentiation step, hematopoietic cells become committed to a more restrictive lineage until cell undergo terminal differentiation to form fully mature functional blood cells. The "classic" models for the hematopoietic system suggested that the earliest lineage decisions categorized hematopoietic cells into either lymphoid or myeloid cells. This thinking has recently been revised to account for greater plasticity among early lineage-committed progenitors than was originally recognized (Ceredig et al., 2009; Doulatov et al., 2010). This includes the characterization of a multilymphoid progenitor (MLP) that, in addition to giving rise to all cells of the lymphoid lineage, is also capable of producing monocytes, macrophages, and dendritic cells.

1.3 Characterizing Hematopoietic Cells

The classification of hematopoietic cell populations is important for understanding the biology of hematopoiesis but also for identifying the cell populations that have clinical benefit in the
transplantation setting. Two aspects of this characterization include standardizing a means to identify specific cell populations and evaluating the relevance of these cell types upon transplantation. The quantification and characterization of cell populations is important for all cell therapy applications but becomes particularly crucial when assessing cells that have been manipulated in vitro, as lineage skewing or the loss of specific cell sub-populations that go unaccounted for may give undesired clinical outcomes. The identification and characterization of hematopoietic cell populations is complicated by heterogeneity and dynamics within the hematopoietic system. Many functional and phenotypic assays have been developed as techniques to identify and quantify specific cell populations. Each comes with advantages as well as limitations.

1.3.1 Functional assays

In vitro and in vivo functional assays provide that most rigorous definitions of hematopoietic cell populations. A cell type must ultimately be specified based on its function and being able to functionally validate the ability of a cell is the only unequivocal means of characterization. Characterizing the HSC population is a critical but non-trivial task. In vivo assays provide a stringent, albeit context dependent, measure of the stem cell characteristic of cells. Strictly, the multipotent potential of an HSC is defined by it having the ability to rescue a lethally irradiated recipient from hematopoietic failure through the regeneration of all myeloid, lymphoid, and erythroid lineages. The self-renewal potential of an HSC is confirmed by its successful engraftment into multiple secondary recipients, thus demonstrating that it has undergone symmetric self-renewal divisions (Benveniste et al., 2003; Jordan and Lemischka, 1990).

The competitive repopulation unit (CRU) assay is most commonly used as the best measure of HSC potential for murine cells. Test cells are injected into a lethally irradiated recipient mouse along with excess bone marrow cells and recipients are scored positive for CRUs based on the proportion of their regenerated hematopoietic tissue that is derived from the test population. Multilineage potential of test cells can be assessed by measuring contribution to the myeloid, lymphoid, and erythroid lineage (Eaves et al., 1999). For human HSCs, immune-deficient xenograft mouse models provide a means to assess stem cell properties in a similar manner. The xenograft models enable SCID-repopulating cell (SRC) assays to be performed, in which recipient mice are sublethally irradiated and human HSCs are transplanted. The level of
Engraftment can be determined by measuring the human pan-blood cell marker CD45 in the BM of recipients. When this assay is performed at limiting dilution, quantification of HSCs can be calculated with Poisson statistics.

The nonobese diabetic/severe combined immunodeficient (NOD/SCID) mouse model was developed by backcrossing the SCID mutation onto nonobese diabetic (NOD) mice with innate immunity defects (Shultz et al., 1995). These animals support high levels of human engraftment and allow for HSC quantification. The major limitations of this mouse model are the presence of active host NK cells and the fact that these animals are subject to high incidences of thymic lymphoma, which limits their lifespan and thus the length with which the transplanted cells can be tracked. In the last decade, several groups have developed xenograft models with improved characteristics. The NOD/SCID/IL-2Rg-c null (NSG) mouse is a popular model in which the deletion of the IL-2R gamma chain results in the loss of NK cells (Shultz et al., 2005). These mice also have much longer lifespans which facilitates longer term follow up studies. Several humanized mouse models have also been developed recently through the expression of various human cytokines, in an effort to enable the recipient mice to better support human cells of all lineages (Rongvaux and Willinger, 2011; Willinger et al., 2011).

Despite improved mouse models, several limitations with the SRC assay exist. The technical and biological details of the assay create large inter-laboratory variability. For instance, it has been reported that female NSG mice engraft low doses of human cells with significantly higher sensitivity than males recipients, suggesting that sex-specific factors such as steroid hormones may be contributing to HSC regulation (Notta et al., 2010). The mode of cell transplantation is also critical; intravenous injection requires proper homing of the human cells to murine BM, while intra-femoral injections eliminate this requirement and result in higher engraftment levels (Mazurier et al., 2003). The greatest limitation of the xenograft mouse models is likely the uncertainty of whether any mouse model can truly identify a human cell that has the ability to survive and self-renew for the lifespan of a human. It may be that the cell which read out during the 8-24 week period post-transplantation when NSG mice are usually assessed is not actually a cell with true human long-term repopulating potential (Horn et al., 2003).
Transplantation of HSCs into large animal models such as non-human primate models have been used by some groups to assess HSC engraftment kinetics, homing, and safety consideration over a longer time period in a more closely related species (Feng et al., 2008; Goessling et al., 2011; Zhang et al., 2006). One study has described the development of an allogeneic macaque monkey UCB model as an option to study intra-species HSC expansion and gene therapy strategies (Watts et al., 2012). These large animal models may provide a more accurate depiction of the behaviour of human HSCs in patients. Longer-term follow-ups in large animal models may allow for the identification of chronic adverse effects that would not have been identified in mouse models, such as the identification of myeloid leukemia developed in 3 of 4 large animals transplanted with retroviral over-expressed HOXB4 two years following transplantation (Zhang and Beard, 2008). Despite some advantages of large animal models, the cost and time-frame of these studies is very often prohibitively high. For translational studies, the specific safety concerns of a given therapeutic approach will dictate the relevance of a large animal study.

Measuring the expansion of primitive progenitor cells or STR-HSCs is important for assessing the cells that are contributing to rapid engraftment after transplantation, however functional assays for these cell populations are in many ways even less defined than for the LTR-HSC population. Standard functional assessments often consist of short-term (10 day to 4 week) read-outs in xenograft models as well as in vitro assessment of progenitor cell content. A recent study demonstrated the development of a mouse model that better reads-out STR-HSC engraftment by constitutively producing human IL-3, GM-CSF, and SCF (Miller et al., 2013). This emphasizes that different xenograft models may be better suited for the assessment of specific cell populations.

In vitro functional assays provide a means to assess and quantify progenitor cell content. The most widely used and standardized assay is the colony forming cell (CFC) assay, which measures committed progenitor cells (Coulombel, 2004). The assay consists of culturing cells in a semi-solid methylcellulose media supplemented with hematopoietic growth factors for 14 days. Colonies can be quantified and categorized as granulocyte, macrophage, erythroid, megakaryocyte, or the mixed granulocyte-erythroid-macrophage-megakaryocyte (CFU-GEMM) colony, which is the most primitive cell population that can be measured by a CFC assay (Broxmeyer et al., 2006). Although often used in the clinical setting as a surrogate for HSC
content, the correlation between the CFC assay and the SRC assay may not be particularly strong (Kirouac et al., 2009). The long term culture-initiating cell (LTC-IC) assay identifies a more primitive progenitor population than the CFC assay. In an LTC-IC assay, cells are co-cultured with an irradiated stromal cell line for at least five weeks before being plated in methylcellulose and scored for colonies after an additional 14 days (Liu et al., 2013). Notably, both the CFC and the LTC-IC assay are only able to measure myeloid-erythroid progenitors and not cells with lymphoid potential. Thus the in vitro functional assessment of cells with true multi-lineage potential remains one of the major limitations of hematopoietic cell characterization.

1.3.2 Phenotypic characterization

Using phenotype as a means of accurately and precisely characterizing hematopoietic cells is highly desirable. Characterizing hematopoietic cells based on surface marker proteins provides a method to rapidly identify a particular cell population by flow cytometry and prospectively characterize and sort cells to isolate the population of interest. Precise phenotype identification of specific cell populations would eliminate the high costs, long time-frames, and technical difficulties of functional assays. However, accurately correlating phenotype to function under all contexts and for all populations of interest has been extremely challenging in the hematopoietic field. Regardless, a great amount of progress has been made in recent years to identify and rigorously validate complex marker combinations that identify functionally distinct HSPC populations.

In the murine system, HSCs can be purified to near homogeneity using a combination of markers such as c-kit<sup>+</sup>Thy-1<sup>−</sup>Sca-1<sup>−</sup>Lin<sup>−</sup>, Endoglin<sup>+</sup>Sca-1<sup>−</sup>Rho<sub>lo</sub>, or CD34<sup>−</sup>CD150<sup>−</sup>CD48<sup>−</sup>CD41<sup>−</sup>Flt3<sup>−</sup>CD49b<sub>lo</sub> (Benveniste et al., 2010; Chen et al., 2003; Doulatov et al., 2012). Early studies of phenotype in the human hematopoietic system led to the realization that phenotypic definitions of specific hematopoietic populations are very poorly conserved between mouse and human. Until recently, phenotypic characterizations of human HSPCs have been poorly defined. However, by initially identifying markers that define broad heterogeneous HSPC populations and gradually identifying additional markers that can further divide these populations based on function, the field is beginning to reach a consensus of marker combinations that can be used to identify more distinct primitive cell populations.
CD34+ was first identified as a surface marker that successfully enriched the human HSC population from total nucleated hematopoietic cells (Civin and Strauss, 1984). CD34+ cells comprise approximately 1% of adult bone marrow and are a very heterogeneous population, marking every cell type from LTR-HSCs to committed lineage progenitors. CD34+ cell selection is widely used to enrich for primitive cell populations to be used clinically or for in vitro studies. CD133 is a marker that identifies a population overlapping with the CD34+ population but it may be restricted to a more primitive subset of CD34+ cells. An alternative method of enriching for a similar primitive cell population is the negative selection based on the exclusion of mature lineage markers, such as glycophorin A, CD2, CD3, CD14, CD19, CD24, CD56 and CD66b. The resulting Lin- population is enriched for HSCs and primitive progenitors, and typically consists of >50% CD34+ cells.

Further enrichment of HSCs can be achieved with the CD34+CD38- population, however a true LTR-HSC is reported to still be only ~1/600 of this cell population (Bhatia et al., 1997). Thy1+ (CD90+) and CD45RA- have been shown to subdivide the CD34+CD38- population, leading to a population more highly enriched for HSCs characterized by CD34+CD38-CD90+CD45RA- (Doulatov et al., 2012). More recent work, spearheaded by the John Dick lab, have built on the CD34+CD38-CD90+CD45RA- phenotype in order to more accurately characterize and isolate specific HSC and primitive progenitor populations from human UCB. These populations were carefully validated for their functional ability with limiting dilution and single cell transplantation of highly sorted cell populations into xenograft models (Doulatov et al., 2010; Notta et al., 2011). These studies have given rise to the most comprehensive phenotypic identification of human HSPC populations to date which defines a HSC as CD34+CD38-CD90+CD45RA-CD49f+ and a MPP as CD34+CD38-CD90+CD45RA-CD49f-. Single cell transplantation studies indicated that this HSC phenotype enriches cells to a point where at least 1 of 5 cells has long-term functional repopulating ability (Notta et al., 2011). These studies have also identified marker combinations for specific progenitor populations, including MLPs, common myeloid progenitors (CMPs), granulocyte and monocyte progenitors (GMPs) and megakaryocytic and erythroid progenitors (MEPs), to better define the downstream hierarchy (Doulatov et al., 2010, 2012). A summary of the functional and phenotypic assays currently used to characterize the hematopoietic hierarchy is shown in Figure 1-1.
Phenotypic characterization still suffers from limitations. The most significant limitation may be the uncertainly of the relevance of phenotypes and their correlations to function following cell manipulation or outside of healthy homeostatic conditions (Zhang and Lodish, 2005). Using these newly identified cell characterization strategies to study and categorize cells in the disease setting or following ex vivo culture will require further validation.

Figure 1-1: Schematic of the hematopoietic hierarchy highlighting cell population characterization.

The major hematopoietic stem and progenitor populations are displayed. Surface marker phenotypic characterization of the current definitions of cell populations (Doulatov et al., 2012) are displayed in blue and on the left hand side. Grey arrows on the right hand side indicate which cell categories are read-out by selected functional assays.

1.4 Regulation of HSC Self-renewal

The fate choice of the HSC to either self-renew or differentiate is controlled by a complex interplay between intrinsic mechanisms and extrinsic signals from the microenvironment (Huang
et al., 2007). Models of HSC self-renewal are numerous; some propose self-renewal is driven primarily extrinsically by growth factors, extracellular matrix, and other external influences, while others propose a more intrinsic stochastic model of lineage selection and self-renewal, although both components are clearly critical (Glauche et al., 2007; Zandstra et al., 2000).

Symmetric self-renewal divisions occur when each dividing cell gives rise to two identical daughter cells with equivalent developmental potential. This mechanism is required for the expansion of the HSC pool, as occurs during embryogenesis and following HSPC transplantations. Asymmetric self-renewal occurs when daughter cells with different characteristics are produced, resulting in the maintenance of the HSC pool and the production of progenitor cells. It has been shown that some proteins segregate differentially when HSC divide asymmetrically, given rise to two different daughter cells, one with more primitive characteristics than the other (Beckmann et al., 2007). Extrinsic factors regulate the transition between symmetric and asymmetric division programs.

Cell fate decisions require the integration of external cues from the microenvironment, the activation of intracellular signaling pathways, transcriptional regulation, and post-transcriptional modifications. It is important to understand these regulatory cascades during embryonic and fetal hematopoietic development, during homeostatic adult hematopoiesis, and during times of recovery from physiological stress. Different factors are important in each of these scenarios as the cells are subjected to different demands. One approach for the ex vivo expansion of HSPCs is to identify and modulate cell fate regulators that would normally be active during hematopoietic development, thus mimicking the in vivo regulation of cell fate decisions during a time of rapid self-renewal (Ross and Li, 2006; Zon, 2008). Alternative strategies seek to over-ride the HSPC maintenance machinery in order to increase symmetric self-renewal divisions. In the following sections, we will summarize and highlight some of the transcription factors and signaling pathways that have shown to be important for hematopoietic regulation.

1.4.1 Transcription factors influencing HSC self-renewal

Stem cell fate decisions regarding self-renewal vs. differentiation and proliferation vs. quiescence are controlled in part by transcription factor regulation, in response to cues from the cell niche. Many transcription factors have been identified as participating in the regulation process of cell fate decisions in the hematopoietic system. A large number of transcription
factors critical to the maintenance of the healthy hematopoietic system are also involved in chromosomal translocations or somatic mutations in human hematopoietic malignancies; thus, HSC fate is very closely linked to the origins of leukemia (Orkin and Zon, 2008). Transcription factors that are important to the hematopoietic system can be categorized in a number of ways, including factors that are required for hematopoietic development, factors that are required for adult hematopoietic maintenance, factors that dictate self-renewal vs. differentiation decisions, cell-cycle related factors, and factors that dictate lineage decisions. A selected set of important transcription factors and nuclear regulators are summarized in Table 1-1. Several factors may have roles in multiple categories.

Table 1-1: Selected transcription factors and nuclear regulators important to the hematopoietic system

<table>
<thead>
<tr>
<th>Category</th>
<th>Selected Transcription Factors</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hematopoietic Development</td>
<td>SCL, Lmo2, Runx1, Gata2</td>
<td>(Lacaud et al., 2002; Zhu and Emerson, 2002)</td>
</tr>
<tr>
<td>Fetal HSCs</td>
<td>Sox17, Rae-28, Ikaros</td>
<td>(Bowie et al., 2007; Kim et al., 2007)</td>
</tr>
<tr>
<td>Adult HSC Maintenance</td>
<td>Gata2, Gata3, Hoxb4, Bmi1, Sall4, Evi1, Meis1, Cited2, FoxO</td>
<td>(Kataoka et al., 2011; Ku et al., 2012; Rizo et al., 2008; Tothova and Gilliland, 2007; Yang et al., 2012; Zhu and Emerson, 2002)</td>
</tr>
<tr>
<td>Cell Cycle Regulation</td>
<td>Pbx1, Gfi1, Gata2, p53, c-Myc</td>
<td>(Li, 2011; Wilson et al., 2009)</td>
</tr>
<tr>
<td>Early Lineage Regulation</td>
<td>Gata1/PU-1, Gata2, C/EBPα, E2A</td>
<td>(Burda et al., 2010; Iwasaki et al., 2006; Mercer et al., 2011)</td>
</tr>
</tbody>
</table>

Many factors have recurrent roles throughout the hematopoietic system and at multiple developmental stages. Runx1 is most commonly known for its role in hematopoietic commitment in the hemangioblast (Lacaud et al., 2002), but it has also been implicated in lineage decisions in the adult hematopoietic system (Lorsbach et al., 2004). Similarly, Gata2 has been shown to be necessary for both the embryonic development of the hematopoietic system and the lineage regulation of hematopoietic cells in adult BM (Zhu and Emerson, 2002). Fetal liver derived HSCs have a much greater expansion potential than BM derived HSCs following transplantation (Pawliuk et al., 1996), which speaks to the high self-renewal propensity of fetal HSCs. Sox17 and Rae-28 are two factors that are highly expressed in fetal HSCs but down-regulated as cells switch to a more quiescent state three to four weeks after birth (Bowie et al.,
These types of factors may be good targets for ex vivo HSC expansion where high self-renewal is desired.

Regulation of self-renewal and differentiation in the adult hematopoietic system is accomplished in part by several transcription factors which have roles in the control of HSC maintenance, survival, cell-cycle entry, and self-renewal. Several members of the Hox family of homeobox genes are differentially expressed during hematopoietic differentiation, with Hoxb4 being of particular interest as it is up-regulated in very primitive cells (Sauvageau et al., 1995) (see next section for more detail on Hoxb4). Gata3 is a transcription factor that is highly expressed in LTR-HSCs in both mouse and human, rapidly down-regulated with differentiation, and shown to be important for HSC maintenance (Ku et al., 2012; Zhong et al., 2005). Several oncogenes are closely associated with cell fate and cell cycling regulation. Polycomb group genes (PcG) are known to be involved in the maintenance of normal and malignant stem cells. Bmi1 is one member of the PcG family that has been shown to be important for the long-term maintenance and self-renewal of human HSCs (Rizo et al., 2008). Other transcription factors that have been identified as having a role in adult HSC self renewal regulation include Sall4, Ev1, Meis1, Cited2, and the FoxO family (Kataoka et al., 2011; Tothova and Gilliland, 2007; Yang et al., 2012; Zhong et al., 2005).

Hematopoietic transcription factors also regulate lineage differentiation. In some instances, lineage commitment is determined by transcription factor switches, such as the role of PU-1 and Gata-1 in myeloid/erythroid fate decisions (Burda et al., 2010; Roeder and Glauche, 2006). The interaction between regulatory factors can be highly nuanced, as in one study that showed that the order of expression of the transcription factors Gata2 and C/EBPα was critical for the regulation of lineage commitment (Iwasaki et al., 2006). It is thought that lineage commitment is largely a result of epigenetic changes in DNA and chromatin, which serve to modulate patterns of gene expression (French et al., 2002; Zardo et al., 2008). Interestingly, the over-expression or repression of lineage specification transcription factors in the right context can generate lineage conversion. (Orkin and Zon, 2008). For instance, the inhibition of Pax5 has resulted in the dedifferentiation of B-cells, which have then been re-differentiated into T-cells or macrophages (Cobaleda et al., 2007; Xie et al., 2004) and the forced expression of Gata1 in
granulocyte/macrophage progenitors redirects their commitment to megakaryocyte/erythroid progenitors (Iwasaki and Akashi, 2007).

Despite the identification of transcription factors that play a variety of critical roles in hematopoiesis, no single "master regulators" of HSCs have been identified. Instead, it appears that it is a complex combination of regulators, many of which have redundant or complementary roles, that maintain the hematopoietic system in its homeostatic state. Transcription level regulation acts in tandem with several other players, including microRNA and post-transcriptional modifiers (Butler and Dent, 2013; Chen et al., 2004), which all contribute to a complex and highly interconnected regulatory system. Genomics and bioinformatics techniques have allowed for a more global investigation of the transcriptional regulation networks that governs the hematopoietic system and studies have illustrated that the network is densely interconnected, with many transcription factors acting in multiple roles throughout the hematopoietic hierarchy (Novershtern et al., 2011). This type of analysis highlights the power of global top down approaches to elucidate regulatory nodes and provides insight into key factors for further experimental manipulation. However, these approaches are somewhat limited by the heterogeneous and dynamic nature of the hematopoietic system. Methods to mathematically deconvolve heterogeneous cell populations (Qiao et al., 2012) provide one approach to be coupled with network analysis in order to more accurately identify regulatory factors specific to individual cell populations or dynamic states.

1.4.2 The transcription factor HOXB4

One transcription factor that has been shown to be very relevant in the regulation of primitive hematopoietic cells in Hoxb4. Hoxb4 is one of 39 Hox genes present in mammals, subdivided into four clusters (A, B, C, D). The Hox genes are involved in embryonic development and axial patterning along the anterior-posterior axis (Grier et al., 2005). Each Hox protein contains a 60 amino-acid homeodomain (HD), which consists of an N-terminal arm and three helices, two of which form a helix-turn-helix motif (Wilson and Desplan, 1999). Several Hox genes in clusters A, B, and C play an important role in both normal and diseased hematopoiesis (Lowney et al., 1991; Magli et al., 1991).
In particular, Hoxb4 has been shown to be preferentially expressed in very primitive hematopoietic cells and is down-regulated upon differentiation (Sauvageau et al., 1995). Hoxb4 consists of 361 amino acids with all but 9 being conserved between human and mouse (Sauvageau et al., 1995). In loss of function mice models, it has been found that Hoxb4 knock-out mice experience only slight proliferation deficiency in HSC development. The deficit of Hoxb4 results in an increase in expression of Hoxa4, suggesting a redundancy in the Hox genes, mitigating phenotypic loss in knock-out studies (Brun et al., 2004).

To study the effect of Hoxb4 over-expression, retroviral transduction was used to over-express Hoxb4 in murine BM cells. Serial transplantation studies demonstrated that Hoxb4 transduced cells enhanced the regeneration of the most primitive HSC compartment by 50 fold (Sauvageau et al., 1995). Retroviral induced Hoxb4 in human Lin- UCB cells caused a 10 fold increase in LTC-ICs and a 4 fold increase in SRCs as compared to control cells (Buske et al., 2002). Hoxb4 over-expression was also found to achieve a high level of in vitro expansion of HSCs. When murine cells transduced with the Hoxb4 retroviral vector were cultured for 14 days, a 40 fold increase in HSCs was seen by CRU assay, while control cultures experienced significant HSC decrease (Antonchuk et al., 2002). Furthermore, Hoxb4 importantly does not impair homeostatic control of HSC population size or the production rate and maintenance of mature hematopoietic cells (Antonchuk et al., 2001). In over-expression experiments, HSC levels did not ever rise above that which is typically seen in un-manipulated mice (Sauvageau et al., 1995), suggesting that Hoxb4 over-expression does not override the regulatory mechanisms within the stem cell niche that maintain the HSC pool size. This is in contrast to many Hox genes, whose over-expression has been shown to have oncogenic properties and lead to uncontrolled expansion (Owens and Hawley, 2002).

The HSC expansion ability of Hox fusion proteins has also been investigated. Nup98-Hox fusions are identified oncogenes found in myeloid leukemia (Pineault et al., 2004). The forced expression of NUP98-HOXA10 was shown to produce up to a 10,000 fold HSC expansion of murine bone marrow cells following a 17 day culture period, surpassing the ability of Hoxb4. Moreover, using only the homeodomain portion of Hoxa10 (Nup98hoxa10hd) proved to also be successful in achieving high expansion, while retaining normal myeloid-lymphoid reconstitution (Ohta et al., 2007).
It is thought that Hoxb4 has a therapeutic window of activity and that perturbed differentiation is seen if the transcription factor is present above a certain threshold (Klump et al., 2005). It has been noted that at increasingly high levels of Hoxb4, T-lymphoid levels become reduced and erythroid development is suppressed (Pilat et al., 2005). Additionally, adenoviral expression of Hoxb4, which exposes the cells to much higher expression levels than retroviral transduction, was found to lead to a reduction in both CFCs and LTC-ICs (Brun, 2003). It has been shown that Hoxb4 sensitizes cells to microenvironmental cues and inhibits cell growth in a dose-dependent manner (Will et al., 2006), suggesting that there may be an optimum dose of Hoxb4 which is context dependent.

It has also been shown that transduction of Hoxb4 can enhance the differentiation of both mouse embryonic stem cells (mESCs) and human embryonic stem cells (hESCs) to the hematopoietic lineage (Bowles et al., 2006; Helgason et al., 1996; Unger et al., 2008). One study demonstrated that a tPTD-HOXB4 fusion protein could be used to effectively differentiate hESCs to hematopoietic progenitor cells, as demonstrated by a significant increase in the number of CFCs generated by these cells as compared to control conditions (Lu et al., 2007). Interestingly, it has been reported that in the developing hematopoietic system, Hoxb4 may not only target the hematopoietic cells, but also the formation of niche components through the modulation of the secreted Wnt-binding protein FRZB (Jackson et al., 2012).

The biological mechanism by which Hoxb4 acts to achieve HSC expansion remains poorly understood. It has been suggested that TPO positively regulates Hoxb4 expression by reducing the ubiquination of hypoxia inducible factors (Kaushansky, 2005; Kirito et al., 2003). Mobility shift assays with K562 extracts have shown the upstream stimulating factors 1 and 2 (USF-1, USF-2) bind to the Hoxb4 promoter and the promoter is induced in response to signals stimulating stem cell self-renewal, through activation of the mitogen-activated protein kinase pathway. Thus, the binding of USF-1 and USF-2 and the expression of Hoxb4 may be favoured by cytokines promoting self-renewal versus differentiation (Giannola et al., 2000). The trimeric transcription factor, nuclear factor-Y (NF-Y) has been shown to activate the Hoxb4 promoter in cooperation with USF-1 and USF-2 (Zhu et al., 2003). The Polycomb group of proteins are thought to maintain long term repression of Hox genes (Kennison, 1995). Downstream effectors
have also been difficult to pinpoint. One large gene expression study of factors up-regulated by
Hoxb4 identified 133 Hoxb4-regulated genes in murine bone marrow LSK cells, including genes
associated with the Wnt, Notch, and FGF pathways. Many studies have looked at cell cycle
regulators as downstream effectors. It has been suggested that cells over-expressing Hoxb4
undergo fewer cell divisions and it is proposed that Hoxb4 impacts HSC expansion by affecting
cell fate decisions through the increase in frequency of symmetrical self-renewal divisions
(Cełlot et al., 2007).

It has also been reported that Hoxb4 over-expression enhances megakaryocyte development in
human UCB cells (Zhong et al., 2010). The mechanism for this effect is not known, although the
known links between TPO and HoxB4 provide one possible connection to megakaryopoiesis.
Hoxb4 has also been shown to down-regulate Geminin (Ohno et al., 2010), and the deletion of
Geminin has been linked to enhanced megakaryocyte production (Shinnick et al., 2010),
suggesting that Geminin may be one downstream target through which HOXB4 modulates
megakaryocytes.

Transplantation studies in mouse models suggested that injecting animals with Hoxb4 transduced
cells allowed for the complete repopulation ability of the lympho-myeloid lineages, and did not
result in any instances of leukemia (Sauvageau et al., 1995). Initial preclinical studies on large
animal models indicated that transplantation of Hoxb4 induced cells improved engraftment in
both dogs and non-human primates, although a skewing towards the myeloid lineages was seen
(Zhang et al., 2006). The Hoxb4 transduced cells led to a large increase in early engraftment and
a small but significant increase in maintained long-term engraftment. However, a longer term
follow-up on these studies found that 3 of the 4 large animals transplanted with the Hoxb4 cells
developed myeloid leukemia within 2 years of transplantation (Zhang and Beard, 2008). These
findings illustrate that the integrating over-expression of viral Hoxb4 poses a significant risk of
leukemogenesis and using Hoxb4 clinically would require safer means of delivering the
transcription factor to cells.

Efforts to expose hematopoietic cells to Hoxb4 without viral transduction have been achieved
with a number of different approaches. One study co-cultured UCB cells with a Hoxb4
expressing MS-5 stromal cell line. After 5 weeks of culture, there was a net increase of 3-fold in
CFCs and 4.9-fold in LTC-ICs as compared to controls (Amsellem et al., 2003). Another method involved the production of a TAT-HOXB4 fusion protein, which enabled the nuclear uptake of a soluble protein form of Hoxb4 (Huang et al., 2010; Krosl et al., 2003). Transactivator of transcription (TAT) is an 86 amino acid protein that can function as a cell penetrating peptide, meaning it has the capability of transporting cargo (including proteins, plasmids, liposomes and oligonucleotides) into cells (Chauhan et al., 2007). The major limitation of TAT-HOXB4 was the poor stability of the protein, which limited its nuclear effectiveness and required cumbersome delivery strategies. As a result, the levels of HSPC expansion generated with the soluble factors in these studies were modest.

1.4.3 Signaling pathways influencing HSC self-renewal

Upstream of transcriptional control, receptor-ligand complexes activate signaling pathways in response to external cues to regulate nuclear factor activities. Identifying and understanding the relationship among transcription factors and upstream components of signaling pathways provides a method to identify nodes in the system which may be externally modulated to ultimately manipulate HSC self-renewal decisions. The identification of important signaling pathways in the hematopoietic system has primarily been performed by investigating interactions in the in vivo bone marrow niche throughout HSC development as well as during maintenance in the adult. The roles of important signaling pathways in hematopoietic regulation are typically very context dependent and identifying key regulatory nodes has remained a challenging task. Several major signaling pathways are known to be critical for the regulation of the hematopoietic system, including the Notch, Wnt, JAK-STAT, and Smad pathways.

1.4.4 The Notch pathway

The Notch pathway was first identified as playing an important role in cell fate decisions in invertebrates (Artavanis-Tsakonas, 1999). In the mammalian system, there are 4 Notch receptors (Notch 1-4) and 5 ligands (Jagged- 1 & 2, Delta- 1, 3, 4) (Bray, 2006). Notch ligands are transmembrane proteins, which act through juxacrine signaling on direct neighbouring cells. Upon binding of the extracellular domain of a Notch receptor to a ligand on an adjacent cell, a series of proteolytic cleavage events occurs to release the intracellular domain of the Notch receptor, which enters the nucleus and modulates the transcription factor CSL. This results in the
activation of the Hes1 and Hes5 genes, the most well-studied downstream targets of Notch signaling (Bray, 2006). The Notch pathway plays many dynamic roles during mammalian development and is well studied for its roles in neural (Ryoichiro and Ohtsuka, 1999), cardiac (De la Pompa and Epstein, 2012), bone (Tao et al., 2010) and hematopoietic (Bigas et al., 2010) development and disease.

In the adult hematopoietic system, the Notch pathway is involved in the regulation of many cell lineages. Notch1 activation by Delta-1 or Delta-4 enhances T-cell production at the expense of B-cell production in lymphoid progenitor cells (Pui et al., 1999; Radtke et al., 1999). In the myeloid lineage, Notch2 appears to inhibit myeloid differentiation (Varnum-Finney et al., 2011). Identifying the role of the Notch pathway on HSPCs has proven more complex. Several groups have reported that canonical Notch signaling or the expression of either Notch1 or Notch2 are not required for HSC maintenance at homeostasis (Maillard et al., 2008; Varnum-Finney et al., 2011). The in vivo-induced deletion of either Notch1 or Notch2 resulted in no effect on primitive hematopoietic cell population numbers or frequencies, and no impact on the repopulating ability of the stem cell population. However, in contrast, mice with the Notch2 receptor deletions had less robust recovery from 5FU stress. These animals also had a slower rate of recovery of HSC and progenitors following transplantation. In this study, mice with the Notch1 deletion were comparable to the control, suggesting that the Notch2 receptor is of specific importance for HSC regulation following injury (Varnum-Finney et al., 2011). Thus it appears that the Notch pathway is dispensable in steady-state adult hematopoiesis but is important to maintain appropriate recovery following physiological stress.

In ex vivo culture, the Notch pathway can be activated with Delta ligand expressing feeder cells or with the immobilized presentation of Delta ligands. Adding Delta ligands in soluble form is not effective, and can in fact be inhibitory to Notch pathway activation (Varnum-Finney et al., 2000). The presentation of immobilized Delta-1 in the human hematopoietic system produces many lineage skewing effects, including an increase in CD7+ lymphoid cells, a decrease in CD14+ and CD15+ myeloid cells, and an increase in progenitors and STR-HSCs (Delaney et al., 2005; Yamamura et al., 2007). The effect of Delta-1 on the primitive cell population is seen at low concentrations, while higher concentrations of Delta-1 are needed for T-cell activation. This suggests that Delta-1 may be able to bind to Notch2 at lower concentrations but, at higher
concentrations, there may be preferential binding between Delta-1 and Notch1 (Dahlberg et al., 2011).

1.4.5 The Wnt pathway

The Wnt pathway has been implicated in hematopoietic development, and Wnt5A has been shown to stimulate self-renewal and proliferation of fetal HSPCs (Austin et al., 1997). In adult human hematopoiesis, Wnt5A has been identified in the hematopoietic bone marrow niche and expressed on CD34+ primitive cells (Van Den Berg et al., 1998). Modulation of the Wnt pathway for ex vivo HSPC expansion has also been investigated and studies have reported that the over-expression of beta-catenin or the addition of Wnt3a protein enhanced expansion of murine HSPCs (Reya et al., 2003; Willert et al., 2003).

The downstream mechanisms by which Wnt factors act on HSPCs have not been well understood and conflicting reports of the importance of the canonical Wnt pathway under different hematopoietic contexts have been presented (Blank et al., 2008). A recent study illustrated that activation of the canonical Wnt pathway in addition to suppression of the mTOR pathway led to the maintenance of HSPCs without the need for any exogenous cytokines (Huang et al., 2012), which provides strong evidence for the important role of Wnt regulation. Although its precise mechanism remains unknown, there does appear to be an interaction between the Wnt and Notch pathways in the hematopoietic system (Duncan et al., 2005).

1.4.6 The JAK-STAT pathway

Activation of the JAK-STAT pathway by soluble ligands has been well studied as a means of in vitro hematopoietic regulation. Knockout studies of Jak or Stat components have demonstrated a variety of hematopoietic impairments, including defective lymphoid development, un-regulated myelopoiesis, depleted erythropoiesis, loss of interferon responses, and impairment of proliferation signaling responses (Ward et al., 2000). STAT3 and STA5 are activated in response to a number of hematopoietic cytokines including IL-6, IL-11, IL-3, G-CSF, GM-CSF, Tpo, Epo, and the interferon family. Many of these cytokines are commonly added to culture to enhance proliferation and expansion of primitive hematopoietic cells, although their specific target cell populations are highly variable. One study in murine cells indicated that activation of STAT5 in
primitive cells led to significant expansion of MPPs and enhanced HSPC self-renewal, while STAT3 activation had no effect on in vivo maintenance of HSPCs and appeared to promote lineage commitment in vitro (Kato et al., 2005). However, it has also been reported that STAT3 activation promotes HSPC self-renewal and expansion in the early stages of recovery following transplantation (Chung et al., 2006), suggesting that STAT3 may act in a more transient role in times of physiological stress.

The JAK-STAT pathway is particularly well-studied in the context of hematological disease. Mutations or fusions of Jak1, 2, or 3 have been indicated in very many myeloproliferative diseases, acute leukemias, and immunodeficiency diseases (Vainchenker et al., 2008). The Tel-JAK2 fusion, which constitutively activates the JAK-STAT pathway, has been found in patients with acute lymphoblastic leukemia and chronic myeloid leukemia, and the Jak2 mutation, JAK2V617F, is a contributing factor to many myeloiproliferative neoplasms, by skewing the lineage proliferation potential of HSPCs or more committed progenitors (Kota et al., 2008). As such, JAK-STAT pathway inhibition therapy has become a major area of research for hematologic malignancies.

1.4.7 The SMAD pathway

The Smad pathway consists of a very diverse set of components that have highly divergent roles in hematopoiesis. TGF-β is a key negative regulator of in vitro HSPC expansion and appears to act in part by modulating HSPC quiescence (Challen et al., 2010; Madlambayan et al., 2005; Yamazaki et al., 2009). One study demonstrated that TGF-β inhibits lipid raft clustering and induces hibernation of murine HSCs (Yamazaki et al., 2009). Interestingly, it has been reported that HSCs that are categorized as either myeloid-biased or lymphoid-biased respond differently to TGF-β (Challen et al., 2010). In contrast to the significant effects that are observed when TGF-β is exogenously supplied to HSCs, mice that have a TGF-β receptor knock-out appear to have a completely normal hematopoietic system both under homeostasis and in response to stress (Larsson et al., 2003). This suggests a redundancy among other ligands in vivo, with one candidate being Activin, as it acts through the same R-Smad pathway (Blank et al., 2008).

BMPs are a set of Smad pathway factors which are critical during hematopoietic development, while their role in adult hematopoiesis is less well studied. BMP-4 has been shown to promote
maintenance of HSPCs in vitro, although its ability appears to be limited to maintaining HSPCs and not expanding the pool (Bhatia et al., 1999). A recent report describes a Smad-independent mechanism of BMP-4 which enhances homing of in vitro cultured HSPCs to the BM niche, generating improvements in engraftment following transplantation (Khurana et al., 2012). As BMPs are linked to signaling cascades from BM niche cells, their role may likely be indirect and involve cross-talk with other contributors.

1.4.8 HSC Homing

Homing of HSCs to the bone marrow niche is essential during hematopoietic development when cells first migrate to the bone marrow, but also during adult hematopoiesis, particularly in response to physiological stress or following HSPC transplantation (Chute, 2006; Lapidot et al., 2005). Homing is a rapid process thought to be coordinated by a number of components, including signaling via SDF-1 and SCF ligands, activation of CD44, cytoskeleton rearrangement, and MMP activation and secretion of MMP2/9 (Lapidot et al., 2005). Functional interactions between the CXCR4 receptor and its ligand, SDF-1 are required for effective homing in mouse models. CXCR4 can be up-regulated on CD34+ cells through stimulation with SCF, IL-6, or HGF, ultimately leading to enhanced SDF-1 mediated migration. Modulation of CXCR4 is an avenue of great clinical interest. Efforts to enhance HSC homing following transplantation are being explored either as an alternative to ex vivo HSC expansion or as a complementary strategy, and these approaches primarily rely on regulation of CXCR4 or its downstream targets (North et al., 2007; Peled et al., 2012). Conversely, plerixafor, a CXCR4 antagonist, is used very effectively in the clinic to mobilize CD34+ cells into the peripheral blood (Jantunen, 2011).

1.5 Cell-cell Interactions and Feedback Signaling

In addition to cell intrinsic self-renewal regulators, HSCs are also controlled by cell-cell interactions and endogenously produced paracrine signaling factors. These interactions are critical in the in vivo niche in order for HSCs to respond to context specific needs and physiological demand. In the adult hematopoietic system, there exists set-points for the number and proportion of specific cell populations and the HSCs act in response to feedback control mechanisms to maintain these populations at the appropriate levels. It has been shown that following HSPC transplantation, cell expansion negatively correlates with transplanted cell dose,
suggesting that cells are responding to their environment in order to replenish cell numbers (Iscove and Nawa, 1997). Interestingly while mature cells and progenitors return to pre-transplantation levels, the HSC population does not (Pawliuk et al., 1996). Although the reason for this has not been clearly demonstrated, one explanation is that the different dynamics of mature cell production following transplantation, as compared to during normal development, produces a microenvironment where the rapid expansion of mature cells feedback to limit HSC production before it has returned to baseline levels.

These cell-cell interactions are also extremely important in ex vivo culture processes where the accumulation of these endogenously produced signaling factors can dominate over exogenous factors, thus convoluting the development of HSC expansion strategies. It has been observed that hematopoietic populations secrete large numbers of soluble signaling factors including many growth factors, cytokines, and chemokines which bind to receptors on other hematopoietic cell populations (Billia, 2001; Majka, 2001). These soluble factors create non-linear cell-cell interactions in vitro, as cell populations emerge at different rates and stages of culture, and concentrations of endogenously produced soluble factors are thus highly dynamic and lead to interactions that are not easily predictable (Natarajan et al., 2006). Cytokines can interact with multiple cell types, often in opposing ways or with very different dose responses. Certainly, dose dependent interactions have been well studied among common exogenous hematopoietic cytokines (Audet et al., 2002) and the complexity of these interactions is similar, although cell well understood, among endogenously produced factors.

The production of both progenitors and mature cells throughout culture creates a signaling microenvironment that is in a continuous state of flux. HSPCs integrate stimulatory and inhibitory factors in order to make cell fate decisions (Cashman et al., 1990) and it has been demonstrated that certain mature cell types act in a predominantly positive or negative manner towards HSPC expansion. Studies have reported that monocytes, macrophages, and mature red blood cells secrete factors that inhibit stem and progenitor cells (Cheshier et al., 2007; Kirouac et al., 2010; Xu et al., 2000), while megakaryocytes, platelets and NK cells have been reported to produce stimulatory factors (Fardoun-Joalland et al., 1994; Foss et al., 2008; Kirouac et al., 2010). In vitro expansion strategies typically generate large numbers of mature cells but there
has been little consideration of how the quantities and proportions of these cell populations may in turn be impacting the stem and progenitor populations.

Although the specific feedback effect of individual factors is highly variable, it has been clearly demonstrated that the accumulation of mature blood cells as a whole act in a predominantly inhibitory manner towards HSPC expansion (Gilmore et al., 2000; Madlambayan et al., 2005). This intrinsic cell-level process control mechanism is crucial for maintaining steady state conditions in vivo, but it is a barrier for the in vitro expansion of HSPCs, as these negative regulators inhibit HSPC self-renewal and induce differentiation (Kirouac and Zandstra, 2006; Majka et al., 2001). Although some key negative regulators have been identified, including TGF-β, MIP-1α, and MIP-1β (Kirouac et al., 2010; Madlambayan et al., 2005), it is the global milieu of these secreted factors that acts to inhibit the HSPC populations. Some groups have investigated the manipulation of mature cell populations or the factors that they secrete as a means of regulating the microenvironment. Most in vitro culture strategies include an initial enrichment of primitive human cells, typically with a selection for CD34⁺, CD133⁺, or Lin⁻ cells. This significantly increases the expansion that can be achieved as compared to culturing un-enriched hematopoietic cells. However, even in highly enriched cell populations, mature cells begin to accumulate once culture is initiated. A re-selection for primitive cells partway through culture is one method to control the levels of mature cells (Madlambayan et al., 2005), and specifically depleting CD14⁺ or CD3⁺ cells has also been shown to enhance primitive cell expansion (Yang et al., 2010). Feeding regimes that involve frequent media exchanges or perfusion flow-through systems are alternative strategies that act to remove or diminish the accumulation of endogenous soluble signaling factors, without altering the cell composition (Madlambayan et al., 2005; Sandstrom et al., 1995; Schwartz et al., 1991a).

The impact of cell-cell interactions and feedback signaling provides an additional point of control in the regulation of HSPC fate decisions. These intercellular interactions contribute to signaling pathway activation and transcription factor control and they cannot be uncoupled from the intracellular regulatory networks. The interplay between microenvironmental signaling factors and cell intrinsic control mechanisms is an important point of consideration both for the understanding of in vivo HSC regulation and for the development of in vitro manipulation strategies (Oh and Humphries, 2012).
1.6 Ex vivo HSPC Expansion

Clinical motivation for robustly producing large numbers of HSPCs in culture has created the field of ex vivo HSPC expansion. Determining culture strategies that best support the expansion of HSPCs has been an active area of stem cell research for decades. Although there have been many innovative strategies and step-wise development, progress has been slow and in vitro HSPC production has not yet neared the ultimate goal of attaining unlimited stem cell expansion or eliminating the onset of differentiation of stem and progenitor cells in culture. The same regulatory mechanisms that so precisely and robustly control the production of hematopoietic cells in vivo provide an immense roadblock to in vitro production of large numbers of specific desired cell types.

Classic in vitro expansion techniques focused on providing critical growth factors, initially through serum-based culture and stromal cell co-culture systems (Douay, 2001; Robinson et al., 2005). Stromal cells secrete supportive growth factors and are used as a means to mimic the in vivo HSC niche (Shih et al., 1999). However, stromal cell co-cultures and serum containing cultures are undefined and ultimately not desirable for clinical use and so efforts were taken to develop culture conditions comprised only of defined soluble factors, and serum-free culture systems supplemented solely by recombinant cytokines became the focus of the field.

Cytokines important for the maintenance of HSPCs in culture have been discovered by identifying important factors in the bone marrow niche. Numerous cytokines cocktails have been suggested as potential optimized combinations for human HSC expansion, which typically include combinations of stem cell factor (SCF), Flt3 ligand (FL), thrombopoietin (TPO), erythropoietin (EPO), Interleukin (IL)-1, IL-3, IL-6, IL-11, granulocyte colony stimulating factor (G-CSF), macrophage colony stimulating factor (M-CSF) and granulocyte macrophage colony stimulating factor (GM-CSF) (Audet et al., 2002; Conneally et al., 1997; Luens et al., 1998). The impact of these growth factors are highly dose dependent and context specific and the non-linear interactions among these factors has made finding the optimized cytokine combination a challenging undertaking (Audet et al., 1998, 2002). Furthermore, many factors that are traditionally added exogenously to culture are also endogenously produced by hematopoietic cell
populations, further complicating the analysis of the concentrations of these factors that are required.

There is no standardized consensus of the cytokine cocktail that is best for human UCB culture. Most groups working on human HSC expansion include the combination of SCF, FL, and TPO in their culture media, while some groups additionally include IL-6, IL-3, G-CSF, or a combination of these (Boitano et al., 2010; Csaszar et al., 2012; Delaney et al., 2010; Peled et al., 2004; Zhang et al., 2008). SCF binds c-kit receptor, which is an early acting cytokine essential for development of the hematopoietic system. SCF is known to act synergistically with other cytokines to promote self-renewal, improve homing and stimulate colony formation (Broudy, 1997). It has been shown that the level of SCF directly alters the self-renewal potential of LTR-HSCs, within the first 16 hours of culture (Kent et al., 2008). FL interacts with the flt3 receptor tyrosine kinase, which acts to activate signal transduction pathways that control survival and proliferation. It synergizes with SCF to promote survival and HSC expansion (Diehl et al., 2007). TPO signaling acts through the MAPK pathway, and is shown to promote survival and proliferation. It has also been identified as an upstream activator of Hoxb4 (Kaushansky, 2005; Kirito et al., 2003). IL-3, IL-6, and G-CSF all promote rapid proliferation of myeloid cell populations and these factors may help support HSPC populations in certain contexts (Bordeaux-Rego et al., 2010).

The optimization of cytokine combinations has allowed for the expansion of hematopoietic cells in culture, which can be maintained for several weeks. Many culture strategies produce significant increases in progenitor cell populations, as measured by CD34+ cells, CFCs, and in some instances LTC-ICs. However, it was found that the more primitive HSPC populations could not be significantly expanded with traditional cytokine based culture strategies and these populations tended to decline or at best be transiently maintained in culture (Sauvageau et al., 2004). This likely resulted from the fact that self-renewal of HSCs is tightly controlled by complex signalling networks and cell-cell interactions, which cannot be sufficiently regulated with these cytokines alone. In order to maintain and expand the primitive cell populations, novel approaches were needed to override the HSC machinery.
1.7 Current Strategies for UCB Stem and Progenitor Cell Expansion

Recently, several groups have reported new strategies apparently capable of generating clinically relevant increases of HSPCs (Dahlberg et al., 2011) (Table 1-2). Ex vivo expansion strategies include the addition of novel HSPC-enhancing factors, such as the transcription factor HOXB4 (Amsellem et al., 2003; Tang et al., 2009), the copper chelator TEPA (Peled et al., 2004), the growth factors Angiopoietin-like 5 and IGFBP2 (Drake et al., 2011; Zhang et al., 2008), the immobilized Notch Delta-1 ligand (Delaney et al., 2010), or the aryl hydrocarbon receptor antagonist small molecule SR1 (Boitano et al., 2010). These strategies have all cultured UCB cells for 1-3 weeks in the presence of standard hematopoietic cytokine combinations.

Table 1-2: Selected current laboratory strategies for the ex vivo expansion of hematopoietic stem and progenitor cells.

<table>
<thead>
<tr>
<th>Strategy</th>
<th>Starting Population</th>
<th>Culture Conditions</th>
<th>Method of Assessing HSPC Expansion</th>
<th>HSPC Expansion Achieved (time in mouse)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>HOXB4</td>
<td>CD34(^+) UCB</td>
<td>14 day culture co-culture with MS-5 cells</td>
<td>LDA in NOD/SCID mice</td>
<td>2.5-fold (7 weeks)</td>
<td>(Amsellem et al., 2003)</td>
</tr>
<tr>
<td>Copper Chelator TEPA</td>
<td>CD133(^+) UCB</td>
<td>21 day culture SCF, FL, TPO, IL6</td>
<td>% chimerism in NOD/SCID mice</td>
<td>non-quantitative increase (8 weeks)</td>
<td>(Peled et al., 2004)</td>
</tr>
<tr>
<td>Angiopoietin-like 5 and IGFBP2</td>
<td>CD34(^+) CD133(^+) UCB</td>
<td>10 day culture SCF, TPO, FGF-1</td>
<td>LDA in NSG mice</td>
<td>20-fold (12 weeks)</td>
<td>(Drake et al., 2011)</td>
</tr>
<tr>
<td>Notch Delta-1 Ligand</td>
<td>CD34(^+) UCB</td>
<td>17-21 day culture SCF, FL, TPO, IL6, IL3</td>
<td>LDA in NSG mice</td>
<td>15.6-fold (3 weeks) 6.2-fold (9 weeks)</td>
<td>(Delaney et al., 2010)</td>
</tr>
<tr>
<td>Aryl hydrocarbon receptor antagonist SR1</td>
<td>CD34(^+) UCB</td>
<td>21 day culture SCF, FL, TPO, IL6</td>
<td>LDA in NSG mice</td>
<td>17-fold (13-16 weeks)</td>
<td>(Boitano et al., 2010)</td>
</tr>
<tr>
<td>Fed-batch bioreactor</td>
<td>Lin(^-) UCB</td>
<td>12 day culture SCF, FL, TPO</td>
<td>LDA in NSG mice</td>
<td>11-fold (16 weeks)</td>
<td>(Csaszar et al., 2012)</td>
</tr>
</tbody>
</table>

Other groups have used approaches whereby factors are identified that may enhance HSC homing or survival once transplanted. Prostaglandin E2 (PGE2) was identified initially in zebrafish, and a brief incubation (1-2 hr) of cells with PGE2 prior to transplantation led to
significantly higher numbers of repopulating HSCs (North et al., 2007). Other strategies that appear to enhance homing of transplanted cells include fucosylating cells prior to transplant (Robinson et al., 2012) or culturing cells in the presence of nicotinamide (Peled et al., 2012).

Advances have also been made to bring some of these ex vivo expansion approaches into early stage clinical trials (Table 1-3). These trials have demonstrated the important precedence for the safety of transplanting ex vivo expanded HSPCs into patients (Jaroscak et al., 2003; de Lima et al., 2008; Shpall et al., 2002). Clinical data are also beginning to validate the predicted benefits of transplanting expanded UCB units. The trial described by Delaney and colleagues (Delaney et al., 2010) demonstrated that by transplanting an ex vivo expanded UCB unit in combination with an un-manipulated unit, the median time to absolute neutrophil count ≥ 500/μL was significantly shortened. A recent report of the clinical results with a strategy in which UCB cells were co-cultured with mesencymal cells also showed improved neutrophil engraftment in the patients who received the ex vivo expanded cells (De Lima et al., 2012). Importantly, these first clinical trials will also generate valuable data providing insight into the correlation between clinical results and standard pre-clinical characterization of stem and progenitor cell content.
Table 1-3: Clinical trials using expanded cord blood.

<table>
<thead>
<tr>
<th>Trial</th>
<th>Expansion strategy</th>
<th>Number of patients</th>
<th>Reported findings</th>
</tr>
</thead>
<tbody>
<tr>
<td>M.D. Anderson Cancer Center</td>
<td>One portion of UCB unit was enriched for CD34+ cells and expanded for 10 days with growth factors. Un-manipulated portion of UCB unit transplanted either on day 0 or day 10.</td>
<td>Phase I/II: 37</td>
<td>Safety demonstrated (Shpall et al., 2002)</td>
</tr>
<tr>
<td>Aastrom Biosciences</td>
<td>Major portion of UCB unit transplanted on day 0, remaining portion expanded for 12 using the AastromReplicell System, continuous perfusion device and then transplanted</td>
<td>Phase I/II: 28</td>
<td>Safety demonstrated (Jaroscak et al., 2003)</td>
</tr>
<tr>
<td>Gamida Cell-TEVA NCT00469729</td>
<td>CD133+ enriched cells from portion (20-50%) of UCB unit were cultured for 21 days with StemEx (copper chelator TEPA). Transplanted with remaining un-manipulated portion of UCB unit</td>
<td>Phase I/II:10</td>
<td>Safety demonstrated (De Lima et al., 2008)</td>
</tr>
<tr>
<td>Fred Hutchinson Cancer Research Center NCT00343798</td>
<td>CD34+ enriched cells expanded for 16 days on flasks coated with Notch Delta-1ext-IgG. Transplanted with second un-manipulated cord</td>
<td>Phase I/II:25 (160 patient Phase II/III trial initiated)</td>
<td>Safety demonstrated, decreased time to neutrophil engraftment reported on first 9 patients (Delaney et al., 2010)</td>
</tr>
<tr>
<td>Mesoblast/M.D Anderson Cancer Center NCT00498316</td>
<td>14 day co-culture of un-enriched UCB with off-the-shelf mesenchymal stromal cells. Transplanted with second un-manipulated cord</td>
<td>Phase I/II: 31 (240 patient phase III trial underway)</td>
<td>Safety demonstrated, decreased time to neutrophil engraftment and platelet engraftment (De Lima et al., 2012)</td>
</tr>
<tr>
<td>Fate Therapeutics NCT00890500</td>
<td>UCB unit incubated for 1-2h with ProHema (Prostaglandin E2). Transplanted with a second un-manipulated unit</td>
<td>Phase I/II: 24</td>
<td>Trial initiated</td>
</tr>
<tr>
<td>Novartis NCT01474681</td>
<td>CD34+ enriched cells expanded for 21 days with aryl hydrocarbon antagonist small molecule, LFU-853 (SR1). Transplanted with a second un-manipulated unit</td>
<td>Phase I/II: 15</td>
<td>Trial initiated</td>
</tr>
<tr>
<td>Gamida Cell-TEVA NCT01221857</td>
<td>CD34+ enriched cells expanded for 21 days with NiCord (nicotidamide). Transplanted with a second un-manipulated unit.</td>
<td>Phase I/II: 11</td>
<td>Trial initiated</td>
</tr>
</tbody>
</table>
1.8 Translating in vitro Culture to Clinical Manufacturing Processes

With several successful expansion strategies being developed in the laboratory and assessed in clinical trials, attention must be given to the translational challenges of preparing ex vivo UCB expansion for broad clinical use. Clinical results indicate that even modest expansion of stem and progenitors may provide beneficial outcomes (Barker et al., 2010b; Michel et al., 2003; Rubinstein et al., 1998). As highlighted in Table 1-4, a 15-fold expansion of LTR-HSCs would enable patients of up to 162kg to receive the equivalent levels of LTR-HSCs that are found in the recommended minimum cell dose for an un-manipulated unit. Therefore, focus may not need to be on further increasing absolute LTR-HSC expansion for the purpose of single patient transplantation, but instead working with expansion systems currently capable of producing a 10-20-fold increase in LTR-HSCs, ensuring the presence of other clinically relevant progenitor populations in the graft, and implementing cell growth techniques that can be translated into robust and highly reproducible technologies.

Table 1-4: Calculations of LTR-HSC content in expanded UCB cells.

<table>
<thead>
<tr>
<th></th>
<th>Calculation</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Minimum recommended UCB cell dose</td>
<td>2.5 x 10^7 cells/kg (Gluckman, 2009)</td>
</tr>
<tr>
<td>2</td>
<td>Approximate CD34^+ cells in mononuclear UCB</td>
<td>1 in 100      (D’Arena et al., 1996)</td>
</tr>
<tr>
<td>3</td>
<td>Approximate LTR-HSCs in the CD34^+ fraction</td>
<td>1 in 1000     (Boitano et al., 2010)</td>
</tr>
<tr>
<td>4</td>
<td>Approximate LTR-HSCs in mononuclear UCB</td>
<td>1 in 100,000* (2) x (3)</td>
</tr>
<tr>
<td>5</td>
<td>Aim for LTR-HSC dose in un-manipulated cells</td>
<td>≥250 LTR-HSC/kg (1) x (4)</td>
</tr>
<tr>
<td>6</td>
<td>Average post-selection CD34^+ cells in a clinical UCB unit</td>
<td>2.7 x 10^6 cells/kg (Delaney et al., 2010)</td>
</tr>
<tr>
<td>7</td>
<td>LTR-HSCs in clinical UCB unit</td>
<td>2,700         (6) x (3)</td>
</tr>
<tr>
<td>8</td>
<td>LTR-HSCs following 15-fold ex vivo expansion</td>
<td>40,500        (7) x 15</td>
</tr>
<tr>
<td>9</td>
<td>Maximum patient weight based on aim of 250 LTR-HSC/kg after expansion</td>
<td>162kg         (8) / (5)</td>
</tr>
</tbody>
</table>

* Based on the NOD.Cg-Prkdcsclid Il2rg^tm1Wjl/SzJ (NSG) mouse model (this frequency has been reported to be 1 in 930,000 in the NOD/SCID model (Bhatia et al., 1997)

In order to evaluate the potential utility of expansion strategies, it is important to clarify what level of expansion, and of what specific cell populations, will produce clinical benefit. This will help to identify the critical quality attributes (CQAs) (ie: the characteristics that define the product's quality and impact the patient) of expanded HSPC products. These correlations will be crucial for determining how best to optimize and comparatively evaluate ex vivo expansion strategies and to help determine appropriate release criteria (ie: the targets that the cell product is
required to meet following ex vivo expansion in order to be deemed acceptable for transplantation).

Incorporating fundamental bioprocess design strategies into HSPC expansion technologies should have a positive impact on blood product manufacturing. Considerations for the clinically-relevant production of ex vivo expanded HSPCs include: (1) ensuring appropriate assessment of CQAs in the expanded cell product by determining which parameters are most relevant to clinical outcomes and standardizing the measurements of these parameters; (2) addressing sample-to-sample variability in the input population and throughout the culture; (3) developing strategies that allow for scalability; (4) identifying opportunities for cost reduction and setting target costs to make UCB ex vivo expansion a feasible approach. These considerations are summarized in Figure 1-2.

Figure 1-2: Considerations for translating a cell culture protocol to a clinical manufacturing process
1.8.1 Standardized evaluation of the expanded cell product is needed

For expanded UCB units to be used as a feasible clinical cell product, accurate assessment of the relevant desired outputs is required. This will be important both in the preclinical development stage in order to rigorously evaluate and optimize expansion strategies, and as release criteria for clinical use, where rapid evaluation is required. For both types of assessments, the major challenge is determining which assays provide the best prediction of positive clinical outcomes.

Assessment of expansion strategies is limited by the relevance of the in vitro and in vivo platforms that can be used to characterize and quantify human hematopoietic cells (as discussed in the above sections). It remains to be seen how xenograft model outputs quantitatively correlate with cells associated with short term recovery and long-term graft stability in human recipients. In order to make these comparisons, it will be important during early clinical trials to assess clinically expanded UCB cells in xenograft models (and surrogate assays) alongside clinical transplantations when possible.

It will also be important to correlate functional xenograft assays to end-points that can be measured rapidly, with small cell numbers and at low cost. This rapid assessment can then be used for multiple iterations of optimization during development, and serve as release and product characterization criteria of CQAs in clinical trials. Current assessment of banked un-manipulated UCB prior to transplant generally relies on measuring total cell number, cell viability, and CD34^+ number. Additional parameters including red blood cell counts, white blood cell counts, and CFC assays are recommended by some transplant centres (Page et al., 2011; Spellman et al., 2011) and more quantitative UCB quality scoring scheme have been devised (Page et al., 2012). A threshold of CD34^+ cell viability ≥ 75% has also been recommended for transplantation (Scaradavou et al., 2010). It is unclear how these criteria translate to the clinically relevant characterization of ex vivo expanded cells and, while providing a good indication of cell dose and progenitor content, the current assessment assays do not provide insight into the LTR-HSC content or lineage specific cell population information.

The recent identification and rigorous validation of surface marker based phenotypes that characterize human HSPCs subsets (Doulatov et al., 2010; Notta et al., 2011) has provided a strategy to evaluate more specific sub-populations of un-manipulated cells. Correlating these
populations with clinical outcomes will provide more information on the importance of specific HSPC doses. Extending these findings to evaluate their relevance in ex vivo expanded cell populations will be necessary for the rapid analysis of expansion strategies and would further enable the routine assessment of expanded cell products being used in the clinic prior to transplantation. In addition to CD34+ as a marker of progenitor cell content in ex vivo expanded cells, CD133+CD38- and CD34+CD90+ or CD34+CD133+CD90+ cells appear to correlate well with functional outputs from cultured HSPCs (Csaszar et al., 2012; Danet et al., 2001; Ito et al., 2010). The assessment of major mature cell lineage phenotype markers (CD14+ monocytes, CD7+ lymphocytes, CD41+ megakaryocytes, GyA+ erythrocytes, CD15+ granulocytes) would also be informative to evaluate lineage frequencies following ex vivo expansion. Of note, it has been demonstrated that transplanting CD41+ megakaryocytes produced in vitro into a mouse model led to enhanced platelet recovery (Tijssen et al., 2008), suggesting that the CD41+ phenotype may be particularly important to assess and correlate with clinical outcomes.

1.8.2 Quality control checkpoints of input populations should be introduced

Producing a robust cell product is dependent on the control of desired outputs (cell quantity, phenotype, and functionality) through the manipulation and regulation of system inputs (culture parameters). In vitro hematopoietic culture is a heterogeneous and dynamic system. Its complex network of interacting feedback loops results in a non-linear system, such that there is often not a simple relationship between a change in an input parameter and the resulting effect on the culture output. External input parameters (culture vessel, culture media composition, oxygen levels, cell seeding densities, length of culture period) can be set to help limit variability, although re-optimization of some parameters is required with scale-up.

One source of variability that cannot be eliminated at input is the intrinsic variability of the primary human UCB cell source. In a study of 4,930 banked UCB units, the CD34+ cell number per unit varied from $0.1 \times 10^6 - 43.6 \times 10^6$ with a mean ± SD of $3.7 \times 10^6 ± 3.4 \times 10^6$ (Yang et al., 2011). These input populations may vary based on differences in the frequencies of cell sub-populations and the specific soluble factors that they secrete, and the non-linearity of the system means that small differences in input populations may be rapidly amplified as culture progresses.
(Kirouac et al., 2009). In one clinical expansion study (De Lima et al., 2008), the observed total cell expansion ranged from 2-fold to 620-fold (mean = 219.2-fold), and CFC expansion ranged from 1.6-fold to 85.9-fold (mean = 37.8-fold). Of the 10 expanded units, one unit produced an exceptionally low expansion of 2-fold and would be classified as a poor expander. Based on the input data provided, the very low input CFC frequency of this sample (3/1000 as compared to the mean for all 10 units of 87.1/1000) is one parameter that may provide a retrospective prediction of the poor expansion. However, due to the time-frame of the CFC assay, this information would only have been available after the expansion culture had been initiated.

As with the output cell assessment, input cell populations should be assessed phenotypically to characterize the progenitor and mature cell content of the starting material. Stringent quality assessment of the input material is particularly crucial for cases in which pre-transplantation conditioning regimens must begin before the final expanded cell product can be assessed. Evaluating cells not only at input but also at an early time point during expansion may also provide a check-point as to the potential success of the final expansion. When initiating cultures with CD34+ enriched cells, a minimum threshold of %CD34+ and %CD34+CD38- or %CD133+CD38- may help eliminate units unlikely to expand well, although setting specific clinical thresholds will necessitate additional clinical data to better understand required cell yields.

1.8.3 Dynamic regulation of culture can address sample to sample variability – towards “quality by design”

Input assessment can provide a prediction of the success of a particular UCB sample and help to reject possible poor performers. However, it cannot completely eliminate sample variability that will manifest during culture, and imposing input thresholds that are too strict may result in discarding potentially high quality cells (Andrade et al., 2011). As such, it would be desirable to have a means to monitor and control cell culture as it progresses. Traditional bioprocesses have incorporated sensors and feedback control systems for controlling parameters including dissolved oxygen levels, glucose and metabolite concentrations, and pH. These are important considerations, especially as culture strategies are scaled up and a variety of sensing and monitoring options are available for use in HSPC expansion cultures (Lim et al., 2007).
Control of cell-level parameters including cell heterogeneity, the local microenvironment, and signalling feedback loops have been largely underappreciated aspects that also should be tightly regulated in a manner that can account for the dynamically changing cell culture environment and biological variability of samples. Cell cultures are subject to dynamic changes in environmental conditions and culture composition as cells interrogate and interpret signaling from the microenvironment and neighbouring cells in order to modulate cell fate decisions.

Methods that regulate or limit these inhibitory feedback signals can be pre-established as a set trajectory or based on measurements of soluble factors with ELISA or proteomics technologies. More powerful is the ability to monitor and control these factors or cell sub-types in a real-time manner as part of a responsive regulation approach. One approach is the implementation of a process control system based on the rapid and automated measurement of important soluble factor(s) (Klostranec et al., 2007). This type of system could be integrated into a bioreactor and would measure concentrations of secreted proteins and maintain these concentration below a preset upper threshold through the automated adjustment to a media dilution scheme. Regular monitoring and control in this manner may allow for tighter regulation of feeding regimes in order to address sample variability.

1.8.4 Efficient scale-up can be accomplished by minimizing complexity

Translating a laboratory expansion strategy to routine clinical use necessitates cell culture to be carried out in a standardized and highly reproducible environment. Cultures also have to be scaled up to accommodate clinical-sized UCB units. The culture volume that is needed is dependent on cell densities, length of culture, and total cell expansion. In mammalian cell culture systems, nutrient requirements and oxygenation needs will limit maximum cell densities that can be maintained (Patel and Papoutsakis, 2000; Roy et al., 2012). In the heterogeneous hematopoietic system, inhibitory feedback signaling from soluble factors also acts to practically limit cell densities (Kirouac et al., 2009). Tight dynamic regulation of these parameters can push the limits of culture densities to some extent; however, culture size (or medium throughput in a continuous flow system) ultimately must scale with cell expansion. In the clinical expansion performed with the Notch ligand (Delaney et al., 2010), cultures were initiated at low cell density with an average of 2.7 x 10^6 CD34^+ cells, and an average total cell expansion of ~560-fold was obtained over the culture period while maintaining a cell density of < 1 x 10^6 cells/mL and
adding fresh media and cytokines every 3-4 days. Therefore, during a 16 day clinical culture, ~10 L of culture media was required to expand a unit.

Groups have made use of a variety of bioreactor designs to accommodate the scale-up of cell culture in a manner that enables the production of the required quantity and quality of cells by allowing for control of external parameters (oxygen, pH, glucose, shear), and ease of handling of culture. Bioreactor designs for hematopoietic cell culture have taken many forms with varying levels of sophistication, including gas-permeable culture bags, roller bottles, stirred tank batch reactors, fixed-bed reactors, three-dimensional scaffolds, and media perfusion systems (Cabrita et al., 2003; Kowalczyk et al., 2011). The logistics of clinical HSPC expansion will also dictate the degree of automation and complexity of the bioreactor that is required. A bioreactor with a simple design and a one-time-use culture vessel may be most useful for UCB expansion to be carried out in multiple GMP facilities, with high reliability, and without need for highly specialized handling skills.

Hematopoietic cells can be cultured in suspension in a well mixed bioreactor by utilizing culture strategies that avoid the need for adherent stromal cells, immobilized ligands, or biomaterial scaffolds. These approaches greatly simplify the requirements of the bioreactor design. Combining these minimal requirements with a single-use bioreactor would enable a system capable of handling the cell quantities produced with expansion in a relatively inexpensive manner. Several varieties of disposable bioreactors systems have been used to culture suspension cells in a single-use gas permeable bag, where mixing of soluble factors is achieved with a rocking motion and dissolved oxygen and pH can be monitored (Somerville et al., 2012; Yuk et al., 2011).

1.8.5 The cost of clinical HSPC expansion must be considered at all stages of translation

Economics must be considered throughout the process of clinical translation, in order to understand economic drivers of ex vivo cell expansion and to identify areas of potential cost reduction. HSPC transplantation is an expensive therapy and the use of patient-specific ex vivo
expanded UCB cells will introduce additional costs related to the cell manufacturing process (see Figure 1-3).

![Figure 1-3: Approximate relative costs associated with the transplantation of ex vivo expanded UCB cells.](image)

Costs of HSPC transplantation in the US include: graft acquisition, estimated to be $68,200 for 2 UCB units (Bart, 2010); and hospital costs, estimated to be $243,500, including pharmacy charges of $91,882, room charges of $79,856, and other charges of $71,872 (Kline et al., 1998). Transplanting an ex vivo expanded cell product adds manufacturing costs, including GMP quality reagents, estimated here to be $40,000 based on current approximate costs of reagents needed for clinical-scale expansion, and costs of personnel, facilities, and administration of GMP production, estimated here to be $40,000. Manufacturing costs will vary substantially and will be highly dependent on production scale. By improving time to mature cell engraftment, the expanded cell product may contribute to reduced hospital stay and thus reduced hospital costs.

One controllable cost associated with the manufacturing of clinical-scale UCB cell products will be the reagent cost of GMP expansion. Due to the high costs of clinical grade cytokines and supplements, the expense of complete culture media can be on the order of several thousands of dollars per litre, in addition to any novel factors being added to culture that will be costly to produce in a GMP facility. These costs drive the desire for culture intensification in order to minimize culture volumes, while still producing the desired quantity and quality of cells.
Although researchers traditionally have had the goal of producing as many HSPCs as possible in culture, understanding how HSPC numbers specifically correlate with transplant success will allow for strategies to be fine-tuned such that excess off-target cells are not being unnecessarily produced and driving up costs.

To partially offset the associated manufacturing costs, the transplantation of expanded cells may allow for some cost recovery to the health care system. One major goal of transplanting ex vivo expanded UCB cells is decreasing the time to neutrophil and platelet recovery. Increasing total cell and progenitor levels appear to promote decreased time to mature cell engraftment (Delaney et al., 2010; Laughlin et al., 2001), which still remains significantly delayed in double UCBTs. Accomplishing this goal should reduce the average days of hospitalization for a patient. A analysis of the hospital charges from 49 HSPC transplant patients in the US found that the mean total hospital charge was $243,550, of which $79,856 resulted from room charges (Kline et al., 1998). These costs were positively correlated with length of stay and allogeneic transplant patients had an average daily hospital charge of $7,043 per day. Thus, reducing length of hospitalization is a major source of potential economic savings.

Public UCB banks impose minimum cell number thresholds that act as an initial cut-off criterion on the cells that are being banked (Sun et al., 2010). One study predicted that having a minimum requirement of $9 \times 10^8$ total nucleated cells would ensure that the average stored UCB unit would contain $1.38 \times 10^9$ cells and would meet the minimum cell dose requirement of $2.5 \times 10^7$ cells/kg for patients up to 55kg, but would result in ~45% of collected UCB units being discarded for not meeting the cut-off (Querol et al., 2009). Cost savings and efficiencies to the system would be achieved if smaller UCB units could be banked and expanded before use, or used to supply additional committed progenitors to compliment an expanded graft. For instance, bringing the cell number requirement for banking down to $5 \times 10^8$ total nucleated cells would reduce the discarded units from 45% to 14% (Querol et al., 2009) and would theoretically only require a 2-fold expansion to make those units available to the same 55kg patient.

1.8.6 Forward looking to HSPC derived cell therapies

The initial clinical indications for use of expanded HSPCs will be the malignant and non-malignant blood and immune diseases that are currently being treated by HSPC transplants but
are limited by availability of donors and low cell numbers (Davies et al., 2008; Gyurkocza and Rezvani, 2010; Khaled et al., 2012; McLornan et al., 2012; Oyekunle et al., 2011; Schmit-Pokorny, 2009; Tolar et al., 2012). Approximately 60% of allogeneic HSPC transplants are performed for the treatment of acute myeloid leukemia, acute lymphoblastic leukemia, and myelodysplastic syndrome and myeloproliferative disorders. The wide-spread application of ex vivo expanded HSPCs for the treatment of leukemias represent the first step in what can be considered the stage-wise translation of hematopoietic cell based therapies. The levels of expansion needed to reach targets for these indications are relatively modest and so are the associated scale-up challenges.

It is further important to note that there are theoretical risks associated with transplanting ex vivo expanded UCB cells, which include graft failure, potential increases in GVHD, and engraftment syndrome. To circumvent the risk of graft failure resulting from the ex vivo manipulation of cells, clinical protocols for expanded UCB trials typically transplant a second un-manipulated UCB unit along with the expanded unit. The transplant of the un-manipulated unit and, in some instances the additional infusion of the CD34^- cells from the manipulated unit, also ensure the presence of lymphoid cells that would otherwise be depleted during CD34^+ enrichment and potentially lead to increased risk of infectious complications and graft rejection (Childs et al., 1998; Holmberg et al., 1999). However, future strategies that would allow for the transplantation of only the single expanded unit would significantly reduce material costs. In mPB transplantations, there are some reports that very high CD34^+ cell dose (8-17 x 10^7 CD34^+ cells/kg) may be associated with increased GVHD (Mohty et al., 2003; Remberger et al., 2008). This link has not been made in UCBT, however if very high levels of CD34^+ expansion are achieved, upper thresholds on cell numbers could be implemented to alleviate this concern. Engraftment syndrome is a clinical manifestation related to the release of pro-inflammatory cytokines that occurs immediately prior or at the time of neutrophil engraftment. There have been reports that high cell doses increase the risk of engraftment syndrome, making it a possible concern if transplanting highly expanded cells, although this correlation remains uncertain (Lopes da Silva et al., 2012; Ravoet et al., 1996; Spitzer, 2001).

The ex vivo expansion of UCB cells to generate higher numbers of HSPCs is just one aspect of UCB "graft engineering" that is being explored as a means of making HSPC transplantation
more successful. Transplant patients face serious challenges related to the high instances of infection (particularly with delayed immune reconstitution) and possibility of relapse, and methods to engineer immunotherapy approaches from UCB cells are being explored by several groups as a treatment option to be combined with HSPC transplantation (Delaney et al., 2013). Viral infections, such as cytomegalovirus, Epstein-Barr virus, and adenovirus are strongly associated with morbidity and mortality in UCBT patients. The generation of UCB-derived viral specific memory T-cells to supplement a UCBT has been investigated preclinically (Hanley et al., 2009; Park et al., 2006) and is now being explored in a clinical trial (Delaney et al., 2013). UCB derived T-cells are also be used to target malignancies via chimeric antigen receptors (CARs). These T-cells can be modified to express CD19 or CD20 CARs to target tumour cells is a means of preventing relapse following transplantation (Micklethwaite et al., 2010; Serrano et al., 2006). The ex vivo production of UCB-derived NK cells is another avenue being explored for relapse prevention and disease control (Xing et al., 2010). Although approaches are still in early stages of development, they have the potential to greatly improve patient outcomes.

Looking forward to future capabilities of expanded hematopoietic cell products, the ability to expand much larger numbers of LTR-HSCs, primitive progenitors, or specific mature cell populations, may allow for the generation of "off-the-shelf" blood products available for multiple patients. As our understanding of hematologic and immunologic diseases and treatments expands, and our ability to produce high quantity and quality blood products increases, more therapeutic potentials for blood cell products will emerge. Each of these will come with greater challenges of generating and accurately characterizing the functional cell type(s) of interest and producing cell products at the needed quantity in an economically feasible manner.

Extending UCB expansion techniques to the expansion of mPB would help advance the autologous transplant field. Un-mobilized peripheral blood contains very low levels of CD34+ cells (<0.05% of white blood cells) and the mobilization process can increase this fraction up to ~6%. (Moog, 2008; Vose et al., 2009). Although greater absolute numbers of CD34+ cells can be obtained from mPB as compared to UCB, these cells have less repopulation potency and it is recommended that >2 x 10^6 mPB CD34+ cells/kg be transplanted (Allan et al., 2002). Many patients do not mobilize well, particularly in the autologous setting, and it has been reported that 13% of patients were unable to mobilize 2 x 10^6 CD34+ cell/kg in ≤ 4 apheresis days, using
Plerixafor and G-CSF (DiPersio et al., 2009). Ex vivo expansion can potentially increase HSPC numbers in peripheral blood of poor mobilizers in order to achieve the minimal cell number predicted to lead to successful transplantation. Expansion strategies for mPB are similar to those used for UCB and several HSPC enhancing factors have been shown to be effective on both cell sources, although the absolute self-renewal and expansion capacity of mPB is significantly lower than UCB (Mayani, 2010; Tanavde et al., 2002). Interestingly, it has been reported that CD34+ cells from "poor mobilizers" may have greater expansion potential than those from "good mobilizers" (Ivanovic et al., 2010), although clinical efficacy of these cells remains to be determined.

Using in vitro culture for the large scale manufacture of mature blood cells will require sophisticated culture strategies and scale-up approaches, due to the extremely high cell numbers required. Some of these requirements are highlighted in Table 1-5. Understanding and overcoming the translational challenges for HSPCs will help to pave the way towards tackling the challenge of large scale blood cell product manufacturing to be used for a variety of therapeutic needs.
Table 1-5: Options and challenges for the large scale production of blood cell products.

<table>
<thead>
<tr>
<th>Blood Cell Type</th>
<th>Clinical Need</th>
<th>Possible Solutions</th>
<th>Challenges</th>
</tr>
</thead>
</table>
| Neutrophils              | Neutropenia  
(Neutropenic infection is the most common cause of chemotherapy associated death (Ziglam et al., 2007)) | Neutrophil donors  
Expanding and transplanting patient specific neutrophil precursors (Prince et al., 2004) | Limited by cell number and lack of shelf life  
Limited by donor supply and cost of patient specific therapy  
Large cell requirements (patients may require >1x10^{10} cells daily for several days) (Timmins et al., 2009) |
| Megakaryocytes/Platelets | Thrombocytopenia  
Platelet donors (~2 million units transfused in US annually (Whitaker et al., 2011)) | Ex vivo production of platelets (Reems et al., 2010)  
Ex vivo production of mature neutrophils (Timmins et al., 2009) | Concerns related to low donor rates and infectious disease transmission (Matsunaga et al., 2006)  
Very large cell requirements (each donor unit contains 5.5 x 10^{10} platelets (Timmins and Nielsen, 2009)) |
| Red blood cells (RBCs)   | Blood loss due to trauma, disease, or surgery  
Blood donors (~15 million units collected in US annually at a cost of $223 per unit (Whitaker et al., 2011)) | Ex vivo production of RBCs (Mountford et al., 2010; Timmins and Nielsen, 2009) | Concerns related to infectious disease transmission, low donor rates especially in developing countries, rare erythrocyte phenotypes (Timmins and Nielsen, 2009)  
Massive cell requirements (each donor unit contains 2 x 10^{12} RBCs (Mountford et al., 2010)) |

1.9 Mathematical Modeling of Stem Cell Systems

Mathematical modeling can be an extremely useful and complementary tool in stem cell biology, both for describing and understanding systems and for providing predictions of experimental outcomes that are either not possible to experimentally assess or technically and economically limiting to assess in full. In these cases, a model allows for the system to be interrogated in silico and predictions can be made to guide experimental validations. Many models of hematopoiesis
have been created in order to capture and investigate a specific aspect of the system, with the fundamental rules underlying the model being either stochastic, deterministic, or a combination of the two (Viswanathan and Zandstra, 2003).

Modeling of cell fate decisions has long been used to help explain kinetics and probabilities of hematopoietic cell properties. Stochastic models have been used to explain the frequency of colony formation by hematopoietic progenitors and clonal contribution following HSC transplantation in mice (Nakahata et al., 1982; Roeder et al., 2005). These models empirically fit data, based on the assumption that the process is subject to a component of randomness, or different behaviours resulting from seemingly identical cells. Much of this observed behaviour likely results from the fact that experimental data is often acquired at the population level and variation among individual cells may lead to results that appear to have a large component of randomness.

Given the high level of regulation and responsiveness that is required from the hematopoietic system, it is unlikely that stochastic mechanisms alone could provide the needed level of control to any aspect of the system. The earliest deterministic models of hematopoiesis looked at the kinetics and factors that dictated HSC population size (Kirk et al., 1968; Lajtha et al., 1964). As more biological insight into regulation of the hematopoietic system emerged, modeling portions of the complex hematopoietic system with deterministic models became a more commonly used approach.

Simulating in vitro hematopoiesis has been attempted with multiple modeling strategies. Compartmental based models have been created to model culture dynamics, in which internal parameters of self-renewal and proliferation rate were set assuming constant exogenous stimulus (Peng et al., 1996; Varma et al., 1992). Other models have looked specifically at the impact of exogenous growth factor signaling on cell proliferation (Chaudhry et al., 2004). Models that combine both deterministic and stochastic elements have been developed by setting internal parameters as distributions as opposed to specific values, which allows for the capture of some degree of single cell heterogeneity within a population (Glauche et al., 2007; Kirouac et al., 2009; Viswanathan et al., 2005). In contrast to uni-directional discrete compartmental models, alternative models describe the hematopoietic system as a continuum through phase space,
subject to attractors that represent differentiated cell types (Huang et al., 2005; Kirkland, 2004). This type of model is more consistent with potential tissue plasticity and the ability of hematopoietic cells to travel multiple paths through the differentiation landscape.

As in all fields, all models of hematopoiesis are subject to many assumptions and simplifications that limit their ability. Despite the strides that have been made in understanding the regulatory networks that guide hematopoietic fate decisions, many unknowns remain regarding cell population dynamics, non-linear cell-cell interactions, and the balance between exogenous and endogenous regulators. As new -omics and bioinformatics techniques emerge to allow for the measurement and quantification of some of these parameters, rich datasets are becoming available to be fit into more sophisticated models with more predictive power. Going forward, the role of mathematical modeling in understanding the system and guiding experiment design will continue to expand as a valuable tool in all areas of cell biology including hematopoiesis.
1.10 Thesis Hypothesis and Approach

1.10.1 Hypothesis
The central hypothesis of this work is that feedback mediated mechanisms are critical for the regulation of cell fate decisions and by concurrently manipulating the interplay between important stem cell autonomous and non-stem cell autonomous regulators, we will improve hematopoietic stem cell expansion and generate a greater understanding of cell interactions in complex heterogeneous systems. Specifically, in Chapter 2 we hypothesize that optimizing the delivery of TAT-HOXB4 will enhance HSPC expansion and provide insight into the relationship between HOXB4 and the cell microenvironment. In Chapter 3, we hypothesize that reducing endogenously produced soluble factors will allow for the production of a more supportive microenvironment which will lead to clinically relevant levels of HSPC expansion. In Chapter 4, we hypothesize that controlling both the cell microenvironment and exogenous Notch Delta-1 ligand levels will enable the elucidation of mechanistic information of Notch signalling and generate an improved expansion strategy.

1.10.2 Project summary
The goal of this work to achieve a greater understanding of the feedback mediated control mechanisms that regulate HSC self-renewal in vitro and in vivo. Cell fate decisions are regulated by the balance between the stem cell autonomous factors and feedback signaling from the microenvironment. If the balance of these components can be manipulated and controlled, it is expected that predictive regulation of HSC self-renewal can be achieved. Enhanced HSC expansion in a clinically relevant manner will enable more effective strategies to treat hematologic disease and increase the therapeutic potential of UCB cells. Furthermore, a greater understanding of cell-cell interactions and the impact of non-stem cell autonomous effects within the hematopoietic system will provide a platform for the development of strategies to manipulate and control other heterogeneous cell systems.

The hypotheses of this thesis will be tested through the integration of mathematical modeling and in vitro and in vivo experimental techniques. Mathematical simulations will be used to explore potential experimental outcomes and to evaluate the impact of parameters within the system.
These predictions will be investigated experimentally to develop new strategies for HSPC expansion and generate insights into the fundamental biology of hematopoiesis. In Chapter 2, we explore the potency of the transcription factor fusion protein, TAT-HOXB4, for the expansion of human HSPCs, by generating a set of tools to optimize its delivery. We build an automated delivery system that allows us to reveal the context-dependent nature of TAT-HOXB4. In Chapter 3, we develop a strategy for HSPC expansion based on the global reduction of endogenously produced inhibitory factors and demonstrate how non-stem cell autonomous regulation is a critical component of the system. In Chapter 4, we integrate our expansion system with the Notch Delta-1 ligand and assess how mature cell lineage skewing can alter the feedback signaling microenvironment to which HSPCs are exposed. Finally, in Chapter 5, we outline how these findings may be applied and extended in future studies of hematopoiesis and human HSPC expansion. Figure 1-4 summarizes the major aims of this work. In summary, this body of work demonstrates how cell-cell interactions and feedback mediated signaling are crucial regulators of HSPCs and the manipulation of these regulators can be used to engineer HSPC expansion processes.

**Figure 1-4**: Schematic summary of thesis aims, highlighting importance of feedback mediated effects.
This chapter has been published in *Biotechnology and Bioengineering* (Csaszar et al., 2009). Co-authors include Geneviève Gavigan, Mark Ungrin, Cynthia Thérien, Pascale Dubé, James Féthière, Guy Sauvageau, Denis Claude Roy, and Peter W Zandstra.

**Author Contributions:**
Elizabeth Csaszar - conception and design, collection and assembly of data, data analysis and interpretation, manuscript writing; Geneviève Gavigan - conception and design, collection and assembly of data, data analysis and interpretation; Mark Ungrin - assistance with LabView programming; Cynthia Thérien - assistance with mPB experiments; Pascale Dubé - assistance with mPB experiments; James Féthière - provision of study material; Guy Sauvageau - provision of study material; Denis Claude Roy - conception and design, review of manuscript; Peter W Zandstra - conception and design, data interpretation, manuscript writing.
2.1 Abstract

An automated delivery system for cell culture applications would permit studying more complex culture strategies and simplify measures taken to expose cells to unstable molecules. We are interested in understanding how intracellular TAT-HOXB4 protein concentration affects hematopoietic stem cell (HSC) fate; however, current manual dosing strategies of this unstable protein are labour intensive and produce wide concentration ranges which may not promote optimal growth. In this study we describe a programmable automated delivery system that was designed to integrate into a clinically relevant, single-use, closed-system bioprocess and facilitate transcription factor delivery studies. The development of a reporter cell assay allowed for kinetic studies to determine the intracellular (1.4 ± 0.2 h) and extracellular (3.7 ± 1.8 h and 78 ± 27 h at 37°C and 4°C, respectively) half-lives of TAT-HOXB4 activity. These kinetic parameters were incorporated into a mathematical model, which was used to predict the dynamic intracellular concentration of TAT-HOXB4 and optimize the delivery of the protein. The automated system was validated for primary cell culture using human peripheral blood patient samples. Significant expansion of human primitive progenitor cells was obtained upon addition of TAT-HOXB4 without user intervention. The delivery system is thus capable of being used as a clinically relevant tool for the exploration and optimization of temporally sensitive stem cell culture systems.
2.2 Introduction

Techniques employed to culture primary cells in vitro typically rely on regimented media exchanges to replenish nutrients and other molecules at the same rate. This batch approach does not consider the relative decrease in each molecule’s concentration due to utilization and degradation rates during the culture, and can expose cells to large fluctuations in the concentrations of regulatory molecules. Medium exchange frequency has a significant impact on hematopoietic cell production from bone marrow (Koller et al., 1995; Schwartz et al., 1991a, 1991b) and umbilical cord blood initiated cultures (Koller et al., 1998; Madlambayan et al., 2005). Independent control of media components and their concentration may improve hematopoietic stem cell (HSC) output from these cultures.

TAT-HOXB4 is a recombinant fusion protein utilizing the cell penetrating peptide, transactivator of transcription (TAT), which is responsible for nuclear localization and protein transduction and has been shown useful for transporting cargo such as proteins into cells quickly and without considerable toxicity (Chauhan et al., 2007; Futaki et al., 2003). TAT-HOXB4 has the potential to expand HSCs in vitro; however, it has been reported to have stability constraints, which include the majority of the protein being lost after 4 hours of incubation in serum containing medium and an intracellular half-life of approximately 1 hour, and so the initial culture strategy was to deliver the protein to murine bone marrow cells every 3 hours for 4 days (Krosl et al., 2003). The 4-6-fold net expansion of HSCs achieved with TAT-HOXB4 was significantly lower than the 40-fold net expansion obtained by retroviral over-expression of HOXB4 (Antonchuk et al., 2002). Understanding the intracellular dynamics of TAT-HOXB4 and optimizing its delivery may eliminate the discrepancy between expansions obtained.

We are interested in understanding how transcription factor concentration affects HSC fate. In order to achieve this goal, a technique to accurately control the concentration of the transcription factor of interest in the culture is necessary. Additionally, a method to relate the nuclear concentration of unstable transcription factors to their biological activity, as a function of time and concentration, would enable the optimization of delivery strategies and may increase the expansion of HSCs. Reporter genes, such as the firefly luciferase gene, have high specificity and a broad dynamic range, making them useful tools to quantify transcription factor activity.
(Naylor, 1999). In this study, we describe a luciferase reporter assay that uses a luciferase gene with a promoter containing binding sites for HOX and PBX (a cofactor of HOX genes). The presence of both HOX and PBX is required to drive the expression of the luciferase gene; thus, by maintaining a constant level of PBX through stable transfection, the assay can be used to correlate the luciferase activity to the active nuclear concentration of TAT-HOXB4.

To date, cell culture manipulations have been predominantly done manually, preventing the culture from being a closed-system, and making some culture strategies labor intensive. An attractive solution is automated delivery of cell culture components, which allows studying different culture strategies with ease. Here we present the development and validation of a programmable micro-volume delivery system, combined with a mathematical model, for controlling the delivery of molecules to cells in culture. The use of this system, in combination with a novel reporter gene assay, allows for dynamic predictions of the protein’s biological activity and can be used for quality control. We have integrated this system into a clinically relevant, single-use, closed-system cell culture bioprocess that supports HSC growth (Madlambayan et al., 2006). Our automated delivery system has been designed for delivering unstable molecules to suspension cell culture; however, it may find broader applications. In this study, we present the design and the validation of the delivery system and the reporter assay for the specific use with TAT-HOXB4 fusion proteins.

2.3 Materials and Methods

2.3.1 Physical delivery system design

A micro-volume delivery system was created, as shown in Figure 2-1A. It comprised a 60-80 cm long fused silica capillary (#TSP100375, Polymicro Technologies, Phoenix, AZ, USA) with an internal diameter of 100 µm. At the input end, the capillary was fed with a luer-lock tip stock syringe (#309585, BD, Franklin Lakes, NJ, USA) to which it was connected via a PEEK luer to a MicroTight adapter (#P-662, Upchurch Scientific, Oak Harbor, WA, USA) The stock syringe was loaded on a Model 33 Twin Syringe Pump (#553333, Harvard Apparatus, St. Laurent, QC, Canada), kept in a refrigerator at 4°C. At the output end, the capillary entered the bioreactor: a two port FEP cell culture bag (#2PF-0002, VueLife, American Fluoroseal Corporation, Gaithersburg, MD, USA) placed on an orbital shaker (#7744, Bellco Glass Inc., Vineland, NJ,
USA) in a cell culture incubator at 37°C with 5% CO₂. The capillary was fed through a self-sealing rubber septa (#B-IIS, InterLink, American Fluoroseal Corporation, Gaithersburg, MD, U.S.A.) with a needle, into the midpoint of the bioreactor. The opposing connector on the bioreactor was replaced with an additional self-sealing rubber septa. Stock syringes and delivery capillaries were blocked overnight before use with a solution of Dulbecco’s phosphate buffered saline (DPBS, #14190, Gibco, Invitrogen, Burlington, ON, Canada) supplemented with fetal bovine serum (FBS, 10%, #12483-020, Gibco), to prevent protein adhesion to syringe and capillary walls.

National Instruments LabVIEW 7.1 was used to create a program, InfusionController, to control the syringe pump’s injections. The program’s basic dosing strategy was an initial injection to concentration $L_1$ followed by wait time $t_1$ and subsequent injections, each to concentration $L_2$ and followed by wait time $t_2$, repeated for $n$ times. The user inputs the flow rate, the stock concentration, the initial culture volume, the desired concentration after injections, the time between injections, and the total number of injections.

2.3.2 Delivery system validation experiments
To validate the ability of the delivery system and evaluate non-specific losses, TAT-FITC (#27043, AnaSpec, San Jose, CA, USA) was reconstituted in water and diluted in a carrier solution of 2% FBS in DPBS. A human myeloid leukemia cell line K562 (CCL-243, American Type Culture Collection, Manassas, VA, USA) was cultured at 37°C with 5% CO₂ and cells were maintained in Iscove’s modified Dulbecco’s medium (IMDM, #12440, Gibco) supplemented with 10% FBS. Cells were resuspended at a density of 5 x 10⁵ cells/ml and the delivery system was connected to a 2 ml bioreactor that contained either carrier solution (for cell-free trials), or cell suspension (for cell uptake trials). For cell-free studies, specific concentrations of TAT-FITC were pumped through the capillary and fluorescent intensities were measured with a SpectraMax Gemini XS Spectrofluorometer (Molecular Devices, Ramsey, MN, USA), with absorbance and emission wavelengths set to 493 nm and 522 nm, respectively. Values were compared to a calibration curve, produced by diluting TAT-FITC to the appropriate concentrations (20, 10, 5, 2.5, 1.25, 0.625 uM). For cell uptake studies, TAT-FITC uptake into cells was measured using a protocol modified from one described previously (Manceur et al., 2007). TAT-FITC was added
to the cells with either the delivery system, or by manually pipetting in the control condition. Cells were incubated at 37°C for 30 minutes, after which wash steps were carried out as described (Manceur et al., 2007). A FACS Canto cytometer (BD Biosciences, Mississauga, ON, Canada) was used to assess TAT-FITC uptake by comparing the average fluorescent intensity from treated cells with that from unstained cells. Samples in which TAT-FITC was delivered with the automated system were compared to samples in which TAT-FITC was manually delivered with pipette directly to cell culture.

2.3.3 Development of TAT-HOXB4 reporter assay

TAT-HOXB4 protein was produced and characterized as described (Krosl et al., 2003). The \textit{pML(5xHOX/PBX)} luciferase reporter vector, the \textit{E2A-PBX} vector, and \textit{β-gal} internal control were generously donated by the Mark Featherstone lab, Mark Kamps lab, and Tak Mak lab, respectively. The luciferase reporter vector, \textit{pML(5xHOX/PBX)} is comprised of a luciferase backbone vector, with a promoter containing binding sites for HOXB4 and PBX, as described (Phelan et al., 1995). Vectors were amplified by being transformed into One Shot INVαF’ competent \textit{E. coli} cells (#C2020-06, Invitrogen), following the manufacturer’s protocol. Transformed bacteria were spread on lysogeny broth (LB) agar plates containing ampicillin (#M0025, Fermentas, Burlington, ON, Canada) and grown overnight. Clones were picked and amplified in liquid LB supplemented with ampicillin (#M0015, Fermentas) for 16 h at 37°C. For DNA isolation, QIAprep Spin Mini-Prep Kits (#27106, QiaGen, Mississauga, ON, Canada) were used and DNA was quantified with a NanoDrop spectrophotometer (#ND-1000, NanoDrop Technologies Inc., Wilmington, DE, USA).

The murine fibroblast cell line NIH-3T3 (CRL-1658, American Type Culture Collection) was cultured at 37°C with 5% CO₂. Cells were maintained in Dulbecco’s modified Eagle’s medium (DMEM, #11960, Gibco) supplemented with 10% FBS, 1% Penicillium-Streptomycin (#15140-122, Invitrogen), and 1% GlutaMAX-1 (#35050-061, Invitrogen). Reporter cells were produced by stably transfecting NIH-3T3 cells with the vector \textit{E2A-PBX} using FuGENE6 (#11814443001, Roche, Mississauga, ON, Canada), following manufacturers guidelines and stably transfected cells were selected by cell sorting for the GFP positive cells with the FACS ARIA instrument (BD Biosciences). Reporter cells were plated in 12-well plates at a density of $2.5 \times 10^4$ cells/well
in antibiotic-free medium. After 24 h of culture, transient transfection of a vector cocktail containing 0.2 μg/ml each of pML(5xHOX/PBX) vector and β-gal vector was performed using FuGENE6. For positive control wells, HOXB4 vector, constructed as previously described (Antonchuk et al., 2001), was added to the vector cocktail at 0.4ug/mL.

TAT-HOXB4 protein was delivered to the cells in fresh media, 44 h post-transfection, at 100 nM, unless noted otherwise. Reporter cell lysis ensued 48 h after transient transfection using a reporter lysis buffer (#E4030, Promega, Madison, WI, USA). TAT-FITC peptide and TAT-SOX2 protein (#110-OT3, PeproTech Inc., RockyHill, NJ, USA) were used as additional controls, delivered at the same doses as TAT-HOXB4. For intracellular time course studies, the TAT-HOXB4 protein was delivered to cells at various time intervals before cell lysis, while maintaining the total time between transient vector transfection and cell lysis of 48 h. For time course studies in cell-free media, TAT-HOXB4 aliquots were kept in serum-free, cell-free media at the specified temperature (4°C or 37°C) for the noted time before being delivered to cell culture.

Luciferase reporter activity was quantified by adding 100 μl of luciferase assay reagent (#E4030, Promega) to 20 μl cell lysate. Bioluminescence was quantified with a luminometer (#TD-20/20, Turner Designs, Sunnyvale, CA, USA) with a sensitivity level of 47.9%. 20 μl of cell lysate was combined with 180 μl of β-gal assay buffer and dispensed into a round-bottom 96-well plate. β-gal assay buffer was made by combining 75% 0.1 M sodium phosphate, 24% o-Nitrophenyl β-D-galactopyranoside (#N1127, Sigma, Oakville, ON, Canada) in a 4 mg/ml solution in 0.1 M sodium phosphate, and 1% 100 fold solution (10% 1 M magnesium chloride solution, 32% β-mercaptoethanol and 58% distilled water). The plate was incubated at 37°C for 5 min and absorbance values at 420 nm were obtained with the VERSAmax spectrophotometer microplate reader (Molecular Devices). The luciferase activity was determined as the ratio of the luciferase activity to the β-gal activity and the luciferase induction of each sample was found by normalizing the luciferase activity to the negative control, consisting of transected cells with no protein addition.
2.3.4 Protein uptake kinetic studies

TAT-HOXB4 protein at 40 µM was labeled with the FITC fluorescent dye, using a FITC labeling kit (#53004, Pierce, Rockford, IL, USA), according to manufacturers guidelines. The human epithelial carcinoma cell line, HeLa S3 (CCL-2.2, American Type Culture Collection) was cultured similarly to the NIH 3T3 cell line, described above. Cells were plated at $5 \times 10^4$ cells/well in 96-well plates and were cultured for 24 h. Nuclear staining was carried out by incubating cells with the nuclear dye Hoechst 33342 in 10% FBS in DPBS for 2 h. Culture media was replaced with fresh media containing FITC labeled TAT-HOXB4 at 1 µM for time periods varying from 5 min to 3 h, and cells were subsequently washed several times in DPBS. FITC fluorescent intensity was imaged and quantified using the Cellomics Arrayscan instrument (Thermo Scientific, Waltham, MA, USA) and the Target Activation software application. Similar time course studies were performed for human mobilized peripheral blood CD34+ cells and human umbilical cord blood Lin− cells, both in serum free media at $1 \times 10^5$ cells/mL. For these cells, average FITC intensity levels were quantified with the FACS Canto instrument. To test the effect of cell number on protein uptake, HeLa cells were suspended at varying cell densities, ranging from $1 \times 10^4$ cells/ml to $1 \times 10^7$ cells/ml in 100 µl of DPBS with 10% FBS. The uptake of 500 nM FITC labeled TAT-HOXB4 was measured with FACS Canto as described above for FITC-TAT uptake studies. A luciferase assay experiment was performed as described above, after 100 nM of TAT-HOXB4 was exposed to HeLa cells of varying cell density for 2 h, by delivering the resulting cell culture media to reporter cells.

2.3.5 Mathematical model of intracellular protein concentration

A mathematical model of ordinary differential equations was constructed to estimate the intracellular concentration of molecules under various dosing strategies. It has been found that TAT-protein internalization predominantly occurs via endocytosis (Chauhan et al., 2007; Soane and Fiskum, 2005; Tünnemann et al., 2006) and so fluid-phase uptake was modeled as the internalization mechanism. Equations 1-3 describe this system and were solved in Matlab 7.0. In these equations: $L$ (mol/L) is the concentration of molecules in the medium; $C$ (molecules/cell) is the intracellular concentration of the molecule; $X$ (cell/L) is the cell density; $k_{\text{medium}}$ and $k_{\text{intracell}}$ are the decay constants for the molecule in the medium and in the cell, respectively; $k_{\text{growth}}$ is the
growth rate for the cells in culture; $k_{uptake}$ is the rate constant for fluid internalization; and $N_A$ is Avogadro’s number.

\[
\frac{dL}{dt} = -(k_{medium} L) - k_{uptake} LX \quad [1]
\]

\[
\frac{dC}{dt} = -(k_{intracell} C) + k_{uptake} N_A L \quad [2]
\]

\[
\frac{dX}{dt} = k_{growth} X \quad [3]
\]

The rate constant parameters pertaining to protein degradation, $k_{medium}$ and $k_{intracell}$, were obtained by curve fitting exponential decays to the experimental luciferase reporter assay time-course studies. The uptake rate constant, $k_{uptake}$, was experimentally determined from the uptake kinetic study. The initial cell density and cell growth rate were set based on the experimental conditions being modeled.

2.3.6 TAT-HOXB4 delivery to mobilized peripheral blood cells

Human mobilized peripheral blood cells were collected from healthy volunteers after obtaining signed informed consent in accordance with Hôpital Maisonneuve-Rosemont ethical committee regulations. CD34$^+$ cells were isolated using EasySep CD34 positive selection cocktail (#18056 StemCell Technologies, Vancouver, BC, Canada), according to manufacturer’s protocol, and frozen. Thawed cells were resuspended at $1 \times 10^6$ cells/mL in X-vivo 15 media (#04744Q, BioWhittaker, Walkersville, MD, USA), supplemented with 100 ng/mL stem cell factor (SCF, #3191401 Stemgen, Amgen, Thousand Oaks, CA, USA), 100 ng/mL thrombopoietin (TPO, #300-18, PeproTech, Rocky Hill, NJ, USA), 100 ng/mL Flt-3 ligand (FL, #300-19, PeproTech), 20 ng/mL interleukin-6 (IL-6, #200-06, PeproTech), 20 µg/mL iron saturated transferrin (#T-8158, Sigma), 10 µg/mL insulin (#I-1882, Sigma), and 0.1 µM β-mercaptoethanol (#M13475 American Chemical ltd. Laurent, QC, Canada). The cell suspension was injected into a 2 ml bioreactor bag and the automated delivery system was set up to deliver TAT-HOXB4, diluted to a 2 µM stock solution. The initial delivery achieved a concentration of 40 nM, with subsequent deliveries of 20 nM every 4 h for 2 d. On day two, cells were removed from the culture bag,
collected by centrifugation and counted, and resuspended in fresh media before being returned to the culture bag, where the same delivery scheme was repeated for the next 48 hours. In the control case, unmodified X-vivo 15 media was delivered to cells using the same delivery scheme. For the manual delivery comparison, cells were cultured in 12 well plates and TAT-HOXB4 was delivered manually every 4h by pipette. Colony-forming cell (CFC) assays were performed on day 0, 2, 3 and 4 as previously described (Madlambayan et al., 2005). To measure the intracellular TAT-HOXB4 concentration, cells at 49h and 95h were lysed using NE-PER Nuclear and Cytoplasmic Extraction Reagents (#78833, Pierce), according to the manufacturer’s directions. Nuclear extracts were delivered to cell culture media of reporter NIH 3T3 cells and the luciferase reporter assay was carried out as described above. A calibration curve for this study was produced by delivering known quantities of TAT-HOXB4 to mobilized peripheral blood cell culture and performing the nuclear lysis after 1 h.

2.3.7 Statistical analysis
Statistics were computed based on a Student’s t-test (paired or unpaired, where appropriate). Error bars on plots represent the standard deviation of three or more replicates (n≥3). Asterisks (*) indicate statistical significance of P<0.05.

2.4 Results
2.4.1 The automated system achieves accurate delivery without non-specific losses
The *InfusionController* program was used to control injections of TAT-FITC into a bioreactor, set-up as in Figure 2-1A. In cell free studies, the fluorescent intensity of each specified dose agrees with its corresponding point on the calibration curve, as shown in Figure 2-1B, indicating that the system is able to deliver the concentrations of TAT-FITC specified by the program with a high level of accuracy, and non-specific losses of protein in the delivery system are negligible. In studies with K562 cells, the average uptake of TAT-FITC into cells in the delivery system was equivalent to cellular uptake in control conditions, as shown by the overlapping distributions of fluorescent intensities (Figure 2-1C). Furthermore, cell viability was not impacted by the use of the automated system, as greater than 90% viability was seen in both cases (data not shown).
Figure 2-1: The delivery system design and validation.

[A] The micro-volume delivery system. The stock syringe is kept at 4°C and is attached via a capillary to the bioreactor, maintained on an orbital shaker at 37°C. Insert shows magnified view of the stock syringe and the bioreactor. [B] Controlled delivery of TAT-FITC to a cell-free culture. The InfusionController program was used to direct the delivery from a 40 µM TAT-FITC stock solution to the bioreactor and achieve a desired concentration dose. [C] Histogram showing the distributions of K562 cells after incubation with 2.5 µM FITC-TAT in the delivery system condition (dotted line) and in the manual control condition (solid line). Unstained cells are represented by the shaded distribution.
2.4.2 Reporter assay analysis indicates that protein degradation occurs mainly intracellularly

The development of the luciferase reporter system established an activity-based assay to measure concentration and the degradation kinetics of TAT-HOXB4 under different conditions. The luciferase vector was driven by a HOX/PBX promoter and by stably transfecting the cells with \( E2A-PBX \), the addition of TAT-HOXB4 was required to activate the luciferase gene. A linear trend was evident between the concentration of TAT-HOXB4 and the resulting induction, illustrating that this reporter assay can be applied as a tool to quantify the biological activity of the recombinant protein (Figure 2-2A). Furthermore, neither TAT-FITC nor TAT-SOX2 showed any significant luciferase induction, confirming that the induction did not result from the presence of the TAT peptide or from non-specific TAT fusion proteins. The specificity of the reporter assay was additionally confirmed by showing that the transient transfection of the \( HOXB4 \) vector resulted in luciferase induction. Figure 2-2B shows the results of the time course studies of protein in cell-free, serum-free media, at 37ºC and 4ºC. An exponential decay fit was applied to each plot leading to the findings that TAT-HOXB4 has a half-life of approximately 3.7 ± 1.8 h at 37ºC and 77 ± 27 h at 4ºC in serum-free media. The results of the intracellular time course are shown in Figure 2-2C and by fitting a curve to these data, the intracellular half-life of TAT-HOXB4 was determined to be 1.4 ± 0.2 h. The short intracellular half-life is comparable to the results found in previous studies (Krosl et al., 2003), and emphasizes the need for frequent periodic delivery of TAT-HOXB4 to culture, in order to maintain the desired intracellular concentration, while the relative stability of the protein in cell-free media at 4ºC confirms the merit of the automated delivery system design. All half-life parameters were incorporated into the mathematical model to refine its predictive ability of the dynamic intracellular concentration of TAT-HOXB4.
Figure 2-2: Luciferase reporter cell assay for TAT-HOXB4.

[A] Dose-response of TAT-HOXB4 (squares) using the luciferase reporter assay. TAT-FITC peptide (rhombuses) and TAT-SOX2 (triangles) do not cause a luciferase induction. Transfected HOXB4 vector is shown as a positive control. [B] Time course showing temporal stability of TAT-HOXB4 at 37°C and 4°C in cell-free, serum-free media, normalized to induction value at t=0 h from luciferase reporter assay, with exponential decay best fit to experimental data. [C] Intracellular time course of TAT-HOXB4, normalized to the largest induction value from luciferase reporter assay. Model was best fit to the experimental data to capture all kinetic parameters, by minimizing the sum of squares.
2.4.3 Quantitative immuno-fluorescence demonstrates that protein uptake kinetics are cell density dependent

The use of FITC-labeled TAT-HOXB4 allowed for the uptake of the protein into cells to be visualized and quantified. The images in Figure 2-3A illustrate the cellular uptake of the protein over time. From the mean intensities of FITC fluorescence at each time point, a kinetic plot of TAT-HOXB4 uptake was produced and best fit to a curve (Figure 2-3B). From this fit, an uptake rate constant, \( k_{\text{uptake}} \), of \( 4.8 \times 10^{-10} \) L/min.cell was determined and incorporated into the mathematical model. Uptake studies with different cell types (HeLa cells, umbilical cord blood lin- cells, and peripheral blood CD34+ cells) confirm that this uptake rate constant is largely invariant across the cell types of interest. Since HSC culture can expand 30-fold over an 8 day culture period (Madlambayan et al., 2005) and previous studies have demonstrated that the peptide-to-cell ratio is a critical factor in the uptake kinetics of cell penetrating peptides such as TAT (Hällbrink et al., 2004), it was desired to determine whether intracellular protein concentration would be affected by the increasing number of cells per culture volume. When the cell density was increased from \( 1 \times 10^4 \) cells/ml to \( 1 \times 10^7 \) cells/ml, a significant difference \((p<0.05)\) in the intracellular FITC fluorescence was measured (Figure 2-3C), suggesting that as the total cell number expands while the extracellular protein concentration and the culture volume are maintained, each cell is exposed to less protein, leading to a decrease in the intracellular protein concentration. Furthermore, TAT-HOXB4 levels, as measured by the luciferase assay, are significantly depleted from cell culture media when the protein is exposed to cells for 2h (Figure 2-3D), suggesting that the trends seen due to cell density arise primarily because individual cells are exposed to limited protein levels at high cell densities.
Figure 2-3: Intracellular uptake kinetics of TAT-HOXB4.

[A] Immuno-fluorescence illustrating kinetics of cellular uptake of TAT-HOXB4; (i) negative control (no protein), (ii) 5 min, (iii) 20 min, (iv) 60 min. 10x magnification, insert 40x magnification, scale bar = 50 µm.

[B] Quantification of TAT-HOXB4 uptake kinetics, based on mean FITC intensity values for HeLa cells (squares), umbilical cord blood (UCB) lin- cells (circles), and peripheral blood (PB) CD34+ cells (triangles). Curve was best fit to the experimental data by minimizing the sum of squares.

[C] Intracellular concentration of 500 nM FITC labeled TAT-HOXB4 in cultures of varying cell densities.

[D] TAT-HOXB4 levels
measured by luciferase assay, showing depletion of protein in cell culture media after 2 h exposure to cells at varying cell densities.

2.4.4 Mathematical model predicts dynamic intracellular concentration of TAT-HOXB4

The mathematical model was created to provide an approximation of the dynamic intracellular concentration of TAT-HOXB4, so that delivery strategies could be devised and optimized. All model parameters were estimated from experimental findings described above. The intracellular concentration resulting from the optimum dosing strategy originally employed for TAT-HOXB4 delivery (Krosl et al., 2003) is presented in Figure 2-4A. This delivery scheme consists of 10 nM of TAT-HOXB4, delivered to a cell culture initiated at 3 x 10^5 cells/ml, every 3 h for 4 days. It is evident that injections every 3 hours exposed the cells to a range of concentrations of protein; cells may only experience the optimal concentration for a fraction of the total cell culture time. Additionally, the intracellular concentration per cell can be seen to decrease slightly over time as the total cells expanded in culture while the concentration of each protein addition remained constant. In this case, the effect of increasing cell number is minimal, as total cell expansion was approximately 2-fold over the 4 day culture period, and protein was delivered in a dilute concentration such that the culture volume would increase over time. However, as illustrated in Figure 2-4B, if the delivery of TAT-HOXB4 is to be used in conjunction with the 8 day culture process of umbilical cord blood as previously described (Madlambayan et al., 2005), the effect of cell expansion will be more extreme, as total cell expansion of 30 fold has been obtained with this protocol.

2.4.5 Automated delivery of TAT-HOXB4 to mobilized peripheral blood cells in a clinically relevant closed system bioreactor

We next used the automated system to deliver TAT-HOXB4 to human mobilized peripheral blood cells, and compared the outcome achieved with the automated system to the outcome using a manual delivery strategy. The cell culture outcome was assayed by enumerating total mononuclear cell number (MNC), CFCs, and colony forming units-granulocytes, erythroblasts, macrophages and megakaryocytes (CFU-GEMM) outputs. The delivery strategy of an initial
dose of 40 nM of TAT-HOXB4 followed by subsequent doses of 20 nM every 4 h, is one that has been previously established with manual delivery for the peripheral blood system, allowing for direct comparison of the ability of the automated system. Importantly, when this delivery scheme was used with manual delivery to peripheral blood culture, a significant difference was found in the CFU-GEMM count between the TAT-HOXB4 case and the control case; these studies have also confirmed that this delivery scheme correlates to in vivo repopulating hematopoietic stem cell expansion (unpublished data). The model simulation for the validation experiment is shown in Figure 2-4C(i). The results of the associated luciferase reporter assay confirm that the levels of intracellular TAT-HOXB4 in the hematopoietic cells correlate well to what would be predicted based on model’s prediction of the intracellular TAT-HOXB4 levels and the luciferase induction calibration (Figure 2-4C(ii)). The results of the four day culture with either the automated system or with manual protein delivery are summarized in Figure 2-4D. The addition of TAT-HOXB4 following this delivery scheme did not have an effect on the expansion of total cells or the total number of CFCs. However, the number of CFU-GEMMs, which are a measure of primitive progenitor cells (Broxmeyer et al., 2006), was significantly greater (p<0.05) when TAT-HOXB4 was delivered to cells, as compared to the control case. Figure 2-4E shows a representative image of the large, heterogeneous CFU-GEMM colonies that were seen in cultures supplemented with TAT-HOXB4. These findings are consistent with previous studies that suggest that HOXB4 targets the expansion of more primitive cell types, while mature progenitors remain unaffected (Antonchuk et al., 2002; Krosl et al., 2003). Furthermore, all trends that were observed using the automated system were comparable to findings using the corresponding manual delivery strategy, indicating that the automated system is able to achieve a comparable outcome to manual delivery and it is capable of being effectively used for protein delivery to HSC culture.
Figure 2-4: Delivery system and model simulations for primary human progenitor cell expansion.

All parameters used for simulations were obtained from experimental kinetic data and culture conditions. [A] Model simulation of TAT-HOXB4 delivery strategy employed in (Krosl et al., 2003) consisting of 10 nM delivered to cell culture initiated at 3 x 10^5 cells/mL every 3 h for 4 days. [B] Model simulation of the same delivery strategy being applied to an 8 day culture process of 1 x 10^5 cells, in which 30 fold total cell expansion is achieved, based on conditions found in (Madlambayan et al., 2005), resulting in steady decrease in protein concentration per
cell. [C] (i) Model simulation of delivery scheme used in peripheral blood experiments, consisting of an initial delivery of 40 nM, and subsequent doses of 20 nM every 4 h to cells seeded at 1 x 10^6 cells/ml for a 2 d culture period, followed by a media exchange and the repeat of the original scheme for an additional 2 d. (ii) Luciferase assay performed on peripheral blood cell lysates at 49h and 95h. Experimental results are compared to predicted values. [D] At the end of the 4 d culture period, the expansions of total mononuclear cells (MNC) and CFU-GEMMs, were analyzed using in vitro functional assays. Expansion values were calculated by normalizing to a starting population of 1 x 10^6 CD34+ cells. Input CFU-GEMM numbers were 6.7 ± 2.1 per 1000 starting CD34+ cells. The fold expansion values shown are in comparison to CD34+ mobilized peripheral blood cells on day 0 of culture. [E] Representative image of CFU-GEMM colony from cell culture supplemented with TAT-HOXB4, 5x magnification, scale bar = 250 µm.

2.5 Discussion

The creation of a controlled delivery system for cell culture allows for more complex culture strategies without labour intensive measures. Furthermore, manual administrations to alter cell culture conditions prevent these cultures from being closed systems, a preferable approach for clinical trials. The automated delivery system is ideal for dosing unstable molecules to cells in suspension at specified intervals, which will assist in investigating the relationship between transcription factor dynamic concentration and HSC fate. Previous studies assessing this relationship have met demanding culturing strategies, and exposed the cells to ranges of concentrations. With these previous strategies, our model simulations predict a fluctuating intracellular concentration; this wide concentration range is particularly important because HOXB4 has been found to have a therapeutic window of activity (Klump et al., 2005).

The delivery system was developed to be adaptable and modular, allowing it to be customized for specific applications, and it achieves several critical sought-after properties. Firstly, protein degradation is kept to a minimum because the stock syringe is maintained at 4°C in the refrigerator. The volume of the capillary delivery tube is small, (3-5 µl total) ensuring that if degradation occurs in the portion of the delivery tube at temperatures above 4°C, this would be minimal. Secondly, for cell culture and clinical applications, the apparatus is a closed-system
with sterilizable components. Finally, the system has the capability to connect multiple syringe pumps in parallel, providing the potential for multiple experimental conditions to be directly compared. Microsphere encapsulation, which is an alternative method often employed for controlled delivery, does not have the ability to slow degradation by protecting the protein from the culture temperature, and the encapsulation process may result in inactivation of the protein (Putney and Burke, 1998; Reis et al., 2006); thus, our automated delivery has the benefit of overcoming these challenges. Validation data showed that the controlled micro-volume delivery system accurately injected the correct concentrations of TAT-FITC peptide into the bioreactor and we did not find significant peptide loss in the system. The cellular uptake data showed that the TAT-FITC uptake into K562 cells was proportional to its concentration, agreeing with observations from others (Thorén et al., 2003). Both cell viability and TAT-FITC uptake into K562 cells attained with the delivery system were equivalent to those in the control condition.

The development of the luciferase reporter assay allowed for a linear dose response of TAT-HOXB4 to be obtained. The reporter system can be used to compare batches of TAT-HOXB4 to investigate production details and to validate the active protein concentration of various aliquots before administration to cell culture. It may also find future use as a quality control tool for clinical use of TAT-HOXB4. Furthermore, the reporter assay permitted half-lives of the protein in various conditions and cellular compartments to be measured, allowing for the model to be refined by incorporating these parameters. The half-lives that were obtained from the time course studies strengthened the motivation for using automated delivery to control TAT-HOXB4 concentrations in HSC culture. The short half-lives of the intracellular molecules confirm the need for frequent delivery in order to maintain a stable intracellular concentration. The relatively large half-life found at 4°C provides evidence that the physical set-up of the delivery system, in which TAT-HOXB4 is stored at 4°C for a few days at a time, will limit the degradation of the unstable protein.

The uptake studies provided kinetic data, allowing the rate constant of TAT-HOXB4 cellular uptake to be estimated, which further refined the predictive power of the model. It was also determined that protein uptake, and thus the resulting intracellular protein concentration, was dependent on the cell density. As the number of cells increased within an essentially constant culture volume, the intracellular concentration per cell decreased, largely due to depletion of the
protein from cell culture media when working with high cell densities and low protein concentrations. This finding was consistent with previous work (Hällbrink et al., 2004), which suggested that the intracellular concentration of cell penetrating peptides is affected more by cell density than by the external peptide concentration. In our system, this effect can be counteracted by increasing the protein dose over time to account for cell proliferation.

The mathematical model was developed to predict the dynamic intracellular concentration of the unstable TAT-HOXB4 molecule so that the delivery scheme could be easily optimized. One model simplification was that protein internalization was modeled bulk fluid phase endocytosis. However, it has been shown that positively charged amino acid residues in TAT interact electrostatically with negatively charged components on the cell membranes (Soane and Fiskum, 2005; Tünemann et al., 2006); thus, the local molecule concentration at the cell surface may be greater than that in the bulk. Furthermore, the fluid phase uptake term assumed that the rate of intracellular uptake is only dependent on the molecule’s concentration in the medium and the cell density. For simplicity, the model was not designed to capture ligand transportation to the cell surface, cytosolic to nuclear transport, or the possibility of molecule saturation in the cell. The degradation rate constants are independent of media composition, secretion of toxic metabolites, and cell number. This model is most applicable to serum-free culture conditions.

The progenitor cell study demonstrated that the automated system has the ability to achieve comparable results to a manual delivery strategy with the added benefit of being a closed system and eliminating labor intensive methods. Furthermore, the experimental results of the TAT-HOXB4 intracellular levels during the experiment validated the model predictions. The significant increase seen in large CFU-GEMM colonies from culture supplemented with TAT-HOXB4 illustrates the potential of TAT-HOXB4 to expand primitive progenitor cells and motivates future optimization of the protein delivery scheme to maximize its potential to expand HSCs. The system has the capability to achieve semi-continuous protein delivery, such that periodic fluctuations in intracellular concentration can be essentially eliminated. By increasing the concentration of the protein delivery as cell proliferate, the effects of increasing cell density can be counteracted, which will be particularly critical when working with longer culture times and rapidly proliferating cells. Thus, the system can be used to achieve stable intracellular transcription factor levels over many days, a capability that may enable identification of
intracellular TAT-HOXB4 concentrations that further enhance the in vitro expansion of human progenitor cell numbers.

This system has been initially designed to be used for TAT-HOXB4 delivery to HSC culture, however it may find use in simplifying the study of other temporal mechanisms critical to stem cell fate. It has been reported that introducing transcription factors (Oct4, Sox2, c-Myc, and Klf4) to mouse embryonic cells and adults fibroblast cells in culture, can induce these cells into pluripotent stem cells (Takahashi and Yamanaka, 2006). This group discussed the temporal dynamics of transcription factor activity and it was proposed that each factor may have a narrow range under which it operates optimally. With a programmable delivery system, injection timing and dose can be easily controlled to investigate signaling dynamics. Ultimately, we have developed and validated a programmable automated delivery system for cell culture applications that is a sterilizable closed-system, and may be tailored to its specific application due to modular components. This system will find initial use in the optimization of TAT-HOXB4 delivery and its versatility will allow it to be used to explore and enhance other areas of in vitro cell culture.

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3 RAPID EXPANSION OF HUMAN HEMATOPOIETIC STEM CELLS BY AUTOMATED CONTROL OF INHIBITORY FEEDBACK SIGNALING

This chapter has been published in Cell Stem Cell (Csaszar et al., 2012). Co-authors include Daniel C. Kirouac, Mei Yu, WeiJia Wang, Wenlian Qiao, Michael P. Cooke, Anthony E. Boitano, Caryn Ito, and Peter W. Zandstra.

Author Contributions:
Elizabeth Csaszar - conception and design, collection and assembly of data, data analysis and interpretation, manuscript writing; Daniel C. Kirouac - mathematical modeling, review of manuscript; Mei Yu - technical assistance with in vivo studies; WeiJia Wang - assistance with data collection; Wenlian Qiao - assistance with data collection; Michael P. Cooke - provision of study material; Anthony E. Boitano - provision of study material; Caryn Ito - conception and design, review of manuscript; Peter W Zandstra - conception and design, data interpretation, manuscript writing.
3.1 Abstract

Clinical hematopoietic transplantation outcomes are strongly correlated with the numbers of cells infused. Novel anticipated therapeutic implementation of hematopoietic stem cells (HSCs) and their derivatives further increase interest in strategies to expand HSCs ex vivo. A fundamental limitation in all HSC-driven culture systems is the rapid generation of differentiating cells and their secreted inhibitory feedback signals. Herein we describe an integrated computational and experimental strategy that enables a tunable reduction in the global levels and impact of paracrine signaling factors in an automated closed-system process by employing a controlled fed-batch media dilution approach. Application of this system to human cord blood cells yielded a rapid (12-day) 11-fold increase of HSCs with self-renewing, multi-lineage repopulating ability. These results highlight the marked improvements that control of feedback signaling can offer primary stem cell culture and demonstrate a clinically-relevant rapid and relatively low culture volume strategy for ex vivo HSC expansion.
3.2 Introduction

Emerging data suggests that robustness and responsiveness in hematopoiesis is a property of the system, not an individual cell. The hematopoietic system is able to dynamically maintain appropriate proportions of cells of all hematopoietic lineages throughout the lifetime of the individual, both during homeostasis and in response to regenerative demand. This regulation depends on intercellular (between cell) communication networks, resulting from both local and systemic factor secretion (Kirouac et al., 2010). Hematopoietic stem cells (HSCs) must respond to and integrate cues from the microenvironment to ensure sustained production of all hematopoietic lineages (Rizo et al., 2006). As such, self-renewal vs. differentiation decisions of HSCs critically depend on feedback mediated paracrine factors, and the associated signaling networks, both in vivo and in vitro.

Several decades of successful bone marrow transplantations have demonstrated the therapeutic importance of HSCs. The use of non-invasively accessible umbilical cord blood (UCB) derived HSCs provides many advantages over bone marrow, including enhanced long-term immune recovery, and decreased graft vs. host disease (Gluckman, 2009; Wagner and Gluckman, 2010). However, as clinical studies have indicated that the most important factor for patient survival following UCB transplantation is infusing a cell dose above a minimum threshold of $3 \times 10^7$ cells/kg (Gluckman, 2009), low cell numbers in single UCB units have limited the suitability of UCB transplantation for adult patients. Methods to robustly increase the number of cells that give a rapid and sustained blood count recovery would enable the use of UCB in all patients (Hofmeister et al., 2007).

Strategies to expand HSC numbers in vitro have focused on identifying molecules that specifically target the stem cell population. Recent data demonstrating that there are multiple sub-populations of HSCs and that these cells are molecularly closely related (Benveniste et al., 2010; Dykstra et al., 2007), suggest that the opportunity of finding molecules that uniquely expand the long-term HSC pool (Antonchuk et al., 2002; Boitano et al., 2010; Delaney et al., 2010; Durand and Zon, 2010; Zhang et al., 2008) without impacting the distribution and growth of their more restricted progeny is challenging, especially after extended periods of time in...
culture. Herein we propose a fundamentally different and complementary approach, examining the impact of feedback signaling control on HSC output.

Feedback control is complicated by the fact that the hematopoietic system is regulated by a complex hierarchy of cellular and molecular networks. We have previously performed cell-cell signaling network analysis to identify and experimentally validate the mode of action of endogenously produced HSC-inhibitory factors generated during in vitro UCB culture (Kirouac et al., 2010). Recognition that large numbers of these factors are produced in a time-dependent manner makes the addition of binding and signaling inhibitors challenging. In a novel and alternative approach we sought to identify a strategy to globally control feedback regulation. We performed computational simulations of in vitro hematopoiesis, and iteratively predicted and evaluated candidate strategies for the reduction of endogenously produced negative regulators. In silico optimization resulted in the identification and development of a “fed-batch” culture strategy for HSC growth enhancement. By linking dynamic cell growth and endogenous factor secretion to a tunable media dilution algorithm, we experimentally confirmed predictions of significant enhancements in stem and progenitor growth, including an 80-fold increase in CD34+ cells and an 11-fold increase in NOD/SCID/IL-2Rgc-null (NSG) repopulating cells (detected at limiting dilution after 16 weeks) within a 12-day culture period. Furthermore, this platform complements the mechanism of known HSC-enhancing factors, and has provided insight into their mode of action. The integration of our strategy into an automated and closed-system bioreactor has produced a clinically relevant system for HSC expansion.

3.3 Materials and Methods

3.3.1 Mathematical simulations

The model that we previously developed and described (Kirouac et al., 2009) was used to run all culture simulations with MATLAB 2009 software (Mathworks, Natick MA, USA). The model simulates in vitro hematopoietic culture by incorporating self-renewal and differentiation of cell populations and soluble factors secreted by mature cells. For this study, the model was adapted to simulate fed-batch and perfusion strategies by adjusting the rate of change of secreted factor concentrations. The fed-batch strategy incorporated a continuous input stream, which resulted in an increase in culture volume and a dilution of all cells and all endogenously produced secreted
factors. The perfusion strategy included a continuous input and output stream, resulting in a constant cell culture volume and a continuous reduction of endogenously produced soluble factors.

3.3.2 Umbilical cord blood cell collection and processing
UCB samples were collected from consenting donors according to ethically-approved procedures at Mt Sinai hospital (Toronto, ON, Canada). Mononuclear cells were obtained as previously described (Kirouac et al., 2009). Lineage negative (Lin⁻) progenitor cells were isolated from the mononuclear cell fraction using the StemSep system or EasySep system with the human progenitor cell enrichment kit (Stemcell Technologies, Vancouver, BC, Canada), following the manufacturer’s protocol.

3.3.3 Cell seeding and in vitro culture
Freshly isolated Lin⁻ cells were seeded at an initial density of 1 x 10⁵ cells/mL in serum free IMDM media (Gibco, Rockville MD, USA) with 20% BIT serum substitute (Stemcell Technologies) and 1% Glutamax (Gibco). The media was supplemented with 100ng/mL Stem Cell Factor (SCF, R&D Systems, Minneapolis, MN, USA), 100ng/mL FMS-like Trysine Kinase 3 Ligand (FL, R&D Systems), 50ng/mL Thrombopoietin (TPO, R&D Systems), and 1ug/mL low-density lipoproteins (LDL, Calbiochem, LaJolla, CA, USA). The syringe loaded pumping system was assembled and connected to the cell culture bag, as previously described (Csaszar et al., 2009). The initial cell suspension was injected into a 2-port 12mL culture bag (VueLife, American Fluoroeseal Corporation, Gaithersburg MD, USA) and maintained on an orbital shaker at 37°C and 5% CO₂. The pump was set to deliver the desired volume of media (based on the user-defined dilution rate) to the cell culture. Media delivery was automated to occur at 0.5h intervals at a flow-rate of 30uL/min, for a semi-continuous delivery. For SR1 studies, SR1 was added to fresh media at 0.75uM as previously described (Boitano et al., 2010). For TAT-HOXB4 studies, TAT-HOXB4 was produced and delivered semi-continuously as previously described (Csaszar et al., 2009; Krosl et al., 2003).
3.3.4 Cell assays
Colony forming cell (CFC) assays and long term culture-initiating cell (LTC-IC) assays were performed as previously described (Kirouac et al., 2009). Surface marker staining was performed with conjugated human antibodies: CD4, CD7, CD8, CD14, CD19, CD33, CD34, CD38, CD41, CD49f, CD56, CD90, CD133, & GyA (BD Biosciences, San Jose CA, USA). 7-AAD dye was added to assess cell viability and isolate live cells for quantification. All samples were analyzed on a FACSCanto flow cytometer (BD Biosciences).

3.3.5 Sorted cell assay
Freshly isolated Lin⁻ cells were sorted for Rho⁺CD34⁺CD38⁻CD45RA⁻CD49f⁺ with a FACSAria flow cytometer (BD Biosciences), following the gating strategy previously described (Notta et al., 2011). 40 sorted cells were dispensed per well in a 96 well plate in the above described media and cultured for 7 days. Following culture, cells were assessed for total cell number and number of CD34⁺CD133⁺CD90⁺ cells by flow cytometry.

3.3.6 Limiting dilution transplantation studies
All animal studies were performed according to procedures approved by appropriate animal ethics boards. Female NOD/SCID/IL-2Rgc-null (NSG) mice were sub-lethally irradiated (250 rad) <24h before transplantation. Uncultured Lin⁻ cells (n=26), or cells cultured for 8 days (n=50) or 12 days (n=25) were transplanted at limiting dilution via tail vein injection. Mice were sacrificed 16 weeks after transplantation and bone marrow was collected from femurs and tibias. Cells were assessed by flow cytometry. Mice were scored positive for human repopulation if at least 0.5% of BM cells were positive for both human CD45 and human HLA-ABC. For secondary transplantations, 33% of the harvested bone marrow cells of positively engrafted mice were transplanted into secondary NSG recipients. Bone marrow harvest and analysis was performed after 16 weeks. All limiting dilution analysis was performed with the L-calc software (Stemcell Technologies). Limiting dilution analysis was based on the combined data of two independent transplantation studies.
3.3.7 Secreted factor analysis
Secreted factor concentrations were sampled in duplicate from conditioned media samples using the Human Cytokine 30-Plex panel (Invitrogen, Burlington ON, Canada), designed for the Luminex microsphere detection platform (Luminex Co. Austin, TX, USA), to screen for: EGF, Eotaxin, FGF-β, G-CSF, GM-CSF, HGF, IFN-α, IFN-γ, IL-1β, IL-2R, IL-4, IL-5, IL-6, IL-7, IL-8, IL-10, IL-12, IL-13, IL-15, IL-17, IP-10 (CXCL10), MCP-1 (CCL2), MIG (CLCL9), MIP-1α (CCL3), MIP-1β (CCL4), RANTES (CCL5), TNF-α, and VEGF. Samples were prepared and assessed using a BD FACSCanto flow cytometer, as previously described (Kirouac et al., 2009). TGF-β1 was analyzed separately in parallel, using a TGF-β1 Quantikine ELISA Kit (R&D Systems, Minneapolis MN, USA), following the manufacturer’s protocol.

3.3.8 Glucose and lactate assay
Conditioned media samples were analyzed for glucose levels, using the Amplex Red Glucose/Glucose Oxidase Assay kit (Invitrogen), following the manufacturer’s protocol. In the subsequent glucose normalization study, D-glucose was added to the D=0 cell culture media every two days. Lactate concentrations were analyzed with a L-lactate assay kit (Eton Bioscience Inc, San Diego, CA, USA), following the manufacturer's protocol.

3.3.9 Statistical analysis
Statistical significance was computed using a student t-test. All error bars represent the standard deviation of three or more biological replicates. Asterisks (*) indicate statistical significance between conditions of p<0.05.

3.4 Results
3.4.1 In silico design of a “fed-batch” media dilution strategy
Hematopoietic culture systems generate a large number of endogenously produced soluble factors as a result of the rapid production of mature blood cells (Kirouac et al., 2010; Majka et al., 2001; Sautois et al., 1997). In order to investigate the impact of these secreted ligands, we first measured a sampling of the secreted factor profile under our previously optimized in vitro
expansion conditions (Madlambayan et al., 2005) and found the rapid accumulation of many
physiologically relevant ligands, and large fluctuations resulting from periodic media exchanges
(Figure 3-1A). Many of these factors have been reported to have inhibitory effects on human
hematopoietic stem and progenitor cell expansion (Bonnet et al., 1995; Broxmeyer and Kim,
1999; Broxmeyer et al., 1995; Cashman et al., 1998; Fortunel et al., 2000; Zhang et al., 1995)
and our previous studies have validated that several factors which are present at high levels in the
culture system have a significant inhibitory effect on hematopoietic progenitor expansion,
including TGF-β, MCP-1 (CCL2), MIP-1α (CCL3), MIP-1β (CCL4) and IP-10 (CXCL10)
(Kirouac et al., 2010).

The large number and non-linear nature of the secreted factor profiles limit the likely success of
using molecularly targeted approaches to reduce the inhibitory impact of these factors and,
instead, necessitates a global and unbiased strategy for feedback regulation. In order to identify
and optimize an appropriate strategy, we performed computational simulations of cell population
dynamics to investigate and predict the effect of candidate culture manipulations. Our
computational approach (Kirouac et al., 2009) incorporated the effect of feedback signaling from
differentiated cells on stem and progenitor cell expansion. This feedback is coded through
different classes of paracrine signaling loops, as depicted in Figure 3-1B.

As a first step, we explored in silico strategies of regulating the effective concentration of the
different classes (SF1-SF4) of secreted ligands. Under baseline conditions, with a full media
exchange every 4 days, simulations depict a predominant accumulation of inhibitory factors (SF1
and SF2) (Figure 3-1C). As such, a net increase in stem and progenitor proliferation was
predicted to result from minimizing the entire set of endogenously produced ligands.
Rationalizing that the frequency of conditioned media removal would be an important parameter
(Madlambayan et al., 2005), we first compared the effects of frequent full or partial media
exchanges with our control culture process. The model predicted that a full media exchange
every day (Figure 3-1D) or a half media exchange every 12 hours (Figure 3-1E) would
outperform less frequent exchanges, emphasizing the importance of strong and frequent secreted
factor regulation.
To limit periodic fluctuations and intermittent exposure to high concentrations of secreted ligands, and to attain a continuous and tunable mode of media regulation, we simulated the effects of perfusion and fed-batch culture systems. A perfusion system (Figure 3-1F) is characterized by a continuous input of fresh media and output of spent media, while maintaining a constant volume, whereas a fed-batch system (Figure 3-1G) contains an input stream only, resulting in a continuous increase in culture volume. These cell culture systems are commonly used as feeding strategies for biopharmaceutical production (Farid, 2006), and previous studies have explored the use of perfusion systems for HSC culture (Koller et al., 1998). Simulations predicted that a fed-batch media dilution approach would achieve the most effective enhancement in stem and progenitor expansion and was predicted to outperform perfusion cultures and frequent media exchange strategies (Figure 3-1C-Figure 3-1G). Although all strategies act by reducing the concentration of accumulating secreted factors, the increasing culture volume of the fed-batch strategy additionally maintains lower cell densities, thereby slowing the rate and impact of endogenous factor accumulation.

The dilution rate (D) of a fed-batch culture system is defined as the input flow-rate divided by the culture volume. This rate can be constant throughout the reaction period, proportional to the current volume of the culture or tuned based on predicted or measured parameters. As shown in Figure 3-1H, the volume of a culture initially containing 1 mL, will increase continuously with time depending on the defined dilution rate. Simulations predicted that the dilution rate would regulate the concentration of all secreted factors (Figure 3-1I), which in turn would regulate expansions of total nucleated cells (TNCs), colony-forming cells (CFCs), long term culture-initiating cells (LTC-ICs), and SCID repopulating cells (SRCs) (Figure 3-1J). The slopes of the predicted increases in expansions are greatest from D=0 to D=1, suggesting a significant impact on HSC expansion should be achievable at moderate dilution rates, while maintaining moderate media and cytokine needs.
Figure 3-1: Computational simulations predict a fed-batch strategy, at moderate dilution rates, to greatly reduce secreted factor concentrations and give a significant enhancement of expansion.
[A] Time course of experimentally measured secreted factor concentrations from UCB culture, with complete media exchange every 4 days. [B] Schematic of mathematical model, indicating the presence of paracrine feedback signaling. Groups of secreted factors are categorized depending on whether they act in a stimulatory or inhibitory manner and whether they impact stem cell self-renewal or proliferation. [C-G] Simulated volume, secreted factor concentrations, and relative expansions under different media manipulation strategies: [C] ”control” culture with complete media exchange (ME) every 4 days; [D] culture with complete media exchange every 24h; [E] culture with 50% media exchange every 12h; [F] perfusion culture with one unit of media perfused every 24h; [G] fed-batch culture with one unit of media added every 24h. Conditions [D-G] are normalized to same media & cytokine requirements (one additional unit of media every 24h). [H] Media volume requirements for a fed-batch culture at different constant dilution rates, assuming a 1mL initial volume. [I] Predicted effect of increasing constant dilution rate of fed-batch strategy on secreted factor concentrations. [J] Predicted effect of increasing constant dilution rate of fed-batch strategy on population expansions.

3.4.2 Automated closed system fed-batch culture enhances progenitor cell expansion

We next undertook experiments to test the predicted superiority of the in silico optimized fed-batch strategy. These studies were facilitated with the use of an automated media delivery system (Csaszar et al., 2009). This system allows the user to control media input flow-rates and enables the entire culture process to be performed in a closed system with minimal manual intervention. A constant flow-rate, corresponding to a dilution rate of D=1 was compared to our previously described optimal culture strategy, referred to here as D=0 (Madlambayan et al., 2005) (Figure 3-2A).

We first confirmed that the D=1 fed-batch strategy significantly outperformed the D=0 control at our previously optimal day 8 endpoint (Figure 3-6). We next computationally predicted that the D=1 strategy would achieve maximum expansions after 12 days of in vitro culture, as the regulation strategy inherent in the fed-batch system would allow for a positive rate of expansion to be maintained for a greater length of time (Figure 3-6). As shown in Figure 3-2B, the D=1 strategy reached 12-day expansions of 179-fold (range: 105-344 fold) of TNCs, 64-fold (22-166
fold) of CFCs, and 29-fold (14-53 fold) of LTC-ICs. Phenotypic analysis by flow cytometry (Figure 3-2C) indicated that the percentage of progenitor cells as measured by CD34+ and CD133+CD38- remained unchanged between the D=0 and D=1 cultures. However, the increased total cell expansion with the D=1 strategy led to a significant increases in the absolute numbers of CD34+ cells (80-fold) and CD133+38- cells (135-fold). Similar results were found with CD34+CD90+ and CD34+CD49fhi cells (Figure 3-6).

To further validate our modeling predictions, we compared the fed-batch strategy to alternative strategies for the reduction of endogenously produced factors by investigating frequent media exchange approaches (Figure 3-7). Although small apparent improvements over the D=0 cultures were achieved with frequent feeding strategies, these strategies produced significantly lower expansions than the D=1 strategy, despite having the same overall media usage. We also assessed the effect of varying the dilution rate of the fed-batch culture. As predicted, the rate of increased expansion began to slow at dilution rates greater than D=1, leading to diminishing returns of cell expansion at the expense of rapidly increasing media needs (Figure 3-2D). Taken together, these findings confirm our computational predictions of enhanced progenitor expansion achieved with a robust media dilution approach.
Figure 3-2: The fed-batch strategy is experimentally validated in vitro to give significantly improved expansion of progenitor cells.

[A] Schematic of experimental set-up comparing control (D=0) strategy with 100% media exchange (ME) every 4 days to fed-batch (D=1) strategy. [B] Expansions of TNC, CFC & LTC-IC, following 12 days of culture with Lin- cells. n>5. [C] (i, iii) Surface marker analysis of CD34+ and CD133+CD38- frequencies throughout in vitro culture. (ii, iv) Expansion of CD34+ cells and CD133+CD38- cells, accounting for total cell expansion. n>5. [D] Normalized expansion of TNC, CFC, & LTC-IC after 12 days of culture, dependent on dilution rate. n=3. Data are expressed as mean ± SD.
3.4.3 Global maintenance of sub-threshold levels of inhibitory factors enables fed-batch mediated expansion of primitive UCB cells

The observed enhancement in progenitor expansion was predicted to result from a global reduction in inhibitory paracrine factor levels in the culture media. To test this hypothesis, we investigated the effect of the fed-batch strategy on the output of specific mature blood cell types. Analysis of hematopoietic cell lineage markers did not reveal any change in the relative frequency of any of the differentiated cell types assessed (CD14⁺, CD7⁺, CD41⁺, GyA⁺, CD33⁺, CD19⁺, CD56⁺, CD8⁺, CD4⁺) with the D=1 strategy, as compared to the D=0 control. Although the absolute number of cells in each of these populations was greater with the D=1 strategy, due to the enhanced TNC expansion, the lower cell densities achieved with the D=1 strategy resulted in lower concentrations (cells/mL) of each mature cell population (Figure 3-8).

We next asked how the reduction in mature cell concentrations would impact soluble factor concentrations. Figure 3-3A depicts the degree by which the concentration of each measured soluble factor was changed with the D=1 strategy, as compared to D=0. The non-linear nature of factor accumulation means that the dynamics of individual factors and the degree by which the fed-batch strategy affects the concentration of specific factors varies; however, the net global reduction in factor concentrations provides an overall minimization of feedback inhibition in the culture system. Figure 3-3B highlights the concentration dynamics for several factors known to impact stem and progenitor growth, demonstrating the reduction of ligand concentrations achieved with the fed-batch strategy.

The functional effect of secreted ligand reduction on cell growth was further tested by culturing cells in media that had been previously conditioned for 8 days in a culture maintained with either the D=0 or the D=1 strategy. Exogenous cytokines (SCF, FL, TPO) were added to the conditioned media to ensure that the difference between the fresh and conditioned media was the presence of endogenously produced soluble factors. Both conditioned media led to a significant reduction in TNC expansions as compared to cultures utilizing fresh media, as expected. However, the expansion achieved with the D=1 conditioned media were significantly greater than with the D=0 conditioned media (Figure 3-3C). This shows the ability of reduced endogenous factor concentrations to better sustain primitive UCB cells in culture.
It remained unclear if the fed-batch strategy was acting by simply regulating one or a few key inhibitory ligands or whether a more comprehensive (global) manipulation of the culture environment was important. To test this directly, we compared the D=1 strategy to the targeted inhibition of TGF-β1, a known endogenous inhibitor of HSC growth (Fortunel et al., 2000). Targeted inhibition of TGF-β1 was achieved by adding the small molecule SB431542 to the culture every 4 days; a manipulation that yielded increases in progenitor cell expansions that approached those achieved by the fed-batch strategy on day 8. However, after 12 days of culture, TGF-β1 inhibition no longer had a significant effect on progenitor expansion (Figure 3-3D). This demonstrated that although some degree of culture regulation could be initially provided through the inhibition of one key endogenously produced factor, the impact of this approach is limited, particularly at the later time points of culture when the accumulation of many inhibitory factors is high, and targeting one factor alone is no longer effective.

In previous studies (Kirouac et al., 2010), we used expression analysis to identify 74 factors whose genes were up-regulated during UCB culture. Many of these factors were hypothesized to be inhibitory to HSC growth and seven of these (TGF-β, TNFSF9, MIP-1α, MIP-1β, IP-10, NAP-2, SPARC) were experimentally validated to have an inhibitory effect on LTC-IC expansion. To investigate whether these factors contribute to HSC inhibition in an additive manner, we performed a combinatorial analysis of inhibitory soluble factors on Lin−RhoαCD34+CD38−CD45RA−CD49f+ cells, which have been reported to be highly enriched for HSCs (Notta et al., 2011). The addition of TGF-β1 (10ng/mL), MIP-1α (100ng/mL), MIP-1β (100ng/mL), and IP-10 (100ng/mL) individually each caused a reduced expansion of this population following 7 days of culture, and the simultaneous addition of these four inhibitors produced a significant reduction of expansion, demonstrating that multiple inhibitory factors present in the culture system act in an additive manner (Figure 3-3E). Importantly, when lower concentrations (10ng/mL each) of MIP-1α, MIP-1β, and IP-10 were used, the effect of the individual factors was negligible, however, the combination of the three led to reduced cell expansions (Figure 3-3F). This study illustrates how sub-threshold levels of factors which individually do not provide significant inhibition can produce an inhibitory effect when acting in combination, highlighting the global nature of feedback inhibition.
In order to rule out the possibility that the fed-batch strategy was primarily acting by preventing critical metabolites from becoming limiting and inhibiting cell growth (Collins et al., 1997; Patel and Papoutsakis, 2000), we monitored the glucose and lactate concentrations of the media. Glucose levels did not become limiting under any of the culture conditions tested, including the D=0 control, as glucose concentrations below 7 mM were never observed (Figure 3-9). Furthermore, when glucose was added to the D=0 culture in order to normalize the glucose levels to the corresponding D=1 culture, no effect on expansion levels was observed (Figure 3-9). Lactate levels reached a maximum of 15 mM in the D=0 cultures and the pH did not drop below 7.0, both of which were well within the range that support normal hematopoietic cell proliferation (Patel and Papoutsakis, 2000) (Figure 3-9). Collectively, these studies support the interpretation that the global reduction of inhibitory ligands was providing the supportive conditions for improved progenitor growth.
Figure 3.3: The fed-batch strategy enhances expansion through the global reduction of endogenously produced inhibitory secreted factors.

[A] Heat map of secreted factor concentration ratios (D=1/D=0) showing the effect of the D=1 strategy on secreted factor profiles as compared to the D=0 strategy. [B] Representative samples of reduction in secreted factor concentrations for: (i) TGF-β1; (ii) RANTES; (iii) IL-8; (iv) HGF with the D=1 strategy as compared to the D=0 strategy. [C] TNC expansion following 8 days of D=0 culture of Lin⁻ cells with either fresh media, or conditioned media (CM) from day 8 of previous D=0 or D=1 cultures. [D] Comparison of the fed-batch D=1 strategy to the addition of a TGF-β1 small molecule inhibitor, SB431542, based on expansions of TNC, CFC, and LTC-IC at
day 8 and (iii, iv) day 12. [E] Inhibitory factors, TGF-β1 (10ng/mL), MIP-1α (100ng/mL), MIP-1β (100ng/mL), and IP-10 (100ng/mL) were added to a sorted HSC population (Lin− Rho(lo)CD34+CD38−CD45RA−CD49f+), and total cell expansion was assessed following 7 days of culture. [F] Low concentrations of inhibitory factors, MIP-1α (10ng/mL), MIP-1β (10ng/mL), and IP-10 (10ng/mL) were added to a sorted HSC population (Lin− Rho(lo)CD34+CD38−CD45RA− CD49f+), and total cell expansion was assessed following 7 days of culture. n=3. Data are expressed as mean ± SD.

3.4.4 Transplantation studies show an 11-fold SRC expansion under fed-batch conditions

To determine whether the enhanced expansions observed with the progenitor populations would also apply to long-term repopulating HSCs (LTR-HSCs), we performed transplantation studies using the NSG mouse model (McDermott et al., 2010). Repopulation was quantified by bone marrow analysis for human hematopoietic cell contribution 16 weeks after transplantation and freshly isolated (day 0) Lin− UCB cells were compared to cells cultured for 8 or 12 days. Figure 3-4A shows representative flow cytometry plots of repopulated and non-repopulated mice. For each condition tested, a dose response of average human contribution was seen, as determined by the quantification of human CD45 & HLA-ABC double positive populations (Table 3-1).

All positively repopulated mice were found to have multi-lineage repopulation, as indicated by human cells that were positive for a myeloid lineage marker (CD33), a B-cell lymphoid lineage marker (CD19), a T-cell lineage marker (CD3), and an erythroid lineage marker (GyA). Representative plots are shown in Figure 3-4B & 3-4C and full details are presented in Table 3-2. Human progenitor cells were detected from 16-week transplanted mice by both surface marker analysis (CD34+ and CD133+) and CFC assays (Figure 3-4D & 3-4E). Cells from 8 mice repopulated for 16 weeks with day 12 D=1 cells (19-62% human cells in the marrow of primary recipients) were re-transplanted into secondary mice to determine whether the culture-expanded cells had retained LTR-HSC activity. Five out of 8 secondary mice were positive for human cells (human contribution ranging from 0.5% to 2.1%), all of which showed multi-lineage repopulation (Table 3-3), indicating that the expanded cells are able to maintain their long-term repopulation potential in vivo.
In order to quantify LTR-HSC expansion, limiting dilution analyses were performed (Figure 3-4F). The frequency of LTR-HSCs in the fresh Lin⁻ cells was 1 in 14,700 (95% CI: 1/8659 to 1/24979, n=26). After 8 days of culture, the D=1 culture produced a corrected LTR-HSC frequency of 1 in 1,940 (1 in 110,000 corrected for the concomitant 57-fold expansion of TNCs, 95% CI: 1/1195 to 1/3149, n=33), giving a 7.6-fold LTR-HSC expansion, relative to the fresh cells. The D=0 strategy gave a 3.6-fold LTR-HSC expansion, as reported previously (Ito et al., 2010; Madlambayan et al., 2005) (1 in 121,000 corrected for a 28-fold TNC expansion to give a 1 in 4,330 corrected frequency, 95% CI: 1/2223 to 1/8428, n=17). We predicted that the 12 day culture would give the greatest LTR-HSC expansion, based on both computational simulations and progenitor assays. Indeed, the corrected LTR-HSC frequency after 12 days of culture was 1 in 1,334 (after a 1 in 233,000 frequency was corrected for 178x total cell expansion, 95% CI: 1/759 to 1/2345, n=24), producing an 11 LTR-HSC expansion. LTR-HSC frequencies were used to determine SRC numbers per 10⁶ cells (Figure 3-4G).

Collectively, these results demonstrate that the fed-batch D=1 culture strategy is effective at expanding clinically relevant numbers of mature cells, progenitor cells and LTR-HSCs in a short (12 day) culture time using an automated closed system bioprocess.
Figure 3-4: In vivo SRC assay with D=1 fed-batch strategy shows an 11-fold multi-lineage expansion of LTR-HSCs.

[A] Representative flow cytometry images of bone marrow analysis of (i) a non-repopulated and (ii) a repopulated recipient mouse. The quantification of the double positive quadrant was used to determine the percentage donor contribution for each recipient. [B, C] Representative images showing multi-lineage repopulation of recipients, as measured by CD33+, CD19+, GyA+, & CD3+. [D] Quantification of progenitor cells in repopulated recipient mice. [E] Representative
images of CFCs formed with human cells from repopulated mice. [F] Limiting dilution curves quantifying LTR-HSC contribution of: (i) uncultured (day 0) Lin- cells; (ii) day 8 cells cultured with the D=0 strategy; (iii) day 8 cells cultured with the D=1 strategy; (iv) day 12 cells cultured with the D=1 strategy. LTR-HSC frequencies, corrected to day 0 equivalent cell numbers are indicated for each. Data are fit with exponential curves. [G] LTR-HSC expansions were used to calculate SRC numbers, based on 1 x 10^6 Lin^- cell input. Results show the pooled data from two independent experiments. Data are expressed as mean ± 95% CI.

3.4.5 The fed-batch strategy complements the effects of other of HSC enhancing factors and provides insight into their modes of action

The fed-batch strategy provided a means to assess the effect of feed-back signaling under different conditions and thus serves as a platform to interrogate the mode of action of factors known to enhance blood stem and progenitor cell growth (Figure 3-5A). We hypothesized that HSC culture additives could be classified into two major categories depending on whether they act directly on HSC self-renewal, or act indirectly on a mature cell population, which feeds back positively on HSCs. If the mode of action is HSC self-renewal specific, the fed-batch strategy should minimize inhibitory feedback signals, providing an enhancing environment for HSCs growth. Alternatively, if the mode of action is non-stem cell autonomous, the fed-batch strategy should dilute the mature cell population and dilute and soluble signaling molecules that have been produced. In this case, the fed-batch strategy should reduce the impact of the factor added.

To test this hypothesis, we investigated the interaction between the fed-batch culture and two known HSC growth-supportive factors, the aryl hydrocarbon receptor antagonist (SR1), and the transcription factor HOXB4. SR1 has been shown to enhance CD34^+ and HSC outputs by inhibiting HSC differentiation (Boitano et al., 2010). We thus predicted that the fed-batch strategy would complement the effect of SR1 by reducing the impact of endogenous inhibitory feedback signaling. Figure 3-5B demonstrates that under our culture conditions, SR1 produced an increase in LTC-IC expansion with both the D=0 and D=1 strategy, and the absolute levels of LTC-IC expansion were significantly enhanced with the D=1 strategy. Primitive cell phenotypes were also enhanced with the addition of SR1 (Figure 3-10). To validate that this factor was acting directly on a population with a very primitive phenotype and not through a feedback
mediated response, we added the molecule to the Lin\(^{\text{lo}}\)CD34\(^+\)CD38\(^-\)CD45RA\(^-\)CD49f\(^+\) population. Figure 3-5C shows that treatment with SR1 leads to a significant increase in primitive (CD34\(^+\)CD133\(^+\)CD90\(^+\)) cells in this assay, illustrating that it is directly targeting a population that is highly enriched in HSCs. Given the apparent additive impact of SR1 and the fed-batch strategy on the output of primitive cell phenotypes, it is possible that combining these technologies under the conditions described herein will enhance the number of LTR-HSCs above that which has been obtained with the D=1 strategy alone (Figure 3-10). However, as the CD34\(^+\)CD133\(^+\)CD90\(^+\) phenotype has not been validated under these culture conditions, limiting dilution long-term transplantation studies are required to confirm the potential additive effects between these two technologies.

We next investigated the transcription factor, HOXB4, which has been shown to increase stem and progenitor expansion by either viral over-expression or delivered as a TAT-HOXB4 soluble protein (Antonchuk et al., 2002; Csaszar et al., 2009; Krosl et al., 2003). HOXB4 has been shown to cause increases in the production of CD41\(^+\) megakaryocytes (Zhong et al., 2010), a finding that we reproduced in our culture system with the TAT-HOXB4 protein (Figure 3-10). Notably, we have previously reported that CD41\(^+\) cells have a stimulatory feedback effect on LTC-IC expansion (Kirouac et al., 2010). Thus, we hypothesized that the mode of action of HOXB4 on UCB HSCs was, at least in part, due to an indirect feedback mediated effect, as has also been suggested in ES cell derived hematopoiesis (Jackson et al., 2012). As Figure 3-5D shows, TAT-HOXB4 produced a significant increase in LTC-ICs when added to D=0 conditions but the impact of the molecule was reduced with D=1 conditions. Furthermore, TAT-HOXB4 did not produce a significant increase in expansion when added to Lin\(^{\text{lo}}\)Rho\(^{\text{lo}}\)CD34\(^+\)CD38\(^-\)CD45RA\(^-\)CD49f\(^+\) cells (Figure 3-5E). Collectively, these data provide further insight into the feedback signaling control mechanism which underpins the HSC-supportive effects of the fed-batch system.
Figure 3-5: Fed-batch system complements the effects HSC-enhancing molecules and provides insight into their mode of action.

[A] Schematic of in vitro expansion mode of action: (i) Under base conditions, static (D=0) culture systems balance HSC self-renewal and feed-back inhibition. The fed-batch (D=1) strategy minimizes feed-back, while allowing for self-renewal; (ii) The addition of a factor that targets HSC self-renewal gives increased self-renewal under both D=0 and D=1 conditions but the impact will be in enhanced with D=1 conditions due to the reduced feed-back; (iii) The addition of a factor with a positively acting indirect mode of action will increase stimulatory feed-back signaling while maintaining self-renewal. Under D=1 conditions, this stimulatory feed-back will be reduced which will reduce the impact of the added factor. [B] The addition of the aryl hydrocarbon receptor antagonist (SR1) to Lin⁻ cells gives a significant increase in LTC-IC expansion under both D=0 and D=1 conditions. [C] When added to a sorted HSC population (Lin⁻Rho⁰CD34⁺CD38⁻CD45RA⁻CD49f⁺), SR1 shows an increase in CD34⁺133⁺90⁺ numbers. [D] The addition of TAT-HOXB4 to Lin⁻ cells yields a significant increase in LTC-IC expansion under D=0 conditions but not D=1 conditions, suggesting a non-HSC mode of action. [E] TAT-HOXB4 shows minimal effect when added to the sorted Lin⁻Rho⁰CD34⁺CD38⁻CD45RA⁻CD49f⁺ population. n=3. Data are expressed as mean ± SD.
3.5 Discussion

A robust strategy to generate ex vivo expanded HSCs will enable the use of UCB for transplantation in patients for whom a single cord blood unit would not contain the desired progenitor content and ensure that a much greater proportion of current and future banked UCB units are applicable for use by any patient meeting the HLA matching criteria. In this study, we have computationally interrogated and experimentally validated a highly-tunable hematopoietic progenitor cell expansion strategy that can produce 11-times more blood stem cells than originally present over a 12 day culture period. The short culture time and continuous tight regulation of cell densities also allows this system to have reduced media volume needs as compared to other expansion strategies, which is an important feature for cost effective clinical implementation. This system has been designed to be adaptable for direct scale-up to accommodate cell numbers needed for human transplantations and we are planning to integrate this technology into clinical trials in the near future.

The fed-batch strategy relies on simplifying the complexity of dynamic and heterogeneous hematopoietic culture systems. In doing so, the need for targeted inhibition of individual endogenously produced factors is eliminated. We have previously shown evidence that mature hematopoietic cells and their associated secreted factors have a net inhibitory effect on stem cell self-renewal during in vitro culture (Madlambayan et al., 2005). Strategies to overcome this typically involve a significant amount of undesired manipulation and handling and provide only a temporary solution, as undesired factors will quickly re-accumulate after each manipulation. Perfusion cultures have been shown to enhance UCB progenitor cell expansion (Koller et al., 1998), however these cultures are subject to the challenge of high cell densities and rapid factor re-accumulation. This challenge can be overcome with the use of higher perfusion flow rates, but this then fails to maintain desired usage limits of media and cytokines. The fed-batch strategy has the benefit of slowing the rate of factor accumulation through the continuous dilution of both endogenous factors and the cells that secrete these factors.

All current expansion strategies that rely on the direct regulation of the HSPC populations are subject to the unregulated accumulation of inhibitory endogenous factors which, if unaccounted for, limit achievable expansion levels. Recent reports of human HSC expansion using apparently
stem-cell autonomous factors include SR1, producing a 17-fold LT-HSC expansion (analyzed 16-weeks post-transplantation) following 21 days of in vitro culture, the immobilized Notch Delta-1 ligand described by Delaney and colleagues (Delaney et al., 2010) producing a 15.6-fold and 6.2-fold in vivo repopulation cell expansion (analyzed at 3 and 9 weeks post transplantation, respectively) following 17-21 days of culture, and the growth factors Angiopoietin-like 5 and IGFBP2 (Zhang et al., 2008), producing a 14-fold repopulating cell expansion (analyzed at 8 weeks post transplantation in NOD/SCID recipients) following 10 days of culture. The fed-batch strategy reported here gives a LT-HSC expansion of comparable magnitude with a 12 day culture time, and a conservative 16-week post transplantation analysis. As this strategy affects non-autonomous feedback regulation and acts on the microenvironment of the culture system, it suggests strong potential to synergize with primarily autonomously acting expansion strategies, the combinations predicted to lead to greater and more sustained cell expansions.

It is clear that in vitro (and in vivo) hematopoiesis is dynamic and regulated, at least in part, through non-linear feedback control. The trajectories of factor secretion vary widely amongst individual factors, and do not always correlate with the exponential trajectory of total cell expansion. Factor concentration dynamics may follow the dynamics of specific lineage sub-populations or may result from multiple interacting feedback networks. Moving to non-linear media dilution strategies is one example of how more sophisticated dilution dynamics can be predicted to further tune regulatory interaction and HSC growth in the system. In order to maximize this control, a feedback regulated system in which a set of critical factors is measured, either “off-line” by ELISA assay, or “on-line” with the adoption of current technologies to quantify multiple soluble factors in real-time (Klostranec et al., 2007), could be linked to a threshold level process control mechanism. This should allow for the dilution strategy to be tuned in real-time and would provide a means to account for sample-to-sample biological variability, ensuring that optimal expansion can be achieved for each specific cord blood unit.

The fed-batch strategy is a globally-acting expansion strategy amenable for clinical use, alone or in combination with other expansion protocols. It also provides a platform with which to more closely study the dynamic nature of the in vitro hematopoietic cell culture system. We have illustrated how the fed-batch strategy has different effects when acting in combination with factors that directly or indirectly enhance HSC growth. These studies provide insight into the
mode of action of the aryl hydrocarbon receptor antagonist, SR1 and the TAT-HOXB4 protein, through the differing effect that the D=1 strategy has on their efficacy. More broadly, they demonstrate that regulating feedback signaling can act to reduce inhibitory feed-back thereby allowing factors that target HSC self-renewal to act with maximal impact.

The ability to modulate secreted factor concentrations and measure corresponding functional outputs of cell expansions will allow for a more precise study of links between specific endogenous protein secretion and lineage sub-populations and their associated cell-cell interactions. This strategy serves as a robust clinically-relevant system for rapid and automated in vitro cell expansion as well as a platform for further study the regulation of cell-cell interactions in vitro and in vivo.

3.6 Acknowledgements

The authors thank the donors, and the Research Centre for Women’s and Infants’ Health BioBank of Mount Sinai Hospital for the human specimens used in this study. The authors thank Dr. Connie Eaves and members of the Zandstra laboratory for their helpful discussion. This work was funded by the Leukemia and Lymphoma Society of Canada, the Canadian Stem Cell Network, and the Ontario Ministry of Research and Innovation. E.C. and D.C.K. were supported by NSERC Graduate Scholarships. P.W.Z. is the Canada Research Chair in Stem Cell Bioengineering.
3.7 Supplementary Information

3.7.1 Supplementary figures

Figure 3-6: Additional computational and experimental expansion data showing predicted and validated expansions achieved with the fed-batch strategy.

[A] Expansions of TNC, CFC & LTC-IC, following 8 days of culture with Lin⁻ cells. n>5. [B-C] Model simulations of the expected dynamics of TNC, CFC, LTC-IC, & SRC expansions with [B] the D=0 strategy, and [C] the D=1 strategy. [D, E] Phenotype analysis of [D] CD34⁺CD90⁻ and [E] CD34⁺CD49fhi cells. n=3. Data are expressed as mean ± SD.
Figure 3-7: Experimental expansion results confirm that the fed-batch D=1 strategy significantly outperforms alternative feeding strategies involving frequent complete (100%) or partial (50%) media exchanges.

[A] Expansions after 8 days of *in vitro* culture with Lin⁻ cells. [B] Expansions after 12 days of *in vitro* culture with Lin⁻ cells. n=3. Data are expressed as mean ± SD.
Figure 3-8: Phenotypic analysis of hematopoietic lineages shows no skewing of frequency with D=1 strategy, but overall reduction in concentration of mature cell populations.

[A] Flow cytometry analysis of surface marker frequency measured at day 0, day 4, day 8, & day 12 of culture with Lin⁻ cells: (i) CD14⁺; (ii) CD7⁺; (iii) CD41⁺; (iv) GyA⁺; (v) CD33⁺; (vi) CD19⁺; (vii) CD56⁺; (viii) CD8⁺; (ix) CD4⁺. [B] Total expansion of cell populations, accounting for total cell expansion as well as phenotype frequency. [C] Concentration (cells/mL) of cell populations, accounting for culture volume. n=3. Data are expressed as mean ± SD.
Figure 3-9: Glucose and lactate levels are not limiting in cultures and are not a significant contributor to the enhanced expansions achieved with the fed-batch strategy.

[A] Glucose concentration measurements from different culture strategies. [B] Expansions resulting from normalizing glucose levels in the D=0 culture to the levels seen in the D=1 culture. [C] Lactate concentration measurements from different culture strategies. n=3. Data are expressed as mean ± SD.
Figure 3-10: Increases in primitive phenotypes are seen with the addition of SR1 and an increase in megakaryocytes is seen with the addition of TAT-HOXB4.

[A] The addition of SR1 causes an increase in the expansion of (i) CD34⁺ cells, (ii) CD133⁺CD38⁻ cells, and (iii) CD34⁺CD133⁺CD90⁺ cells, as quantified by flow cytometry, following 12 days of culture with Lin⁻ cells using the D=0 or D=1 strategy  

[B] The trend of CD34⁺CD133⁺CD90⁺ cell expansion is compared to SRC expansion from limiting dilution analysis for different culture conditions. 

[C] CD41⁺ megakaryocytes were quantified by flow cytometry with the D=0 strategy in the absence or presence of TAT-HOXB4. n=3. Data are expressed as mean ± SD.
3.7.2 Supplementary tables

Table 3-1: Summary of bone marrow analysis from two independent transplantation studies.

<table>
<thead>
<tr>
<th>time-point</th>
<th>cell dose</th>
<th>transplanted mice</th>
<th>repopulated mice</th>
<th>% donor contribution (average [range])</th>
</tr>
</thead>
<tbody>
<tr>
<td>day 0</td>
<td>5.00E+03</td>
<td>4</td>
<td>1</td>
<td>1.5 [0.1-5.7]</td>
</tr>
<tr>
<td></td>
<td>1.00E+04</td>
<td>6</td>
<td>3</td>
<td>9.1 [0.1-42.9]</td>
</tr>
<tr>
<td></td>
<td>1.50E+04</td>
<td>10</td>
<td>7</td>
<td>29.2 [0.1-54.8]</td>
</tr>
<tr>
<td></td>
<td>2.00E+04</td>
<td>6</td>
<td>4</td>
<td>29.5 [0.1-68.5]</td>
</tr>
<tr>
<td>day 8 D=0</td>
<td>1.25E+05</td>
<td>6</td>
<td>4</td>
<td>5.4 [0.1-16.0]</td>
</tr>
<tr>
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<td>2.50E+05</td>
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<td>5</td>
<td>13.2 [0.1-42.2]</td>
</tr>
<tr>
<td></td>
<td>5.00E+05</td>
<td>5</td>
<td>5</td>
<td>17 [13.6-33.4]</td>
</tr>
<tr>
<td>day 8 D=1</td>
<td>5.00E+04</td>
<td>5</td>
<td>2</td>
<td>7.3 [0.1-21.7]</td>
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<tr>
<td></td>
<td>1.25E+05</td>
<td>11</td>
<td>7</td>
<td>8.5 [0.1-30.4]</td>
</tr>
<tr>
<td></td>
<td>2.50E+05</td>
<td>11</td>
<td>10</td>
<td>21.4 [0.1-47.2]</td>
</tr>
<tr>
<td></td>
<td>5.00E+05</td>
<td>6</td>
<td>6</td>
<td>27.5 [2.6-50.4]</td>
</tr>
<tr>
<td>day 12 D=1</td>
<td>1.25E+05</td>
<td>5</td>
<td>1</td>
<td>0.6 [0.1-2.7]</td>
</tr>
<tr>
<td></td>
<td>2.50E+05</td>
<td>4</td>
<td>3</td>
<td>6.2 [0.1-23.3]</td>
</tr>
<tr>
<td></td>
<td>5.00E+05</td>
<td>10</td>
<td>9</td>
<td>16.1 [0.1-38.2]</td>
</tr>
<tr>
<td></td>
<td>1.00E+06</td>
<td>6</td>
<td>6</td>
<td>37.6 [12.2-62.2]</td>
</tr>
</tbody>
</table>

Quantification of the number of transplanted and repopulated mice, for each condition and cell dose, shown as the combined data from two independent experiments. Mice were scored positive for human repopulation if at least 0.5% of BM cells were positive for both human CD45 and human HLA-ABC. Average percentage donor contribution shows cell dose response of human contribution.
Table 3-2: Summary of multi-lineage engraftment of all positively repopulated primary recipients.

<table>
<thead>
<tr>
<th>time-point</th>
<th>cell dose</th>
<th>%CD33+/%CD45+ (average [range])</th>
<th>%CD19+/%CD45+ (average [range])</th>
<th>%CD3+/%CD45+ (average [range])</th>
<th>%GyA+/%CD45+ (average [range])</th>
</tr>
</thead>
<tbody>
<tr>
<td>day 0</td>
<td>5.00E+03</td>
<td>16.7 [N/A]</td>
<td>57.8 [N/A]</td>
<td>3.2 [N/A]</td>
<td>4.3 [N/A]</td>
</tr>
<tr>
<td></td>
<td>1.00E+04</td>
<td>14.2 [11.2-19]</td>
<td>53.3 [48.5-55.6]</td>
<td>1.6 [0.9-2.1]</td>
<td>2.4 [1.7-3]</td>
</tr>
<tr>
<td></td>
<td>1.50E+04</td>
<td>12.7 [8.5-20.5]</td>
<td>47.9 [32.2-58.1]</td>
<td>2.8 [1.0-4.8]</td>
<td>2.4 [1.0-4.9]</td>
</tr>
<tr>
<td></td>
<td>2.00E+04</td>
<td>19.0 [9.7-30.7]</td>
<td>46.1 [36.3-59.9]</td>
<td>2.7 [0.8-5.7]</td>
<td>3.5 [1.0-6.2]</td>
</tr>
<tr>
<td>day 8 D=0</td>
<td>1.25E+05</td>
<td>13.7 [4.4-23.3]</td>
<td>50.2 [32.8-69.9]</td>
<td>3.9 [0.4-13.6]</td>
<td>2.6 [1.8-3.9]</td>
</tr>
<tr>
<td></td>
<td>2.50E+05</td>
<td>16.4 [8.4-25.2]</td>
<td>51.9 [21.7-68.1]</td>
<td>2.3 [0.5-4.6]</td>
<td>3.2 [1.0-5.8]</td>
</tr>
<tr>
<td></td>
<td>5.00E+05</td>
<td>15.5 [12.6-22.2]</td>
<td>56.3 [48.7-63.5]</td>
<td>2.1 [0.6-4.4]</td>
<td>3.4 [2.3-4.5]</td>
</tr>
<tr>
<td>day 8 D=1</td>
<td>5.00E+04</td>
<td>17.3 [10.3-24.3]</td>
<td>55.9 [50.3-61.4]</td>
<td>2.8 [0.9-4.7]</td>
<td>4.1 [3.6-4.5]</td>
</tr>
<tr>
<td></td>
<td>1.25E+05</td>
<td>15.4 [7.8-24.5]</td>
<td>52.9 [42.2-63.4]</td>
<td>2.5 [0.7-5.4]</td>
<td>3.7 [1.0-7.4]</td>
</tr>
<tr>
<td></td>
<td>2.50E+05</td>
<td>12.0 [6.6-20.3]</td>
<td>56.8 [45.1-69.0]</td>
<td>2.7 [0.4-5.4]</td>
<td>2.8 [1.0-5.4]</td>
</tr>
<tr>
<td></td>
<td>5.00E+05</td>
<td>19.2 [8.9-39.2]</td>
<td>51.5 [25.1-65.5]</td>
<td>2.2 [0.6-4.5]</td>
<td>4.7 [1.0-8.4]</td>
</tr>
<tr>
<td>day 12 D=1</td>
<td>1.25E+05</td>
<td>17.9 [N/A]</td>
<td>58.8 [N/A]</td>
<td>3.1 [N/A]</td>
<td>3.9 [N/A]</td>
</tr>
<tr>
<td></td>
<td>2.50E+05</td>
<td>13.6 [5.1-19.6]</td>
<td>57.7 [51.9-63.3]</td>
<td>2.0 [1.6-2.6]</td>
<td>2.1 [1.2-3.3]</td>
</tr>
<tr>
<td></td>
<td>5.00E+05</td>
<td>14.1 [8.7-20.0]</td>
<td>50.2 [20.1-63.1]</td>
<td>2.6 [1.1-5.3]</td>
<td>2.8 [0.8-5.0]</td>
</tr>
<tr>
<td></td>
<td>1.00E+06</td>
<td>10.8 [5.2-19.8]</td>
<td>51.6 [41.7-61.2]</td>
<td>1.5 [0.5-3.4]</td>
<td>2.3 [1.1-3.9]</td>
</tr>
</tbody>
</table>

Averages and ranges of lineage markers measured by CD33, CD19, CD3, and GyA, for each condition and cell dose. Lineage values are shown as the percentage of the positively repopulated human cells.
Table 3-3: Details of the recipients used for secondary transplantations and the corresponding repopulation results.

<table>
<thead>
<tr>
<th>primary recipient #</th>
<th>primary cell dose</th>
<th>primary engraft (%)</th>
<th>% CD34+</th>
<th>secondary engraft (y/n)</th>
<th>secondary engraft (%)</th>
<th>%CD33+/ %CD45+</th>
<th>%CD19+/ %CD45+</th>
<th>%CD3+/ %CD45+</th>
<th>%GyA+/ %CD45+</th>
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<td>60</td>
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<td>23.3</td>
<td>7</td>
<td>n</td>
<td>0.1</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>65</td>
<td>5.0E+05</td>
<td>30.5</td>
<td>15.8</td>
<td>y</td>
<td>0.9</td>
<td>22.3</td>
<td>51</td>
<td>1.3</td>
<td>1.7</td>
</tr>
<tr>
<td>66</td>
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<td>y</td>
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<td>6.6</td>
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<tr>
<td>69</td>
<td>1.0E+06</td>
<td>62.2</td>
<td>20.8</td>
<td>y</td>
<td>2.1</td>
<td>23.8</td>
<td>48.7</td>
<td>1</td>
<td>4.2</td>
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<tr>
<td>70</td>
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<td>33.4</td>
<td>12.4</td>
<td>y</td>
<td>0.7</td>
<td>16.1</td>
<td>57.9</td>
<td>2.2</td>
<td>0.8</td>
</tr>
<tr>
<td>71</td>
<td>1.0E+06</td>
<td>26.9</td>
<td>8.6</td>
<td>y</td>
<td>0.5</td>
<td>12.3</td>
<td>54.4</td>
<td>2.7</td>
<td>5.7</td>
</tr>
</tbody>
</table>

Secondary SR1 frequency (corrected for 178x day 12 TNC expansion): 1/4438 (95%CI: 1/1734-1/11359)

Quantification of secondary human repopulation and multi-lineage potential of positively repopulated secondary recipients. 8 secondary transplantations were performed, each using 33% of the harvested bone marrow cells from positively repopulated mice from the day 12 D=1 condition.
3.7.3 Supplementary experimental procedures: derivation of fed-batch and perfusion ODEs:

The secreted factor ODEs in the previously described computational model (Kirouac et al., 2009) were adjusted to describe fed-batch or perfusion culture systems, based on the following mass-balance derivations.

**Model Variables**

- $P$ = secreted protein (pg)
- $[P]$ = secreted protein concentration in media (pg/ml)
- $r_p$ = rate of protein $P$ secretion (pg/cell.d)
- $X$ = cells
- $[X]$ = cell density (cell/ml)
- $V$ = culture volume (ml)
- $V_o$ = initial culture volume (ml)
- $F$ = flow rate (ml/d)
- $D = \text{dilution rate} = F/V_o$

**Fed-Batch ODE derivation:**

$$\frac{dP}{dt} = r_p \cdot X$$

$$\frac{d[P]}{dt} = \frac{r_p \cdot X}{V}$$

IF: $F$ = constant, $D = F/V_o$

$$\frac{dV}{dt} = F$$

$$\int_{V_o}^{V_f} dV = F \int_{t_o}^{t_f} dt$$

$$V_f - V_o = F(t_f - t_o)$$

$$V(t) = V_o + F \cdot t$$

$$\frac{d[P]}{dt} = \frac{r_p \cdot X}{V_o + F \cdot t} \times \frac{1/V_o}{1/V_o}$$

$$\frac{d[P]}{dt} = \frac{r_p \cdot [X]}{1 + D \cdot t}$$

**Perfusion ODE derivation:**

$$\frac{dP}{dt} = P_{in} - P_{out} + P_{gen}$$

$$\frac{d[P]}{dt} = \frac{F}{V}([P]_{in} - [P]_{out}) + \frac{r_p \cdot X}{V}$$

$$\frac{d[P]}{dt} = \frac{r_p \cdot [X]}{1 + D \cdot [P]}$$
This chapter is currently submitted for peer review and publication. Co-authors include Tatiana Usenko, Wenlian Qiao, Colleen Delaney, Irwin Bernstein, and Peter W. Zandstra.

**Author Contributions:**
Elizabeth Csaszar - conception and design, collection and assembly of data, data analysis and interpretation, manuscript writing; Tatiana Usenko - technical assistance with in vivo studies; Wenlian Qiao - gene expression analysis; Colleen Delaney - technical advice, review of manuscript; Irwin Bernstein - provision of study material, review of manuscript; Peter W Zandstra - conception and design, data interpretation, manuscript writing.
4.1 Abstract

Increasing evidence supports the importance of cell extrinsic regulation in somatic stem cell fate control. Rare hematopoietic stem cells must be responsive both to local signals from their niche and, directly or indirectly, to systemic feedback. The Notch ligand Delta-1 (DL1) is a key component of the stem cell niche and has been used clinically for in vitro progenitor expansion. DL1 produces a progenitor cell supportive environment by reducing myeloid cell production despite activating STAT3. Here we show that DL1 causes the loss of membrane bound IL-6R, resulting in the conversion from IL-6 cis- signaling to IL-6 trans-signaling and subsequent mature cell lineage skewing. By concurrently reducing IL-6 trans-signaling through continuous microenvironment regulation, we show that DL1 can generate a supportive environment for stem cell expansion. The identification of DL1-mediated IL-6 rewiring demonstrates how changing cell-type specific signal responsiveness can affect microenvironment feedback control and alter primitive cell fate.
4.2 Introduction

Niche-mediated signaling effects that impact hematopoietic stem and progenitor cells (HSPC) are often complex and context dependent. Notch signaling in the hematopoietic system exemplifies this complexity. The mammalian Notch family consists of 4 receptors (Notch 1-4) and 5 ligands (Jagged- 1 & 2, Delta- 1, 3, 4) (Bray, 2006), which can have complementary, redundant, or opposing roles, depending on context (Weber and Calvi, 2010). Osteoblasts regulate HSPCs in the bone marrow niche in a Notch mediated manner, however the precise role of Notch signaling in HSPC regulation and its necessity in adult hematopoiesis is less clear (Weber and Calvi, 2010). In vitro, the Notch pathway has been shown to regulate several hematopoietic cell lineages in a highly dose dependent manner. Binding of the Notch1 receptor is critical for T-cell development and differentiation (Pear et al., 1996; Radtke et al., 1999) and the Delta-1 ligand (DL1) can be used at high concentrations in culture to enhance the expansion and in vivo reconstitution of T-cell progenitors (Dallas et al., 2007; Zakrzewski et al., 2006). In the myeloid lineage, DL1 causes a decrease in monocytes and granulocytes through the binding of Notch2 (Delaney et al., 2005; Varnum-Finney et al., 2011; Yamamura et al., 2007) and, at low concentrations, DL1 has been shown to enhance CD34+ cells and primitive progenitors (Delaney et al., 2005). Importantly, DL1 significantly enhances short-term SCID-repopulating cells (SRCs) and early clinical data indicates that patients transplanted with cells cultured in the presence of DL1 had improved time to neutrophil repopulation following transplantation (Delaney et al., 2010).

Much like the Notch pathway, the IL-6-JAK-STAT pathway has also been implicated in impacting both HSPC expansion as well as mature cell lineage skewing in a context-dependent manner (Kimura et al., 2000; Yamamura et al., 2007). IL-6 can act in a cis-signaling manner on cell populations expressing the membrane bound IL-6R (mIL-6R), where the IL-6 ligand first binds to mIL-6R, and the complex then associates with gp130 to initiate signaling (Taga and Kishimoto, 1995; Viswanathan et al., 2002). During hematopoiesis, mIL-6R expression is typically restricted to myeloid lineage cells (Tajima et al., 1996) and IL-6 has been shown to significantly enhance the production of monocytes and granulocytes (Yu et al., 2006). In addition to cis-signaling, IL-6 can act via trans-signaling by binding to soluble IL-6R (sIL-6R). This soluble complex can then initiate signaling in additional cell types via gp130, which is
ubiquitously expressed (Jones and Rose-John, 2002). For instance, it has been shown that expansion of erythroid lineage cells, which do not express mIL-6R, can be achieved though IL-6 trans-signaling in the presence of both IL-6 and sIL-6R (Sui et al., 1996). Interestingly, there is evidence of cross-talk between the Notch pathway and the JAK-STAT pathway both in the hematopoietic system and other systems (Choi et al., 2012; Kamakura et al., 2004; Yamamura et al., 2007). However, it has not been demonstrated how this cross-talk mechanistically impacts either mature cell phenotype changes or the expansion of HSPCs. Understanding this mechanism would enable the determination of the signaling regulation underpinning adult hematopoiesis and the identification of key nodes for HSPC manipulation.

Methods to expand HSPCs in vitro traditionally have focused on identifying factors that directly target HSPC self-renewal (Amsellem et al., 2003; Boitano et al., 2010; Delaney et al., 2010; Peled et al., 2004; Zhang et al., 2008). The impact of these culture strategies on the emerging mature cell populations is often either not considered, or assumed to be distinct from the effects on the primitive cells. However, we have previously shown that feedback from mature cells in culture have a significant impact on HSPC expansion and the impact of this feedback is specific to the mature cell type (Csaszar et al., 2012; Kirouac et al., 2010). The role of lineage skewing on local and system-wide feedback to the HSPC populations may significantly contribute to the regulation of HSPCs in vitro and in vivo. As both the Notch pathway and the IL-6-JAK-STAT pathway are linked to specific changes in mature lineage frequencies, understanding how these signaling pathways are modulated in response to stimuli will provide a critical link between mature cell production and the resulting impact of the soluble factor microenvironment on HSPCs.

Herein, we sought to understand the link between the Notch pathway's impact on mature cell lineage skewing and primitive cell fate modulation during human hematopoiesis. Building upon our previously described platform that allows for feedback mediated regulation (Csaszar et al., 2012), we are able to track cell population dynamics to explore the impact of changing mature cell populations on the feedback signaling environment. We demonstrate that the myeloid cell lineage skewing observed in the presence of DL1 is predominantly attributed to Notch signaling-mediated regulation of IL-6R expression. This lineage skewing then creates a microenvironment that alters the feedback signals to which the HSPCs are exposed. By manipulating this feedback
environment to be highly supportive of HSPCs with the combination of DL1 and the fed-batch culture system, we can generate significant expansion of SRCs.

4.3 Materials and Methods

4.3.1 Umbilical cord blood cell collection and processing
UCB samples were collected from consenting donors according to ethically-approved procedures at Mt Sinai hospital (Toronto, ON, Canada). Mononuclear cells were obtained by first mixing the UCB sample with 10% pentastarch at a 1:5 ratio. The sample was then centrifuged for 10min at 50 g, and the upper (leukocyte rich) plasma layer was collected and centrifuged for 10min at 400 g to obtain a cell pellet. Red blood cells were depleted by mixing the cells with a red blood cell lysis buffer for 5min. Lineage negative (Lin⁻) progenitor cells or CD34⁺ cells were isolated from the mononuclear cell fraction using the EasySep system with the human progenitor cell enrichment kit or human CD34⁺ enrichment kit (Stemcell Technologies, Vancouver, BC, Canada), following the manufacturer’s protocol.

4.3.2 DL1 preparation
The Notch Delta-1 ligand (DL1) was produced as previously described (Varnum-Finney et al., 2000). 2.5 ug/mL of DL1 and 5 ug/mL of retronectin (Takara Shuzo, Otsu, Japan) were diluted in chilled PBS, coated onto non tissue culture treated plates at 0.16mL/cm² and kept overnight at 4°C. The coated dishes were then blocked with PBS + 10% FBS for at least one hour at 37°C and washed with PBS prior to cell seeding.

4.3.3 Cell seeding and in vitro culture
Freshly isolated CD34⁺ cells were seeded on DL1 coated plates at an initial density of 1 x 10⁵ cells/mL in serum free IMDM media (Gibco, Rockville MD, USA) with 20% BIT serum substitute (Stemcell Technologies) and 2mM Glutamax (Gibco). The media was supplemented with 100ng/mL Stem Cell Factor (SCF, R&D Systems, Minneapolis, MN, USA), 100ng/mL FMS-like Trysine Kinase 3 Ligand (FL, R&D Systems), 50ng/mL Thrombopoietin (TPO, R&D Systems), and 1ug/mL low-density lipoproteins (LDL, Calbiochem, LaJolla, CA, USA). Plates
were cultured on an orbital shaker to aid in mixing of the system. To mimic the feeding scheme of the previously described fed-batch system (Csaszar et al., 2012), cultures were fed every 24 h with one unit volume of fresh media, to maintain a dilution rate of $D=1$. Cells were transferred to plates with freshly coated ligand every 4 days. Where indicated, IL-6 (R&D) was used at 50ng/mL, sIL-6R (R&D) was used at 1000ng/mL, JAKi (Calbiochem) was used at 50nM. SR1 was generously donated by Michael Cooke (Novartis) and was used at 750nM. For non-fed-batch conditions, where indicated, CD34$^+$ cells were cultured at $1 \times 10^5$ cells/mL, and a full media exchange was performed every 4 days.

4.3.4 Cell assays

Colony forming cell (CFC) assays and long term culture-initiating cell (LTC-IC) assays were performed as previously described (Kirouac et al., 2009). Surface marker staining was performed with conjugated human antibodies: CD7, CD14, CD15, CD34, CD41, CD45RA, CD90, mIL-6R (CD126), CD133, GyA (all antibodies BD Biosciences, San Jose CA, USA). 7-AAD dye was added to assess cell viability and isolate live cells for quantification. All samples were analyzed on a FACSCanto or FACS LSR Fortessa flow cytometer (BD Biosciences).

For the sorted cell assay, freshly isolated Lin$^-$ cells were sorted for Rho$^{lo}$CD34$^{+}$CD38$^{-}$CD45RA$^{-}$CD49f$^+$ with a FACSARia flow cytometer (BD Biosciences), following the gating strategy previously described (Notta et al., 2011). 40 sorted cells were dispensed per well in a 96-well plate in the above described media, or in conditioned media where indicated, and cultured for 7 days. To assess DL1 in this assay, cells were sorted directly into a 96-well plate that had been pre-coated with DL1, as described above. Following culture, cells were assessed by flow cytometry. For the assessment of paracrine signaling from mature cell populations, conditioned media from mature cell populations was produced by culturing Lin$^-$ cells for 10 days in the above described culture media and then sorting for cells positive for CD14, CD15, CD7, CD41, or GyA. The mature cells were seeded at $5 \times 10^5$ cells/mL for 7 days, at which point the media was collected and used to culture the sorted primitive cells as above.

4.3.5 Gene Expression Analysis

Raw gene expression data were obtained from the NCBI’s Gene Expression Omnibus (GEO) through accession number GSE42414 (Laurenti et al., 2013) and GSE24759 (Novershtern et al.,
Quality of the GSE24759 arrays was assessed using the simpleaffy (v2.32.0) and AnnotationDbi (v1.18.4) packages of BioConductor. The two datasets were combined by following a 4-step procedure. First, quantile signals of GSE42414 and GSE24759 datasets were obtained using the normalizeQuantiles() function and the justRMA() function in the limma package (v3.12.3) of BioConductor, respectively. Second, genome-wide normalization was performed with respect to a common population between the two. Third, for each dataset, the average value of probes that target the same gene was calculated. Lastly, the two datasets were combined through Entrez ID. All the analyses were performed in R. Cell populations were labeled as follows: HSC = CD34⁺CD38⁻CD90⁻CD49f⁺CD45RA⁻, MLP = Lin⁻CD34⁺CD38⁻CD90⁺CD45RA⁺CD7⁻CD10⁻, CMP = CD34⁺CD38⁺CD135⁺CD45RA⁻CD7⁻CD10⁻, GMP = CD34⁺CD38⁺CD135⁺CD45RA⁺CD7⁻CD10⁻, MEP = CD34⁺CD38⁺CD135⁺CD45RA⁻CD7⁻CD10⁻, CFU-M = CD34⁺CD33⁺CD13⁻, MONO = FSC hi SSC lo CD14⁺CD45 dim, CFU-G = CD34⁺SSH hi CD11b⁻CD16⁻, NEUT = FSC hi SSC hi CD16⁺CD11b⁺, CFU-MK = CD34⁺CD41⁺CD61⁺CD45⁺, MEGA = CD34⁺CD41⁺CD61⁺CD45⁺, CFU-E = CD34⁺CD71⁺GlyA⁻, ERY = CD34⁻CD71⁻GlyA⁺.

4.3.6 pSTAT3 analysis
CD34⁺ cells cultured for 8-12 days were starved of cytokines and DL1 overnight at low cell densities. Following starvation, cells were stimulated with soluble factors by adding one or more of IL-6 (50ng/mL, R&D Systems), sIL-6R (1000ng/mL, R&D Systems) or JAK inhibitor I (50nM, Calbiochem). For DL1 simulation, starved cells were transferred to plates pre-coated with DL1 (2.5ug/mL) to initiate the stimulation period. After the commencement of stimulation, cells were fixed at the time-points indicated with 4% paraformaldehyde. Following fixation, cells were permeabilized for 5min with chilled methanol and stained with pSTAT3 antibody (Cell Signaling Technology, Danvers MA, USA) and an AF-647 secondary antibody.

4.3.7 Secreted factor analysis
Conditioned media from cells cultured for 12 days in the indicated conditions was collected and frozen. Secreted factor concentrations were sampled in duplicate from thawed conditioned media.
aliquots using IL-6 and sIL-6R Quantikine ELISA Kits (R&D Systems, Minneapolis MN, USA), according to the manufacturer’s directions.

4.3.8 Limiting dilution transplantation studies

All animal studies were performed according to procedures approved by appropriate animal ethics boards. Female NOD/SCID/IL-2Rγc-null (NSG) mice were sub-lethally irradiated (250 rad) 24h before transplantation. Uncultured CD34⁺ enriched cells, or cells cultured for 12 days or 16 days were transplanted at limiting dilution via tail vein injection. Cohorts of mice were sacrificed at 3, 9, 16, and 24 weeks following transplantation and bone marrow was collected from tibias. Cells were assessed by flow cytometry. Mice were scored positive for human repopulation if at least 0.5% of bone marrow cells were positive for both human CD45 and human HLA-ABC. All limiting dilution analysis and associated statistical analysis was performed with the L-calc software (Stemcell Technologies).

4.3.9 Mathematical simulations

The model that we previously developed and described (Csaszar et al., 2012; Kirouac et al., 2009) was used to run all culture simulations with MATLAB 2012 software (Mathworks, Natick MA, USA). The model simulates in vitro hematopoietic culture by incorporating self-renewal and differentiation of cell populations and soluble factors secreted by mature cells. For these studies, specific mature cell lineages were incorporated into the model and each mature cell lineage was given a weighting on negative and positive feedback from 0 to 1, based on experimental findings. The internal parameters of the model were fit with the fed-batch control data of total cell expansion, CD34⁺ expansion, CD34⁺CD90⁺ expansion, and LTR-HSC expansion. For each culture condition (fed-batch control, DL1, or IL-6/sIL-6R) the probability of cells entering each lineage was set based on experimentally derived phenotype frequencies. The internal parameters that dictate maximum proliferation rate of Lin⁺ and Lin⁻ cells were multiplied by a scaling factor, determined by experimental data. (Input values for each condition are listed in Table 4-1). The model was then run with the inputs from each culture condition in order to predict the population expansions that would result based on the mature cell frequencies and proliferation rates. These model outputs were compared to experimental data of population expansions for each condition.
4.3.10 Statistical analysis
Statistical significance was computed using student t-test and ANOVA. All error bars represent the standard deviation of three or more biological replicates. Asterisks (*) or number signs (#) indicate statistical significance between indicated conditions (or if not indicated, between the test condition and control) of p<0.05.

4.4 Results
4.4.1 DL1 mediated lineage skewing creates a HSPC supportive environment.
Immobilized DL1 was combined into our fed-batch culture platform, which can be used to regulate paracrine signaling (Csaszar et al., 2012). Figure 4-1A illustrates that the addition of DL1 causes a significant increase in primitive cells, as measured both by phenotype (CD34⁺ and CD34⁺ CD90⁺) and by functional in vitro assays (colony forming cell (CFC) assay and long term culture-initiating cell (LTC-IC) assay). The greatest impact of the ligand was seen on the more primitive progenitor populations (CD34⁺CD90⁺ and LTC-IC). During the first several days of culture, we observed that DL1 caused a decrease in total cell number due to slower proliferation, as demonstrated by CFSE staining (Figure 4-5). In this heterogeneous culture system, there was not an immediate enhancement in the frequency of progenitor cells, as compared to the control; however, as the culture progressed, the anticipated enhancement of HSPC output upon DL1 supplementation emerged. We have previously demonstrated that the endogenously produced microenvironment is a powerful stimuli for HSPCs in longer term culture systems (Csaszar et al., 2012; Madlambayan et al., 2005). Thus, given the divergence of HSPC expansion after several days of culture with DL1 as compare to the control, we were interested in whether DL1 was impacting the cell microenvironment in addition to the HSPCs themselves.

To investigate this hypothesis, we compared the observed impact of DL1 in the heterogeneous system to its impact on purified primitive cells that had been sorted for a phenotype shown to be highly enriched for HSCs (Lin⁺Rho⁺CD34⁺CD38⁻CD45RA⁻CD49f⁺) (Notta et al., 2011). After 7 days of culture of the Lin⁺Rho⁺CD34⁺CD38⁻CD45RA⁻CD49f⁺ population, cells were assessed by
flow cytometry. In this assay, the addition of factors that directly target the self-renewal of the highly enriched HSC population without influence by feedback from other cell populations, such as the small molecule SR1 (Boitano et al., 2010), will enhance the expansion of the primitive CD34+CD90+ population (Csaszar et al., 2012). When DL1 was added to this population, there was not a significant increase in expansion above what was seen with the cytokine control, and the expansion was lower than that demonstrated with SR1. (Figure 4-1Bi). In contrast, in the 12 day culture of the more heterogeneous CD34+ cell population, where HSPCs are influenced by both directly acting stimuli and changes to the microenvironment, the expansion of the CD34+CD90+ population produced by DL1 was equivalent to that produced by SR1 and was significantly greater than the cytokine control (Figure 4-1Bii). Therefore, this data suggests that DL1 may not be acting by directed target of HSPC self-renewal, but the enhanced HSPC self-renewal may result from the manipulation of the cell microenvironment.

In order to help determine which cell types may be receptive to DL1 (and thus candidates for indirect feedback to HSCs), we mined gene expression datasets of sorted hematopoietic populations (Laurenti et al., 2013; Novershtern et al., 2011) to assess the expression levels of Notch1 and Notch2, the known receptors of DL1 (Dahlberg et al., 2011). Figure 4-1C shows that both of these Notch receptors are expressed at varying levels on many hematopoietic cell populations, indicating that there are likely many mature cells that are being influenced by DL1-mediated Notch activation and are, in turn, modulating the cell microenvironment to which the HSPCs are exposed. We next sought to understand how these lineage-committed populations were affected by DL1, and how this related to the effects seen on the more primitive cells.

It has previously been reported that DL1 changes HSPC derived mature lineage output (Delaney et al., 2005; Yamamura et al., 2007). We predicted that this lineage skewing may be contributing to the effects seen on HSPC expansion in a paracrine signaling feedback-mediated manner. Figure 4-1D shows that the addition of DL1 to CD34+ cells led to a significant and sustained reduction in the production of CD14+ monocytes and CD15+ granulocytes. In contrast, there was an increase in CD7+ lymphocytes, as has been previously reported (Delaney et al., 2005). We did not observe any significant effects on the CD41+ megakaryocyte lineage or the GyA+ erythrocyte lineage. Mature cells in hematopoietic culture secrete paracrine signaling factors that act in a feedback mediated manner on HSPCs. We have previously shown that the net impact of these
mature cells in our control culture system is inhibitory, however each mature cell population will have an individual impact on the HSPC population (Csaszar et al., 2012; Madlambayan et al., 2005). Skewing of mature cell lineages will change the feedback signaling profile that is being produced during culture and will thus alter the net impact of feedback signaling. The reduction of CD14+ and CD15+ cells seen with the addition of DL1 is particularly interesting as our group has shown that the addition of CD14+ cells to a purified progenitor population causes a significant reduction in LTC-IC expansion (Kirouac et al., 2010) and it has been demonstrated that the depletion of CD14+ cells in culture enhances total cell and CD34+ cell expansion (Yang et al., 2010).

To better assess the feedback signaling impact of specific mature cell populations, we mixed sorted mature cell populations or their conditioned media, with LinRhoCD34−CD38−CD45RA−CD49f− cells in a 7-day co-culture assay. Figure 4-1E demonstrates that similar paracrine effects on the primitive cells were seen whether they were mixed with the mature cells themselves or with the mature cell conditioned media, confirming that these paracrine effects are soluble factor mediated. We then compared the expansion of the LinRhoCD34−CD38−CD45RA−CD49f− cells cultured in media conditioned from specific mature cell lineages (Figure 4-1F). Conditioned media from CD14+, CD15+, and GyA+ cells caused a significant decrease in expansion of the CD34+CD90+ cells, while the effect of CD7+ cells was neutral and CD41+ cells caused a trend towards increased primitive cell expansion, as previously reported (Kirouac et al., 2010). These results confirmed the differential effects of mature lineage populations on HSPC expansion and highlighted the inhibitory effect of CD14+ and CD15+ cells.

Collectively these studies illustrate that the effects of DL1 addition on HSPCs are predominantly regulated via indirect changes in mature cell output. The emerging mature cells produce a soluble factor based feedback signaling microenvironment that is highly dependent on the frequencies of specific mature cell populations. DL1 reduces the CD14+ and CD15+ cell populations that are most inhibitory towards HSPCs and this altered signaling environment contributes to the sustained expansion of the HSPC population in the presence of DL1. We next sought to understand how DL1-mediated signaling altered myeloid cell production.
Figure 4-1: DL1 generates a microenvironment that is supportive towards HSPC expansion.

[A] The addition of DL1 to CD34^+ cells in the fed-batch culture system enhances the expansion of: (i) CD34^+ cells; (ii) CD34^+CD90^+ cells; (iii) colony forming cells (CFCs); (iv) long-term culture-initiating cells (LTC-ICs).

[B] (i) When DL1 is added to purified cells highly enriched for HSCs (Rho^0CD34^-CD38^-CD45RA^-CD49f^+), there is not a significant increase in the primitive cell population after 7 days of culture. In contrast, the small molecule SR1 significantly
increases primitive cell expansion. (ii) In contrast to the purified cell assay, the addition of DL1 to a heterogeneous CD34+ population causes equivalent levels of primitive cell expansion as SR1 and significantly more than the control, indicating that DL1 targets more than just the purified primitive cells. [C] Microarray analysis of ≥3 representative samples indicates that the Notch1 and Notch2 receptors are expressed at varying levels in many uncultured hematopoietic populations. [D] DL1 causes lineage skewing in cultured CD34+ cells, most notably a decrease in CD14+ cells and CD15+ cells, and an increase in CD7+ cells. [E] When CD14+ cells, or the conditioned media (CM) from these cells are cultured with Rho<sup>lo</sup>CD34+CD38-CD45RA-CD49f<sup>+</sup> cells, there is an inhibitory effect on the proliferation of the CD34+CD90<sup>+</sup> population after 7 days of culture. [F] The conditioned media from the specified mature cell lineages (CD14<sup>+</sup>, CD15<sup>+</sup>, CD41<sup>+</sup>, GyA<sup>+</sup>, CD7<sup>+</sup>, other = CD34<sup>+</sup>CD14<sup>+</sup>CD15<sup>+</sup>CD41<sup>+</sup>GyA<sup>+</sup>CD7<sup>+</sup>) were added to the Rho<sup>lo</sup>CD34+CD38-CD45RA-CD49f<sup>+</sup> cells and cultured for 7 days. Different lineages have varying impacts on the expansion of CD34+CD90<sup>+</sup> cells. Effects are categorized as negative (red region), neutral (grey region), or positive (green region) as compared to conditioned media from unsorted cell populations (all). All error bars indicate standard deviation. n ≥ 3, * = p < 0.05

4.4.2 DL1 activates the JAK-STAT pathway in a similar manner as IL-6, but skews mature cell lineages in an opposing way

Given the inhibitory nature of CD14<sup>+</sup> and CD15<sup>+</sup> cells, reducing the production of these cells provides a method to generate a cell microenvironment that is more supportive for HSPC expansion. Therefore, we were particularly interested in understanding the mechanism by which DL1 reduces the generation of CD14<sup>+</sup> and CD15<sup>+</sup> cells. The production and differentiation of macrophages and granulocytes is known to be largely modulated by gp130 signaling and cytokine-mediated STAT3 activation (Jenkins et al., 2008; Metcalf, 1997; Minami et al., 1996; Zhang et al., 2010). Furthermore, there have been reports of interactions between Notch signaling and STAT3 activation (Choi et al., 2012; Kamakura et al., 2004). Thus, we hypothesized that the DL1-mediated effect on myeloid cell production may be acting through modulation of the JAK-STAT signaling pathway.

To investigate the impact of DL1 on STAT3, we measured its dynamic activation in response to different stimuli. As expected, stimulation of cultured CD34+ cells with IL-6 caused a significant
up-regulation of pSTAT3. This effect was amplified by stimulating the cells with a combination of IL-6 and sIL-6R and could be diminished by the addition of a JAK inhibitor (Figure 4-2Ai). When cultured CD34+ cells were stimulated with DL1, we saw a rapid activation of STAT3 that mimicked that seen with IL-6 and sIL-6R (Figure 4-2Aii). The combination of DL1 and IL-6 stimulation produced an additive signaling effect on STAT3 activation (Figure 4-2Aiii). The JAK-STAT pathway is known to act in an auto-regulatory manner, such that targets of STAT3 up-regulate the further production of IL-6 and related ligands (Ichiba et al., 1998). To test whether the activation of STAT3 by DL1 affected IL-6 production, we measured the levels of soluble IL-6 family members during culture with or without DL1 by ELISA. Media from CD34+ cells cultured in the presence of DL1 contained significantly higher concentrations of both IL-6 and sIL-6R as compared to cultures without DL1 (Figure 4-2B). Notably, DL1-mediated IL-6 production was abrogated by the addition of a JAK inhibitor. Conversely, levels of sIL-6R were only somewhat dampened by a JAK inhibitor, suggesting that the DL1-mediated increases in sIL-6R were at least partially JAK independent.

Our observation that DL-1 was modulating sIL-6R led us to consider whether DL1 was also changing the population of cells that were responsive to IL-6. IL-6 can initiate JAK-STAT signaling in one of two ways (Figure 4-2C). IL-6 cis-signaling results when the IL-6 ligand binds to the membrane bound IL-6R, which then associates with gp130 to activate the signaling cascade. In the hematopoietic system, it is known that mIL-6R, and thus IL-6 cis-signaling, is restricted to myeloid lineage cells (Tajima et al., 1996), and we observed that the addition of IL-6 to CD34+ cells produced a significant increase in CD14+ and CD15+ cell production (Figure 4-2D). In cells that do not express mIL-6R, IL-6 trans-signaling can occur when IL-6 binds to sIL-6R, and the soluble complex then binds to the ubiquitously expressed gp130 receptor. When both IL-6 and sIL-6R were added to culture, in addition to the increase in CD14+ and CD15+ cells, we also observed an increase in GyA+ cells (Figure 4-2D). This is consistent with a previously reported finding that IL-6 trans-signaling enhances erythroid cell production (Yu et al., 2006). These mature cell phenotype changes resulting from IL-6/sIL-6R addition could be rescued with the addition of a JAK inhibitor, confirming that they were indeed JAK-STAT dependent (Figure 4-6).
IL-6/sIL-6R increased the production of CD14⁺, CD15⁺, and GyA⁺ cells, all of which act in an inhibitory manner towards HSPC expansion. Of note, we found that the addition of IL-6 or IL-6/sIL-6R led to a significant increase in total cell expansion but a corresponding decrease in the frequency of CD34⁺ cells. As such, the total expansion of CD34⁺ cells was not significantly different with IL-6/sIL-6R as compared to the control (Figure 4-6). The high levels of inhibitory mature cells in these cultures likely contribute to the decrease in CD34⁺ frequency that is observed with IL-6/sIL-6R. Therefore, despite the similar propensities of DL1 and IL-6/sIL-6R on STAT3 activation, these two stimuli produce very different effects on both mature cell populations and primitive cell expansions.

It was puzzling that both DL1 and IL-6/sIL-6R activated STAT3 but that the effects of the signaling resulted in completely different cell population dynamics. Specifically, IL-6/sIL-6R enhances the production of CD14⁺ and CD15⁺ cells, while DL1 reduces the production of these cells. Moreover, when exogenous IL-6/sIL-6R was added in combination with DL1, CD14⁺ and CD15⁺ cell production remained at the low levels that were seen with DL1 addition alone (Figure 2Di,ii), indicating that DL1 had the dominant effect on CD14⁺ and CD15⁺ cell production and its impact could not be rescued by higher levels of IL-6/sIL-6R. Conversely, consistent with previous findings (Yamamura et al., 2007), DL1 in combination with IL-6/sIL-6R enhanced GyA⁺ levels above what is seen with either IL-6/sIL-6R or DL1 alone (Figure 4-2Diii). Therefore, it appears that DL1 reduces IL-6 cis-signaling and enhances IL-6 trans-signaling.
Figure 4-2: DL1 activates STAT3 similarly to IL-6 but impacts mature cells in opposing way.

[A] (i) IL-6 stimulation activates STAT3 in CD34⁺ cells that had been cultured for 8-10 days. This effect is enhanced with the combination of IL-6 and sIL-6R and is diminished with the addition of a JAK inhibitor. * indicates statistical comparison of IL-6 stimulation to unstimulated condition, # indicates statistical comparison of IL-6/sIL-6R stimulation to IL-6 stimulation. (ii) DL1 stimulation rapidly activates STAT3 in CD34⁺ cells that had been cultured for 8-10 days. This effect is diminished with the addition of a JAK inhibitor. (iii) There is an additive effect of DL1 and IL-6 on STAT3 activation. [B] DL1 up-regulates the secretion of (i) IL-6 and (ii) sIL-6R. Soluble factor quantification was performed on conditioned media from CD34⁺ cells.
cultured for 12 days. [C] Schematic of IL-6 cis-signaling and trans-signaling. IL-6 ligand can initiate JAK-STAT signaling in the absence or presence of sIL-6R on cell types that express mIL-6R (cis-signaling). On cells that do not express mIL-6R, IL-6 mediated signaling can occur only in the presence of sIL-6R (trans-signaling). [D] IL-6 produces an increase in CD14$^+$ cells and CD15$^+$ cells when added to the culture media of CD34$^+$ cells. The combination of IL-6 and sIL-6R produces the same effect as well as an additional increase in GyA$^+$ cells. The lineage skewing caused by DL1 is shown for comparison. When IL-6/sIL-6R are added in combination with DL1, DL1 inhibits the production of: (i) CD14$^+$ cells and; (ii) CD15$^+$ cells, generated by IL-6 cis-signaling, and enhances the production of: (iii) GyA$^+$ cells, generated by IL-6 trans-signaling. * indicates statistical comparison of IL-6 to control condition, # indicates statistical comparison of IL-6/sIL-6R to IL-6 condition. All error bars indicate standard deviation. n ≥ 3, *,# = p < 0.05.

4.4.3 DL1 converts HSPCs from IL-6 cis-signaling to trans-signaling through loss of mIL-6R

DL1 prevents the IL-6-mediated production of mature myeloid cells and we hypothesized that the reduction of IL-6 cis-signaling was resulting from the loss of mIL-6R in the presence of DL1. The loss of the membrane bound receptor via receptor shedding would also be consistent with the observed increase in sIL-6R (Figure 4-2Bii). To test this hypothesis, we measured cell population specific mIL-6R levels as a function of DL1 activation. In freshly isolated CD34$^+$ cells, there was no mIL-6R expression, however, upon culture, mIL-6R expression was very quickly (within 6 h) up-regulated (Figure 4-3A). With the addition of DL1, the levels of mIL-6R were significantly lower than in the control culture. The decreased mIL-6R expression in the presence of DL1 occurs rapidly, before the first cell division, suggesting that this effect is signaling mediated, as opposed to the being due to the emergence of a new cell population or due to a difference in cell composition between conditions. Cell counts at these early time-points also confirmed that there were no differences in cell numbers between the control and DL1 condition, confirming that the decreased levels of mIL-6R with DL1 were not the result of apoptosis of a mIL-6R expressing cell population (data not shown). The rapid DL1-mediated mIL-6R reduction was seen on both the more primitive CD34$^+$CD45RA$^-$ cell population and the more committed CD34$^+$CD45RA$^+$ cell population, indicating that both these cell populations are being
targeted by DL1. (Figure 4-3Ai,ii). On the highly purified CD34+CD38-CD45RA-CD90+ population, a similar trend was seen, although the overall mIL-6R expression was lower and more variable (Figure 4-3Aiii).

Throughout culture, mIL-6R was present at a significantly lower frequency on CD34+ cells cultured in the presence of DL1, as compared to those cultured in the control condition (Figure 4-3B). This difference was maintained on both the CD34+CD45RA- subset and the CD34+CD45RA+ subset (Figure 4-3C). The addition of either exogenous IL-6/sIL-6R or a JAK inhibitor did not significantly alter the levels of mIL-6R seen with or without DL1 on the CD34+ cells, demonstrating that the loss of mIL-6R was occurring via a JAK independent mechanism (Figure 4-3D). This JAK independent mechanism is consistent with the JAK independent sIL-6R production observed in DL1 culture (Figure 4-2Bii).

It has previously been shown that CD34+ mIL-6R+ cells are myeloid-restricted progenitors (Yamamura et al., 2007). Therefore, the loss of mIL-6R on these myeloid progenitors prevents IL-6 cis-signaling-mediated production of mature monocytes and granulocytes. In the absence of mIL-6R, myeloid progenitor cells can still respond to IL-6 via IL-6 trans-signaling in the presence of sIL-6R. However, the fed-batch culture system reduces sIL-6R levels as compared to less regulated culture systems (Figure 4-3E). As a result, the addition of DL1 in combination with the fed-batch approach minimizes both IL-6 cis-signaling and IL-6 trans-signaling and thus the production of CD14+ and CD15+ cells is significantly reduced.

Taken together, these data indicate that both DL1 and IL-6/sIL-6R activate the JAK-STAT pathway. Activation through IL-6/sIL-6R leads primarily to IL-6 cis-signaling acting on myeloid progenitor cells which enhances production of CD14+ and CD15+ cells. This results in the generation of a non-supportive microenvironment and high levels of feedback inhibition. Conversely, the JAK independent loss of mIL-6R on progenitor cells in the presence of DL1 inhibits IL-6 cis-signaling, despite JAK-STAT activation. Instead, activation of the JAK-STAT pathway by DL1 leads primarily to IL-6 trans-signaling, although trans-signaling is reduced under fed-batch culture conditions. Together, this results in a decrease in CD14+ and CD15+ cell production, which generates a more supportive microenvironment and a reduction in feedback inhibition. A summary model of the system is shown in Figure 4-3F.
Figure 4-3: DL1 causes the loss of mIL-6R which prevents IL-6 cis-signaling

[A] The membrane bound IL-6R is not expressed on freshly enriched CD34^+ cells, but it is rapidly up-regulated within 6 h of culture, prior to the first cell division. With DL1, the mIL-6R levels is consistently lower than in the control culture. The mIL-6R expression was measured on (i) CD34^+CD45RA^- cells; (ii) CD34^+CD45RA^+ cells; (iii) CD34^+CD38^-45RA^- CD90^+ cells. [B] (i) The addition of DL1 causes a significant decrease in mIL-6R on CD34^+ cells throughout culture; (ii,iii) Representative FACS plots of CD34^+ cells after day 16 of culture: (ii) without DL1 and; (iii) with DL1. [C] The impact of DL1 on mIL-6R is similar on the more primitive CD34^+CD45RA^- and less primitive CD34^+CD45RA^+ populations. [D] The addition of
endogenous IL-6/sIL-6R or JAKi does not affect the DL1 mediated impact on mIL-6R on culture CD34+ cells, indicating that this is occurring via a JAK independent mechanism. [E] Fed-batch (FB) conditions reduce sIL-6R production as compared to non-fed-batch conditions. [F] Schematic of proposed mechanism. In the absence of DL1, STAT3 activation results in high levels of cis-signaling and the generation of mature myeloid cells, which inhibit HSPC expansion. DL1 causes the loss of mIL-6R. The loss of mIL-6R reduces IL-6 cis-signaling, and thus reduces the production of CD14+ and CD15+. Instead, only IL-6 trans-signaling can occur. Regulating the soluble environment with the fed-batch (FB) platform reduces sIL-6R and IL-6 trans-signaling, further reducing the production of myeloid cells and results in a microenvironment that is more supportive for HSPCs. All error bars indicate standard deviation. n ≥ 3, * = p < 0.05.

4.4.4 Paracrine signaling controlled DL1 supplemented cultures support the expansion of short-term and long-term HSCs

DL1 modulates IL-6-JAK-STAT signaling to produce a supportive mature cell microenvironment. To better evaluate the impact that this microenvironment has on the HSPCs, we used a previously described mathematical model (Csaszar et al., 2012; Kirouac et al., 2009) that was designed to assess feedback mediated effects in hematopoietic culture under different conditions. To investigate the impact of altering specific mature cell lineages, we revised the model such that mature CD34+ cells were categorized to be CD14+, CD15+, CD7+, CD41+, GyA+, or other (all CD34+ cells not categorized by one of the five specified phenotypes). We then assigned each lineage a unique impact on feedback signaling, by adding a weighting from 0 to 1 for each lineage to both the stimulatory and inhibitory feedback loops based on the experimental data in Figure 4-1F. The internal model parameters were then fit using the genetic algorithm method previously described (Kirouac et al., 2009).

Having set-up the model, we simulated the microenvironments generated by different culture conditions with two unique inputs (Table 4-1). The first input was the probability of cells entering each mature cell lineage based on average experimental frequencies of mature cell lineages for each culture condition as measured by flow cytometry in Figure 4-2E. The second input was the relative proliferation rate under each culture condition, as observed from
experimental CFSE analysis (Figure 4-5). The proliferation rate alters the microenvironment as not only were the proportion of mature cells changed, but the quantity of them was also affected. The CFSE data was used to determine a scaling factor that was applied to the proliferation rate parameters (Table 4-1). Once these inputs were made to mimic the microenvironment of each culture condition, the model simulations were then used to output the predicted population expansions that would result. Figure 4-4A shows that the predicted expansions of total cells, CD34+ cells and CD34+CD90+ cells were well aligned with what had been experimentally measured with each culture condition, indicating that the changes to population expansions that were observed with the DL1 or IL-6/sIL-6R could be linked to the microenvironmental changes caused by each stimuli.

Given the impact of DL1 on the progenitor cell populations, we wanted to assess the effect on the repopulating stem cell populations. One prediction that emerged from the model simulations was that the combination of the fed-batch system and DL1 would lead to significant expansion of both short-term and long-term SRCs (Figure 4-4B). The expansion of the short-term SRC population by DL1 has been previously demonstrated, by quantification of human engraftment at 3 weeks and 9 weeks post-transplantation (Delaney et al., 2010). This study did not see a significant expansion of the longer term SRCs with DL1. However, the fed-batch system provides a more highly controlled base culture system than that used in previous studies, and this produces a different microenvironment context onto which DL1-induced Notch signaling is added.

To experimentally assess the expansion of the SRC populations, we transplanted cells expanded with or without DL1 into NSG mice, and quantified human cell repopulation in the bone marrow at the indicated time-points. We first confirmed that DL1 showed a trend towards enhancement of human engraftment at 3- and 9-weeks post-transplantation (Figure 4-4C), which correlated to both our model predictions and the previously described short-term expansion with DL1 (Delaney et al., 2010). We next assessed a longer-term SRC population. CD34+ cells were cultured for 12 or 16 days in the fed-batch system with or without DL1, before being transplanted. We assessed SRC expansion by limiting dilution analysis at 16-weeks post-transplantation. As shown in Figure 4-4D, the combination of fed-batch and DL1 led to a significant expansion of week 16 SRCs as compared to the uncultured CD34+ cells that were
transplanted. Furthermore, the addition of DL1 led to a trend of enhanced SRC expansion above what was seen with the fed-batch control system. Full details of the in vivo study are summarized in Table 4-2.

These results demonstrate the potency of DL1 in addition to the fed-batch culture system, in the expansion of both the short-term and long-term SRC populations. Moreover, these findings highlight how specific microenvironment changes can impact HSPC expansion in an indirect manner. Taken together, the findings in this study demonstrate a novel mechanism for signaling modulation through the conversion from IL-6 cis-signaling to IL-6 trans-signaling, which alters the cell populations that are able to respond to JAK-STAT signaling. This regulation alters the soluble factor microenvironment that is generated and allows for supportive conditions to enable enhanced expansion of HSPCs.
Figure 4-4: The impact on SRCs by feedback mediated DL1 is predicted and validated.

[A] Comparison of day 12 model simulation predictions and experimental expansion outputs from the fed-batch control culture, the DL1 culture, and the IL-6/sIL-6R culture of CD34⁺ cells for: (i) total cell expansion; (ii) CD34⁺ cell expansion; (iii) CD34⁺CD90⁺ cell expansion. [B] (i) Model simulation predictions of day 12 short-term SRC expansions under each culture conditions. (ii) Model simulation predictions of long-term SRC expansions, following 12 days and 16 days of culture. [C] Time-course of human engraftment after transplantation of day 12 cultured cells into NSG mice (3 animals per condition at each time-point). Bone marrow was assessed at 3 weeks, 9 weeks, 16 weeks, and 24 weeks following transplantation with CD34⁺ cells cultured in the fed-batch control or the fed-batch plus DL1 culture conditions. [D] Quantification of week 16 SRC expansion, based on limiting dilution analysis performed 16 weeks post-transplantation. CD34⁺ cells were cultured for 12 days or 16 days in the fed-batch system with or without DL1 before transplantation. Expansions indicated compare cells cultured in vitro to transplantation of the uncultured CD34⁺ cells.
4.5 Discussion

The Notch pathway plays many important roles in human hematopoiesis, including the in vitro enhancement of primitive cell expansion, which can be achieved through presentation of immobilized DL1 ligand at low concentrations. However, the mechanisms through which this effect is achieved in a heterogeneous in vitro system are not fully understood. Here, we demonstrate a non stem cell autonomous role whereby DL1 acts in an indirect manner by skewing mature cell lineage production and generating a more supportive microenvironment with reduced feedback inhibition. DL1 produces significant changes in frequency of several mature cell lineages, including a reduction in CD14$^+$ and CD15$^+$ cells, which have known inhibitory feedback effects on the HSPC population.

The generation of monocytes, macrophages, and granulocytes is known to be governed in a large part by cytokine-mediated gp130 signaling and STAT3 activation. As there is evidence of links between the Notch pathway and the IL-6-JAK-STAT pathway in the hematopoietic system (Choi et al., 2012; Yamamura et al., 2007), we predicted that the myeloid lineage skewing observed with DL1 resulted from modulation of the JAK-STAT pathway. However, despite similar STAT3 activation by DL1 and IL-6/sIL-6R, the resulting impact of these two stimuli on mature cell populations was very different. IL-6/sIL-6R increased CD14$^+$ and CD15$^+$ cell production while DL1 decreased the production of these populations. We hypothesized that this difference was due to the modulation of IL-6 signaling at the level of mIL-6R and we showed that the addition of DL1 causes the loss of mIL-6R from CD34$^+$ myeloid progenitor cells. This prevents these cells from signaling via IL-6 cis-signaling. Instead, gp130 signaling can only be maintained via IL-6 trans-signaling. Furthermore, the fed-batch culture strategy reduces sIL-6R so that IL-6 trans-signaling is also reduced. This reduction in IL-6 signaling prevents the production of the highly inhibitory mature myeloid cells and results in the generation of more supportive microenvironment conditions.

It has not yet been explored how DL1 causes the loss of mIL-6R. The levels of mIL-6R on progenitor cells are not affected by either the addition of IL-6/sIL-6R or JAK inhibition, indicating that this loss is not occurring in a JAK-dependent manner. The shedding of mIL-6R is consistent with the increase in sIL-6R that was observed in the presence of DL1. mIL-6R shedding is known to be facilitated by the metalloproteases, ADAM17 and ADAM10 (Garbers et
al., 2011). These factors are also responsible for the cleavage of the extracellular domain of Notch receptors in order to activate Notch signaling (Tian et al., 2008), thus it is possible that the formation of the DL1-Notch complex results in the activation of ADAM17 and ADAM10, which initiates mIL-6R shedding.

Mathematical model simulations allowed us to further explore how changes to the microenvironment contribute to HSPC expansion. Using this model, we observed that the cell population outputs from each culture condition (fed-batch control, DL1, IL-6/sIL-6R) could be accurately simulated by altering the mature cell lineage frequencies and the maximum proliferation rate. This indicated that the observed changes to the microenvironment under each stimuli play a critical role in the resulting primitive cell expansions. The model was used to predict that the microenvironmental conditions generated by the combination of fed-batch and DL1 would lead to significant expansion of both the short-term and long-term SRC populations. This prediction was validated with in vivo transplantation studies in NSG mice. The level of long-term SRC expansion observed here was not seen in previous studies with DL1 (Delaney et al., 2010) and it is likely that the combination of the tightly regulated fed-batch system and the addition of DL1 provided a supportive microenvironment such that self-renewal of the LTR-HSC population could be achieved over 12 days of culture. The reduced levels of sIL-6R in the fed-batch system provides one example of how the culture platform can interact with exogenous factors.

The Notch pathway likely acts through multiple mechanisms to impact hematopoietic cells. Here we demonstrate a novel mode of action by which DL1 cause skewing of mature cell lineages, which then alters the microenvironment to which the HSPCs are exposed, resulting in an indirect regulation of the HSPCs. We show that important and previously unexplored indirect mechanisms of action are contributing to the effects of DL1 in human hematopoiesis. This study identifies a new level of control of IL-6-JAK-STAT signaling through the conversion of cis-signaling to trans-signaling. This allows for different cell populations to become responsive to IL-6 proliferation signals. This study highlights the importance of understanding context dependency and cell-cell interactions when manipulating a complex cell signaling pathway. By accounting for the significant impact of feedback signaling, a greater understanding of the mechanisms behind the supportive role of the HSPC niche can be achieved. Furthermore, with
the knowledge gained herein, we have been able to demonstrate further enhancement in the
generation of progenitors as well as short-term and long-term SRC.

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Bioengineering.
4.7 Supplementary Information

4.7.1 Supplementary figures

**Figure 4-5: Comparison of cell division rate among culture conditions.**

CFSE staining was used to assess average cell division number of [A] CD34+ and [B] CD34- cells when Lin- cells were cultured in the fed-batch system under control, DL1, or IL-6/sIL-6R conditions. The addition of DL1 decreases cell division rates while the addition of IL-6 and sIL-6R increases cell division rates as compared to controls.
Figure 4-6: The effect of IL-6/sIL-6R on mature cell production and primitive cell expansion.

[A] The addition of IL-6/sIL-6R to CD34⁺ cells increases production of (i) CD14⁺ cells; (ii) CD15⁺ cells; and (iii) GvA⁺ cells. This effect can be reversed by the addition of JAKi, confirming that the phenotype changes are JAK-STAT dependent. [B] The addition of IL-6/sIL-6R to CD34⁺ cell culture causes: (i) a significant increase in total cell expansion but; (ii) a decrease in the frequency of CD34⁺ cells. This results in (iii) no significant difference in the CD34⁺ expansion with IL-6 or IL-6/sIL-6R as compared to the fed-batch control.
4.7.2 Supplementary tables

Table 4-1: Inputs to mathematical model for each culture condition, to simulate microenvironmental conditions.

<table>
<thead>
<tr>
<th>Mature Cell Frequencies (weightings for stimulatory/inhibitory feedback)</th>
<th>Control</th>
<th>DL1</th>
<th>IL6/sIL6R</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD14+ (0/1)</td>
<td>20%</td>
<td>5%</td>
<td>30%</td>
</tr>
<tr>
<td>CD15+ (0/1)</td>
<td>15%</td>
<td>5%</td>
<td>25%</td>
</tr>
<tr>
<td>CD7+ (0.5/0.5)</td>
<td>2%</td>
<td>10%</td>
<td>2%</td>
</tr>
<tr>
<td>CD41+ (1/0)</td>
<td>30%</td>
<td>30%</td>
<td>20%</td>
</tr>
<tr>
<td>GyA+ (0/1)</td>
<td>10%</td>
<td>10%</td>
<td>20%</td>
</tr>
<tr>
<td>Other (0.5/0.5)</td>
<td>23%</td>
<td>40%</td>
<td>3%</td>
</tr>
<tr>
<td>Max Proliferation Rate Scaling Factor</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lin- cells (u_{max})</td>
<td>1</td>
<td>0.8</td>
<td>1.8</td>
</tr>
<tr>
<td>Lin+ cells (u_{+})</td>
<td>1</td>
<td>0.8</td>
<td>1.8</td>
</tr>
</tbody>
</table>
Table 4-2: Summary of week 16 in vivo data used for limiting dilution analysis.

<table>
<thead>
<tr>
<th>Cell Dose</th>
<th>Day 0 equivalent</th>
<th>Engrafted mice/Total mice</th>
<th>HSC frequency</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fresh</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1000</td>
<td>1000</td>
<td>1/4</td>
<td>1/6115</td>
</tr>
<tr>
<td>5000</td>
<td>5000</td>
<td>3/4</td>
<td></td>
</tr>
<tr>
<td>15000</td>
<td>15000</td>
<td>3/4</td>
<td></td>
</tr>
<tr>
<td>25000</td>
<td>25000</td>
<td>4/4</td>
<td></td>
</tr>
<tr>
<td>Day 12 FB</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>50000</td>
<td>320.5</td>
<td>1/4</td>
<td>1/754</td>
</tr>
<tr>
<td>125000</td>
<td>801.3</td>
<td>3/4</td>
<td></td>
</tr>
<tr>
<td>250000</td>
<td>1602.6</td>
<td>3/4</td>
<td></td>
</tr>
<tr>
<td>500000</td>
<td>3205.1</td>
<td>4/4</td>
<td></td>
</tr>
<tr>
<td>Day 12 FB+DL1</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>50000</td>
<td>362.3</td>
<td>3/5</td>
<td>1/550</td>
</tr>
<tr>
<td>125000</td>
<td>905.8</td>
<td>5/5</td>
<td></td>
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<tr>
<td>250000</td>
<td>1811.6</td>
<td>4/5</td>
<td></td>
</tr>
<tr>
<td>500000</td>
<td>3623.2</td>
<td>3/3</td>
<td></td>
</tr>
<tr>
<td>Day 16 FB</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>50000</td>
<td>121.1</td>
<td>0/5</td>
<td>1/1540</td>
</tr>
<tr>
<td>125000</td>
<td>302.7</td>
<td>0/5</td>
<td></td>
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<td>605.3</td>
<td>2/4</td>
<td></td>
</tr>
<tr>
<td>500000</td>
<td>1210.7</td>
<td>2/3</td>
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</tr>
<tr>
<td>Day 16 FB+DL1</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>50000</td>
<td>114.4</td>
<td>1/4</td>
<td>1/969</td>
</tr>
<tr>
<td>125000</td>
<td>286.0</td>
<td>0/5</td>
<td></td>
</tr>
<tr>
<td>250000</td>
<td>572.1</td>
<td>3/5</td>
<td></td>
</tr>
<tr>
<td>500000</td>
<td>1144.2</td>
<td>2/3</td>
<td></td>
</tr>
</tbody>
</table>
4.7.3 Supplementary experimental procedures: changes made to computational model

The previously described computational model (Csaszar et al., 2012; Kirouac et al., 2009) was adjusted to simulate the individual feedback roles of specific mature cell lineages. The following changes were made to the model:

1. Cell exiting the CD34⁺ compartments [1-13] and becoming CD34⁻ enter into one of the following lineages, where they remain for compartments [14-20]:
   - CD14⁺
   - CD15⁺
   - CD41⁺
   - GyA⁺
   - CD7⁺
   - Other (CD14⁻CD15⁻CD41⁻GyA⁻CD7⁻)

2. The probabilities (P) of any cell entering each lineage are based on the experimental frequencies summarized in Table 4-1 and are specific to each culture condition.

3. Each mature cell lineage contributes to either the stimulatory feedback loops, the inhibitory feedback loops or both, depending on whether the impact of the mature lineage cells had experimentally been classified at positive, negative, or neutral. For the secreted factor loop A:

\[
\frac{d[SF_A]}{dt} = r_p \cdot \left( w_{A,CD14}[X_{CD14}] + w_{A,CD15}[X_{CD15}] + w_{A,CD7}[X_{CD7}] + w_{A,CD41}[X_{CD41}] + w_{A,GyA}[X_{GyA}] + (w_{A,Other}[X_{Other}]) \right) / (1 + D \cdot t)
\]

where:
- \([SF] = \text{secreted protein concentration in media}\)
- \(r_p = \text{rate of protein } P \text{ secretion}\)
- \([X_Y] = \text{cell density of lineage } Y\)
- \(w_{A,Y} = \text{weighting of lineage } Y \text{ towards secreted factor loop A}\)
- \(D = \text{dilution rate}\)
5 CONCLUSIONS AND FUTURE WORK
5.1 Summary of Results

Cell-cell interactions are a critical and often unexplored component of all complex cell systems. This is particularly true in heterogeneous hierarchical systems like the hematopoietic system, in which highly regulated interactions are required to maintain tight control over the development, proliferation, and differentiation of each blood cell population. Only by interrogating these dynamic and complex interactions are we able to understand the in vivo regulation that occurs both during homeostasis and in response to physiological demand, and ultimately use this knowledge to perform in vitro manipulations to the hematopoietic system.

This work was motivated by the 50 years of success in the HSPC transplantation field and by the challenges that still remain to be solved. In particular, we aimed to develop clinically relevant strategies whereby UCB-derived HSPCs could be expanded in vitro. This challenge was undertaken by focusing on regulation of the cell microenvironment and the endogenous intercellular interactions that dictate cell fate decisions. By learning from in vivo hematopoiesis and translating the knowledge to the in vitro culture scenario, we explored and developed techniques whereby the manipulation of the cell microenvironment led to enhancement of the HSPC populations.

We investigated the use of TAT-HOXB4 to expand human hematopoietic cells, as a means of exposing cells to high levels of HOXB4 without genetic manipulation. With the fusion between the HOXB4 transcription factor and the TAT cell penetrating peptide, HOXB4 could be delivered to the cell nucleus in a transient and non-integrating way. However, the major challenge to this process was the extremely short half life of TAT-HOXB4 and uncertainty about its dynamic intracellular concentration, which prevented regulated exposure of the cells to the molecule. To overcome these limitations we developed a luciferase reporter assay which, combined with cell uptake studies, allowed us to quantify the kinetics of delivery of TAT-HOXB4. This information was put into a mathematical model that enabled us to simulate the intracellular concentration of TAT-HOXB4 under different experimental culture conditions. In parallel, we created an automated delivery system to facilitate the continuous delivery of unstable factors to suspension cell culture and we integrated this system into a closed-system bioreactor, producing a clinically relevant hematopoietic culture process. We were then able to
use this system in combination with outputs from our model to deliver TAT-HOXB4 to human primary blood cells in a controlled and automated manner.

In combination with the addition of exogenous factors that target HSPC expansion, we were interested in developing a more global means of regulating the endogenous factors in hematopoietic culture. Through model simulations, we predicted that a fed-batch delivery strategy may produce a culture environment in which the impact of endogenously produced inhibitory secreted factors and the mature cells that secrete these factors were minimized, thus allowing for a microenvironment that was more supportive of HSPC expansion. The delivery system that was developed for the TAT-HOXB4 studies was repurposed to experimentally validate the utility of a fed-batch culture system. By delivering fresh media and cytokines in a controlled continuous manner, we were able to significantly reduce the concentration of endogenous soluble factors and achieve a significant enhancement of stem and progenitor cell expansion. With this system, we could expand the LTR-HSC population 11-fold, with 12 days of in vitro culture. This level of expansion was comparable with the most successful UCB expansion strategies that have been reported.

The advantage of our fed-batch delivery system was that it achieved high levels of HSPC expansion in a clinically relevant closed system bioreactor and in a non-stem cell autonomous manner. This meant that it could serve as a highly regulated base culture system platform onto which additional HSPC enhancing factors could be added. Interestingly, in doing so, the system provided us with additional mechanistic information. Factors that directly targeted the HSPC population would be enhanced by the fed-batch system, as it provided a supportive microenvironment. In contrast, factors that indirectly targeted the HSPC population by primarily modulating a mature cell population that then positively interacted with the HSPCs would not be enhanced by the fed-batch system. This platform allowed us to identify TAT-HOXB4 as acting primarily in this indirect manner in our system, through the increased production of megakaryocytes. We also determined that we could achieve greater levels of expansion with our fed-batch system alone than we could with the more complex system which included TAT-HOXB4 delivery.
Other HSPC enhancing factors had recently been identified and validated and we predicted that we would be able to synergize our fed-batch system with these novel factors. We combined our system with the Notch ligand, DL1, which had been shown to significantly enhance a STR-HSC populations and is has been used in a promising clinical trial. By studying DL1 in our fed-batch conditions, we were able to rigorously track its effect on both primitive and mature cell populations. We identified a novel mode of action by which DL1 caused the loss of the membrane bound IL-6R and a conversion from IL-6 cis-signaling to trans-signaling. This modulation of the IL-6-JAK-STAT3 pathway resulted in lineage skewing and, in particular, reduced the production of mature myeloid cells. The change in mature cell environment altered inhibitory feedback signaling factors produced by these cells, leading to a more supportive microenvironment for HSPC expansion. Therefore, we demonstrated an important, and previously unknown, non-stem cell autonomous role for DL1 in human hematopoietic regulation and highlighted an example of regulation via the conversion from IL-6 cis- to trans- signaling. We also showed that the combination of the fed-batch system and DL1 produces an environment that is supportive for the expansion of STR-HSCs and LTR-HSCs, above what was achieved with the fed-batch system alone.

In summary, we have developed mathematical and experimental techniques to investigate strategies for the ex vivo expansion of HSPCs and have identified highly robust and clinically relevant approaches whereby LTR-HSCs and critical progenitors can be expanded in a rapid and reproducible manner. We have demonstrated how the understanding of cell-cell interactions and microenvironmental regulation can produce non-cell autonomous approached to culture control. This work represents a set of critical bioengineering tools for ex vivo HSPC expansion and a deeper understanding of the biology underlying the hematopoietic system.

5.2 Future Directions

This work has combined the development of in vitro HSPC expansion systems with analyses of the cellular biology that underpins these systems. From the conclusions of each of the preceding chapters, there are several avenues to extend on both the biological understanding and the practical applications that have been described. It is important to note that there remains many limitations and unknowns to the results that have been presented here, which pave the way for
future investigations. In particular, the use of in vitro assays and xenograft models for the study of human cell populations is limited by the clinical relevance of the assays used. Until long-term clinical data can be clearly correlated with measured laboratory outputs, it cannot be confirmed that the HSPCs that are expanded in vitro will act as true LTR-HSCs for the lifespan of the human transplant recipient. There always remains the possibility that the expanded cells have been altered or pushed towards senescence in ways that current hematopoietic assays are unable to capture. Updated xenograft models, techniques for in vitro and in vivo clonal cell tracking, and better correlation of phenotype to function are providing enhanced methods to interrogate and understand cells that have been produced in vitro and these strategies will be important for investigating current limitations and extending on current expansion strategies.

The human hematopoietic field remains a vastly expanding area of biological knowledge and the pioneering system for developing cell therapies. Global top-down molecular studies and bioinformatics approaches are providing links between the cell-cell interactions that have been observed at the population level and the intracellular molecular level signaling that is governing the response of individual cells. The genetic and epigenetic networks of hematopoietic cells are being elucidated and an understanding of the interactive roles of both exogenous and endogenous factors is increasing at a rapid pace. The application of HSPC expansion strategies for UCB-derived cells is at an exciting stage, as clinical benefits of transplanting expanded HSPCs are being realized and the translation of laboratory expansion strategies to the clinic is gaining momentum. The combination of HSPC expansion strategies with techniques for enhanced homing or immunotherapy graft engineering is emerging as an approach to improve patient outcomes. In the following sections, we present some specific studies that we have initiated or conceptualized to extend on our above described work, with regards to both enhanced biological understanding and clinical translation.

5.2.1 Extending the TAT-HOXB4 studies

We used the model and delivery system that were developed for the delivery of TAT-HOXB4 to optimize the effect of TAT-HOXB4 in an 8 day UCB culture system. By performing a semi-continuous delivery of the fusion protein and increasing the concentration during culture to account for rapid cell proliferation, we achieved a significant improvement in CFU-GEMM and
LTC-IC expansion, as compared to that which was generated with the un-optimized system (Figure 5-1). This highlighted the importance of the controlled automated delivery approach as a means to eliminate periodic fluctuations in intracellular concentration and retain HOXB4 levels within a tightly controlled range. Increasing the concentration of delivered TAT-HOXB4 as culture progressed also helped to account for rapid cell proliferation in the UCB system, which was not a concern in the mPB cultures. This ensured stable levels of intracellular HOXB4 throughout culture.

Figure 5-1: The optimization of TAT-HOXB4 in 8 day UCB culture.

[A] Schematic of TAT-HOXB4 delivery schemes that were compared. The original delivery scheme (blue) based on the manual TAT-HOXB4 delivery designed for mouse BM culture consists of 40nM of TAT-HOXB4 delivered every 4h (Krosl et al., 2003). The optimized delivery scheme (red) consists of 1.5nM of TAT-HOXB4 delivered every 30min for 4 days, followed by an increase to 6nM delivered every 30min. [B] Mathematical model output of intracellular protein concentration of the different delivery schemes. The original delivery scheme resulted in large periodic fluctuations and a decrease in effective per-cell concentration resulting from cell proliferation. These are both corrected with the optimized delivery scheme, which provided stable levels of TAT-HOXB4 throughout culture. [C] The addition of TAT-
HOXB4 to UCB culture using either the original or optimized delivery scheme was assessed. Lin+ cells were cultured for 8 days, using the D=0 conditions described in Chapter 2. The original scheme enhanced CFU-GEMM and LTC-IC above what was achieved with the buffer control and the optimized delivery scheme further enhanced these populations above the levels achieved with the original scheme.

However, despite much effort into the optimization of TAT-HOXB4 delivery, including assessing a wide range of intracellular concentrations, we were unable to achieve levels of primitive cell expansion that neared those seen with TAT-HOXB4 in the mouse, and we were orders of magnitude below the levels achieved in the mouse with viral over-expression. These studies identified that there was a more fundamental limitation in the use of TAT-HOXB4 in human hematopoietic culture.

Several additional strategies to enhance the impact of TAT-HOXB4 have been investigated. These included the development of different mutants of the TAT-HOXB4 fusion protein that were designed to have greater stability or greater nuclear uptake (Huang et al., 2010). We assessed the stability and functionality of similar TAT-HOXB4 mutant proteins that had been developed to have enhanced stability from the Guy Sauvageau lab and a version of TAT-HOXB4 developed by the Jean Gariepy lab with an eta domain and a protein transduction domain that were designed to help with endosome escape. In each case, we observed that the nuclear activity as measured by luciferase assay and the effect on UCB culture was not significantly different than what was seen with the original TAT-HOXB4 protein.

We also explored the ability of the TAT-NUP98HOXA10 (TAT-NA10) fusion protein. The NUP98-HOX fusions were first identified in leukemias and have been shown to cause dramatic proliferation of HSPCs. One study investigated the use of NUP98-HOXB4 and NUP98-HOXA10 over-expression for the ex vivo expansion of murine HSCs and showed that NUP98-HOXA10 led to 10,000-fold expansion of murine HSCs (Ohta et al., 2007), which was significantly greater than the expansion achieved by HOXB4 over-expression. Based on these promising findings, efforts were made to expand human HSCs with NUP98-HOXA10 and TAT-NA10 was developed to explore the non-integrating delivery of this factor. We found that the degradation kinetics of TAT-NA10 were different than those of TAT-HOXB4, as TAT-NA10
had a more than 4-fold increase in stability as compared to TAT-HOXB4. In order to compare the difference in biological function between these molecules, we developed individual delivery schemes such that the intracellular concentration of TAT-HOXB4 and TAT-NA10 were normalized. When this was done, we did not observe any significant difference between the two molecules in terms of their effect on primitive cell expansion. (Figure 5-2). Varying the delivery scheme and dosage of TAT-NA10 did not result in an further significant enhancements of this factor above what could be reached with the optimized delivery of TAT-HOXB4. Furthermore, it was observed that viral over-expression of NUP98-HOXA10 in human UCB cells only provided minimal (2-4-fold) expansion of LTR-HSCs, despite the enormous expansion that was seen in the mouse (unpublished data from Keith Humphries lab). Thus the species specific difference appear to extend to the use of multiple HOX genes for ex vivo expansion.

Figure 5-2: Comparison of TAT-HOXB4 and TAT-NA10 gives comparable effects on primitive cell expansion.

[A] The intracellular stability of TAT-HOXB4 and TAT-NA10 as measured by luciferase assay. The intracellular half-life of TAT-HOXB4 was calculated to be 1.4 ± 0.2h while the intracellular half-life of TAT-NA10 was calculated to be 6.2 ± 0.9h. [B] The delivery schemes of the two molecules were normalized such that they would give equivalent intracellular concentrations. To do so, a delivery of TAT-HOXB4 of 40nM every 4h (the original delivery scheme) was
compared to a delivery of TAT-NA10 of 10nM every 4h. The effect of TAT-HOXB4 and TAT-NA10 on UCB cell expansion was assessed using the normalized delivery schemes. Lin^− cells were cultured for 8 days, using the D=0 conditions described in Chapter 2. This resulted in no significant difference in the expansion of CFU-GEMMs and LTC-ICs with TAT-NA10 as compared to TAT-HOXB4.

Our optimization studies exposed the limitations of TAT-HOXB4 and TAT-NA10 in the UCB expansion system. Low levels of expansion were originally attributed to issues of stability and delivery issues of the TAT fusion proteins. However, our optimization studies were able to correct for the short half life of TAT-HOXB4 and maintain the intracellular HOXB4 concentration at a stable level, but did not result in HSPC expansion that was near what had been observed with viral over-expression in the mouse. Recent studies with lentiviral over-expression of HOXB4 and NUP98-HOXA10 in human hematopoietic culture have led to modest expansion of primitive progenitor and stem cell populations, but the levels achieved have remained orders of magnitudes below the expansion produced in the murine system (unpublished date from the Keith Humphries lab). Therefore, it appears that there is likely a mechanistic limitation of HOXB4 in human hematopoietic cells, which is distinct from any limitations caused by the TAT fusion proteins. This lack of conservation between species that is seen with HOXB4 was also recently reported for several nuclear factors that were identified in a murine gain-of-function screen. In this study, it was found that all the novels factors identified to have very large effect on murine HSC expansion did not show a significant effect on human HSCs (Deneault et al., 2013). These findings illustrate the limitations of relying on the murine system for the identification of factors important for human hematopoiesis.

Although it may no longer be a top candidate for UCB expansion, TAT-HOXB4 continues to be used for the expansion of mPB in a study by Denis-Claude Roy's lab that is now being translated to the clinic for autologous transplantations of poor mobilizers. For this purpose, only modest levels of expansion are required and TAT-HOXB4 offers a strategy to provide enhancement of expansion. This study is in the translational stage and is making use of the delivery system that was developed as part of this work to automate the delivery of TAT-HOXB4.
HOXB4 remains a very interesting factor for investigation within the hematopoietic system. Recent studies have revealed an interesting new role for HOXB4 in enhancing the long-term repopulating potential of short or intermediate term HSC populations in the mouse (unpublished data from Norman Iscove's lab), and it would be very interesting to see how these findings transfer to the human system, especially given the difference in stem cell expansion potency between species. Our studies in which we combined our fed-batch system with TAT-HOXB4 revealed that the impact of the protein is highly dependent on the microenvironment and potentially acts in a partially non-stem cell autonomous manner. Specifically, it appears that HOXB4 increases the production of megakaryocytes in the human system. Megakaryocytes, in turn, secrete signaling factors that positively feed back to enhance HSPC expansion. Exploring the possibility of using TAT-HOXB4 for high yield megakaryocyte production is of interest.

There continues to be interest in the use of TAT to bring protein and peptide cargo into cells in a transient and non-integrative manner. For potential cell therapy applications, there is the desire to convert many techniques that were originally demonstrated via viral over-expression to methods in which factors can be presented to cells transiently and without genetic modification. Somatic cell reprogramming is one area where controlled transient transcription factor over-expression is particularly relevant. The use of TAT as a cell penetrating peptide is one option that is being explored for this purpose (Bosnali and Edenhofeher, 2008; Pan et al., 2010).

One limitation with TAT has been an uncertainty of how well it delivers nuclear factors and, in particular, some reports suggest that the majority of the cargo ends up trapped in endosomes. Endosome disruption poses a method of releasing the fusion proteins and increasing the nuclear uptake of the cargo molecules (Caron et al., 2004; Shiraishi and Nielsen, 2006). Recently, there have been reports of peptides that have been developed which can be used in conjunction with TAT to enhance intracellular delivery of cargo (Salomone et al., 2012). Another report has illustrated one such peptide to dramatically increase the nuclear activity of TAT-HOXB4, as measured by luciferase assay, by releasing the protein from endosomes. These data suggests that the nuclear activity of TAT-HOXB4 can be increased 10-50-fold above what has been previously seen (unpublished work from the Jean-Philippe Pellois lab). This opens the door for exploring TAT-HOXB4, possibly for megakaryocyte production, as well as investigating the use to TAT-fusion proteins to deliver a variety of cargo.
5.2.2 Combining the fed-batch system with HSPC enhancing factors

The fed-batch system provides a non-stem cell autonomous strategy for expanding HSPCs. One benefit of this system is that it is likely to synergize with HSPC enhancing factors that have been identified and validated in other culture systems. Thus, in addition to having potential as a stand-alone culture system, the fed-batch system can act as a base culture platform onto which additional factors can be added. These combined strategies may lead to more potent HSPC expansion and produce an optimized strategy that could be translated to the clinic. Due to the non-stem cell autonomous mode of action of the fed-batch system, it should not negatively interfere with factors that directly target HSPC expansion. For factors that may partially act by manipulating the cell microenvironment, the interactions with the fed-batch system may be more complex, as evidenced by the observations with HOXB4 and DL1 in Chapter 4.

We added some of the most potent HSPC enhancing factors in the literature to the fed-batch system to test the combined effects. Many of these factors were developed in UCB culture strategies with non-ideal baseline conditions and we hypothesized that translating these molecules to the fed-batch system may enhance the overall expansion effect as well as provide a clinically relevant expansion strategy. To explore combining HSPC enhancing factors with the fed-batch system, we used a factorial design approach to assess the combination of the fed-batch system with the small molecule SR1 (Boitano et al., 2010), the Notch ligand DL1 (Delaney et al., 2010), and an unpublished small molecule UM729/UM171, from the Guy Sauvageau lab, which appeared to have similar potency as SR1. We cultured the combinations of these factors for 12 or 16 days under fed-batch conditions and assessed the expansion of primitive cell populations. (Figure 5-3). All three factors individually enhanced the expansion of primitive cells above what was seen with the fed-batch system alone. When we combined the factors, fed-batch+SR1+UM729 gave the greatest expansion of CD34<sup>+</sup> cells and CFCs, while the fed-batch+SR1+UM729+DL1 gave the greatest expansion of CD34<sup>+</sup>CD90<sup>+</sup> cells and LTC-ICs. This was consistent with our observation that DL1 most specifically enhances the CD34<sup>+</sup>CD90<sup>+</sup> and LTC-IC populations. Therefore, it appeared that the fed-batch system provided a strong base-line culture platform onto which UM729, SR1, or DL1 could be added to produce high levels of progenitor expansion.
Figure 5-3: The combination of the fed-batch system with HSPC-enhancing factors reveals synergies.

[A] Factorial design study used to assess the combination of the fed-batch system with UM729, SR1, and DL1 (-1 = 0ng/mL or 0nM, +1 = 500nM for UM729, 750nM for SR1, 2.5ug/mL for DL1. Midpoint concentrations were also assessed but data is not shown). [B] The CD34+ frequency and [C] CD34+ cell expansion after 12 days of culture of CD34+ cells. The highest levels were seen with the combination of fed-batch +UM729+SR1. [D] CD34+CD90+ frequency
and CD34+CD90+ cell expansion after 12 days of culture of CD34+ cells. The highest levels were seen with the combination of fed-batch +UM729+SR1+DL1.

We decided to further explore the combination of the fed-batch system with the UM729 molecule as a strategy that may have potential to be translated to a clinical trial. This approach appeared very promising due to the significant enhancement in the primitive progenitor expansion and the technical ease of using a soluble small molecule additive. A collaborative effort was initiated with the Guy Sauvageau lab to rigorously test this combined culture strategy and carry out the development work required for clinical translation. UM171, a more potent analog of UM729 was produced and it was demonstrated to achieve similar levels of expansion at ~20-fold lower concentration than UM729. We assessed this molecule in the fed-batch system and quantified expansion of primitive cell populations over time (Figure 5-4A). The fed-batch + UM171 combined strategy gave a significant enhancement of CD34+ cells and CD34+CD45RA- cells at both 12 and 16 days of culture.

To assess the impact of this combined strategy on the HSC population, we transplanted NSG mice with cells cultured in the fed-batch system with or without UM171 for 12 or 16 days and analyzed the bone marrow for human engraftment at 3 weeks, 9 weeks, and 16 weeks (Figure 5-4B,C,D). We found that the cells expanded with UM171 enhanced expansion of the HSC populations above what was seen with the fed-batch system alone and, at 16 weeks post-transplantation, limiting dilution analysis indicated that there was a 18.4-fold expansion of LTR-HSCs with 12 days of culture and a 9.4-fold expansion with 16 days of culture. Interestingly, although the progenitor cells continued to expand between day 12 and day 16 of culture, the stem cell population appeared to have peaked by day 12. These data indicated that the culture of UCB cells with the fed-batch + UM171 strategy for 12 days gives a significant expansion of stem and progenitor cells.
Figure 5-4: Assessment of stem and progenitor expansion of cells cultured with the fed-batch + UM171 strategy.

[A] Expansion of (i) total cells, (ii) CD34+ cells, and (iii) CD34+CD45RA- cells after in vitro culture of CD34+ cells with the fed-batch strategy alone or with the fed-batch + UM171 strategy. The addition of UM171 (35nM) significantly enhances the expansion of CD34+ cells and CD34+CD45RA- cells. [B] Comparison of human engraftment, as measured by human CD45+ cells, in the bone marrow of female NSG mice that had been transplanted with expanded UCB
cells. Data is shown for the engraftment resulting from 500,000 expanded cells at (i) 3 weeks, (ii) 9 weeks, and (iii) 16 weeks post transplantation. [C] Limiting dilution analysis at 16 weeks post transplantation of cell expanded for: (i) 12 days with the fed-batch control system; (ii) 12 days with fed-batch +UM171; (iii) 16 days with the fed-batch control system; (iv) 16 days with fed-batch +UM171. The equivalent d0 HSC frequencies are indicated, as calculated with the Lcalc software. [D] Expansion in SRC number, as an indication of LTR-HSCs at 16 weeks post-transplantation, with the indicated culture conditions.

Therefore, it appears that the combined culture strategy of fed-batch + UM171 is a promising approach to assess in a Phase I/II clinical trial. This strategy can expand both LTR-HSCs and primitive and committed progenitor cells to levels that appear to be clinically relevant and can be done with a method that is more rapid and very likely less costly than other strategies currently being investigated in clinical trial (Delaney et al., 2010; de Lima et al., 2012). Translational work is ongoing to develop this strategy and carry out the necessary safety and efficacy studies to seek regulatory approval from Health Canada. Translational work includes switching to reagents that contain no animal derived products and are GMP quality where possible, assessing the toxicity and biodistribution of cells expanded in the culture system, and scaling up the system so that it can accommodate a clinical sized UCB unit with a required final culture volume of approximately 1.5 L.

5.2.3 High yield mature blood cell production from expanded cells

There is much clinical motivation for the large scale in vitro generation of mature blood cells, including red blood cells, platelets, and neutrophils (Douay and Andreu, 2007; Reems et al., 2010). We have demonstrated that the fed-batch expansion system can achieve ~80-fold expansion of CD34+ cells after 12 days or culture and this can be doubled with the addition of other HSC enhancing factors like SR1 or UM171. Therefore, we hypothesized that these expanded cells may be a useful input population that can be used in mature cell differentiation systems in order to produce large numbers of mature blood cells from UCB. Since the fed-batch system is integrated into a clinically relevant bioreactor, it can act as the first stage of a scalable cell differentiation process.
We have partnered with collaborators to explore the production of neutrophils and platelet-producing megakaryocytes from progenitor cells that have been expanded in the fed-batch system in the presence or absence of UM171. It has been demonstrated that functional neutrophils and platelets can be produced from freshly isolated CD34+ cells from UCB or mPB (Chen et al., 2009; Matsunaga et al., 2006; Timmins et al., 2009), and so we predicted that by significantly increasing these progenitor cells in our HSPC expansion system, we could significantly increase the yield of the output mature cells. Importantly, the ex vivo expanded cells retain a >50% proportion of CD34+ cells after 8-12 days of culture with UM171. However, it was not clear whether these cells would still retain the same functional propensity as the fresh CD34+ cells to differentiate into specific mature blood cells. Additionally, despite the expansion of total CD34+ cells in our culture system, the frequency of CD34+ cells does diminish with culture and it was unclear whether a more heterogeneous input population would affect the differentiation process.

We have begun to investigate the production of mature megakaryocytes from our expanded cells in a collaboration with the Bill Miller laboratory. In these experiments, we have cultured UCB CD34+ cells under fed-batch conditions with or without UM171 for 8 to 12 days and then initiated megakaryocyte differentiation. Megakaryocyte production can be enhanced following the initial progenitor proliferation stage by removing the FL from the cytokine cocktail while retaining high levels of SCF and TPO and adding nicotinamide, to help induce polyploid megakaryocytes. Preliminary results indicate that with an 8 day expansion of CD34+ enriched UCB cells with UM171, following by the removal of FL and UM171, we can achieve >1000 CD41+ cells/input cell and a maximal mean megakaryocyte ploidy of 2.5N (Figure 5-5). The addition of nicotinamide on day 9 increases the maximal mean megakaryocyte ploidy to ~4N. These levels are equivalent or better than what has been reported in the literature (Chen et al., 2009; Feng et al., 2005; Matsunaga et al., 2006; Proulx et al., 2003). The addition of UM171 for the first 8-12 days of culture appears to enhance viability of cells as culture progresses and maintains a higher frequency of CD34+ cells, leading to greater overall megakaryocyte production.
Figure 5-5: Demonstration of polyploidy megakaryocyte production from UCB cells cultured with the fed-batch +UM171 strategy.

[A] CD41+ cells generated per input CD34+ cells. Lin- cells were cultured for 8 days with the fed-batch +UM171 culture strategy with TPO, SCF, and FL (TSFU). On day 9, FL and UM171 were removed, while TPO and SCF were maintained (TS), and in one condition nicotinamide (Nic) was added. [B] The mean ploidy of the CD41+ megakaryocytes. [C] The total DNA content of the megakaryocytes per input cell. Total DNA content was calculated by: (Total cells) x (%CD41+) x (mean ploidy). [D] Representative fluorescent image of a megakaryocyte expanded with this protocol (green=β-tubulin, blue=dapi).

It is expected that further optimization of this strategy will enable greater expansion of high ploidy megakaryocytes. In subsequent studies, the quantification of platelet production will also be assessed by daily harvesting and flow cytometry analysis of DNA- platelet-like particles. These platelet will also be analyzed by immunocytochemistry for morphology and functional response to agonist treatment (Matsunaga et al., 2006). Carrying out the megakaryocyte...
differentiation with freshly isolated CD34+ cells as a control will help to compare the relative ability of the ex vivo expanded CD34+ cells at producing megakaryocytes.

The promising results from these initial preliminary studies are encouraging and suggest that mature blood cell production from cells expanded in the fed-batch system is a potential avenue that should be further explored. We have also initiated a collaborative study with the Lars Nielsen lab to investigate the production of neutrophils from expanded UCB cells. The scalability of our fed-batch system makes it a platform that is highly amenable for cell generation and we anticipate that an initial progenitor expansion step could serve as the basis for high yield blood cell production.

5.2.4 Culture regulation through real-time process control

The fed-batch system provided a strategy to reduce the impact of endogenously produced soluble inhibitory factors. The feeding scheme that was used for this study was a constant dilution rate of one unit volume of media being added over each 24h period (D=1). The dilution rate was determined from model simulations as achieving a balance between providing significant reduction in soluble factors and requiring relatively small amounts of media in order to consider cost requirements and technical limitations for clinical scale-up. This feeding scheme worked well for the 12-day UCB culture, however it was not rigorously optimized. Moreover, as we began to explore combined culture strategies extending beyond 12 days or those that include the addition of factors which affect proliferation rate or lineage skewing, we observed that the constant D=1 dilution scheme that was developed for the fed-batch system was not always effective. The feeding scheme can be re-optimized for each unique culture condition, however, it is clear that this can become cumbersome and having a system that is flexible to respond to different culture conditions without significant optimization would be ideal.

When considering optimizing the feeding scheme, it is also important to account for the fact that the culture of primary human cells is subject to sample to sample variability that cannot be eliminated. Numerous studies have explored the variability of freshly isolated UCB cells, and their compositions vary in terms of primitive progenitors as well as more mature cell populations (Cairo et al., 2005; Yang et al., 2011). In addition, the primitive cell enrichment process that
precedes most culture strategies can lead to very high variability in the purity of the input cell population. This variation manifests itself during in vitro culture, as the non-linearity of the system means that small variations at input can significantly affect culture dynamics, mature cell accumulation, and the feedback signaling environment that is generated. Therefore, even the replication of the identical culture conditions will not necessarily respond identically to the same feeding scheme each time. Sample variation may lead to higher than average levels of critical inhibitory factors during a particular experimental run such that the pre-established D=1 dilution scheme is not the ideal feeding strategy. Conversely, if inhibitory factors are lower than average, overfeeding the system will result in excess media and cytokine use, which becomes important when working on a clinical scale. Therefore, what is desired is a feeding regimen that would respond to variation among each UCB sample and among different culture conditions and provide an optimized dilution each time (Figure 5-6A).

To meet these demands, we are exploring methods of "on-line" process control that could be adapted to our culture system. Process control systems are widely used in industry to keep the desired product within specifications. In the biotechnology field, real-time monitoring and control of macro components such as dissolved oxygen, pH, and glucose are widely used and these have been implemented in some hematopoietic culture bioreactor systems (Lim et al., 2007). However, as we have demonstrated, in addition to controlling macro components of the system, soluble signaling factors should also be tightly regulated and maintained to the desired specifications. To our knowledge, real-time process control strategies have not been used to regulate endogenously produced signalling factors in hematopoietic culture, but we predicted that designing such a system would allow for enhanced and responsive regulation of the expansion system.

The endogenous factor milieu that contributes to inhibitory feedback signaling is comprised of many factors and indeed we have shown that controlling the global set of factors, as opposed to an individual factor, is critical to achieve sustained regulation of the system. In order to demonstrate proof-of-principle of a real-time monitor and control system, we decided to choose one factor that we could monitor throughout culture, the concentration of which would act as a surrogate for all inhibitory factors. The fed-batch dilution rate would then be adjusted to maintain the levels of the surrogate factor within specification and, in doing so, all inhibitory
factors in the culture system would be diluted. Although secretion of specific factors follow unique trajectories, the majority of measured inhibitory factors accumulate roughly in an exponential manner. Therefore, we chose TGF-β1 (or its peptide surrogate, LAP) as one candidate for a real-time monitor and control system, as it is a potent inhibitor of HSPC expansion and accumulates at high levels (>1ng/mL) in our culture system with an exponential trajectory.

In collaboration with the Warren Chan lab, we developed a monitoring system based on the rapid measurement of LAP concentration. The goals of the detection system included: 1) the measurement would be performed in <1 h; 2) the sensitivity of the assay would be < 0.5 ng/mL; 3) the assay would require < 10 uL of culture media. We developed a detection system using a sandwich antibody assay on microbeads that met these goals (Figure 5-6B). A 10uL media sample is mixed with labeled microbeads for 30min, and the beads are read via flow cytometry to quantify LAP concentration. A dose response curve using this assay was performed to determine that the assay gave a linear response with sensitivity <0.1ng/mL (Figure 5-6C).

We next performed a proof of principle real-time control (RTC) study with this detection assay by measuring the concentration of LAP every 12 h in a 16 d cell culture system that was initiated with CD34+ cells at three different cell seeding densities (low=100,000 cells/mL, medium=300,000 cells/mL, high=500,000 cells/mL). If the LAP concentration was measured to be > 0.1 ng/mL, the cell culture was diluted 2-fold. If the concentration was < 0.1 ng/mL, no feeding was done. This RTC scheme maintained the LAP concentration around 0.1ng/mL for the duration of culture, with little fluctuation, regardless of the initial cell density. In contrast, the constant D=0 and D=1 control feeding scheme resulted in very high LAP levels, especially beyond day 12 or with the medium or high cell seeding densities (Figure 5-6D). With the constant D=1 feeding scheme, the higher cell seeding densities result in a reduction in primitive cell expansion after 16 days of culture, as this dilution rate was not sufficient to regulate inhibitory signaling factors at these higher cell densities. In contrast, the RTC feeding scheme was able to achieve comparable day 16 primitive cell expansion, regardless of cell seeding density (Figure 5-6E).
One limitation observed with the RTC proof of principle study was that the RTC scheme required comparably very large media volumes to achieve the observed progenitor cell expansion. This issue may be overcome by adjusting the LAP concentration upper threshold and using a more sophisticated feedback scheme (such as a PID control algorithm) with more frequent sampling times. This should minimize fluctuations of LAP levels and provide for tighter control with less media usage. The detection system could also be multiplexed to monitor either multiple secreted signaling factors, particularly ones which may have different accumulation kinetics than TGF-β1, or other endogenous or exogenous factors that have fluctuating levels in culture.

**Figure 5-6:** Proof-of-principle of a real-time process control system for secreted factor concentration regulation.
[A] Schematic of real-time control monitor and control system. A cell culture media sample is taken from the culture vessel to the detection assay, where the concentration of one or more soluble factors is measured. The concentration value is fed to the controller which uses an algorithm to convert the concentration into a dilution rate. The dilution rate is fed to the pump which controls the input of fresh media into the culture vessel. [B] Schematic of microbead assay used for detection system. Beads are labeled with an antibody specific to the soluble factor of interest and mixed with the cell culture supernatant. [C] The sensitivity and linearity of the detection assay were demonstrated by measuring the concentration of LAP standards. [D] LAP concentration measurements were taken every 12 h during a 16 day CD34+ UCB culture. The fresh media negative control (NC) and the concentration threshold positive control (PC, 0.1ng/mL LAP) are shown in black. The real-time control (RTC) scheme using the detection assay was compared to a culture with no media dilution (D=0) or a constant dilution rate of one unit volume per day (D=1). The measurements were performed in culture with a cell seeding density of: (i) 100,000 cells/mL (L); (ii) 300,000 cells/mL (M); (iii) 500,000 cells/mL (H). [E] For each of the cultures described above, the expansion of progenitor cells was measured after 16 days of culture. (i) CD34+ cell expansion; (ii) CD34+ CD45RA− cell expansion; (iii) CD34+ CD90+ cell expansion. The RTC control scheme enabled the enhancement of progenitor cell expansion over the D=1 scheme, particularly at the higher cell seeding densities.

The ultimate goal for this technology is to develop a fully automated process control system that could be integrated into our closed system fed-batch bioreactor. An "on-chip" image based detection system, similar to one previously described (Klostranec et al., 2007), would allow for the automation of the detection system. The concentration reading could then be fed back into the controller which would convert the measured concentration into a dilution rate and would be used to adjust the media inlet flow rate to the cell culture vessel. The development of such a system requires solving several outstanding challenges, including ensuring appropriate sensitivity levels with the on-chip detection system, developing a means of automated media sample that is compatible with the bioreactor system, and converting the concentration measured by the detection system to an instruction of a media input rate that is received by the controller. This type of control system would enable an optimized feeding scheme to be used for each individual UCB sample, and would also account for any unexpected dynamic fluctuations during
culture. This system would not only be relevant to the hematopoietic culture system but would find utility in many dynamic and heterogeneous cell systems.

5.3 Broader implications and significance

The importance of cell-cell interactions and non-cell autonomous mechanisms or regulation were paramount throughout this work. This is often an overlooked aspect of heterogeneous systems and here we sought to highlight the importance of identifying, understanding, and manipulating this aspect of a cell system in order to achieve regulation that cannot be done by targeting a single cell type. Findings presented here demonstrated how this aspect of the cell system can provide important mechanistic insights into the underlying biology as well and provide a means to manipulate the system to achieve the desired outcome of enhanced stem and progenitor expansion. The hematopoietic system served as an example of a complex and dynamic system, but these strategies and considerations apply to all cell systems and are of particular consideration across the stem cell field, where heterogeneity and dynamic interactions often dominate the culture system.

Each aspect of this work combined mathematical modeling and experimental strategies. Mathematical simulations were used both for understanding the system and for predicting new experimental approaches. In vitro and in vivo experimental studies were performed to validate model predictions and interrogate the underlying biology. The combination of simulations and experiments allowed for the exploration of techniques and concepts that would not be feasible with either approach alone. This integration speaks to the importance of bioengineering strategies bridging the gap between what can be analyzed with fundamental biology techniques and what can be innovated through engineering design.

UCB transplantation provides an opportunity to make curative HSPC transplantations available for patients who otherwise would not have access to a stem cell donor. The ex vivo expansion of UCB provides a strategy for which UCB transplantations may achieve a greater success rate in all patients. Translating the laboratory techniques of HSPC culture to the clinical setting through strategies of scale-up, automation, and quality assurance processes are critical steps to ensure that basic medical science research is reaching its ultimate target of helping patients. The work
presented here is an important step towards the development of a robust method for the ex vivo expansion of UCB in a clinically relevant manner and contributes toward the positive impact of achieving cures to hematologic disease.
6 REFERENCES


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Copyright Acknowledgements

**Chapter 1:**

**Chapter 2:**

**Chapter 3:**