

**REGULATION OF HUMAN HEMATOPOIETIC CELLS WITH
SHORT-TERM *IN VIVO* REPOPULATING ACTIVITY**

by

PAUL H. MILLER

BAppSc, Queensland University of Technology, 2002

A THESIS SUBMITTED IN PARTIAL FULFILLMENT OF
THE REQUIREMENTS FOR THE DEGREE OF

DOCTOR OF PHILOSOPHY

in

THE FACULTY OF GRADUATE AND POSTDOCTORAL STUDIES
(Experimental Medicine)

THE UNIVERSITY OF BRITISH COLUMBIA

(Vancouver)

April 2016

© Paul H. Miller, 2016

Abstract

Transplantation of hematopoietic cells is a critical component of treatments used to cure a range of malignancies and congenital disorders. Cells with rapid but short-term repopulating ability (STRCs) are the main source of neutrophils and platelets early post-transplant, but methods to increase their availability are lacking. In this study the use of NOD/SCID-IL-2Receptor- γ chain-null mice engineered to produce human interleukin-3, granulocyte-macrophage colony-stimulating factor and Steel factor as hosts of CD34⁺ cells was shown to improve the quantification of human STRCs. This mouse was found to support a 5-fold higher human myeloid cell output at early time-points post-transplant, sufficient to assess total engraftment from an analysis of circulating human cells. This strategy was then used to determine the optimal protocol for mobilizing STRCs in normal adult human donors, comparing administration of granulocyte colony stimulating factor (G-CSF) plus plerixafor (P) to administration of P alone. Blood and marrow samples were obtained from 10 normal adult donors before, during, and after treatment and then evaluated for their content of CD34⁺ cells, colony-forming cells (CFCs), long-term culture-initiating cell activity, and cells with *in vivo* STRC activity. The results show all activities were maximally increased in the blood 4 hours after administration of P, with or without pre-treatment with G-CSF. *In vivo* assessment showed that administration of P led to a 30-fold increase in STRC activity over baseline levels, with further enhancement (90-fold over baseline) by prior G-CSF treatment. The ability of currently available growth-factor (GF)-containing HSC expansion protocols to support the expansion of STRCs was then assessed. The results revealed a significant (10-fold) loss of STRC activity after being cultured for 7 to 10 days, in spite of an extensive increase in CD34⁺ cells and CFCs. This GF-induced loss of *in vivo*

STRC activity occurred within 24 hours, and was paralleled in time and amount by a loss of CFC homing to the bone marrow. Together these findings provide a further understanding of STRC biology and provide a foundation for developing improved yields of manipulated STRCs for clinical use.

Preface

Under the supervision of my supervisor, Dr. Connie Eaves, I designed and conducted all experiments that contributed to the results presented in Chapters 2, 3, and 4, except for the methods and studies outlined as follows. Staff of the British Columbia Cancer Agency Stem Cell Assay Laboratory assisted in processing and cryopreserving blood and marrow samples. Kirk Schultz, David Jones, Stephen Couban and Connie Eaves designed the protocols used to treat donors with G-CSF and Plerixafor for the studies described in Chapter 3. Naoto Nakamichi executed LTC assays in Chapter 3. Margarita MacAldaz assisted with some of the CD34⁺ expansion experiments described in Chapter 4. Glenn Edin, Margaret Hale, Gabrielle Rabu, Kiran Dhillon, and Shabnam Rostamirad helped with blood and marrow analysis of some of the transplanted mice. Alice Cheung, Philip Beer, David Knapp and Suzan Imren gave advice on xenotransplantation methodology, and David Knapp and Davide Pellicani gave advice on and shared scripts for data analysis using R. For all chapters, I analyzed and interpreted the data and wrote the associated manuscripts with the assistance of Dr. Eaves.

The following publications arose from this thesis work and are incorporated into the material presented in Chapters 1 and 2, respectively:

Miller, P.H., Knapp, D.J.H.F., and Eaves, C.J. (2013). Heterogeneity in hematopoietic stem cell populations: implications for transplantation. *Current Opinion in Hematology* 20, 257–264.

Miller, P.H., Cheung, A.M.S., Beer, P.A., Knapp, D.J.H.F., Dhillon, K., Rabu, G., Rostamirad, S., Humphries, R.K., and Eaves, C.J. (2013). Enhanced normal short-term human myelopoiesis in mice engineered to express human-specific myeloid growth factors. *Blood* 121, e1–e4.

Chapter 3 has been submitted as a manuscript entitled, “Improved prediction of optimally mobilized cells from normal human donors”, for publication in a peer-reviewed journal.

Chapter 4 has been submitted as a manuscript entitled, “Early production of human neutrophils and platelets post-transplant is severely compromised by growth factor exposure”, for publication in a peer-reviewed journal.

All animal experiments were carried out in accordance with the policies and guidelines presented by the University of British Columbia Animal Care Committee. Canadian Council on Animal Care Approval was granted under the certificate numbers #A10-0331 and #A15-0223.

Table of Contents

Abstract.....	ii
Preface.....	iv
Table of Contents	vi
List of Tables	x
List of Figures.....	xi
List of Abbreviations	xii
Acknowledgements	xiv
Dedication	xv
Chapter 1: Introduction	1
1.1 Clinical hematopoietic cell transplantation.....	1
1.2 Hematopoiesis.....	2
1.2.1 Repopulating cell subsets and methods of their detection	3
1.2.1.1 Colony-forming cells (CFCs)	3
1.2.1.2 Long-term culture-initiating cells (LTC-ICs).....	5
1.2.1.3 Short term <i>in vivo</i> repopulating cells (STRCs).....	6
1.2.1.4 Long-term <i>in vivo</i> repopulating cells (LTRCs).....	9
1.3 Cell sources	12
1.4 Mobilization of primitive human hematopoietic cells	13
1.5 HSC expansion.....	15
1.5.1 Growth factor (GF)-mediated cell expansion strategies	15
1.5.2 HSC expansion strategies using novel additives or manipulations	16

1.5.3	Enhancement of cell homing	19
1.5.4	<i>Ex vivo</i> manipulated transplants.....	20
1.6	Thesis objectives.....	22
Chapter 2: Enhanced Normal Short-Term Human Myelopoiesis in Mice Engineered to Express Human-Specific Myeloid Growth Factors26		
2.1	Introduction.....	26
2.2	Methods.....	27
2.2.1	Cells	27
2.2.2	Transplantation procedure	27
2.2.3	Lenti-viral transduction and bioluminescence imaging.....	28
2.3	Results.....	29
2.3.1	NSG-3GS mice support a 5-fold increase in myeloid cell outputs from transplanted human STRCs but do not change the frequency of STRCs detected.	29
2.3.2	Increased support of mature human myeloid cell production results in increased blood chimerism and improved monitoring of human chimerism by blood sampling.	31
2.4	Discussion.....	31
Chapter 3: Plerixafor Plus G-CSF is a Superior Mobilizing Regimen for Cells that Produce Neutrophils and Platelets Rapidly <i>In Vivo</i>40		
3.1	Introduction.....	40
3.2	Methods.....	42
3.2.1	Human cell samples	42
3.2.2	Flow cytometry	43
3.2.3	<i>In vitro</i> assays	43

3.2.4	Xenotransplant assays.....	44
3.2.5	Statistical analysis.....	45
3.3	Results.....	45
3.3.1	Higher numbers of hematopoietic progenitors are present in the PB 4 hours after a single injection of P with or without prior G-CSF.....	45
3.3.2	Individual donor assessment reveals superiority of the G-CSF+P mobilization regimen	46
3.3.3	G-CSF+P mobilizes more short term repopulating cell activity than P alone.....	47
3.3.4	Isomap reveals high overlap between in vitro and in vivo myeloid assays	48
3.4	Discussion.....	49
Chapter 4: Early Production of Human Neutrophil and Platelets Post-Transplant is Selectively Compromised by Prior Growth Factor Exposure59		
4.1	Introduction.....	59
4.2	Methods.....	60
4.2.1	Human cells	60
4.2.2	Cell cultures	60
4.2.3	Transplantation procedures.....	61
4.2.4	Flow cytometry.....	61
4.2.5	Statistical analysis.....	61
4.3	Results.....	62
4.4	Discussion.....	63
Chapter 5: Discussion.....69		
5.1	Detection and quantification of human STRCs	69

5.2	Mobilization of STRCs.....	72
5.3	<i>Ex vivo</i> expansion of STRCs.....	74
5.4	Concluding comments	76
References.....		78

List of Tables

Table 2.1. Frequency of human STRC-Ms detected in NSG and NSG-3GS mice based on achievement of detectable BM chimerism 3 weeks post-transplant.....	37
-----------------------------------------------------------------------------------------------------------------------------------------------------	----

List of Figures

Figure 1.1. Multiple human hematopoietic cell types contribute to the mature blood cells produced at different times in heavily myelosuppressed recipients, as modeled in transplanted sublethally irradiated immunodeficient mice.....	24
Figure 1.2. The relative abundance of STRCs and LTRCs in a typical transplant of normal human CB, BM, and G-CSF mPB normalized for the same body weight of the recipient.	25
Figure 2.1. Increased short-term myeloid cell output in NSG-3GS mouse BM and PB.	33
Figure 2.2. Increased short-term mature cell output in NSG-3GS mouse BM and PB.	35
Figure 2.3. Circulating human cell and <i>in vivo</i> bioluminescence counts enable accurate assessment of total human cell engraftment.	38
Figure 3.1. Plerixafor ± G-CSF experimental design	52
Figure 3.2. Hematopoietic progenitors are optimally harvested from the blood 4 hours after plerixafor.....	54
Figure 3.3. Analysis of individual samples shows higher post-plerixafor progenitor increases in donors pre-treated with G-CSF.....	55
Figure 3.4. <i>In vivo</i> assays of individual donor PB samples show higher activity in donors pre-treated with G-CSF.	57
Figure 3.5. Isomap analysis reveals a high degree of concordance between <i>in vitro</i> and <i>in vivo</i> assays of myeloid cell output activity for similar periods of time.....	58
Figure 4.1. Cultured CD34+ CB cells show a profound and selective loss of STRC activity.	65
Figure 4.2. GF-induced loss of STRC activity occurs rapidly and is associated with a similar reduction in CFC homing efficiency to the BM.	67

List of Abbreviations

3GF	TPO + SF + FLT3-L
3GS	IL-3 + G-CSF + SF
5GF	SF + FLT3L + G-CSF + IL-3 + IL-6
BL	baseline
BM	bone marrow
CB	cord blood
CFC	colony-forming cell
CFU	colony-forming unit
CSF	colony stimulating factor
FBS	fetal bovine serum
FLT3L	FMS-like tyrosine kinase 3 ligand
G	granulocyte
GF	growth factor
GFP	green fluorescent protein
GM	granulocyte macrophage
G-PB	G-CSF mobilized peripheral blood
HSC	hematopoietic stem cell
IL	interleukin
LDA	limiting dilution assay
LTC-IC	long term culture initiating cell
LTRC	long term repopulating cell

M	myeloid
mPB	mobilized peripheral blood
NH ₄ Cl	ammonium chloride
NOD/SCID	non-obese diabetic, severe combined immunodeficient
NS	NOD/SCID
NSG	NOD-SCID interleukin-2 receptor gamma null
NRG	NOD-Rag1 null interleukin-2 receptor gamma null
P	plerixafor
PB	peripheral blood
PI	propidium iodide
SF	Steel factor
SFM	serum free media
STRC	short term repopulating cell
WT	wild type

Acknowledgements

I am immensely grateful to my supervisor, Dr. Connie Eaves, for the incredible dedication and commitment she brings as a mentor. I thank her for providing the support wherever needed, as well as for nurturing my independence from early on. Her passion and high standards for quality have been highly influential and the environment of discussion and engagement that she provides have changed my way of thinking.

I also want to thank Dr. Keith Humphries, who has served as a secondary supervisor and has provided rich insight into the questions I've pursued and helped interpret the often confusing results I've produced. Thank you also to the other members of my supervisory committee, Dr. Fabio Rossi and Dr. Ryan Brinkman.

Thank you to the whole Eaves' lab, particularly Philip Beer, Alice Cheung, and David Knapp, as well as to Suzan Imren from the Humphries lab, for their regular and often lengthy discussions and for the sharing and stirring of scientific passion. Thank you to David Knapp for persuading me to learn data analysis in R, and putting in the time to help whenever needed.

Thank you to the University of British Columbia (UBC). I am also grateful for the generous funding from the Canadian Institutes of Health Research (CIHR) Frederick Banting and Charles Best Canada Doctoral Scholarship, the CIHR Transplant Research Training Program, and the UBC Faculty of Medicine Graduate Award.

Thank you to all my parents and brothers for their tremendous support and encouragement over the many years.

And finally, thank you to my wonderful wife Arla for her unending love and support, and for making the journey a joy.

Dedication

I dedicate this thesis to my parents

Chapter 1: Introduction

1.1 Clinical hematopoietic cell transplantation

The clinical use of hematopoietic transplants is an established treatment modality for patients with a variety of diseases. Historically a major consideration has been to keep the procedure as effective as possible generally with minimal *ex vivo* handling of the cells, with success being ultimately determined largely by the cellular composition of the transplant. Broadly speaking, transplants have two major applications. Most frequently, hematopoietic cell transplants are used to rapidly and permanently restore blood cell production in patients, primarily patients with hematological malignancies, whose endogenous supply of stem cells and progenitors is compromised by the intensity of the chemotherapy and/or whole body radiotherapy used to treat the patient. The co-infused immune effector cells in allogeneic transplants offer an additional important therapeutic contribution in such patients as they recognize the residual malignant cells as foreign and assist in their elimination. However, the downside is the ability of these cells to also cause graft-versus-host disease (GVHD) by attacking normal host tissues (Goldman et al., 1988).

The other major use of hematopoietic cell transplants is to correct an inherited genetic disorder or an acquired autoimmune disorder. In such patients, a myeloablative preparative regimen is used to eliminate the recipient's hematopoietic stem cells (HSCs) and derivative progenitors to enable the transplanted cells to regenerate a new and permanent supply of mature blood cells. In both of these scenarios, a prompt resumption of neutrophil and platelet production is a principal determinant of clinical success. Other considerations relate to the choice of donor

and source of the cells to be transplanted. Different types of donors include the patient (autologous transplants) as well as other, normal individuals (allogeneic transplants). Different cell sources now in widespread use include bone marrow (BM), granulocyte-colony-stimulating factor (G-CSF)-mobilized peripheral blood (mPB), and cord blood (CB).

Improving the content of the transplant for all of these applications remains a highly sought goal. To this end, much effort continues to focus on the development of specific and direct methods to quantify the cells able to provide both immediate and sustained outputs of mature blood cells, as well as to understand their engrafting efficiencies, the spectrum and durability of their output capabilities, and methods for their expansion *ex vivo*.

1.2 Hematopoiesis

Experiments in mice were the first to reveal a hierarchy of cell states that enable successive waves of repopulation and production of an increasing range of mature blood cell types (Hodgson and Bradley, 1979; Jones et al., 1990; Magli et al., 1982). This model supports the concept of clonal succession during the initial period post-transplant, but does not exclude a role of stochastic mechanisms or extrinsic factors also contributing to a changing clonal landscape of blood cell production (Lemischka, 1991). Increasingly powerful cell purification and analytical strategies applied to mouse HSCs now suggest that the mechanisms regulating their self-renewal and lineage potential at the single cell level are not as tightly linked as historically assumed (Copley et al., 2012; Muller-Sieburg et al., 2012).

The development of long-lived immunodeficient mice that can be engrafted with human cells has unveiled a comparable multi-step process in the human hematopoietic system with

accompanying inherent and extrinsic sources of clonal variability. Such information has come from cell separation experiments using antibodies against cell surface proteins to segregate the cells of interest (Glimm et al., 2001; Notta et al., 2011), and from clonal tracking studies utilizing analysis of vector integration sites (Guenechea et al., 2001) or barcoded genomic inserts (Cheung et al., 2013). Relevance of these findings to transplants in people is supported by several gene therapy trials where the unique insertion sites of transduced therapeutic genes have been used to track the outputs of multiple clones in individual patients over many months and, in some cases, for several years post-transplant (Adair et al., 2012; Aiuti et al., 2013; Biffi et al., 2013; Cavazzana-Calvo et al., 2010; Payen and Leboulch, 2012). However, interrogation of the biology regulating these clinical results requires the use of assays specific for the responsible classes of repopulating cells.

1.2.1 Repopulating cell subsets and methods of their detection

1.2.1.1 Colony-forming cells (CFCs)

The earliest *in vitro* assays for human clonogenic progenitors were developed following the successful demonstration of such assays for mouse hematopoietic cells. The latter were based on the discovery that colonies of hematopoietic cells could be obtained by plating relatively small numbers of mouse bone marrow cells in a layer of semi-solid agar above a layer of mouse kidney or embryo feeder cells (Bradley and Metcalf, 1966; Pluznik and Sachs, 1965). The use of semi-solid media enabled the clonally generated cells to be spatially restricted and hence enumerated by basic light microscopy. The earliest human CFC assay consisted of a lower layer of mouse

kidney cells and an upper layer of agar containing 10% human serum and the hematopoietic cells to be tested (Senn et al., 1967). These culture conditions were found to support the development of colonies of mature granulocytes at a frequency of 1 in 3000 from adult human BM and the number of colonies obtained was a linear function of the number of initial nucleated cells plated over a certain range of input cell concentrations. The human CFC assay now in widest use involves suspending the test cells in a single layer of a highly viscous methylcellulose-containing medium supplemented with 30% fetal bovine serum (FBS) and the recombinant human growth factors (GFs) erythropoietin (EPO), Steel factor (SF), Interleukin-6 (IL-6), IL-3, granulocyte-macrophage colony-stimulating factor (GM-CSF), and in some cases G-CSF (Hogge et al., 1996). These conditions support the clonal differentiation of a wide range of mature myeloid cells including erythroid cells (from erythroid colony-forming units/cells, CFU-Es; and more primitive erythroid burst-forming units/cells, BFU-Es), GM cells (from CFU-GMs), and cells of both of these lineages (from CFU-GEMMs). However, as the production of human lymphoid cells is not supported under these conditions, this system cannot test for any possible concomitant lymphoid differentiation potential of any of these input cells.

The size of the erythroid, GM or mixed colonies obtained under these conditions correlates with the differentiation state of the initial cell, with many types of experiments demonstrating that larger colonies are derived from cells that overlap phenotypically with cells with other characteristics of very primitive hematopoietic cells (Cheung et al., 2012; Majeti et al., 2007; Sauvageau et al., 1994). However, because no category of CFC consistently correlates with the number of cells with long-term *in vivo* repopulating ability, assays with increased specificity for such primitive cells are needed.

1.2.1.2 Long-term culture-initiating cells (LTC-ICs)

Precursors of CFCs (i.e., detected based on their ability to form scorable colonies within 2-3 weeks in semi-solid medium) can be identified by their ability to generate progeny CFCs for many weeks. This can be achieved by co-culturing human test cells with a preformed adherent layer of irradiated marrow “stromal” cells (Sutherland et al., 1990) or irradiated murine fibroblasts that display equivalent supportive activity (Sutherland et al., 1989). Use of such fibroblasts that have been genetically engineered to permanently produce human SF, IL-3 and G-CSF enhances the number of CFCs present after 6 weeks and hence improves the sensitivity of the assay (Hogge et al., 1996). The 6-week period allows the majority of CFCs in the input to differentiate into more mature cells so that any CFCs then present can be inferred to represent the progeny of a more primitive ancestral population. These cells are referred to as LTC-ICs, and can be prospectively separated away from the bulk of the CFCs based on their different forward light scatter characteristics, readily detectable expression of CD34 and CD90 and lack of expression of CD45RA, CD71 and CD38 (Baum et al., 1992; Craig et al., 1993; Hogge et al., 1996; Sauvageau et al., 1994; Udomsakdi et al., 1992). Further evidence of the distinct biological states of CFCs and LTC-ICs comes from experiments showing the different GF concentrations required to maximize their production in liquid expansion cultures initiated with CD34⁺CD38⁻ cells from either BM or CB (Petzer et al., 1996a; Zandstra et al., 1998, 1997). Colonies plucked from 14-19 day old CFC assays contain cells that proliferate in secondary CFC assays, although they do not contain cells detectable as LTC-ICs. Interestingly, methylcellulose assays of adult cells do not appear to support the growth of LTC-ICs (Petzer et al., 1996b).

The LTC-IC assay detects the ability of a cell to survive, proliferate and differentiate for an extended period of time in LTC conditions. However, to test the more complex requirements of *in vivo* repopulation, more stringent assays are required.

1.2.1.3 Short term *in vivo* repopulating cells (STRCs)

The likely existence of human hematopoietic cells with early but transient repopulating ability was first suggested by experiments in mice that showed most blood cell production after more than a month or two post-transplant did not originate from spleen colony-forming cells, even those with self-renewal ability, but rather, from cells with different physical properties that allow their prospective separation (Jones et al., 1990). Parallel evidence of a similar situation in humans was provided by the observation that *in vitro* pre-treatment of autografts with 4-hydroperoxycyclophosphamide (4-HC) resulted in a consistent delay in the neutrophil and platelet recoveries subsequently obtained when these cells were transplanted back into their donors (Yeager et al., 1986). This idea was further supported by the finding that 4-HC treatment also selectively killed cells with immediate clonogenic activity *in vitro*, with little effect on their more primitive precursors detectable as LTC-ICs (Udomsakdi et al., 1992). Later, when mice with genetically determined immunodeficiencies sufficient to enable human hematopoietic cells to engraft at high efficiency were developed (Kamel-Reid and Dick, 1988; Shultz et al., 2012), it was discovered that human cells with rapid but short term repopulating activity (STRCs) are more sensitive to elimination in mouse hosts by CD122⁺ natural killer (NK) cells than are cells with more durable repopulating activity (Glimm et al., 2001; McKenzie et al., 2005). Cell separation experiments further showed that the earliest wave of repopulation that peaks at 3

weeks post-transplant is derived from cells with a CD34⁺CD38⁺ phenotype which in CB, adult BM and mPB harvests, appear largely myeloid-restricted (Figure 1.1). Accordingly, these cells were called short term repopulating cells-myeloid (STRC-Ms). In these same experiments (Glimm et al., 2001), it was noted that STRC-Ms in CB samples were less well represented than cells with more durable and lympho-myeloid repopulating activity in immunodeficient mice - thus offering a possible explanation for the relatively poor initial rates of neutrophil and platelet recovery seen in patients transplanted with CB cells.

Additional characterization of STRCs has been provided by studies exploiting even more permissive NOD/SCID-IL-2R γ c-null (NSG) mice than the NS- β 2-microglobulin-null mice initially used by Glimm et al. (Glimm et al., 2001) or the NS mice pretreated with anti-CD122 antibody used by the Dick group (McKenzie et al., 2005). These more recent experiments have shown that some of the circulating human platelets and neutrophils produced within the first 3 weeks post-transplant derive from separate populations in the initially transplanted cells, as the input cells responsible for these early outputs do not have identical phenotypes and may be numerically dissociated (Cheung et al., 2012). Quantification of the numbers of these different types of STRCs in human CB and mPB by limiting dilution assays (LDAs) indicates that an average CB harvest contains >20-fold fewer rapid platelet repopulating cells and rapid neutrophil repopulating cells than an average mPB harvest, despite the 3-fold difference in the number of cells able to produce neutrophils in NSG mice many months post-transplant. Interestingly, total cell outputs per STRC have proven to be the same for both CB and mPB STRCs. However, the PB levels of human platelets obtained in NSG mice still seem low given the level of chimerism evident in the BM. This suggests a persistent host macrophage-mediated mechanism causing the premature removal of newly released human platelets (Hu and Yang, 2012). Together, these

experimental findings solidify the concept that human bone marrow STRCs represent a distinct subset of cells that can differentiate into mature blood cells sooner than coexisting cells with sustained repopulating activity with both being required to achieve rapid and sustained high-level chimerism (Figure 1.1).

Several human GF-producing mice have been found to enhance the levels of human myeloid cells and platelets produced from transplanted human hematopoietic cells. These include the so-called MISTRG, a BALB/c mouse in which the cDNAs for human M-CSF, IL-3, GM-CSF and TPO together were knocked-in to their respective mouse loci, with the further addition of a bacterial artificial chromosome transgene encoding human SIRP α (Rongvaux et al., 2014). At 7-12 weeks post-transplant of human CD34⁺ fetal liver cells the total human chimerism seen in the BM (but not in the blood) of the recipients was moderately elevated with a skewing towards human myeloid (CD33⁺) cells in the blood. However, measurements of human cell outputs at earlier post-transplant time-points (i.e., week 3) or of cells of the megakaryocyte lineage at any time point were not performed.

The same group also generated a BALB/c human-TPO knock-in mouse and assessed human platelet levels in these mice 3-4 months after they were transplanted with human CD34⁺ cells CB or fetal liver cells (Rongvaux et al., 2011). The loss of mouse-TPO in these mice resulted in a 2-fold reduction of mouse platelets pre-transplant which was further depressed 3-4 months post-transplant. While the *percentage* of human platelets was elevated compared to control BALB/c mice, there was no difference in the absolute number of human platelets per μ L of mouse blood.

To more closely replicate conventionally used hematopoietic transplants in patients, the development of protocols allowing co-infusion of the CD34 negative fraction of cells will be

needed. However, when immunodeficient mice are injected with human CD3⁺ cells together with human CD34⁺ cells, the mice develop and generally quickly die of a GVHD-like wasting disease. Thus it is not currently possible to investigate a potential role of T-cells on STRC repopulating activity, although this may be addressable in the future with the development of human HLA-engineered immunodeficient mice (Danner et al., 2011).

Experiments with both mouse (Busch et al., 2015; Sun et al., 2014; Wilson et al., 2008) and human (Aiuti et al., 2013; Cheung et al., 2013; Notta et al., 2011) cells demonstrate that the contribution of transplanted LTRCs to hematopoiesis in transplanted recipients can be delayed for an extended period of time with the contribution from co-transplanted cells with transient repopulating ability lasting several months. This underlines the clinical importance of STRCs and highlights the need for assays specific for each class of repopulating cells.

1.2.1.4 Long-term *in vivo* repopulating cells (LTRCs)

The concept of what constitutes a HSC used to rely on the assumption that HSCs represent a discrete and essentially homogeneous population. However, as investigations at the single HSC level have become possible, their biological definition has become increasingly challenging. Studies of some of the earliest transplant recipients of unseparated normal human BM (Mathioudakis et al., 2000) or CB (Gluckman et al., 2011) cells have now clearly shown that these sources contain cells that can restore blood cell production in people for decades. However, the properties, phenotypes, and numbers of cells required to achieve this result remain poorly defined. Recent advances in characterizing different phenotypes of human cells within the CD34⁺ fraction of adult BM or CB have now enabled time course studies of the spectrum and

duration of blood cells each subset produces in irradiated NSG mice (Figure 1.1). Among the multiple distinct populations with increasing multi-lineage differentiation options and increasing self-renewal capability (Cheung et al., 2012; Christ et al., 2007; Glimm et al., 2001; Majeti et al., 2007), a method to obtain highly purified cells with long-term (20 weeks) repopulating activity (10–20% pure) has been reported (Notta et al., 2011).

The pattern of continued mature multi-lineage cell output for an extended, but finite period (4–6 months) mirrors findings in the mouse where hematopoietic cells with similar capabilities can be prospectively separated from those with lifelong activity and serial transplantability (Benveniste et al., 2010; Benz et al., 2012; Dykstra et al., 2007; Kent et al., 2009; Morita et al., 2010). This is a key issue as properties of human HSCs are often inferred from measurements of mature cells produced in NSG mice for less than 16 weeks post-transplant. Choice of this 16-week time point is largely due to historical extrapolation from protocols developed for NS mice. Most NS mice die between 6 and 12 months of age from a fatal endogenous thymoma and hence cannot be followed for more than 4–6 months after a transplant is performed (Shultz et al., 2007). Interestingly, data obtained 20 years ago from preimmune fetal sheep transplanted *in utero* with human cells also suggested that a period of more than 6 months is required before the mature human cells being produced reflect the exclusive output of cells in the original transplant that have durable self-renewal potential (Civin et al., 1996). This concept has been further extended by recent data derived from analysis of NSG mice transplanted with the progeny of phenotypically more refined subsets of human CB cells. The latter have shown that CB cells defined phenotypically as ‘MPPs’ (multi-potent progenitors, i.e., CD34⁺CD38⁻CD45RA⁻CD90⁻CD49f⁻) are capable of initiating the production of differentiating myeloid cells in NSG recipients for up to 20 weeks but poorly after that (Notta et

al., 2011). In contrast, more sustained outputs are derived from CD34⁺CD38⁻CD45RA⁻CD90⁺CD49f⁺ cells.

Importantly, in the mouse, even cells that are capable of repopulating successive generations of irradiated mice are now known to comprise cells with different, intrinsically determined and serially preserved lineage outputs (Copley et al., 2012; Ema et al., 2014). All of these long-term repopulating cells (LTRCs) have sustained myeloid differentiation potential, but have different abilities to produce lymphoid precursors (Dykstra et al., 2007; Muller-Sieburg et al., 2012). This heterogeneity in HSC output specificity is apparent as early as midgestation (E14.5) and persists into old age, but the relative numbers of these biologically different HSCs change over time (Benz et al., 2012). The creation of predominantly myeloid clones does not appear to be explained by a mechanism that causes multipotent HSCs to favor commitment to a myeloid vs a lymphoid fate, but rather an activated inability to support multiple stages of lymphoid differentiation, including the production of common lymphoid progenitors as well as their subsequent production of mature lymphoid cells.

Ontogeny and aging are also associated with a decline in HSC self-renewal and mature cell output (Benz et al., 2012; Bowie et al., 2007; Dykstra et al., 2011; Muller-Sieburg et al., 2012). These are important caveats to inferring changes in HSC numbers from total cell outputs when manipulated or developmentally undefined bulk transplants are being investigated.

Evidence of parallel heterogeneity in very primitive human hematopoietic cells is still preliminary. However, both an age-related shift towards lymphoid-deficient HSCs (Pang et al., 2011) and the existence of occasional clones with marked and sustained lineage biases regenerated and maintained for long periods of time in gene therapy patients has been reported (Cavazzana-Calvo et al., 2011).

1.3 Cell sources

The early reliance on BM for clinical transplants has more recently shifted to the widespread use of G-CSF mPB. mPB collections now account for over 75% of hematological transplants (Anasetti et al., 2012). The advantages of mPB include the fact that the cells can be collected by leukapheresis, thus avoiding the risks of the general anesthesia required for collecting sufficient BM from multiple marrow aspirations (Anasetti et al., 2012; Chao et al., 1993; Schmitz et al., 1995; Sheridan et al., 1992). Later, when CB was discovered to contain similarly high concentrations of CFCs interest arose in its potential use as an even readier alternative to mPB collections (Broxmeyer et al., 1989; Nakahata and Ogawa, 1982; Rubinstein et al., 1998). However, recipients of CB transplants were found to have a longer time to recover minimal neutrophil and platelet levels than is typical of BM or mPB transplants (Rocha et al., 2004), with mPB giving the most rapid mature cell recoveries (Anasetti et al., 2012; Couban et al., 2002; Powles et al., 2000). These differences correlate with differences in the absolute numbers of STRCs present in these differently sourced transplants as indicated by quantitative LDAs of STRC activity in immune-deficient mice (Figure 1.2) (Cheung et al., 2012; Christ et al., 2007; Glimm et al., 2001). Nevertheless, assessments of more primitive cells has indicated that CB contains a higher frequency of LTRCs than BM or mPB (Cheung et al., 2012; Holyoake et al., 1999; Wang et al., 1997). Such differences highlight the intrinsic heterogeneities in absolute as well as relative numbers of transplantable cells present in different sources being considered for clinical use, as well as the need to consider the effects of any culture protocol on perturbing these cell types differentially.

1.4 Mobilization of primitive human hematopoietic cells

Despite the extensive use of mPB as a source of cells for the treatment of patients to be given intensive therapies requiring a transplant, or for gene therapy protocols, there remain subsets of patients that fail to mobilize adequate cell numbers (Haas et al., 1994; To et al., 2011). For these individuals, efforts to increase yields of STRCs and LTRCs continue to be of major interest. Early discovery that some GFs induce mobilization of hematopoietic progenitors into the peripheral circulation led to the adoption of G-CSF administration to donors prior to apheresis (Lapidot and Petit, 2002; Sheridan et al., 1992). Although the mechanisms of mobilization are still not fully understood, several adhesion and GF receptors including the VLA-1, CXCR4 and G-CSF receptors have been shown to be involved (Lapidot and Petit, 2002; Morrison et al., 1997; Papayannopoulou and Nakamoto, 1993).

An interesting feature of mobilization is the range of GFs and cytotoxic agents that can elicit this response, albeit with marked differences in efficacy and timing (Lapidot and Petit, 2002). For at least some of these GFs, mobilization of primitive cells does not require that the cognate receptor be expressed on them, as shown by Liu et al. in mice previously made chimeric with transplants containing both G-CSF receptor-deficient and WT hematopoietic cells (Liu et al., 2000). The indirect action of G-CSF was then shown to be due to an increased release of proteolytic enzymes such as elastase and cathepsin G from mature myeloid cells resident in the BM that cleave VCAM-1 on BM stromal cells, thereby inhibiting interactions with VLA-4 expressed on the surface of hematopoietic progenitors (Lévesque et al., 2001). Further

highlighting the indirect activities of G-CSF is the wide range of cell types mobilized, which include LTRCs, STRCs, CFCs and mature myeloid and lymphoid cells.

More recently, plerixafor (also known as AMD3100) has been adopted for the mobilization of hematopoietic cells in certain clinical scenarios. After initially being investigated for its ability to inhibit CXCR4-mediated HIV infection, plerixafor was found to inhibit the interaction between CXCR4 and its ligand CXCL12 (Donzella et al., 1998), an intriguing finding co-incident with the discovery that hematopoietic repopulation requires transplanted cells to express CXCR4 (Aiuti et al., 1997; Kawabata et al., 1999; Peled et al., 1999). The observation that plerixafor administration induces a mild leukocytosis in recipients led to it being investigated as a mobilizing agent (Liles et al., 2003). Early studies revealed that plerixafor mobilizes primitive hematopoietic cells into the PB within 2-8 hours. It was also shown to synergize with prior G-CSF administration, although documentation of this latter effect on mouse HSCs was initially restricted to CFCs in the case of treated humans (Broxmeyer et al., 2005; Liles et al., 2005). Additional studies showed that in humans, the frequency of cells capable of repopulating immunodeficient NOD/SCID mice for 8-10 weeks was greater following plerixafor administration as compared to G-CSF (Hess et al., 2007). Clinical trials then showed that patients given G-CSF plus plerixafor achieved CD34⁺ harvest thresholds after fewer leukapheresis cycles than patients given G-CSF alone (DiPersio et al., 2009a, 2009b; Nademanee et al., 2012). However, whether plerixafor alone can mobilize numbers of repopulating cells comparable to those mobilized by G-CSF with or without plerixafor is not known.

1.5 HSC expansion

1.5.1 Growth factor (GF)-mediated cell expansion strategies

The aim of *ex vivo* stem cell expansion is to increase the number of repopulating cells available for subsequent analysis and/or transplant purposes. Historically, the survival and proliferation of primitive cells *in vitro* has relied upon the addition of growth factors (GF) to liquid suspension cultures initiated with populations that have been pre-enriched in their content of hematopoietic progenitors (e.g., CD34⁺ cells), with the goal of expanding cells that retain LTRC activity after they have divided. With the recognition that hematopoietic recovery early post-transplant is usually dependent on the additional presence in the graft of more differentiated and biologically distinct cells, the effects of expansion systems on STRCs has also attracted interest (Delaney et al., 2010; Zhang et al., 2006).

Accruing evidence points to the survival and proliferation of primitive mouse hematopoietic cells being separately regulated by external factors (Wohrer et al., 2014), with examples also in the human system of synergistic or inhibitory effects on proliferation dependent on the factor combinations and concentrations to which the target cells are exposed (McNiece et al., 1988; Metcalf, 2008; Metcalf and Nicola, 1992; Muench et al., 1992; Okano et al., 1989). Various GF combinations that include SF and those stimulating the gp130 receptor element support optimal proliferation of primitive mouse cells, with some combinations shown to support the maintenance of stem cell functions (Audet et al., 2001, 2002; Bodine et al., 1992; Kent et al., 2008; Miller and Eaves, 1997). GF combinations that act on primitive human hematopoietic cells have also been investigated. Analysis of the concentrations and combinations of SF, FLT3-

Ligand (FLT3L), IL-3, IL-6 and G-CSF for supporting the *in vitro* production of cells detectable as LTC-ICs and CFCs in 10-day serum-free liquid suspension cultures initiated with CD34⁺CD38⁻ BM cells showed that these endpoints were differentially affected, despite equal numbers of initial CD34⁺CD38⁻ cells proliferating at each GF concentration tested (Petzer et al., 1996a; Zandstra et al., 1997). In addition, CB cells were found to have different GF requirements than adult BM, with FLT3L and gp130 stimulation producing the highest output of LTC-ICs from CB CD34⁺CD38⁻ cells (Zandstra et al., 1998). Maximal expansion of CB CFC numbers was dependent on the addition of SF, with optimal output of myeloid and erythroid CFC numbers being generated in cultures containing FLT3L+SF and hIL-6+SF, respectively.

A complicating influence is the presence of multiple cytokines released by the maturing progeny of the initial precursor cells that exert both positive and negative feedback effects (Csaszar et al., 2012). Such complexities and differential requirements for cells of distinct differentiation states, lineage capabilities, and ontogeny underline the necessity of determining GF combinations that are specific for both the input and desired output cells, with significant consideration required for the choice of endpoint assay. With the increasing clinical use of CB cells and with the recent push for *ex vivo* manipulated cells, there is an urgent need to determine optimal conditions for producing expanded numbers of both STRCs and LTRCs.

1.5.2 HSC expansion strategies using novel additives or manipulations

The historic failure to obtain an expansion of HSCs *ex vivo* in cultures supplemented with GFs alone led to searches for transcription factors and small molecules that might achieve this outcome. Screening assays based on increased numbers of CD34⁺ or CD34⁺CD45RA⁻ cells

unveiled a potential role for small molecules in the regulation of primitive human cells, with SR1 and UM171 being found to expand the number of progenitor cells when used in GF-supplemented cultures (Boitano et al., 2010; Fares et al., 2014). Twenty-one day cultures containing SR1 showed a 17-fold increase in cells able to repopulate NSG mice for 16 weeks post-transplant, with a >9,500-fold increase in CFC numbers after 5 weeks compared to control cultures containing GFs only. In a later study, UM171 was found to have a similar effect, with a 13-fold increase in 20-week NSG repopulating cell activity. However, while the effects of SR1 and UM171 on CFC and LTRC production were systematically assessed, their effects on STRC production has not been investigated. Interestingly, both SR1 and UM171 were also shown to expand non-human primate, but not mouse, progenitor cell populations (Boitano et al., 2010; Fares et al., 2014). This lack of cross-reactivity underlines the need for a species-specific model to discover new molecules that control primitive human hematopoietic cell behaviour. Mechanistically, SR1 antagonizes the aryl hydrocarbon receptor, a ubiquitous cytoplasm-located ligand-induced transcription factors previously associated with the metabolism of toxic dioxins (Casado et al., 2010). The molecular target of UM171 has not yet been determined.

The Hox family of transcription factors have also been investigated for their ability to generate expanded populations of primitive cells based on observations of their effects on hematopoietic proliferation and differentiation. Retroviral-mediated transduction and overexpression of HOXB4 or a NUP98-HOXA10hd fusion protein into primitive mouse cells led to an unprecedented production of LTRCs *ex vivo* without signs of leukemic transformation (Antonchuk et al., 2002; Sekulovic et al., 2011). The 1,000-fold increases obtained with the NUP98-HOXA10hd fusion protein suggest that very high rates of HSC self-renewal divisions had been obtained (i.e., theoretically at least 10 “symmetrical” self-renewal divisions out of a

possible 22 in 14 day cultures, assuming a 15-hour cell cycle time). However, analysis of single-cell cultures suggested some recruitment of non-HSCs into the final output of HSCs. On the other hand, LTC-IC and immunodeficient mouse transplant assays of similarly treated human cells showed much more modest effects in terms of the LTRC expansion achieved with either of these same constructs (Amsellem et al., 2003; Sloma et al., 2012). However, experiments in the pig-tailed macaque model have suggested an ability of HOXB4 to expand STRCs (Watts et al., 2011; Zhang et al., 2006).

Activation of Notch via stimulation with Delta1 ligand has been reported to expand human STRCs (Delaney et al., 2010). Here, immobilized ligand was used with a GF combination consisting of SF, FLT3L, TPO, IL-6 and IL-3, and expansion of STRCs in 17- to 21-day cultures was assessed by transplanting the recovered cells in NOD/SCID mice. NOD/SCID mice showed a 6-fold higher level of chimerism and a 3-fold higher frequency of 3-week repopulating cells when transplanted with cells from cultures that contained Delta1 plus GFs versus GFs alone. Furthermore, both of these conditions enabled higher levels of chimerism to be achieved at this time point than non-cultured control cells, suggesting that GFs alone enhanced STRC production. However, these results are difficult to interpret as STRCs are thought to engraft NOD/SCID mice relatively poorly (Glimm et al., 2001).

CD34⁺ cells from one CB unit cultured in Delta1 plus the same GFs were also transplanted into 10 patients with high-risk acute leukemia in morphologic remission, together with a co-transplant of a second, unmanipulated CB unit. The median recovery of 500 neutrophils per μ L was achieved in 16 days in these recipients, compared to 26 days for similar patients receiving two unmanipulated CB units. DNA polymorphism analysis of the PB neutrophils revealed an almost exclusive contribution from the expanded unit for up to 7 days in

all 8 patients, and up to 14 days in 3 of 8 patients, although only one patient had a >50% contribution from the cultured unit at day 21 post-transplant.

1.5.3 Enhancement of cell homing

Rather than increasing the number of repopulating cells, attempts have also been made to increase the efficiency with which such cells might enter the marrow immediately post-transplant. After a 2-hour treatment with prostaglandin E₂ (PGE₂) was found to increase zebrafish hematopoiesis, experiments with mouse BM cells showed that their brief exposure to PGE₂ could provide a 4-fold enhancement of 12-week repopulation, although this effect was diminished to 2-fold by 24 weeks (North et al., 2007). Later studies suggested that this effect was due to increased homing of treated cells, although increased cell survival and proliferation of both human and mouse cells were subsequently reported also (Goessling et al., 2011; Hoggatt et al., 2009). A 15-minute treatment with the tripeptide Diprotin-A (DPA) has been shown to enhance the BM homing of primitive mouse cells and lead to increased engraftment of immunodeficient mice transplanted with human CD34⁺ cells (Campbell et al., 2007; Christopherson et al., 2004). DPA acts by inhibiting dipeptidyl-peptidase IV (DPP-IV) activity on CD34⁺ cells that otherwise inactivates the chemotactic activity of CXCL12 (Christopherson et al., 2002).

Single-cell transplants with mouse cells have indicated that LTRCs repopulate with near-absolute efficiency (Benz et al., 2012; Kent et al., 2009). This implies that any reported enhancement of LTRC activity is likely to be explained by recruitment of other cell types into

this pool. Further studies are warranted to determine if these agents are active on freshly isolated STRCs or STRCs that have been expanded in the presence of GFs (Wyss et al., 2009).

1.5.4 *Ex vivo* manipulated transplants

Despite over 3 decades of preclinical and clinical research, methods that significantly increase the number of human STRCs or LTRCs are still lacking. Confidence in early culture methods was based on the ability to robustly expand CFC- and LTC-IC-containing populations using defined combinations of GFs. This then led to the initiation of several clinical trials, the results of which were, however, disappointing, with delayed neutrophil and platelet reconstitution and, in some cases, a required use of backup cells (Barnett et al., 1989, 1994; Chang et al., 1986; Engelhardt, 2001, 2001; Holyoake et al., 1997; Shpall et al., 2002).

Syngeneic and congenic mouse transplant experiments using *ex vivo* cultured BM cells later revealed similar decreases in early post-transplant reconstitution after as little as 48 hours of culture (Kittler et al., 1997; Peters et al., 1995, 1996; Spooncer and Dexter, 1983; Vallera and Blazar, 1988), although contradictory examples also exist (Holyoake et al., 1996; Muench et al., 1993). Deleterious effects were also reported for transplants of cultured autologous cells from non-human primates (Takatoku et al., 2001; Tisdale et al., 1998). The development of immunodeficient NOD/SCID and NSG mice that can be engrafted with human cells revealed that human CB or mPB cells cultured for 4-7 days in a variety of GF combinations provided decreased levels of week 3 myeloid and platelet engraftment as compared to the freshly isolated controls (Gan et al., 1997; Guenechea et al., 1999; Holmes et al., 2012; Mazurier et al., 2004; Perez et al., 2001).

A major lesson from these studies was that many *in vitro* assays lose their ability to predict *in vivo* function for hematopoietic cells that have been cultured. What has remained unresolved is the extent to which the *in vitro* assays detect distinct cell types that expand differentially in culture and/or potential negative effects of the culture conditions on cell certain characteristics specifically required for engraftment or survival and proliferation *in vivo*. Another question is whether present culture systems are deficient for certain critical agents or whether the problem is actively induced by the presence of particular factors.

The concept of a GF-induced loss of ability to home into the BM was first suggested by the discovery that the 16-hour seeding of mouse BM CFCs is markedly decreased after their incubation for 2-3 hours in IL-3 or IL-3 + IL-12 + SF (Loo and Ploemacher, 1995). This finding contrasted with several earlier *in vitro* studies demonstrating an increased adhesiveness of GF-exposed cells due to activation of VLA-4 and VLA-5 integrins (Kovach et al., 1995; Lévesque et al., 1995). Later reports also found dramatically reduced mouse CFC homing efficiencies after 9 days of culture with IL-3 + IL-6 + G-CSF + SF (Szilvassy et al., 1999) and human CD34⁺ mPB cells cultured for 2 to 5 days in SF + FLT3L + IL-3 + L-6 displayed a similarly reduced homing into the BM of transplanted NOD/SCID mice (Ahmed et al., 2004).

Subsequent reports of a human GF-induced homing defect encompassed a range of different GF combinations, cell detection methods, and xenotransplant assay systems, suggesting the potential induction of this response by a wide range of stimuli (Bonig et al., 2006; Kallinikou et al., 2012; Larochelle et al., 2012; Wyss et al., 2009). Additionally, Yamamura et al. found that while direct intra-femoral injection of cultured CB cells improved the week 6 engraftment of the injected femur, post-transplant migration of repopulating cells to other marrow sites was greatly diminished as compared to recipients of non-cultured cells (Yamamura et al., 2008).

An important question is whether the homing defect is caused by a loss or a gain of function. A hypothetical example of the latter could be sequestration of cultured cells in the lung, leading to cell death prior to BM seeding. GF-induced lung sequestration of neutrophils is well-documented, with IL-6 exposure leading to increased neutrophil rigidity and induced sequestration in the narrow capillaries of the lung (Doerschuk et al., 1993; Suwa et al., 2001). While transient accumulation of (non-cultured) transplanted cells has been shown in recipient mouse lungs, whether this is exacerbated post-culture has not been directly examined (Ahmed et al., 2004).

An obvious candidate mechanism for decreased cell homing is a change in cell adhesion receptor activity. Several studies of murine and human cells have shown that the expression of integrins associated with progenitor homing is dysregulated after the cells have been in culture for 24 hours (Berrios et al., 2001; Bonig et al., 2006; Kallinikou et al., 2012; Larochelle et al., 2012; Ramírez et al., 2001; Szilvassy et al., 2001). The demonstration of an ability of GFs to modulate integrin function as well as CXCR4 receptor-activated signaling has added further weight to this possibility (Ali, 1999; Hughes and Pfaff, 1998; Wang et al., 2001). However, whether such modulation of such receptors and their downstream signaling is of functional significance to the homing functions of repopulating cells had yet to be determined.

1.6 Thesis objectives

In summary, there exists a subset of repopulating cells that are responsible for a time-limited wave of neutrophil and platelet production early after transplant. These STRCs have distinct biological properties when compared to the cells capable of mature outputs of longer duration.

They are also an important component of transplants in patients receiving myeloablative preconditioning, although their numbers are reduced in CB and in some patient's autologous harvests. The lack of understanding of STRC biology has contributed to a deficit of quantitative assays for their detection and methods to manipulate and or increase their numbers *ex vivo*. There also exists a poor understanding of methods for mobilizing optimal numbers into the circulation to enable their harvest, using GFs or small molecules.

While *in vitro* CFC and flow cytometric-based assays have been available for some time, these are insufficiently predictive of early post-transplant engraftment, particularly when novel mobilizations strategies or *ex vivo* manipulated cells are used. The need for more specific STRC assays is gaining increasing importance with the parallel development of HSC expansion protocols, which will require verification that the STRC content of such transplants remain adequate to ensure rapid neutrophil and platelet recovery. The failure of increased CFC yields with current *ex vivo* expansion strategies to translate into improved clinical practices is poorly understood and justifies their detailed study. In addition, determining protocols for maximizing yields of mobilized STRCs is of great clinical importance.

The overall aims of this thesis were thus as follows:

- 1) To develop a precise and sensitive method to detect and quantify human STRCs *in vivo*.
- 2) To determine the optimal donor mobilization protocol for the maximal yield of human STRCs.
- 3) To quantify and further the understanding of the inability to expand STRC numbers *ex vivo*.

(Sections of a review entitled “**Heterogeneity in hematopoietic stem cell populations: implications for transplantation**” published in *Current Opinion in Hematology* were used to contribute to Chapter 1.)

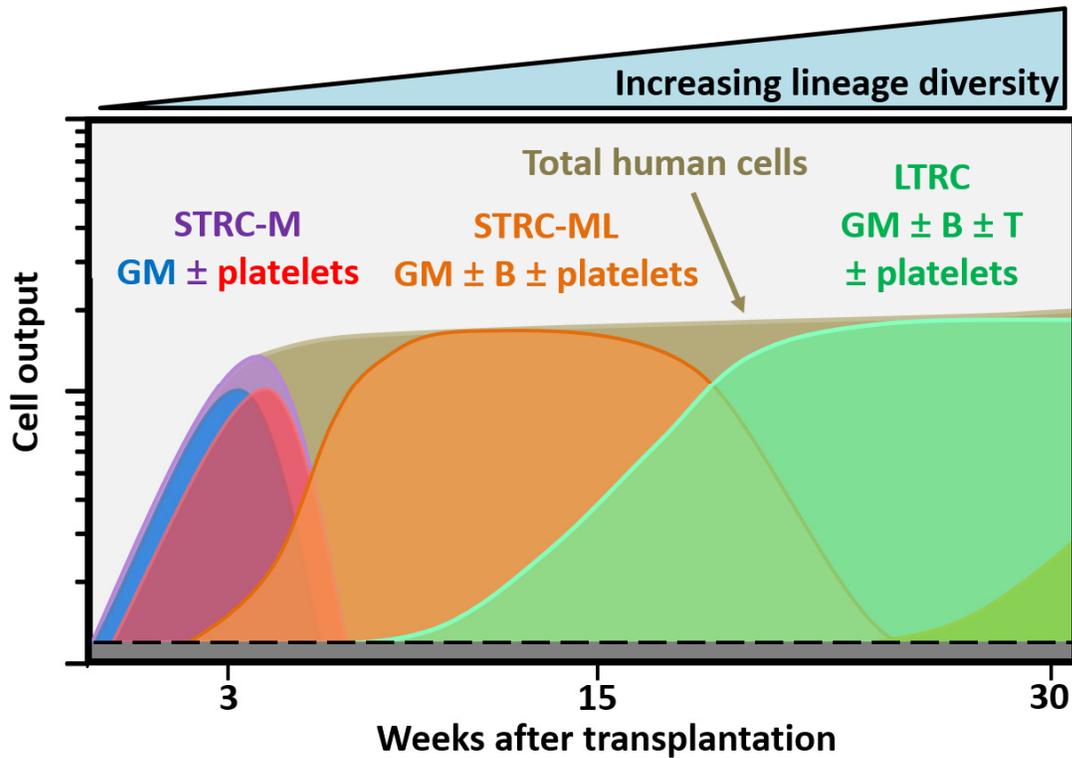


Figure 1.1. Multiple human hematopoietic cell types contribute to the mature blood cells produced at different times in heavily myelosuppressed recipients, as modeled in transplanted sublethally irradiated immunodeficient mice.

Repopulation of highly immunodeficient mice by human cells represents the cumulative outputs of biologically distinct types of repopulating cells with generally increasingly broad and more durable lineage outputs. The first repopulating cells to produce detectable numbers of progeny in this model include separate subsets that are granulocyte-monocyte (GM)- or platelet-restricted and produce clones that disappear within 5 weeks. Subsequently, clones that contain both lymphoid and myeloid cells are more common even though most of these are not sustained beyond 20 weeks. Mature blood cells produced at much later time-points (dark green) are derived from clones that are not apparent above the limit of detection (represented by the dashed line) until more than 20 weeks after transplantation.

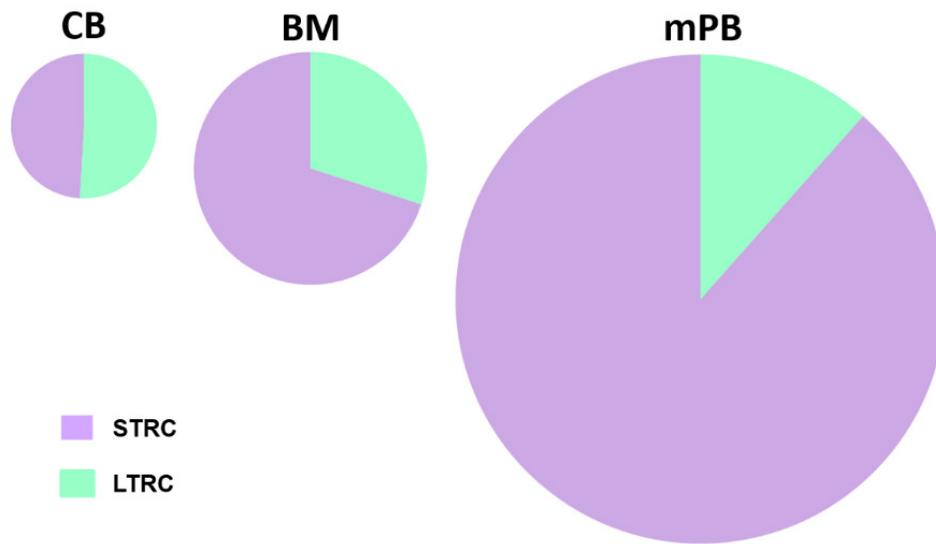


Figure 1.2. The relative abundance of STRCs and LTRCs in a typical transplant of normal human CB, BM, and G-CSF mPB normalized for the same body weight of the recipient.

The circles illustrate the similar LTRC content but variable content of STRCs in these different transplant sources as described (Cheung et al., 2012; Christ et al., 2007; Glimm et al., 2001).

Chapter 2: Enhanced Normal Short-Term Human Myelopoiesis in Mice Engineered to Express Human-Specific Myeloid Growth Factors

2.1 Introduction

Current evidence suggests that human hematopoiesis involves the production of sequential stages of phenotypically separable populations of cells with increasingly restricted lineage options and decreasing self-renewal abilities (Doulatov et al., 2012). These subsets are thus capable of different durations and spectra of differentiated cell outputs upon transplantation into myelosuppressed recipients. Interestingly, the most primitive of these cell types engraft T-cell and B-cell-depleted mice at high efficiency; whereas those with short term repopulating activity (STRCs, i.e., limited to a few weeks) do not unless the NK cells of the mice are also completely eliminated (Eaves et al., 2001; Shultz et al., 2003). Efficient engraftment of STRCs with myeloid-restricted activity (STRC-Ms) is currently most effectively achieved in NSG mice (Shultz et al., 2005). This has heightened interest in the use of NSG mice to design specific and sensitive functional assays for these cells. Characterization of human STRCs and their expansion is of particular interest for clinical transplantation protocols that could benefit from improved methods to predict and accelerate neutrophil (and platelet) recovery in patients (Cheung et al., 2012). However, this has remained a challenge because of the poor output of human neutrophils in NSG mice, which is insufficient for PB sampling to be used to assess transplanted STRC activity. We now show that this difficulty can be overcome using NSG mice bred to previously engineered transgenic mice (Nicolini et al., 2004) to enable their constitutive expression of human IL-3, GM-CSF and SF to enhance stimulation of human hematopoiesis.

2.2 Methods

2.2.1 Cells

CD34⁺ cell-enriched (>80% pure) suspensions of low-density human CB cells were isolated using the EasySep kit (STEMCELL Technologies, Vancouver, BC, Canada) from previously pooled and anonymized samples (collected according to procedures approved by the University of British Columbia Research Ethics Board). Lineage marker-negative (Lin⁻) CD34⁺ cells were isolated as described (Cheung et al., 2012). All samples were cryopreserved until use.

2.2.2 Transplantation procedure

Cells were injected intravenously along with 10⁶ irradiated (15 Gy) human BM cells into 8-10 week-old NSG or NSG-3GS mice (bred and maintained in the Animal Resource Centre of the BC Cancer Research Centre) within 24 hours of their being irradiated (315 cGy ¹³⁷Cs γ -rays). The frequency of human myeloid (CD45⁺CD33/15⁺) cells present in BM aspirates and 50-100 μ L of PB samples was determined by flow cytometry analysis of cells labeled with appropriate antibodies as previously described (Cheung et al., 2012). Absolute values for circulating human myeloid cells were derived using AccuCheck counting beads (Invitrogen, Burlington, ON, Canada). To detect circulating human platelets in the PB, a 20 μ L sample of blood was collected from each mouse. Total mouse platelet counts were recorded with an automated animal blood cell counter (Scil Vet ABC). Blood samples were stained directly with human-specific anti-CD41a (HIP8) and mouse-specific anti-CD41 (MWreg30; both from BD Pharmingen) for 30

minutes at room temperature. Two hundred microliters of 0.8% NH₄OH were then added to each sample to lyse the RBCs present during a 5-minute period of incubation at room temperature. Samples were then diluted with 200 µL of HEPES-Tyrode buffer (10 mM HEPES, 137 mM NaCl, 268 mM KCl, 0.42 mM NaH₂PO₄, 1.7 mM MgCl₂, 11.9 mM NaHCO₃ and 5 mM glucose) and analyzed by flow cytometry immediately. All animal studies were approved by the Animal Care Committee of the University of British Columbia.

2.2.3 Lenti-viral transduction and bioluminescence imaging

CD34⁺ CB cells were prestimulated for 16 hours in a serum-free medium containing BIT (STEMCELL Technologies) and 100 ng/mL FLT3-L (Immunex, Seattle, WA, USA), 100 ng/mL SF and 20 ng/mL G-CSF (STEMCELL Technologies), 20 ng/mL IL-3 (Novartis, Basel, Switzerland) and 20 ng/mL IL-6 (Cangene, Winnipeg, MA, Canada), then incubated for 6 hours in the same medium containing a MND-lentivirus encoding a firefly luciferase cDNA (MND-LUC) and then immediately washed and transplanted. To collect bioluminescent images, anaesthetized mice were injected intraperitoneally with 150 mg/kg D-luciferin (Caliper Life Sciences, Hopkinton, MA, USA) and 10 minutes later placed in the supine position in a Xenogen IVIS Lumina system (f stop 1, binning factor 4, and 60 seconds) with Living Image version 3.0 software (Caliper Life Sciences).

2.3 Results

2.3.1 NSG-3GS mice support a 5-fold increase in myeloid cell outputs from transplanted human STRCs but do not change the frequency of STRCs detected.

NSG-3GS mice were generated by crossing NSG mice with NS-3GS mice (Nicolini et al., 2004) and selecting for F2 progeny homozygous for both the absence of the IL-2R γ c gene and the presence of the human 3GS cDNA transgene insert as carried out independently by the Mulloy lab (Wunderlich et al., 2010). We then transplanted both genotypes of recipients with 10^4 lin $^-$ CD34 $^+$ CB cells to first compare the levels of human chimerism achieved in their BM (Figure 2.1A & Figure 2.2A) and PB (Figure 2.1B & Figure 2.2B) from 3 to 20 weeks later. This period was chosen because it has been previously shown to encompass the sequential outputs of mature cells produced by different subsets of STRCs (Glimm et al., 2001; Hogan et al., 2002; Majeti et al., 2007). The first of these are STRC-Ms which are predominantly CD34 $^+$ CD38 $^+$ (Glimm et al., 2001) and correlate with cells detected *in vitro* as primitive CFCs (Cheung et al., 2012). STRC numbers are inferred from their peak mature cell outputs seen at 3 weeks post-transplant. STRC-M outputs are then followed by the progeny of CD34 $^+$ CD38 $^-$ CD90 $^-$ cells that have lymphoid as well as myeloid potential (STRC-MLs) but, because these have limited self-renewal ability, the production of mature progeny is not sustained beyond 16-20 weeks post-transplant (Glimm et al., 2001; Hogan et al., 2002; Majeti et al., 2007). The results of our time course comparison of cell outputs revealed a markedly elevated output of myeloid cells in NSG-3GS BM and PB during the first 12 weeks. Interestingly, this period also coincides with the time taken for the BM

cellularity of non-transplanted but similarly irradiated NSG recipients to recover maximally (Figure 2.1C).

These results suggest that the NSG-3GS host enhances mature myeloid cell outputs from STRCs at the stage of terminal myelopoiesis. To address this question we focused on a more detailed study of STRC-Ms. A cell dose-response analysis showed that GM outputs in both BM (Figure 2.1D) and PB (Figure 2.1E) were linearly related to the number of CD34⁺ cells injected over a wide range in both host genotypes (up to at least 10⁴ CD34⁺ cells per NSG-3GS mouse and >6x10⁴ CD34⁺ cells per NSG mouse). These results also confirm the ~5-fold higher output of human GM cells in the NSG-3GS mice and show that it is transplant cell dose-independent. As a result, human GM cells are readily detectable in the PB of NSG-3GS recipients even when small numbers of human CD34⁺ cells are transplanted in contrast to NSG mice where human GM cells are close to the limit of detection for transplant doses of <10⁴ CD34⁺ CB cells. Morphological analyses confirmed that the human GM cells in the PB were human neutrophils (Figure 2.1F).

The established linearity of the cell dose-response experiments validated the use of LDA to quantify the efficiency of STRC-M detection in the 2 host genotypes. The results showed that the frequency of STRC-Ms detected in both types of recipient was the same (1/870 and 1/600 CD34⁺ cells, p=0.35, see Table 2.1 for details). Thus the difference in 3-week human neutrophil outputs measured in the 2 strains is entirely attributable to a difference in the clonal outputs from similar numbers of STRCs.

2.3.2 Increased support of mature human myeloid cell production results in increased blood chimerism and improved monitoring of human chimerism by blood sampling.

These findings suggested that NSG-3GS mice enable PB data to be used for LDA measurements of STRC-Ms and thereby circumvent some of the inter-mouse variability expected at early times post-transplant when the host BM is still recovering (Figure 2.1C). To investigate this possibility, we transplanted both genotypes of mice with $4-30 \times 10^3$ CD34⁺ CB cells that had been exposed to a lentivirus encoding the firefly luciferase cDNA. Assessment of their total body bioluminescence 3 weeks later revealed a highly heterogeneous distribution of labeled human cells (Figure 2.3A). A similar heterogeneity was mirrored in the level of human myeloid chimerism measured in paired aspirates of the right and left femurs of individual recipients of both strains (Figure 2.3B). Although a significant correlation between BM and PB values was achieved when the results for both femurs were combined (Figure 2.3C), the correlation between the bioluminescent endpoint and absolute PB counts of human cells was even stronger (Figure 2.3D).

2.4 Discussion

Together these findings demonstrate the ability of NSG-3GS mice to enable more precise and efficient determination of intravenously injected human STRCs using simple PB sampling and assessment methods. This assay will facilitate the characterization of factors that determine their properties and generation *in vivo* and *in vitro*, and thereby lead to improved adult transplant protocols where the STRC content of freshly isolated or cultured/genetically manipulated

transplant harvests remains a serious limitation (Bensinger et al., 2009; Brunstein and Wagner, 2006; Haas et al., 1994; Kallinikou et al., 2012; Rocha and Gluckman, 2006).

(Chapter 2 has been published in the journal *Blood* as a manuscript entitled “**Enhanced normal short-term human myelopoiesis in mice engineered to express human-specific myeloid growth factors**”.)

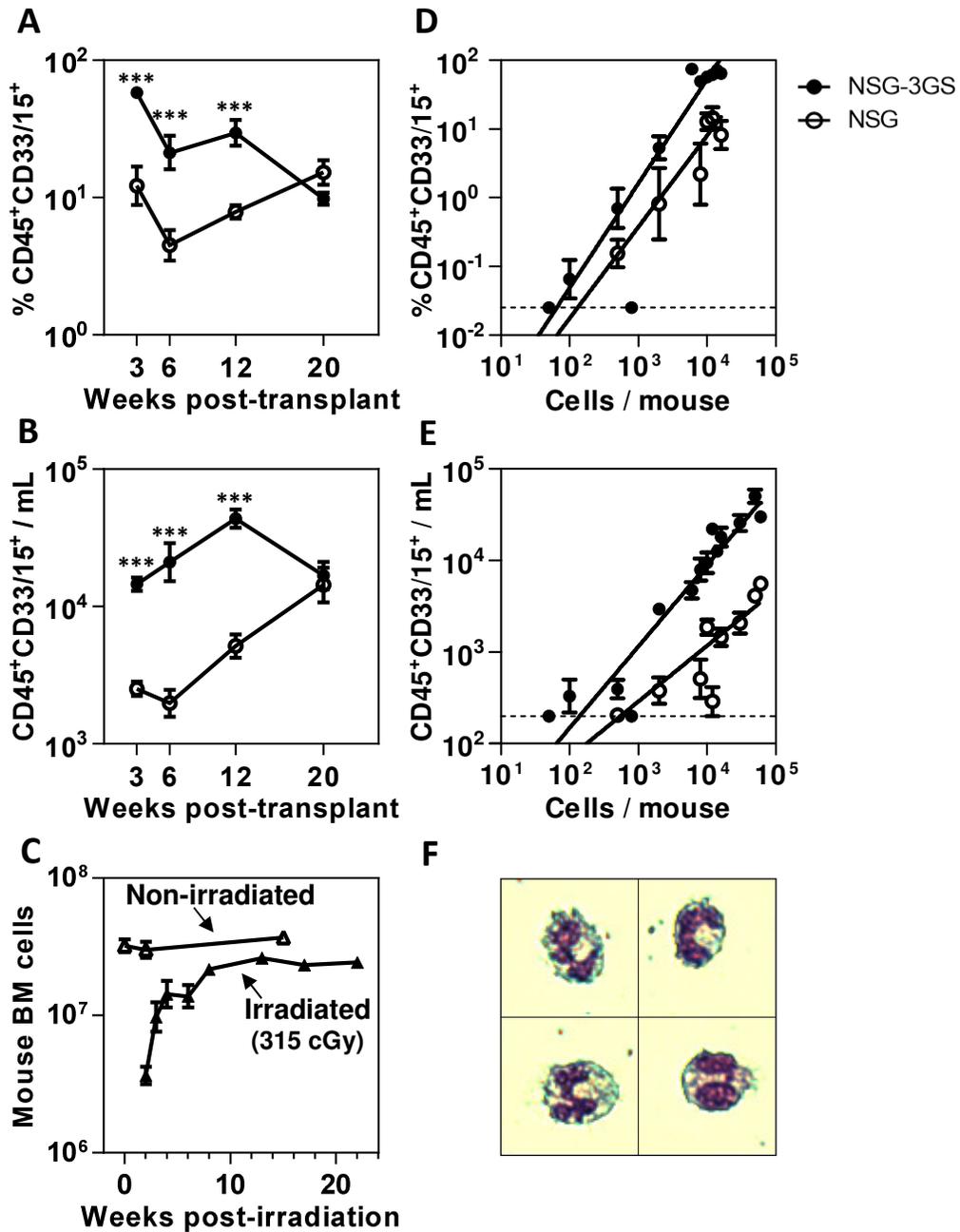


Figure 2.1. Increased short-term myeloid cell output in NSG-3GS mouse BM and PB.

Kinetics of myeloid cell (CD45⁺CD33/15⁺) reconstitution in the BM (A) and PB (B) of NSG (open circles) and NSG-3GS (closed circles) mice after their transplantation with 10⁴ lin⁻CD34⁺ CB cells. Data are the results of 12 mice per group pooled from 2 independent comparison experiments. (C) Total mouse BM cells per 2 femurs plus 2 tibias at the times indicated after

irradiation of mice with 315 cGy ^{137}Cs but no transplanted cells (3-10 mice per time point). (D) Linear relationship between the number of CD34^+ CB cells transplanted and the extent of human myeloid cell chimerism produced in the BM of NSG and NSG-3GS mice 3 weeks after transplantation of $50 - 1.6 \times 10^4$ CD34^+ CB cells (3-33 mice per cell dose). (E) Linear relationship between the number of CD34^+ CB cells transplanted and the absolute number of circulating human myeloid cells in NSG and NSG-3GS mice 3 weeks later (3-33 mice per cell dose). (F) Wright-Giemsa stained human $\text{CD45}^+\text{CD33}/15^+$ cells in the PB of NSG-3GS mice 3 weeks after their transplantation with human CD34^+ CB cells, representing >90% of the sorted $\text{CD45}^+\text{CD33}/15^+$ cells analyzed. All values shown are the mean \pm SEM. The dotted lines in panels D and E indicate the limit of detection of human $\text{CD45}^+\text{CD33}/15^+$ cells in BM and PB samples, respectively. Asterisks indicate statistically significant differences between results obtained in NSG and NSG-3GS mice using the Student t-test (* = $p \leq 0.05$; ** = $p \leq 0.01$; *** = $p \leq 0.001$).

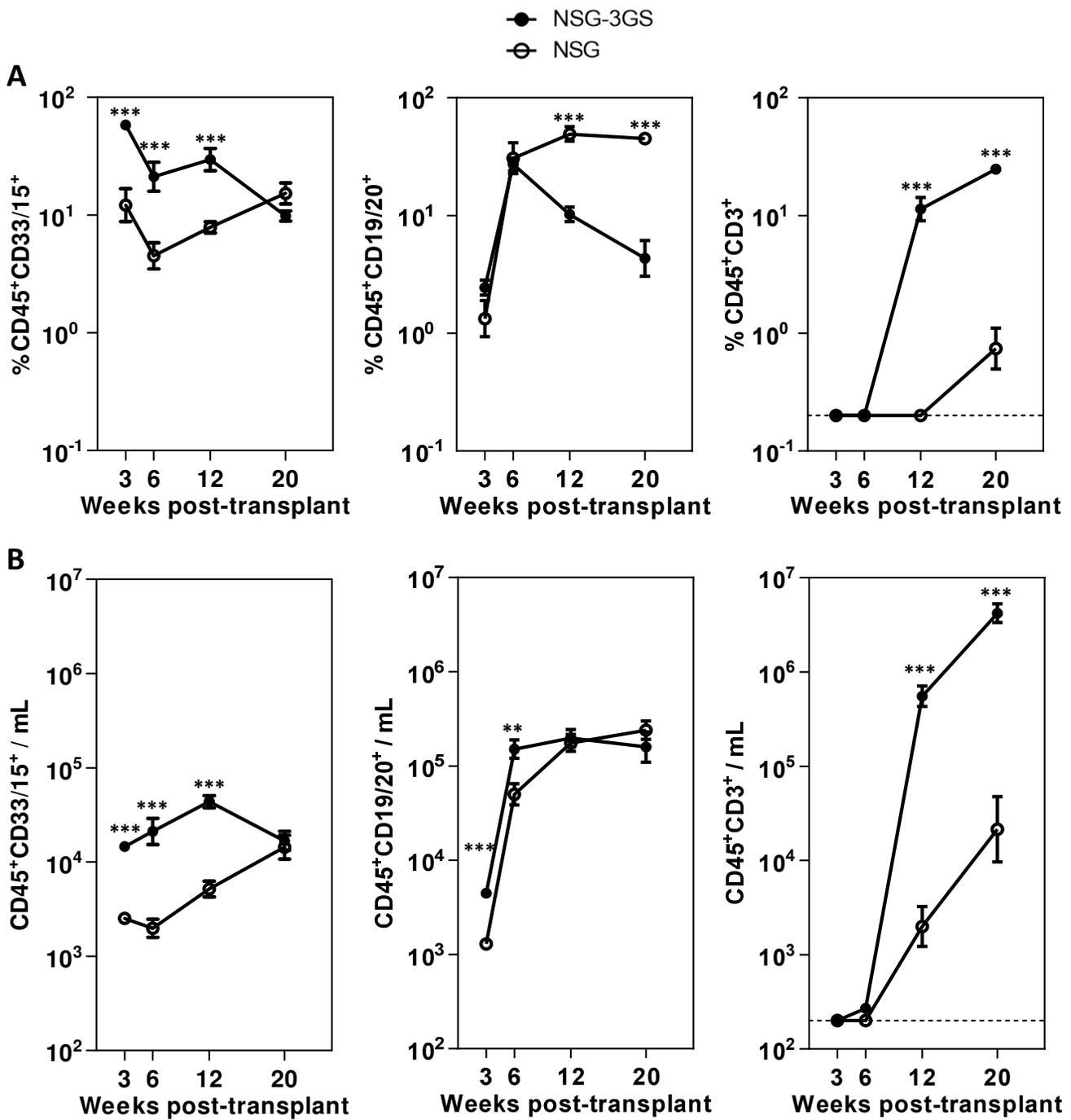


Figure 2.2. Increased short-term mature cell output in NSG-3GS mouse BM and PB.

Kinetics of human GM, B-lineage and T-cell reconstitution in the BM (A) and PB (B) of NSG (open circles) and NSG-3GS (closed circles) mice after their transplantation with 10^4 lin⁻CD34⁺ CB cells. Data are from the same experiments shown in Figure 2.1 (12 mice/group pooled from 2

independent experiments). Asterisks indicate statistically significant differences between the results obtained in NSG and NSG-3GS mice using the Student t-test (* = $p \leq 0.05$; ** = $p \leq 0.01$; *** = $p \leq 0.001$).

Table 2.1. Frequency of human STRC-Ms detected in NSG and NSG-3GS mice based on achievement of detectable BM chimerism 3 weeks post-transplant.

CD34 ⁺ cell dose	No. of positive/Total mice	
	NSG	NSG-3GS
50		0/3
100		2/6
500	9/18	10/15
800		0/3
2000	3/4	7/7
8000	4/4	4/4
Overall frequency	1 in 870	1 in 600
95% CI	1 in 470 - 1 in 1600	1 in 350 - 1 in 1000
Chi-square P-value	0.35	

Mice were injected IV with a range of CD34⁺ CB cell numbers and 3 weeks later the mice were sacrificed and analyzed individually for the presence of viable human CD45⁺CD33/15⁺ cells in the cells harvested from both femurs. Mice were classified as positive if 5 or more human CD45⁺CD33/15⁺ events were detected per 20,000 PI⁺ cells analyzed (i.e., $\geq 0.025\%$ of total viable mouse BM cells). Data from 4 independent experiments have been pooled. Limiting dilution analysis for the calculation STRC-M frequencies was performed using bioinformatics software (<http://bioinf.wehi.edu.au/software/elda/>) from the Walter and Eliza Hall Institute of Medical Research (Hu and Smyth, 2009).

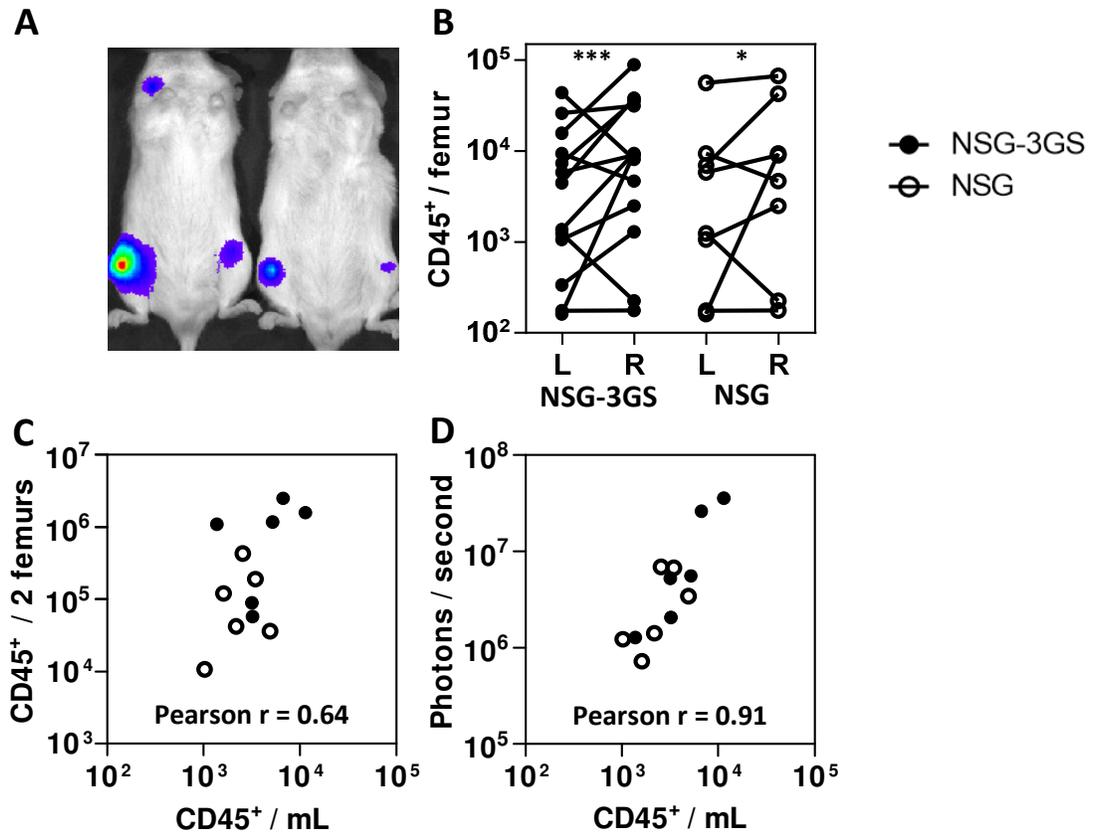


Figure 2.3. Circulating human cell and in vivo bioluminescence counts enable accurate assessment of total human cell engraftment.

NSG-3GS (closed circles) and NSG (open circles) mice were transplanted with $0.4\text{--}3 \times 10^4$ luciferase-transduced CD34⁺ CB cells and 3 weeks later were assessed by bioluminescent imaging. (A) Representative image of transplanted mice (examples shown are NSG-3GS mice). (B) Total human cell counts in the left (L) and right (R) femurs in individually assessed NSG-3GS and NSG mice. Lines connect paired cell counts from individual mice which were significantly different ($P < 0.05$) for both strains. (C) Association between the absolute number of circulating human cells with the total number of human cells in both femurs (Pearson $r = 0.64$, $P = 0.02$). (D) Association between the absolute number of circulating human cells with photons

emitted per second per mouse (Pearson $r = 0.91$, $P < 0.001$). Asterisks indicate statistically significant differences between left and right femurs determined using the Student t-test (* = $p \leq 0.05$; ** = $p \leq 0.01$; *** = $p \leq 0.001$).

Chapter 3: Plerixafor Plus G-CSF is a Superior Mobilizing Regimen for Cells that Produce Neutrophils and Platelets Rapidly *In Vivo*

3.1 Introduction

PB collected by leukapheresis from normal donors given G-CSF is now the most common type of allogeneic transplant used to regenerate hematopoiesis in patients given intensive therapy. Recipients of such G-CSF mobilized PB (G-PB) allografts typically recover protective levels of circulating neutrophils and platelets faster than recipients of bone marrow (BM) cells from unmanipulated donors, with an associated reduction in infectious complications, supportive care requirements, and healthcare costs (Anasetti et al., 2012; Couban et al., 2002; Powles et al., 2000; Smith et al., 1997; To et al., 1992). Nevertheless, to obtain an optimal G-PB collection requires several days of prior G-CSF administration, with occasional associated side effects to the donor and a higher incidence of graft-versus-host disease (GVHD) in the recipients (Cutler et al., 2001; Haas et al., 1994). Additional studies have now suggested that the increased GVHD obtained with G-PB transplants can be abrogated using BM cells from G-CSF-treated donors, but with the accompanying disadvantages associated with harvesting BM (Couban et al., 2000; Morton et al., 2001; Shier et al., 2004).

More recently, plerixafor (also known as AMD3100), a small molecule antagonist of CXCR4 (Donzella et al., 1998), was found to enhance the mobilizing activity of G-CSF. This made it possible to collect the desired yields of CD34⁺ cells in fewer leukapheresis cycles, with similar speeds of neutrophil recovery in the recipients post-transplant (DiPersio et al., 2009a, 2009b; Flomenberg et al., 2005; Nademanee et al., 2012). Indeed, a single plerixafor injection

was found to mobilize sufficient progenitors into the PB within a few hours (Devine et al., 2004; Liles et al., 2003) to produce neutrophil and platelet counts in subsequently transplanted recipients as quickly as is achieved with G-PB (Devine et al., 2008; Flomenberg et al., 2010). Interestingly, despite reports of suboptimal harvests of CD34⁺ cells in plerixafor-PB harvests, the concentration of cells able to repopulate NOD/SCID mice for 7-8 weeks was higher than that measured in G-PB harvests. This suggested that NOD/SCID mouse repopulating activity may be a more relevant and predictive parameter than CD34⁺ cell measurements (Hess et al., 2007). These latter results are perhaps not surprising given the known heterogeneity of the CD34⁺ compartment. They also highlight the need for more predictive assays to anticipate the utility of donor harvests for clinical allografting applications and the potential of functional rather than phenotypic endpoints to serve this purpose. This issue is becoming increasingly important with growing interest and possibilities to use novel sources or manipulated hematopoietic cells for specific transplantation applications.

To address these issues, we designed a study to compare the results of multiple *in vitro* and *in vivo* assays for cells with platelet and neutrophil output potential when these were applied to PB and BM samples obtained from normal adult human donors before and after their treatment either with plerixafor alone, or before and after a course of G-CSF+plerixafor. The results indicate a high concordance in the cells detected by *in vitro* and *in vivo* assays of the same duration and lineage output. They also indicate G-CSF+plerixafor to be superior to plerixafor alone and PB to be superior to BM with an optimal PB collection time 4 hours after plerixafor administration.

3.2 Methods

3.2.1 Human cell samples

Approval was obtained from the Research Ethics Review Boards of Dalhousie University (Halifax, NS, Canada), Memorial University (St Johns, NF, Canada), and the British Columbia Cancer Agency (Vancouver, BC, Canada) to enroll 10 healthy, consenting adult volunteers (18-30 years old) into the study. Five were given a subcutaneous injection of 240 $\mu\text{g}/\text{kg}$ plerixafor (supplied by Genzyme) 24 hours after a 4 day course of 5 $\mu\text{g}/\text{kg}/\text{day}$ G-CSF (from Amgen). The other 5 received no G-CSF prior to the injection of plerixafor. PB and BM samples were obtained pre- (baseline) and post-treatment as shown in Figure 3.1A. Exclusion criteria were any of the following: medical comorbidities (specifically, cardiopulmonary disease, autoimmune disease, chronic skin conditions, sickle cell disease, or obesity), pregnancy or lactation, splenomegaly, known allergy to lidocaine, known hypersensitivity to *Escherichia coli*-derived products, or abnormalities on screening complete blood count or liver enzymes. All volunteers were followed for 30 days post-treatment, and no adverse reactions were noted.

All samples were collected in Halifax, NS and then sent by overnight courier on wet icepacks to Vancouver, BC where low-density cells isolated by centrifugation on Lymphoprep (STEMCELL Technologies) and aliquots assessed directly for their content of total nucleated cells (TNC), CD34⁺ cells, and colony-forming cells (CFCs), either per mL of PB, or per 10⁵ viable BM cells. The remaining cells were cryopreserved in 10% DMSO and >50% fetal calf serum (STEMCELL Technologies) and then stored in a vapour phase liquid N₂ freezer. After all samples were accrued, cells were thawed, depleted of viable T-cells by *in vitro* incubation with

diphtheria-toxin conjugated anti-CD3 antibody (kindly provided by Dr. David Neville, Angimmune LLC, Bethesda, MD), and then assessed for different progenitor types. Calculated equivalent pre-freeze aliquots of like samples were then either combined into a series of pools or, in some cases, also assessed individually, as indicated (Figures 3.1B and 3.3A). Expected cell recoveries after thawing were obtained from a comparison of the values for total starting CD34⁺ cells and CFCs, respectively (obtained by averaging those measured individually on like pre-freeze samples), with the corresponding values for post-thaw cells (obtained on the pooled like post-thaw samples, Figure 3.1C).

3.2.2 Flow cytometry

CD34⁺ cell analyses were performed prior to cryopreservation of the samples according to the ISHAGE protocol (Keeney et al., 1998). Absolute numbers of human neutrophils and monocytes (CD45⁺CD33/15⁺ cells), lymphocytes (CD45⁺CD19⁺ cells), and platelets (FSC^{low}CD41⁺CD61⁺cells) in PB samples obtained from transplanted mice were determined using AccuCheck counting beads (Life Technologies) as previously described (Cheung et al., 2012; Miller et al., 2013a).

3.2.3 *In vitro* assays

CFC assays were performed by plating an equivalent of 20 μ L of whole PB or 5×10^4 BM cells into growth factor-supplemented methylcellulose culture medium (H4435, STEMCELL Technologies) and all colonies present counted 14-16 days later as described (Hogge et al.,

1996). Assays of long term culture-initiating cell (LTC-IC) activity were initiated by plating a T-depleted starting cell equivalent of 80 μL of whole PB or 2×10^5 low-density BM cells into replicate 2.5 mL cultures containing pre-established irradiated feeder layers of murine fibroblasts secreting human G-CSF, IL-3, SF, and FLT3-L (Beer et al., 2014; Hogge et al., 1996). These cultures were maintained in Myelocult (STEMCELL Technologies) supplemented with hydrocortisone as previously described for 3 or 6 weeks prior to harvesting each entire culture and assessing its CFC content which was then used as a retrospective measure of the corresponding input LTC-IC activity.

3.2.4 Xenotransplant assays

8-12 week-old NOD-*Rag1^{null}-Il2r γ ^{null}* mice engineered transgenically to express human IL-3, human GM-CSF, and human SF (NRG-3GS mice) were given a sublethal dose of 900 cGy ^{137}Cs γ -rays delivered over 3 hours. This protocol was found in preliminary experiments to give full long term survival of NRG mice and a pace and level of reconstitution with human cord blood cells equivalent to that obtained in NSG mice given 315 cGy ^{137}Cs γ -rays (unpublished data). Mice were then injected intravenously with test cells the same day. All mice were bred, maintained and used for experiments under SPF conditions in the Animal Resource Centre of the British Columbia Cancer Research Centre according to protocols approved by the Animal Care Committee of the University of British Columbia.

3.2.5 Statistical analysis

Data shown are geometric means \pm SEM. Differences between groups were examined using the Student t-test and significance was associated with a p value ≤ 0.05 . Dimensionality reduction analysis was performed with Isomap (Tenenbaum et al., 2000) using the R package RDRToolbox (Bartenhagen, 2014).

3.3 Results

3.3.1 Higher numbers of hematopoietic progenitors are present in the PB 4 hours after a single injection of plerixafor with or without prior G-CSF

For initial experiments like samples were pooled to reduce the number of assays required. Comparison of the CD34⁺ cell and CFC content of post-thaw pooled donor PB samples (one pool for each treatment time point, see Figure 3.1) with matching pre-treatment (baseline) values for the same samples showed both cell types to be present in increased numbers in all post-treatment samples. Peak increases of 13- and 7-fold, respectively, were seen 4 hours after administration of plerixafor alone, and these were even higher (22- and 19-fold over baseline, respectively), 4 hours after plerixafor when this was preceded by a course of G-CSF administration (Figure 3.2A & 3.2B). To evaluate cells with prolonged mature cell output potential, the same pooled samples were assessed for LTC-IC activity using both 3-week and 6-week CFC output endpoints. The results indicate parallel increases in these activities in the PB of

both plerixafor- and G-CSF+plerixafor-treated donors compared to baseline levels, again with peak increases 4 hours after the administration of plerixafor (2,500- and 2,000-fold increases, respectively after plerixafor alone, and 2,500- and 2,100-fold increases after G-CSF+plerixafor, respectively, Figure 3.2C & 3.2D). Interestingly, there was also an absolute decrease in all cell types 24 hours post-plerixafor that was not seen in donors given prior G-CSF. Similar assays performed on pooled BM cells from the same 10 donors indicated that the frequencies of CD34⁺ cells and CFCs in the BM remained unchanged by both prior plerixafor and G-CSF+plerixafor treatments, and the relative numbers of 3- and 6-week LTC-IC activities were actually decreased several-fold in the post-plerixafor BM samples (Figures 3.2E - 3.2H).

An ability of plerixafor alone or G-CSF+plerixafor to mobilize primitive cells into the PB by several orders of magnitude and the lack of a positive effect on their frequency in BM aspirates suggests that BM would not prove to be as advantageous a source of primitive hematopoietic cells for clinical use. Accordingly, we focussed further analyses on assessments of the activities in the PB samples collected 4 hours after plerixafor or plerixafor preceded by G-CSF.

3.3.2 Individual donor assessment reveals superiority of the G-CSF+plerixafor mobilization regimen

To confirm the effect of plerixafor alone versus G-CSF+plerixafor and also obtain an estimate of the variability between the responses of individual donors, we then assessed each baseline and plerixafor+4 hour sample individually and performed pairwise comparisons between these values (Figure 3.3A). CFC numbers in the PB were increased 10- and 40-fold above baseline levels for

plerixafor- and G-CSF+plerixafor-treated donors, respectively (Figure 3.3B). This result is similar to that obtained for the pooled samples and revealed a high degree of replicability between donors. Assessment of mobilized 3- and 6-week LTC-IC activity also indicated substantial increases in both plerixafor and G-CSF+plerixafor donors, for multiple measures of LTC output at both LTC time-points (Figure 3.3C & 3.3D). Overall, the results showed the G-CSF+plerixafor combination gave a consistently, albeit not always significantly, greater increase in all cell types measured.

3.3.3 G-CSF+plerixafor mobilizes more short term repopulating cell activity than plerixafor alone

To assess the STRC activity of these samples, we transplanted cells from the same PB samples individually into NRG-3GS mice. Each mouse received the processed equivalent of 1.2 mL of the initial PB sample, and the level of human cells present in the blood of the mice was then assessed in samples obtained 3, 6, and 12 weeks later (Figure 3.4). We used NRG-3GS mice for this purpose as experiments in Chapter 2 showed that the early production of circulating mature myeloid cells in NSG-3GS mice (NSG mice carrying the same human transgenes as NRG-3GS mice) given a radiobiologically equivalent sublethal dose of irradiation is significantly enhanced by comparison to NSG mice. Here, the overall level of human CD45⁺ cell chimerism and the numbers of circulating human neutrophils and monocytes were consistently higher from 3 up to 12 weeks post-transplant in the mice transplanted with donor PB samples mobilized by either plerixafor or G-CSF+plerixafor by comparison to the corresponding levels obtained in mice transplanted with the same donors' baseline PB samples. An enhanced early output of human

platelets was also seen in the mice at 3 weeks post-transplant, but this effect then rapidly and continuously decreased. An opposite effect of mobilization was seen on the kinetics of human B-lineage cell production. In this case, there was little enhancement at 3 weeks post-transplant, but an increasing effect at 6 and 12 weeks post-transplant.

We then compared the repopulating activity in the two sets of mobilized PB samples (plerixafor+4 hours and G-CSF+plerixafor+4 hours) as reflected in the levels of different lineages of human cells present in the PB of the mice at the 3 post-transplant time points assessed. These comparisons again showed superior results for cells obtained from donors given G-CSF+plerixafor compared to donors given plerixafor alone. At 3 and 12 weeks post-transplant, the difference for mature human myeloid cell outputs (both 3-fold) achieved statistical significance ($p < 0.05$).

3.3.4 Isomap reveals high overlap between *in vitro* and *in vivo* myeloid assays

Assays able to predict the rapidity of neutrophil and platelet repopulating activity of a transplant are of great interest for many stem cell transplant applications. Here we used the *in vivo* repopulation data in mice as a surrogate for what might be expected in transplanted patients and then compared the relatedness of all *in vitro* and *in vivo* assays employed to identify those that appeared to give the most similar results. For this purpose, we performed a dimensionality reduction using Isomap (Tenenbaum et al., 2000) to analyze ranked measures of activity from the results of 23 assays applied to a total of 20 individual plerixafor+4 hours PB samples. Isomap is a nonlinear dimensionality reduction method based on the relatedness of data points in multi-dimensional space, as defined by the distance between each data point along the shortest path of

a network made up of 5 nearest neighbour connections. Reduction to 2 dimensions already captured >80% of the variance (and reduction to 4 dimensions captured >90% of the variance). The 2-dimension analysis revealed distinct groups that distinguished assays for precursors with similar durations of mature cell output or lineage capabilities, regardless of whether these parameters were measured *in vitro* or *in vivo* (Figure 3.5). These consisted of clusters of short-term granulopoietic precursors, long-term granulopoietic precursors, *in vivo* lymphoid precursors, and *in vivo* platelet precursors. Assays with relatively low specificity (TNC, CD34 and CFC) also clustered together and were centrally placed. The particularly close proximity of the week 3 *in vitro* and *in vivo* myeloid output assays suggests a high degree of overlap and *in vivo* predictive capability of the *in vitro* assay as a surrogate.

3.4 Discussion

Allografting adult patients with leukapheresis products obtained from normal donors is a widely utilized therapeutic modality, but optimizing mobilization of the cells needed is still an ongoing concern (Gratwohl et al., 2015; To et al., 2011). Collections from G-CSF-stimulated donors are now the most commonly used protocol, with plerixafor being more recently investigated as a generally effective agent, particularly when used together with G-CSF. However, more specific and quantitative comparisons of the multiple functionally defined cell types present in harvests obtained from donors treated with plerixafor alone or plerixafor after a course of G-CSF administration have not been performed. Multiple studies of immunodeficient mice transplanted with phenotypically distinct subsets of human cells, or genetically marked human CD34⁺ cells have all indicated that cells able to regenerate different lineages for different periods of time

comprise biologically distinct subpopulations, but with considerable breadth and overlap in their developmental properties (Aiuti et al., 2013; Glimm et al., 2001, 2005; Guenechea et al., 2001; Majeti et al., 2007). Defined subsets able to produce mature neutrophils or platelets within the first 3 weeks post-transplant, but only transiently, have also been identified. These cells are concentrated in the CD38⁺ fraction of CD34⁺ cells, some of which also do not display detectable aldehyde dehydrogenase activity (are ALDH⁻) and are thus generally more prevalent than their much rarer CD34⁺CD38⁻ALDH⁺ counterparts (Cheung et al., 2012; Glimm et al., 2001). It may thus be anticipated that the numbers of these short term repopulating cells are critical elements in determining the speed of neutrophil and platelet recovery in myelo-suppressed recipients of normal allografts.

Here we examined this issue by evaluating the ability of plerixafor alone and plerixafor in combination with G-CSF to mobilize cells with different speeds and durabilities of neutrophil and platelet outputs in a highly sensitive *in vivo* xenograft model. We also investigated the question of whether these treatments had similar or different effects on donor cells that remain in the BM and the optimal time for harvesting cells post-plerixafor administration. Using a 3-week *in vivo* output endpoint, we found that greater progenitor cell increases were evident in the PB as compared to the BM of the donors, that larger increases were elicited by the G-CSF+plerixafor compared to the plerixafor alone mobilizing regimen, and that for both, the optimal collection time-point was 4 hours post-plerixafor.

The range of *in vitro* and *in vivo* assay endpoints used here to investigate the potency of the various test samples included 2-week CFC assays, as well as 3- and 6-week bulk LTC-IC assays. CFC assays detect cells that produce large numbers of mature granulocytes, monocytes and erythrocytes after 2-3 weeks *in vitro* (10-15 amplifying divisions) and the 3- and 6-week

LTC-IC assays detect cells able to extend this ability for another 3-6 weeks (potentially another 20-40 divisions). The *in vivo* assay endpoints included total human, myeloid, B-cell, and platelet outputs at multiple time-points in the myeloid-enhancing immune-deficient NRG-3GS mouse (Miller et al., 2013a). Employment of this diversity of assays allowed a comparison to be made of those most closely correlated with rapid neutrophil or platelet production *in vivo*. A simple comparison of the kinetics of their initial outputs, as well as the results of a dimensionality reduction analysis, confirmed previous evidence that the production of these two lineages in the first 3 weeks likely derive primarily from distinct subsets (Cheung et al., 2012). In addition, the latter analysis indicated a very close relationship between the results of *in vivo* and *in vitro* assays for cells that produce myeloid cells for similar periods of time suggesting that these assays might effectively substitute for one another. In mobilized harvests from normal adults, these associations are less likely to be critical, as an overall correlation in the effect of the mobilizing protocol on all primitive cell types has generally been seen. However, with the growing use of genetically modified and/or other *ex vivo* manipulations of transplants, knowledge of the simplest predictive assays of short-term neutrophil and platelet output potential is assuming greater importance.

In summary, we have shown that human short term *in vivo* repopulating cells are optimally collected from the blood of donors 4 hours after plerixafor administration and after a preceding course of G-CSF, and that these repopulating cells can be predicted using a 3 week *in vitro* LTC-IC assay.

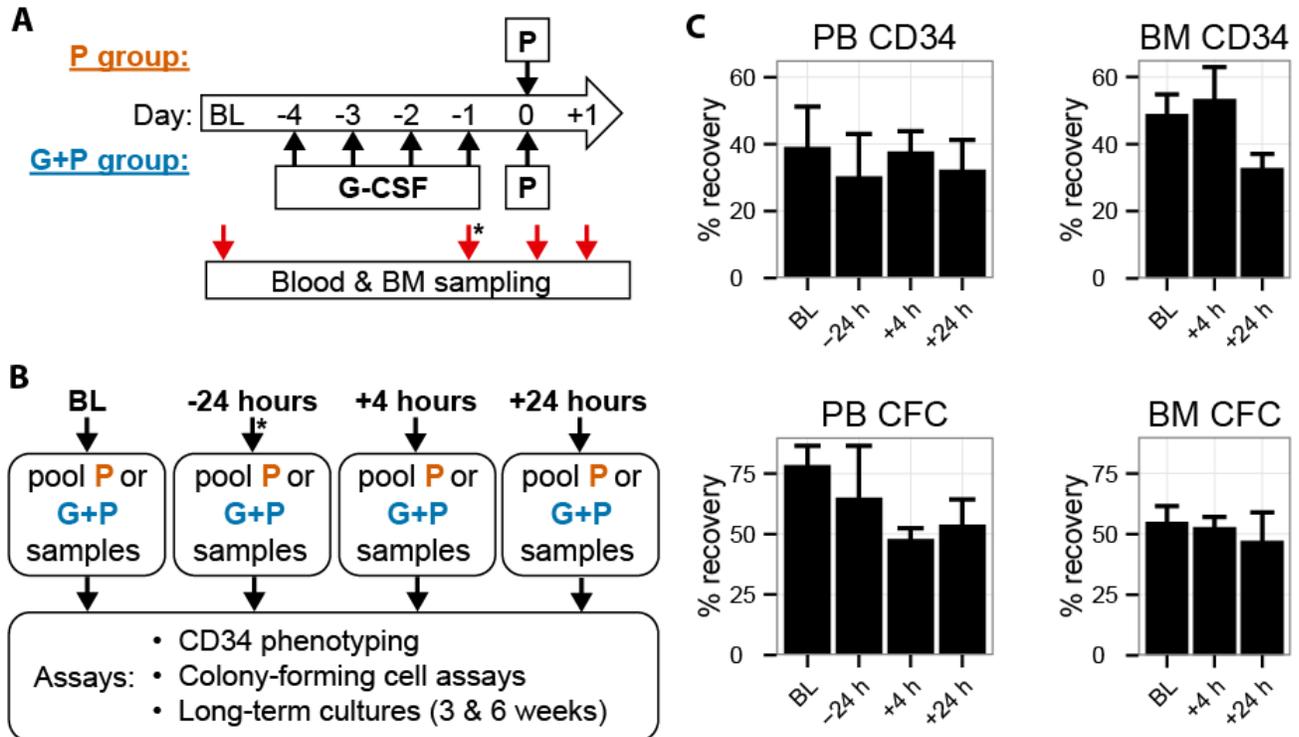


Figure 3.1. Plerixafor ± G-CSF experimental design

(A) Donor treatment and sample acquisition schedule. Five healthy adult donors received a single subcutaneous injection of plerixafor (P). Another 5 received plerixafor 24 hours after the last of 4 daily doses of G-CSF (G+P). Baseline (BL) PB samples were collected as shown. (B) Preliminary assessments of CD34⁺ cells, CFCs, and LTC-IC activity were performed on separate PB and BM pools prepared for each time point for both protocols. For each pool, equal aliquots by volume or cell number were combined from the 5 PB or 5 BM samples, respectively. (C) Recoveries of CD34⁺ cell and CFC numbers post-thaw were determined by comparing the average of individually measured pre-freeze data and corresponding post-thaw values for pooled cells collected at times shown in (A). *- indicates time-point with only G-CSF+plerixafor donor PB collections.

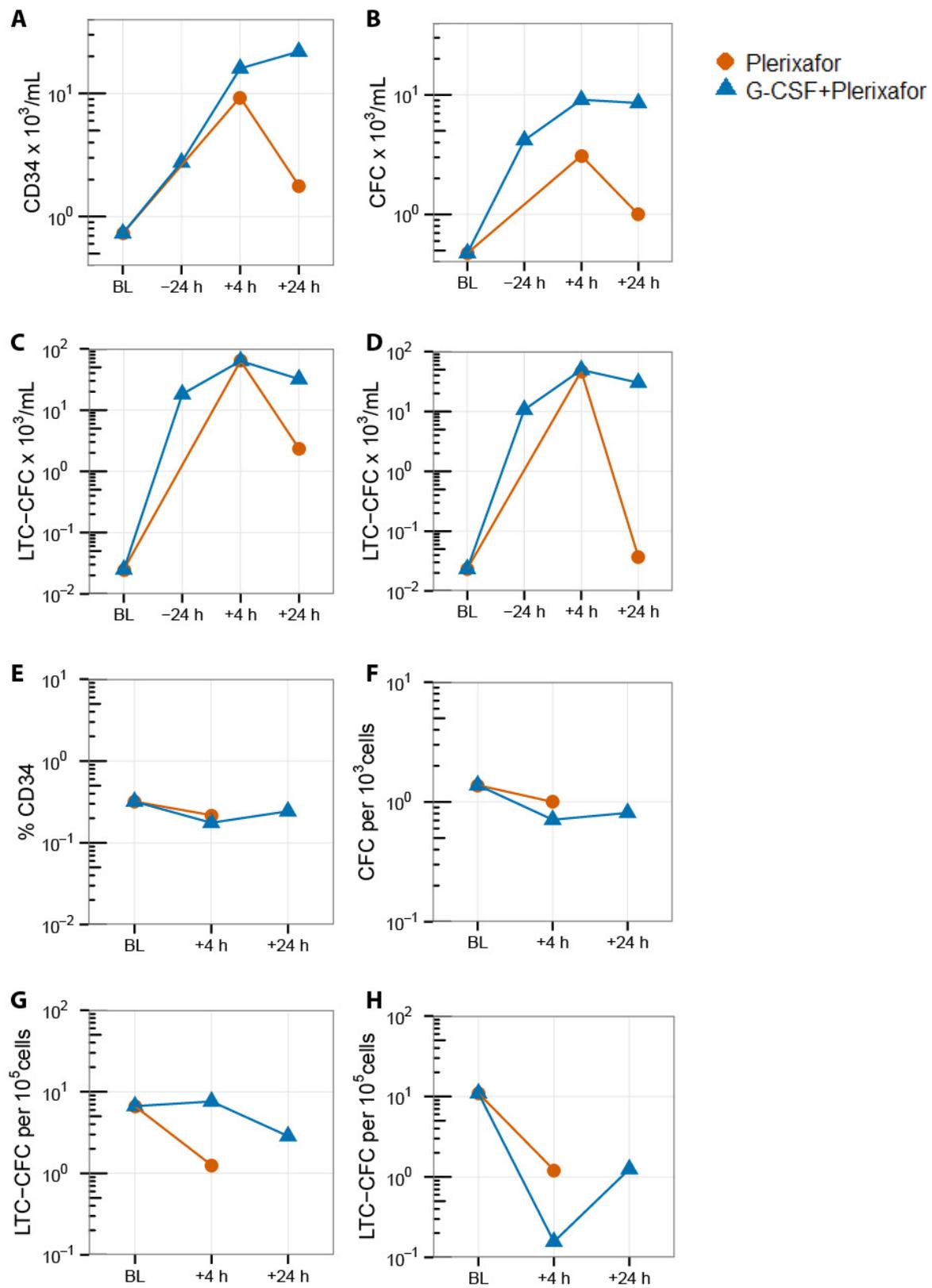


Figure 3.2. Hematopoietic progenitors are optimally harvested from the blood 4 hours after plerixafor.

Kinetics of changes in progenitor levels in matched PB (Figure 3.2A - D) and BM (Figure 3.2E - H) donor samples before treatment (baseline, BL) and after treatment with plerixafor (orange) or G-CSF+plerixafor (blue). Pools were assessed for CD34+ cells (Figure 3.2A & E), CFCs (Figure 3.2B & F), 3-week LTC-ICs (Figure 3.2C & G), and 6-week LTC-ICs (Figure 3.2D & H). Peripheral blood and bone marrow sample analyses were based on initial blood volume and initial TNC counts, respectively.

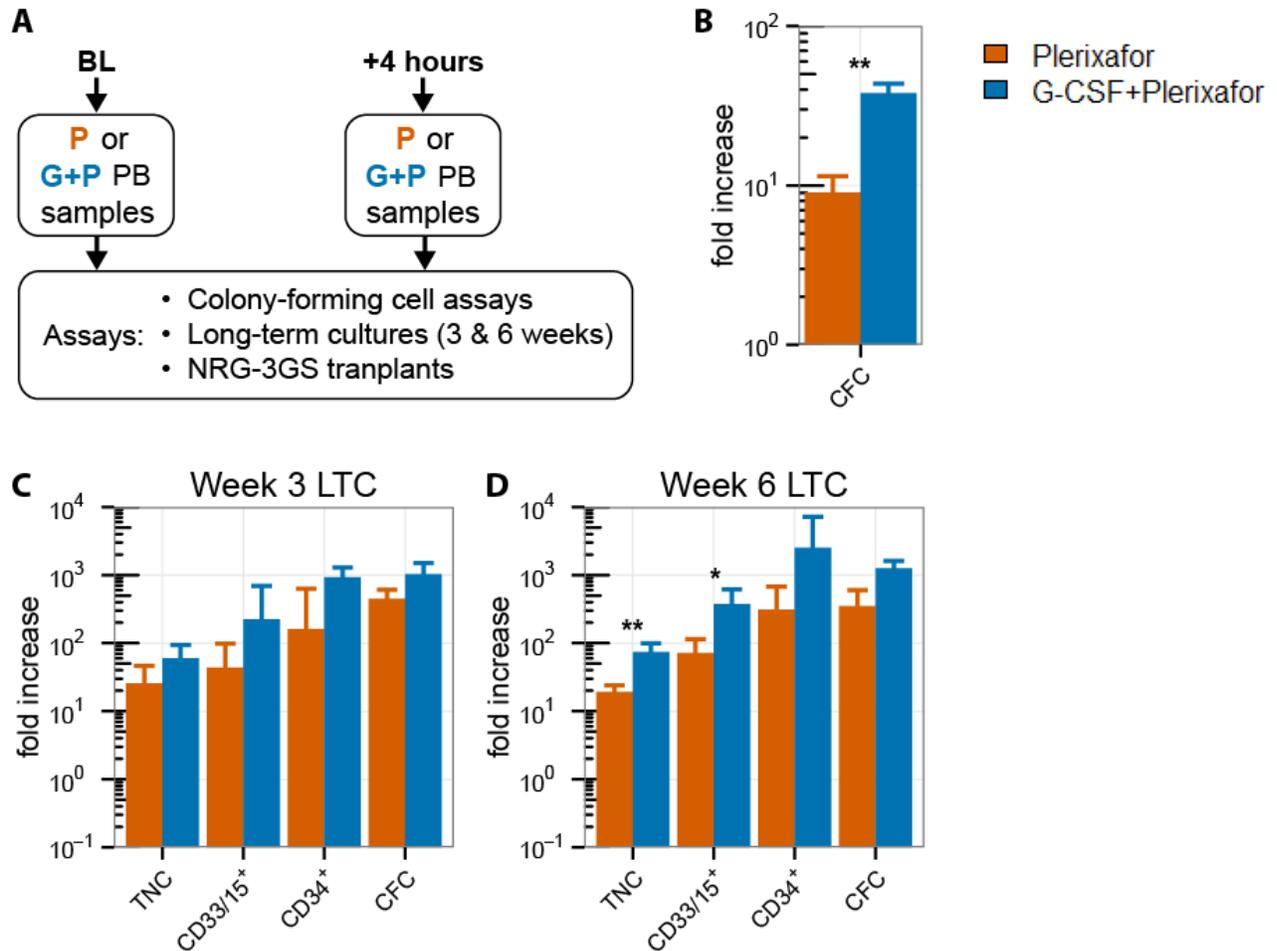


Figure 3.3. Analysis of individual samples shows higher post-plerixafor progenitor increases in donors pre-treated with G-CSF

(A) Pairwise comparisons of the results of multiple measures of hematopoietic activity between the BL and plerixafor (P)+4 hour PB samples from individual donors. (B) CFC assays showed significantly ($p < 0.01$) greater increases in G-CSF (G) +plerixafor (blue) versus BP as compared to donors treated with plerixafor only (orange) (9- and 40-fold increases, respectively, compared to BL). 3- (C) and 6- (D) week LTCs duration showed consistently higher plerixafor+4 hour increases in both mature (TNC, CD33/15⁺) and precursor (CD34⁺, CFC) outputs in LTCs initiated with volume matched aliquots of PB cells from donors given G-CSF+plerixafor as

compared to donors given plerixafor only. Asterisks indicate statistical significance between plerixafor and G-CSF+plerixafor samples determined using the Student t-test (* = $p \leq 0.05$; ** = $p \leq 0.01$).

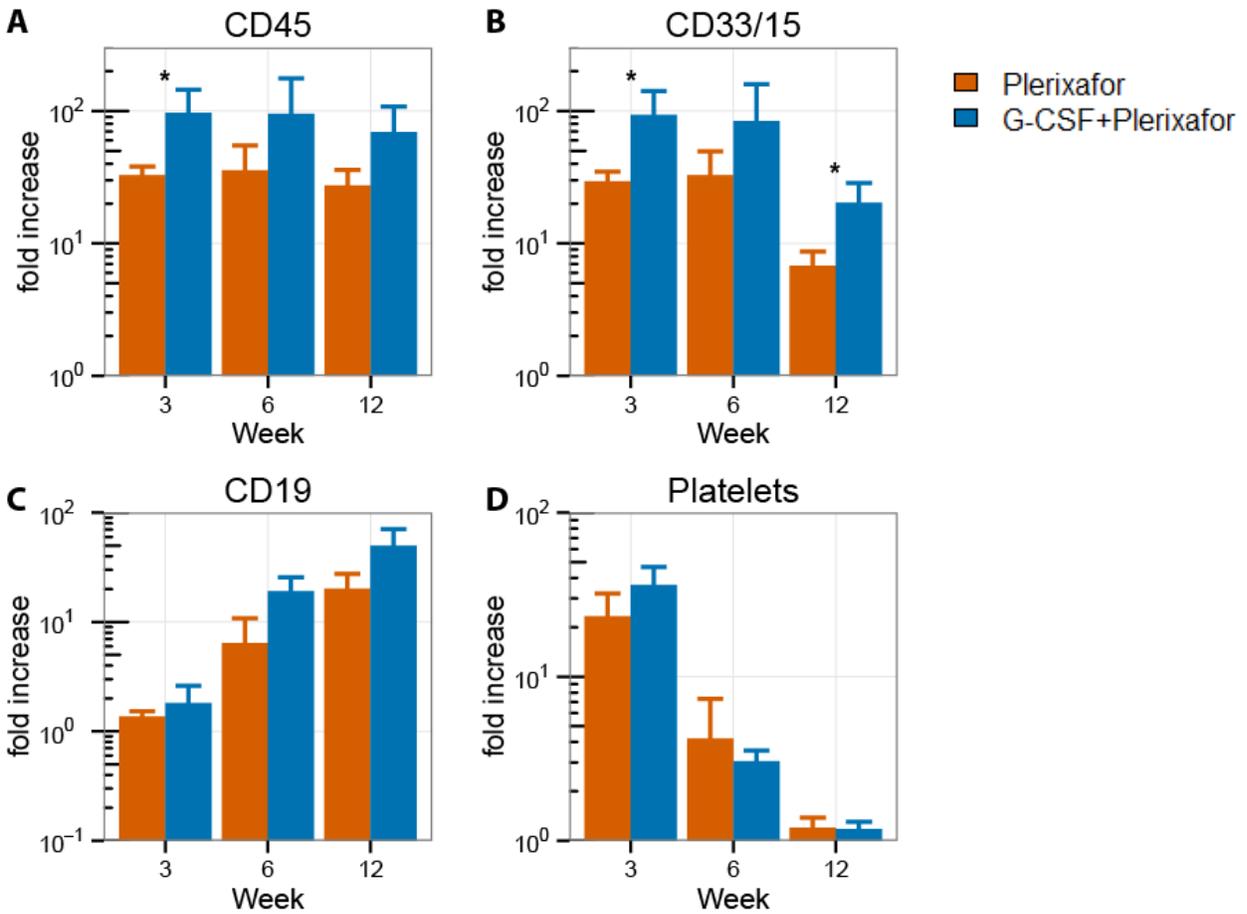


Figure 3.4. *In vivo* assays of individual donor PB samples show higher activity in donors pre-treated with G-CSF.

Individual BL and plerixafor+4 hour PB samples were transplanted into irradiated NRG-3GS mice and 3, 6, and 12 weeks later human (A) total CD45⁺, (B) CD45⁺CD33/15⁺ myeloid, (C) CD45⁺CD19⁺ B cell, and (D) CD41⁺CD61⁺ platelets were assessed in mouse blood. Increases in myeloid cell outputs were greater at week 3 and 12 from G-CSF+plerixafor versus plerixafor-only donor samples ($p < 0.05$). Orange and blue bars indicate results for plerixafor only and G-CSF+plerixafor, respectively. Asterisks indicate statistical significance between plerixafor and plerixafor+G-CSF samples determined using the Student t-test ($* = p \leq 0.05$).

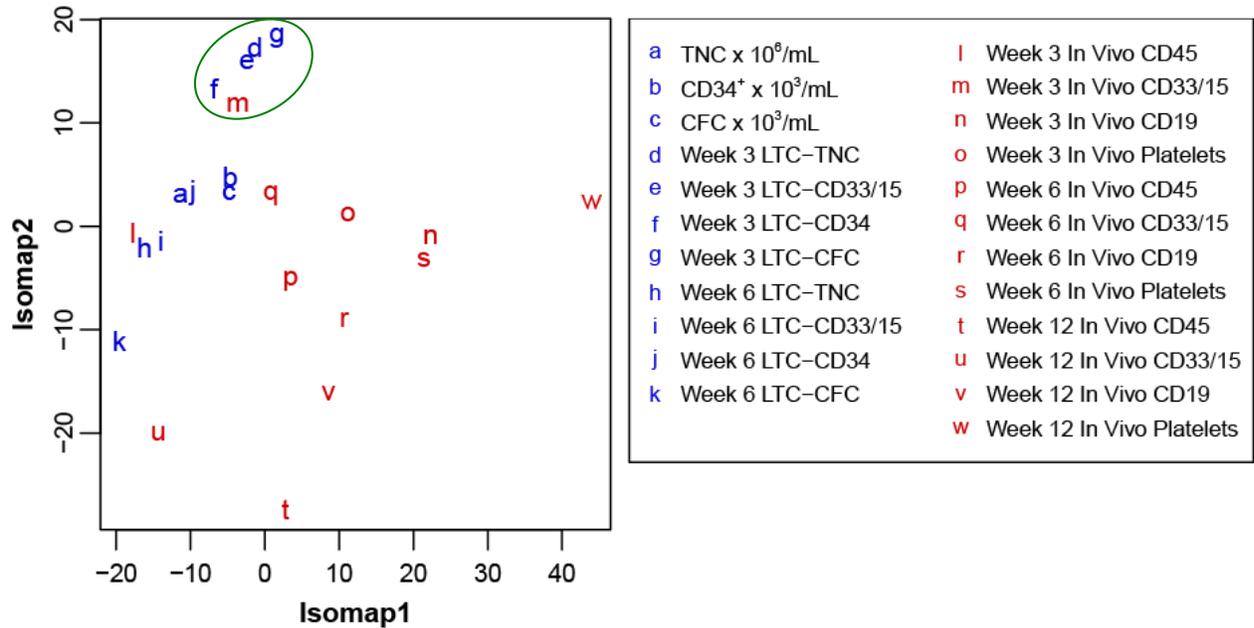


Figure 3.5. Isomap analysis reveals a high degree of concordance between *in vitro* and *in vivo* assays of myeloid cell output activity for similar periods of time.

Isomap dimensionality reduction was performed to characterize the relatedness of 23 *in vitro* and *in vivo* assays, based on rank-ordered measures of the activities measured in 20 paired BL and plerixafor+4/G-CSF+plerixafor+4 samples. Reduction to 2 dimensions of a multidimensional network made of 5 nearest neighbour connections accounted for >80% of the variance. Groups formed among precursor assays with similar durations of mature cell output durations or lineage capabilities, regardless of whether these parameters were measured *in vitro* or *in vivo*. Blue and red letters represent *in vitro* and *in vivo* assays, respectively. The green ellipse encompasses the concordant *in vitro* and *in vivo* myeloid assays.

Chapter 4: Early Production of Human Neutrophil and Platelets Post-Transplant is Selectively Compromised by Prior Growth Factor Exposure

4.1 Introduction

Transplantation of hematopoietic cells is a critical component of treatments that cure many life-threatening hematological disorders. LTRCs are essential for durable recovery, but are unlikely to be the main source of early appearing neutrophils and platelets with conventional transplants. More prevalent STRCs (Cheung et al., 2012; Glimm et al., 2001; Miller et al., 2013b) are less abundant in human CB than in G-CSF-mPB or adult BM which likely contributes to the clinical inadequacy of CB units as transplants for adults (Brunstein and Wagner, 2006; Rocha et al., 2004).

GFs regulate primitive hematopoietic cell survival, proliferation and differentiation (Metcalf, 2008) and can also influence their adherence to BM microenvironment elements (Becker et al., 1999). Initially, GFs anticipated to be useful for the *ex vivo* generation of cells for clinical transplants were identified by their ability to stimulate the production of progenitors identified *in vitro* as colony-forming cells (CFCs) and/or as their precursors (Petzer et al., 1996a; Zandstra et al., 1997). More recent studies have examined the GFs required to produce human cells with intermediate to long-term repopulating activity in immunodeficient mice (Boitano et al., 2010; Fares et al., 2014). However, a corresponding *in vivo* analysis of human STRC outputs has been lacking. This situation is notable as significant improvements in clinical outcomes using transplants of culture-expanded cells have yet to be achieved.

4.2 Methods

4.2.1 Human cells

CD34⁺ enriched (>80%) suspensions of pooled human CB cells were isolated using an EasySep kit (STEMCELL Technologies, Vancouver, Canada) from cryopreserved collections according to procedures approved by the University of British Columbia Research Ethics Board.

4.2.2 Cell cultures

CD34⁺ cells were cultured at 10⁵ cells/mL in serum-free medium containing BIT (STEMCELL Technologies) plus 40 mg/mL low-density lipoproteins and 10⁻⁴ M β-mercaptoethanol (Sigma-Aldrich, Oakville, Canada) (SFM). GFs added were 100 ng/mL FL (Amgen, Thousand Oaks, CA), 100 ng/mL SF and 20 ng/mL G-CSF (STEMCELL Technologies), 20 ng/mL IL-3 (Novartis, Basel, Switzerland), and 20 ng/mL IL-6 (Cangene, Winnipeg, Canada) (5 GFs), or 100 ng/mL FL, 100 ng/mL SF and 50 ng/mL thrombopoietin (STEMCELL Technologies) (3 GFs). Human AML5, HL60 and KG1 cells were cultured in IMDM plus 10% FBS (STEMCELL Technologies) with 5 GFs. CFC assays were performed as described (Hogge et al., 1996).

4.2.3 Transplantation procedures

Cells were injected intravenously into 8-12 week-old NSG mice or NSG-3GS mice irradiated with 315 cGy ^{137}Cs γ -rays. STRC activity was determined on antibody-stained blood samples obtained 3 weeks later. BM homing was assessed by injecting 5×10^4 CD34⁺ CB cells or their cultured derivatives into mice and comparing the proportion of CFCs recovered from 2 femurs 16 hours post-transplant. Mice were maintained under SPF conditions using protocols approved by the Animal Care Committee of the University of British Columbia.

4.2.4 Flow cytometry

Absolute numbers of human CD34⁺ cells, human CD45⁺CD33/15⁺ myeloid cells and FSC^{low}CD41⁺CD61⁺ platelets were determined using appropriate antibodies and AccuCheck counting beads (Life Technologies) (Cheung et al., 2012; Miller et al., 2013a). For assessment of cell line homing, recipient BM cells were stained with anti-human CD45 and also analyzed for fluorescent dyes used to label the cells pre-injection (CFSE, Life Technologies, Burlington, Canada, and Cell Dye eFluor 450, eBiosciences, San Diego, USA).

4.2.5 Statistical analysis

Data shown are geometric means \pm SEM. Differences were examined using the Students t-test and significance associated with a p value ≤ 0.05 .

4.3 Results

CD34⁺ CB cells cultured for 7 days with the 5-GF cocktail showed expected increases (Zandstra et al., 1997) in CD34⁺ cells and CFCs (6- and 12-fold, respectively, Figure 4.1A). In contrast, NSG-3GS mice transplanted intravenously with aliquots of the same cells showed a 10-fold reduction in STRC activity relative to the freshly isolated CD34⁺ cells (Figure 4.1B). Similar results were obtained for cells cultured for 12 days in the 3-GF cocktail (Figure 4.1B). This consistent loss of *in vivo* STRC activity *in vitro* was not reversed by inclusion of 35 nM UM171 (Fares et al., 2014) (STEMCELL Technologies) in the medium or pre-transplant exposure of the cells to 10 μ M 16,16-dimethyl prostaglandin E2 (PGE2, Cayman Chemical, Ann Arbor, USA) for 120 minutes (Cutler et al., 2013) and 5 mM diprotin A (DPA, Sigma-Aldrich) in the last 15 minutes (Christopherson et al., 2004) (Figure 4.1B). Intrafemoral injection of cells cultured in 5 GFs improved myeloid STRC activity 4-fold compared to cells injected intravenously, but still markedly less than predicted by the expansion of CFCs.

Time course experiments showed both myeloid and platelet STRC activities were reduced within 24 hours below input values (Figure 4.2A), despite no change in CFCs (270 \pm 30 versus 250 \pm 40 colonies per 10³ CD34⁺ CB cells, 4 experiments). Interestingly, 3-week platelet outputs were already maximally reduced after 4 hours of GF exposure, whereas matching myeloid outputs were not yet affected.

Comparison of 3-week STRC-derived myeloid outputs in mice transplanted with CD34⁺ cells before and after culture for 24 hours in SFM with different GFs or 2% FBS showed no loss of STRC activity when FBS was substituted for the 5-GF cocktail (Figure 4.2B), as did all groupings of GFs that activate similar downstream pathways (Gilliland and Griffin, 2002;

Rönstrand, 2004; Schindler, 2002) (Figure 4.2C). However, insulin alone, either as a component of SFM (Figure 4.2B), or when present at a 200-fold lower concentration (Figure 4.2C), did not induce a significant loss of STRC activity, indicating that not all GFs have an equal ability to impair engraftment. In the absence of insulin, total cell viability was markedly reduced, leading to a 70% reduction within 24 hours.

We then investigated whether the rapid loss of STRC activity might be caused by an acquired inability to home into the BM. Despite no change in functional CFC numbers after 24 hours in culture with 5 GFs, the proportion of CFCs that homed to the BM was reduced 3-fold (Figure 4.2D). This reduction is similar to the concomitant effect obtained on STRC activity. Moreover, when the CB cells were cultured in SFM with 2% FBS instead of GFs, CFC homing was also little affected (Figure 4.2D). A similar reduction in the BM homing of 3 different intravenously injected human leukemia cell lines was also observed when these were first incubated with the same 5 GFs for 24 hours before being transplanted (Figure 4.2E).

4.4 Discussion

To date no phenotype or *in vitro* assay has proven to be a reliable predictor of the rapidity with which neutrophils or platelets will appear in the blood of patients transplanted with cultured hematopoietic cells. Indeed, there is little information regarding the ability of GFs alone to support the robust expansion of human hematopoietic cells capable of producing large numbers of neutrophils and platelets early after transplant. We have now demonstrated the rapidity with which multiple GFs impair the ability of primitive human hematopoietic cells to regenerate mature neutrophils and platelets within 3 weeks *in vivo*. We also show that the *in vitro* activity of

progenitors (CFCs) that produce neutrophils and macrophages in the same time frame (2-3 weeks) is not affected. This finding and the similarity in speed and magnitude of the GF-induced homing deficiency of CFCs suggests that an initial homing failure, rather than a loss of viability or differentiation capacity, is responsible for the subsequent deficient *in vivo* outputs of neutrophils (and platelets). The differential effect on the cells responsible for producing these different outputs (Cheung et al., 2012) highlights a potential need for cell-specific culture conditions to preserve their *in vivo* activity.

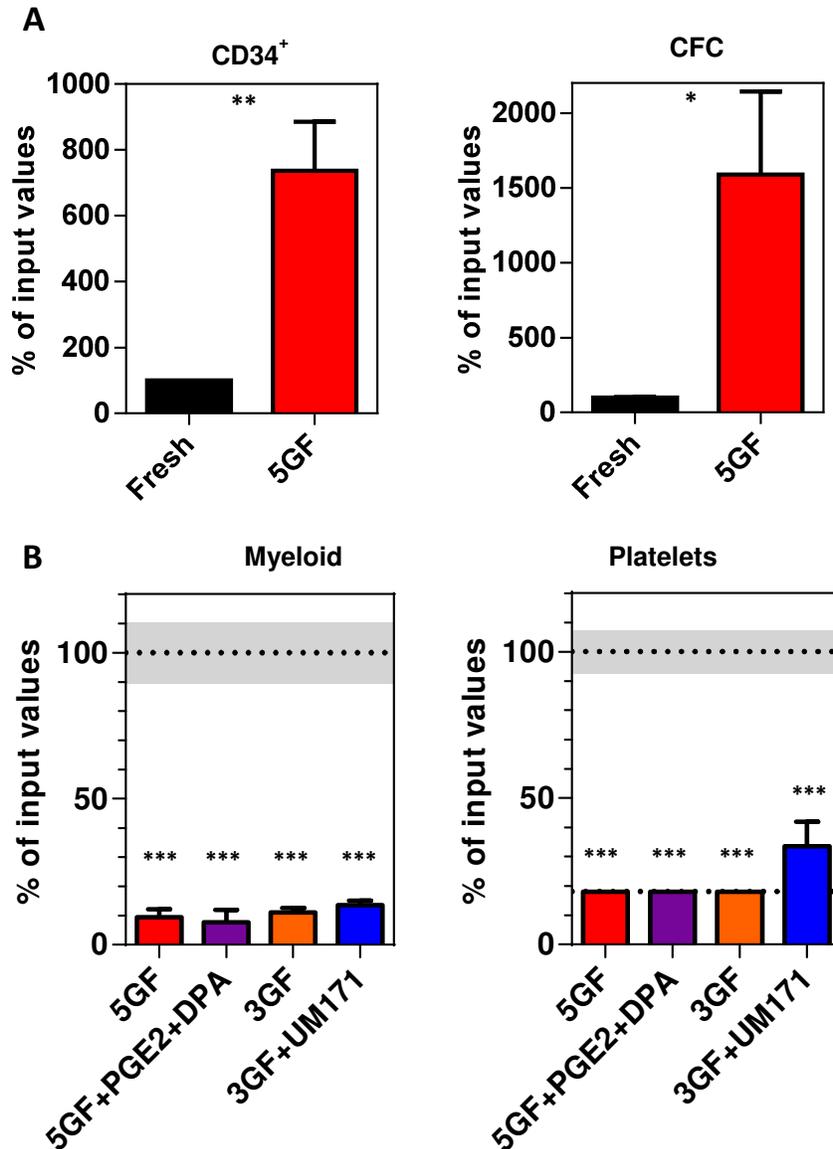


Figure 4.1. Cultured CD34⁺ CB cells show a profound and selective loss of STRC activity.

(A) Fold-increase in CD34⁺ cells (left panel) and CFC numbers (right panel) after 7 days of culture of CD34⁺ CB cells in SFM+5 GFs (4 experiments). (B) Levels of human neutrophils and platelets in the peripheral blood of NSG-3GS mice transplanted 3 weeks before with the progeny of 104 CD34⁺ CB cells (“input”) cultured for 7 days in SFM+5 GFs (9 mice) or SFM+5 GFs+PGE2+DPA (3 mice), or for 12 days in SFM+3 GFs (10 mice) or SFM+3 GFs+UM171 (10

mice), expressed as a percent of the corresponding levels obtained from the cells used to initiate the cultures (20 mice). The gray horizontal bar indicates the range defined by ± 1 SEM of pooled values obtained with the input cells. The dotted line indicates the platelet detection threshold. Asterisks indicate statistically significant differences determined using the Student t-test (* = $p \leq 0.05$; ** = $p \leq 0.01$; *** = $p \leq 0.001$) between each condition and the freshly isolated control.

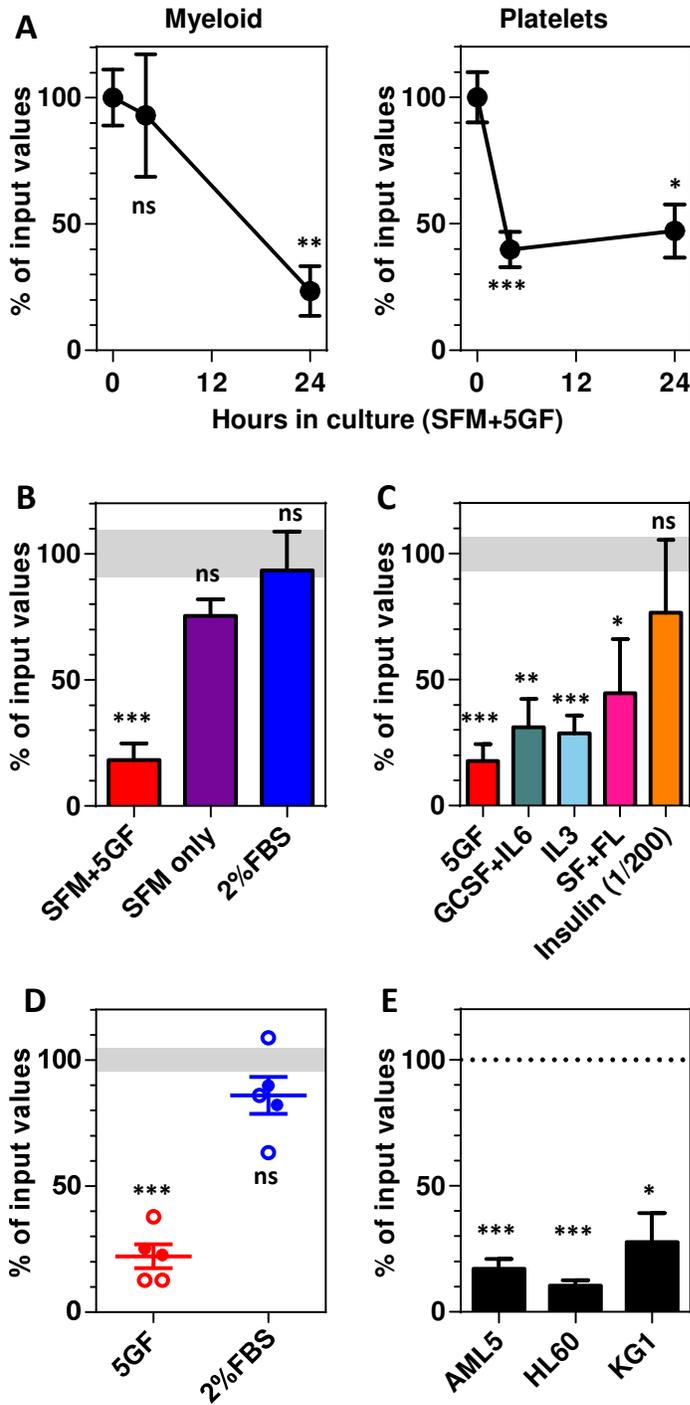


Figure 4.2. GF-induced loss of STRC activity occurs rapidly and is associated with a similar reduction in CFC homing efficiency to the BM.

(A) Kinetics of the culture-induced loss of human myeloid (left panel) and platelet (right panel) STRC activity (2×10^4 CD34⁺ cells injected per NSG-3GS mouse, ~8 mice per point) (B) Effect on human myeloid STRC activity of culturing 2×10^4 CD34⁺ CB cells for 24 hours in SFM+5 GFs (13 mice), SFM only (6 mice), or IMDM+2% FBS (7 mice). Values shown are the chimerism levels in NSG-3GS mice obtained by the cultured transplants expressed as a percent of the chimerism levels obtained from the number of pre-cultured cells (“input”) used to derive the same number of cultured cells tested (17 mice). (C) Effect on human myeloid STRC activity of culturing 2×10^4 CD34⁺ CB cells for 24 hours in SFM plus the indicated GF(s) (5 NSG-3GS mice per group). 1/200 insulin level = 60 ng/mL. Values shown were calculated as in (B). (D) Effect on the BM homing activity of CFCs cultured for 24 hours in SFM+5 GFs or IMDM+2% FBS tested in NSG-3GS (solid circles) or NSG (open circles) recipients. Values shown are the proportion of injected CFCs detected in 2 femurs after injection of the cultured cells expressed as a percent of the proportion of CFCs that homed to 2 femurs from the pre-cultured cells (“input”, 6 mice per group). (E) Effect of culturing the cell lines shown for 24 hours in IMDM+10% FBS+5 GFs versus IMDM+10% FBS alone on their NSG BM homing activity. Pre and post-cultured cells were differentially labeled with CFSE and Cell Dye eFluor 450 and co-transplanted. Values for 5 GF-cultured cells are shown as a percent of FBS alone-cultured values (same starting cell numbers) obtained in the same mice. Asterisks indicate statistically significant differences (* = $p \leq 0.05$; ** = $p \leq 0.01$; *** = $p \leq 0.001$) between each condition and the freshly isolated control determined using the Student t-test.

Chapter 5: Discussion

The experimental results presented in this thesis demonstrate improved detection and assessment of normal human STRCs and an analysis of their activity in mobilized and *ex vivo* manipulated products. STRCs are a highly important component of hematopoietic transplants as they provide the majority of the early neutrophils and platelets produced in patients undergoing treatments involving myeloablative therapy. However, investigation of their numbers in mobilized cell harvests is lacking, with the effect of new and old mobilizing agents on STRCs in the PB and BM being poorly understood. In addition, the under-representation of STRC in CB collections warrants the development of *ex vivo* expansion protocols that will help overcome the delayed hematopoietic recoveries seen in adult recipients of CB.

5.1 Detection and quantification of human STRCs

The results described in Chapter 2 highlight the need to develop methodology specific for the analysis of STRCs, and describe the main assay used to generate the results shown in later chapters. Immunodeficient mouse strains widely used for the detection of human LTRCs have also been the most common choice for detecting human STRCs (Shultz et al., 2012). However, their poor support for human myeloid cell production has undermined the sensitivity for cell types that predominantly produce myeloid cells, such as STRCs. This has required the use of BM aspirations to provide the larger numbers of cells needed when detecting very low chimerism. The findings shown in Chapter 2 show that NSG-3GS mice support 5-fold greater myeloid cell outputs from transplanted STRC than NSG mice, without affecting the frequency of STRCs that

engraft. This increase provides a corresponding increase in human myeloid cells in the circulation and enables their accurate measurement by PB sampling, obviating the sampling error associated with BM aspirations during the post-irradiation recovery period.

Incomplete post-irradiation recovery of mouse hematopoiesis influences the interpretation of human BM chimerism and increases the associated inter-mouse variation. Results in Chapter 2 show that at 3 weeks post-irradiation, recovery of NSG mouse hematopoietic cell outputs is still in progress. Although these data were generated with non-transplanted mice it is assumed that mouse hematopoietic recovery is not significantly enhanced by their being transplanted with human cells. In support of this, results in Chapter 2 show that at 3 weeks post irradiation and transplant of human CB there is considerable inter-femur variation of human chimerism in both NSG and NSG-3GS mice. These latter results indicate that single site BM sampling would lead to inaccurate measures of human chimerism and argue for the use of whole mouse measurements, such as blood sampling, of human chimerism at early time-points.

The duration of enhanced myeloid cell outputs in NSG-3GS, as compared to NSG mice, was sustained for up to at least 12 weeks post-transplant, with a loss of enhancement seen 20 weeks post-transplant. The cause of this late reduction was not investigated, however several processes can be suggested. For instance, the related NOD/SCID-3GS (NS-3GS) mouse, that produces the same human growth factors but was developed prior to the existence of the NSG mouse, has been shown to have negative effects upon transplanted primitive human BM cells (Nicolini et al., 2004). These effects include decreased CFCs, LTC-ICs, and secondary NOD/SCID repopulating cells in the marrow of NS-3GS mice 4-6 weeks post-transplant, as well as increased levels of human CFCs in the mouse circulation. Alternatively, immunological

activity of transplanted (contaminating) or *de novo* generated human T cells may have a deleterious effect on either the host microenvironment or on the engrafted human cells.

Indeed, NSG-3GS mice transplanted with CD34⁺ CB cells produced very high levels of human T cells at 12 and 20 weeks post-transplant as compared to NSG mice. However, whether or not these cells were generated *de novo* or came from contaminating input T cells cannot be definitively established due to the relatively high cell doses used (10^4), despite a cell sorting strategy that included both negative (CD3⁺) and positive (CD34⁺) selection. Levels of circulating B cells were slightly elevated in NSG-3GS mice at weeks 3 and 6, with similar levels as NSG mice at week 12 and 20. B cell levels in the BM appeared lower at these same latter time-points, perhaps due to extramedullary human lymphopoiesis or by effects on total BM cellularity, and therefore percentage values, by infiltrating human T cells.

The increased outputs reveal limitations with commonly used xenotransplantation systems and imply that further modification may lead to additional improvements. For instance, the under-representation of circulating myeloid cells as compared to that expected from BM chimerism levels in both NSG and NSG-3GS mice suggests that an impairment remains in cell trafficking, terminal maturation, or survival after egress from the BM. Similar mechanisms may be responsible for the dramatically low circulating human platelet levels seen in mice engrafted with human cells, with Hu et al. suggesting a mechanism of clearance by mouse macrophages (Hu and Yang, 2012). While these effects are likely not due to the well-studied self-recognition Sirp α /CD47 interaction (Hu and Yang, 2012; Takenaka et al., 2007), discovery of pathways that are responsible will be highly informative in the development of next-generation xeno-recipients.

Parts of Chapter 3 show the high degree of co-variance between the above described STRC assay and several *in vitro* surrogate assays. Total cell, CD34⁺, and CFC assays are widely

used to predict time to engraftment post-transplant in patients, however there is an increasing need to move from correlative assays to more direct assays with the increasing use of a wider source of cells range of *ex vivo* cell manipulations. These new results show that an extended *in vitro* stromal cell based assay had a high concordance with week 3 *in vivo* myeloid activity. The further investigation of such STRC assays that are amenable to widespread use would be highly beneficial for clinical transplanters and their patients.

5.2 Mobilization of STRCs

Harvested mobilized cells are the most common source of cells used for clinical transplants. While G-CSF is the usual mobilizing agent for allogeneic transplants, plerixafor (P) is now gaining popularity for use in combination with G-CSF. Experiments in Chapter 3 sought to determine the optimal timing and treatment combination for the mobilization of normal human STRCs.

In this study a wide range of hematopoietic cell assays were used, including *in vitro* CD34, CFC, and multiple long-term culture endpoints, along with multiple endpoints of *in vivo* activity. This range of assays was used to capture a wider range of cell types than addressed in most studies of hematopoietic cell mobilization. Three and 6 week *in vitro* LTC-IC assays were used to assess cells more primitive than CFCs, while *in vivo* assays were performed with human GF-producing NRG-3GS mice to enhance the assessment of human STRCs. While later confirmatory and *in vivo* assessments were performed using individual patient samples, to reduce the number of assays performed the initial experiments that determined optimal timing were performed on pools of the donor samples.

The results of these preliminary experiments demonstrate that peak levels of CD34⁺ cells, CFCs, and LTC-ICs were mobilized at 4 hours post-P administration, irrespective of whether G-CSF had been administered beforehand. The same pooled samples showed that P and or G-CSF administration did not enhance the BM levels of CD34⁺ or CFCs, with 3 and 6 week LTC-IC levels being decreased by these treatments. While this latter finding coincides with increased levels in the circulation, analysis of these samples for STRC content is required to indicate decreased levels of *in vivo* repopulating subsets.

Interestingly, in the PB of donors treated with P-only there was a sudden drop in all hematopoietic subsets 24 hours post-P that was not seen in donors given the combination G-CSF+P treatment. This decrease may have practical considerations in terms of a narrower window for harvest of mobilized cells. However, this would need to be balanced against the advantage of being able to give P just 4 hours ahead of a cell harvest, unlike the multi-day regimen required for mobilization by G-CSF.

Results from assays performed on individual 4-hour samples confirmed the enhanced mobilization after either treatment as compared to baseline levels. In addition, they showed a high degree of similarity among the 5 donors tested. For all hematopoietic endpoints there was a trend towards greater increases after treatment with G-CSF+P, however these increases reached significance only for some endpoints. These included CFCs, week 6 mature LTC-IC outputs, and week 3 and 12 *in vivo* myeloid cell outputs.

While these results clearly demonstrate enhanced mobilization by use of the combination treatment, these increases should be weighed against practical and clinical considerations. For instance, a moderately reduced cell yield may be acceptable for donors unable to undergo multi-day G-CSF treatment. There is also the possibility of additional enhancement of mobilization

with the use of newer CXCR4 antagonists, or with combination of P with other agents that also act specifically on mediators of cell localization.

5.3 *Ex vivo* expansion of STRCs

The STRC deficiency in most CB collections intended for adult recipients suggests that *ex vivo* expansion of their numbers would improve clinical outcomes. This, along with the ongoing development of HSC gene therapy protocols that require periods of *ex vivo* culture, demands a comprehensive understanding of the biology of STRCs that have been in culture for short or long periods. The lack of improved engraftment times with GF-expanded cells has led to the pursuit of agents able to induce the self-renewal of LTRCs (Boitano et al., 2010; Fares et al., 2014). However, studies indicating that early engraftment is predominantly provided by STRCs suggest that the clinical deficit is not HSC-related (Cheung et al., 2013; Glimm et al., 2001). Nevertheless, most pre-clinical expansion studies addressing delayed hematopoietic recovery are evaluated based on results of assays for LTRCs, with minimal assessment of STRC outputs in the same cultures. Ultimately, these questions will only be revealed by successful application of future expansion strategies. Unfortunately, if simple definitions of clinical recovery are not met, such transplant expansion strategies may be discarded instead of being re-purposed for other LTRC-based applications. The experimental results in Chapter 4 describe a dramatic reduction in STRC activity after brief periods of culture with GFs, suggesting that the engraftment defect seen is not due to the excessive differentiation observed in prolonged cultures. A parallel decrease in CFC marrow homing aligned with previous findings in mouse and human that the engraftment defect is due to impaired homing (Ahmed et al., 2004; Szilvassy et al., 1999).

Experiments in this thesis show that culture in a range of GFs can induce the engraftment defect, with the maintenance of STRC activity in non-GF-supplemented cultures suggesting that the defect is actively induced rather than being due to the absence of critical factors. Moreover, the inability of insulin to invoke a defect suggests that a particular signalling pathway is responsible, rather than general GF stimulation, which raises the possibility of specific manipulation to prevent or reverse the homing defect. Elucidation of the responsible pathways could be uncovered by use of a screening assay for engraftment of cultured cells, such as with an arrayed small molecule inhibition screen or with a pooled shRNA library. Alternatively, transcriptome analysis of 24 hour cultured cells might reveal prospective targets. However, this would likely be enhanced by limiting the comparison to IL-3 and insulin (control)-treated cells to reduce the false positives associated with unrelated GF effects on CD34⁺ cells. As it is unknown whether transcriptional changes are involved in the engraftment defect, analysis of signalling changes after culture may also be required, again using an IL-3 versus insulin comparison. For these latter approaches a further refinement of the STRC isolation phenotype be highly beneficial in reducing noise from contaminating non-STRCs in the CD34⁺CD38⁺ population.

A previous study also showed that homing of human CFCs is reduced upon culture in GFs (Ahmed et al., 2004), but this experimental design does not directly address the homing of cultured STRCs. The negative GF effect precludes the use of a secondary STRC assay in place of the *in vitro* CFC endpoint, although with improved phenotypic identification the specificity could be increased with a flow cytometry based homing assay. This significant caveat is partially overcome by the parallel changes in STRC repopulation and CFC homing in extent and timing, as well as by previous data showing a significant overlap between CFCs and STRCs (Cheung et al., 2012).

Another important question is whether the engraftment defect is simply due to the use of xenograft-based assays. This idea is contradicted not only by the lack of established clinical cell expansion protocols and by several accounts of poor clinical results (Barnett et al., 1989, 1994; Chang et al., 1986; Engelhardt, 2001, 2001; Holyoake et al., 1997; Shpall et al., 2002), but also by studies with syngeneic mice (Loo and Ploemacher, 1995; Szilvassy et al., 1999) demonstrating similar effects of GF culture upon CFC homing and repopulating activity (Kittler et al., 1997; Peters et al., 1995, 1996; Spooncer and Dexter, 1983; Vallera and Blazar, 1988).

5.4 Concluding comments

The results presented in this thesis provide a further understanding of the regulation, assessment, and procurement of human STRCs. Future studies of STRCs should focus on further development of STRC assessment and procurement. Development of rapid assays for the specific assessment of human STRCs would prove vital for both STRC research and clinical use, as current STRC assays are costly and time consuming, and surrogate assays lose their power on manipulated cells. Development of a phenotype that is highly enriched for human STRCs would likely be most useful, as it is rapid and would be compatible with current laboratory protocols. However, use of *in vitro* functional assays that reliably indicate STRC activity would be likely to prove beneficial despite their lengthy duration.

Further optimization of STRC mobilization protocols is likely to prove beneficial, with a focus on reducing the duration of mobilization preparative regimens. Increased cell specificity is likely to reduce the number of co-transplanted GVHD-causing immunological cells, allowing for greater control over clinical transplant outcomes.

Generation of large numbers of transplantable STRCs is also still of high importance, with protocols that expand these cells along with LTRCs without pre-separation likely to be most useful. Based on the findings in this thesis, success will most likely come from understanding and overcoming the mechanisms of the engraftment defect, as simply omission of certain growth factors is unlikely to yield positive results. Development of protocols that lead to the differentiation of pluripotent cells into hematopoietic STRCs may also be a viable option, with the development of the pluripotent differentiation field developing rapidly since its inception (Amabile et al., 2013; Takahashi et al., 2007). However, without the development of *in vitro* STRC assays, alternative methods such as barcoding will be required to increase the throughput of *in vivo* STRC assessments (Bystrykh et al., 2012).

References

- Adair, J.E., Beard, B.C., Trobridge, G.D., Neff, T., Rockhill, J.K., Silbergeld, D.L., Mrugala, M.M., and Kiem, H.-P. (2012). Extended survival of glioblastoma patients after chemoprotective HSC gene therapy. *Science Translational Medicine* 4, 133ra57–ra133ra57.
- Ahmed, F., Ings, S.J., Pizzey, A.R., Blundell, M.P., Thrasher, A.J., Ye, H.T., Fahey, A., Linch, D.C., and Yong, K.L. (2004). Impaired bone marrow homing of cytokine-activated CD34+ cells in the NOD/SCID model. *Blood* 103, 2079–2087.
- Aiuti, A., Webb, I.J., Bleul, C., Springer, T., and Gutierrez-Ramos, J.C. (1997). The chemokine SDF-1 is a chemoattractant for human CD34+ hematopoietic progenitor cells and provides a new mechanism to explain the mobilization of CD34+ progenitors to peripheral blood. *The Journal of Experimental Medicine* 185, 111.
- Aiuti, A., Biasco, L., Scaramuzza, S., Ferrua, F., Cicalese, M.P., Baricordi, C., Dionisio, F., Calabria, A., Giannelli, S., Castiello, M.C., et al. (2013). Lentiviral hematopoietic stem cell gene therapy in patients with Wiskott-Aldrich syndrome. *Science* 341, 1233151.
- Ali, H. (1999). Chemoattractant receptor cross-desensitization. *Journal of Biological Chemistry* 274, 6027–6030.
- Amabile, G., Welner, R.S., Nombela-Arrieta, C., D’Alise, A.M., Ruscio, A.D., Ebralidze, A.K., Kravtsov, Y., Ye, M., Kocher, O., Neubergh, D.S., et al. (2013). In vivo generation of transplantable human hematopoietic cells from induced pluripotent stem cells. *Blood* 121, 1255–1264.
- Amsellem, S., Pflumio, F., Bardinet, D., Izac, B., Charneau, P., Romeo, P.-H., Dubart-Kupperschmitt, A., and Fichelson, S. (2003). Ex vivo expansion of human hematopoietic stem cells by direct delivery of the HOXB4 homeoprotein. *Nat. Med.* 9, 1423–1427.
- Anasetti, C., Logan, B.R., Lee, S.J., Waller, E.K., Weisdorf, D.J., Wingard, J.R., Cutler, C.S., Westervelt, P., Woolfrey, A., Couban, S., et al. (2012). Peripheral-blood stem cells versus bone marrow from unrelated donors. *New England Journal of Medicine* 367, 1487–1496.
- Antonchuk, J., Sauvageau, G., and Humphries, R.K. (2002). HOXB4-induced expansion of adult hematopoietic stem cells ex vivo. *Cell* 109, 39–45.
- Audet, J., Miller, C.L., Rose-John, S., Piret, J.M., and Eaves, C.J. (2001). Distinct role of gp130 activation in promoting self-renewal divisions by mitogenically stimulated murine hematopoietic stem cells. *Proceedings of the National Academy of Sciences* 98, 1757–1762.
- Audet, J., Miller, C.L., Eaves, C.J., and Piret, J.M. (2002). Common and distinct features of cytokine effects on hematopoietic stem and progenitor cells revealed by dose-response surface analysis. *Biotechnology and Bioengineering* 80, 393–404.

- Barnett, M.J., Eaves, C.J., Phillips, G.L., Kalousek, D.K., Klingemann, H.G., Lansdorp, P.M., Reece, D.E., Shepherd, J.D., Shaw, G.J., and Eaves, A.C. (1989). Successful autografting in chronic myeloid leukaemia after maintenance of marrow in culture. *Bone Marrow Transplant.* *4*, 345–351.
- Barnett, M.J., Eaves, C.J., Phillips, G.L., Gascoyne, R.D., Hogge, D.E., Horsman, D.E., Humphries, R.K., Klingemann, H.G., Lansdorp, P.M., and Nantel, S.H. (1994). Autografting with cultured marrow in chronic myeloid leukemia: results of a pilot study. *Blood* *84*, 724–732.
- Bartenhagen, C. (2014). RDRToolbox: A package for nonlinear dimension reduction with Isomap and LLE.
- Baum, C.M., Weissman, I.L., Tsukamoto, A.S., Buckle, A.M., and Peault, B. (1992). Isolation of a candidate human hematopoietic stem-cell population. *PNAS* *89*, 2804–2808.
- Becker, P.S., Nilsson, S.K., Li, Z., Berrios, V.M., Dooner, M.S., Cooper, C.L., Hsieh, C., and Quesenberry, P.J. (1999). Adhesion receptor expression by hematopoietic cell lines and murine progenitors: Modulation by cytokines and cell cycle status. *Experimental Hematology* *27*, 533–541.
- Beer, P.A., Knapp, D.J.H.F., Kannan, N., Miller, P.H., Babovic, S., Bulaeva, E., Aghaeepour, N., Rabu, G., Rostamirad, S., Shih, K., et al. (2014). A dominant-negative isoform of IKAROS expands primitive normal human hematopoietic cells. *Stem Cell Reports* *3*, 841–857.
- Bensinger, W., DiPersio, J.F., and McCarty, J.M. (2009). Improving stem cell mobilization strategies: future directions. *Bone Marrow Transplantation* *43*, 181–195.
- Benveniste, P., Frelin, C., Janmohamed, S., Barbara, M., Herrington, R., Hyam, D., and Iscove, N.N. (2010). Intermediate-term hematopoietic stem cells with extended but time-limited reconstitution potential. *Cell Stem Cell* *6*, 48–58.
- Benz, C., Copley, M.R., Kent, D.G., Wohrer, S., Cortes, A., Aghaeepour, N., Ma, E., Mader, H., Rowe, K., Day, C., et al. (2012). Hematopoietic stem cell subtypes expand differentially during development and display distinct lymphopoietic programs. *Cell Stem Cell* *10*, 273–283.
- Berrios, V.M., Dooner, G.J., Nowakowski, G., Frimberger, A., Valinski, H., Quesenberry, P.J., and Becker, P.S. (2001). The molecular basis for the cytokine-induced defect in homing and engraftment of hematopoietic stem cells. *Experimental Hematology* *29*, 1326–1335.
- Biffi, A., Montini, E., Lorioli, L., Cesani, M., Fumagalli, F., Plati, T., Baldoli, C., Martino, S., Calabria, A., Canale, S., et al. (2013). Lentiviral hematopoietic stem cell gene therapy benefits metachromatic leukodystrophy. *Science* *341*, 1233–1238.
- Bodine, D.M., Orlic, D., Birkett, N.C., Seidel, N.E., and Zsebo, K.M. (1992). Stem cell factor increases colony-forming unit-spleen number in vitro in synergy with interleukin-6, and in vivo in Sl/Sld mice as a single factor. *Blood* *79*, 913–919.

- Boitano, A.E., Wang, J., Romeo, R., Bouchez, L.C., Parker, A.E., Sutton, S.E., Walker, J.R., Flaveny, C.A., Perdew, G.H., Denison, M.S., et al. (2010). Aryl hydrocarbon receptor antagonists promote the expansion of human hematopoietic stem cells. *Science* *329*, 1345–1348.
- Bonig, H., Priestley, G.V., and Papayannopoulou, T. (2006). Hierarchy of molecular-pathway usage in bone marrow homing and its shift by cytokines. *Blood* *107*, 79–86.
- Bowie, M.B., Kent, D.G., Dykstra, B., McKnight, K.D., McCaffrey, L., Hoodless, P.A., and Eaves, C.J. (2007). Identification of a new intrinsically timed developmental checkpoint that reprograms key hematopoietic stem cell properties. *Proc. Natl. Acad. Sci. U.S.A.* *104*, 5878–5882.
- Bradley, T.R., and Metcalf, D. (1966). The growth of mouse bone marrow cells in vitro. *The Australian Journal of Experimental Biology and Medical Science* *44*, 287–299.
- Broxmeyer, H.E., Douglas, G.W., Hangoc, G., Cooper, S., Bard, J., English, D., Army, M., Thomas, L., and Boyse, E.A. (1989). Human umbilical cord blood as a potential source of transplantable hematopoietic stem/progenitor cells. *PNAS* *86*, 3828–3832.
- Broxmeyer, H.E., Orschell, C.M., Clapp, D.W., Hangoc, G., Cooper, S., Plett, P.A., Liles, W.C., Li, X., Graham-Evans, B., Campbell, T.B., et al. (2005). Rapid mobilization of murine and human hematopoietic stem and progenitor cells with AMD3100, a CXCR4 antagonist. *J Exp Med* *201*, 1307–1318.
- Brunstein, C.G., and Wagner, J.E. (2006). Umbilical cord blood transplantation and banking. *Annual Review of Medicine* *57*, 403–417.
- Busch, K., Klapproth, K., Barile, M., Flossdorf, M., Holland-Letz, T., Schlenner, S.M., Reth, M., Höfer, T., and Rodewald, H.-R. (2015). Fundamental properties of unperturbed haematopoiesis from stem cells in vivo. *Nature advance online publication*.
- Bystrykh, L.V., Verovskaya, E., Zwart, E., Broekhuis, M., and de Haan, G. (2012). Counting stem cells: methodological constraints. *Nat Meth* *9*, 567–574.
- Campbell, T.B., Hangoc, G., Liu, Y., Pollok, K., and Broxmeyer, H.E. (2007). Inhibition of CD26 in human cord blood CD34+ cells enhances their engraftment of nonobese diabetic/severe combined immunodeficiency mice. *Stem Cells and Development* *16*, 347–354.
- Casado, F.L., Singh, K.P., and Gasiewicz, T.A. (2010). The aryl hydrocarbon receptor: Regulation of hematopoiesis and involvement in the progression of blood diseases. *Blood Cells, Molecules, and Diseases* *44*, 199–206.
- Cavazzana-Calvo, M., Payen, E., Negre, O., Wang, G., Hehir, K., Fusil, F., Down, J., Denaro, M., Brady, T., Westerman, K., et al. (2010). Transfusion independence and HMGA2 activation after gene therapy of human β -thalassaemia. *Nature* *467*, 318–322.

- Cavazzana-Calvo, M., Fischer, A., Bushman, F.D., Payen, E., Hacein-Bey-Abina, S., and Leboulch, P. (2011). Is normal hematopoiesis maintained solely by long-term multipotent stem cells? *Blood* *117*, 4420–4424.
- Chang, J., Morgenstern, G., Deakin, D., Testa, N.G., Coutinho, L., Scarffe, J.H., Harrison, C., and Dexter, T.M. (1986). Reconstitution of haemopoietic system with autologous marrow taken during relapse of acute myeloblastic leukaemia and grown in long-term culture. *The Lancet* *327*, 294–295.
- Chao, N.J., Schriber, J.R., Grimes, K., Long, G.D., Negrin, R.S., Raimondi, C.M., Horning, S.J., Brown, S.L., Miller, L., and Blume, K.G. (1993). Granulocyte colony-stimulating factor “mobilized” peripheral blood progenitor cells accelerate granulocyte and platelet recovery after high-dose chemotherapy. *Blood* *81*, 2031–2035.
- Cheung, A.M.S., Leung, D., Rostamirad, S., Dhillon, K., Miller, P.H., Droumeva, R., Brinkman, R.R., Hogge, D., Roy, D.C., and Eaves, C.J. (2012). Distinct but phenotypically heterogeneous human cell populations produce rapid recovery of platelets and neutrophils after transplantation. *Blood* *119*, 3431–3439.
- Cheung, A.M.S., Nguyen, L.V., Carles, A., Beer, P.A., Miller, P.H., Knapp, D.J.H.F., Dhillon, K., Hirst, M., and Eaves, C.J. (2013). Analysis of the clonal growth and differentiation dynamics of primitive barcoded human cord blood cells in NSG mice. *Blood* *122*, 3129–3137.
- Christ, O., Lucke, K., Imren, S., Leung, K., Hamilton, M., Eaves, A., Smith, C., and Eaves, C. (2007). Improved purification of hematopoietic stem cells based on their elevated aldehyde dehydrogenase activity. *Haematologica* *92*, 1165–1172.
- Christopherson, K.W., Hangoc, G., and Broxmeyer, H.E. (2002). Cell surface peptidase CD26/dipeptidylpeptidase IV regulates CXCL12/stromal cell-derived factor-1 α -mediated chemotaxis of human cord blood CD34+ progenitor cells. *J Immunol* *169*, 7000–7008.
- Christopherson, K.W., Hangoc, G., Mantel, C.R., and Broxmeyer, H.E. (2004). Modulation of hematopoietic stem cell homing and engraftment by CD26. *Science* *305*, 1000–1003.
- Civin, C.I., Almeida-Porada, G., Lee, M.J., Olweus, J., Terstappen, L.W., and Zanjani, E.D. (1996). Sustained, retransplantable, multilineage engraftment of highly purified adult human bone marrow stem cells in vivo. *Blood* *88*, 4102–4109.
- Copley, M.R., Beer, P.A., and Eaves, C.J. (2012). Hematopoietic stem cell heterogeneity takes center stage. *Cell Stem Cell* *10*, 690–697.
- Couban, S., Messner, H.A., Andreou, P., Egan, B., Price, S., Tinker, L., Meharchand, J., Forrest, D.L., and Lipton, J. (2000). Bone marrow mobilized with granulocyte colony-stimulating factor in related allogeneic transplant recipients: A study of 29 patients. *Biology of Blood and Marrow Transplantation* *6*, 422–427.

- Couban, S., Simpson, D.R., Barnett, M.J., Bredeson, C., Hubesch, L., Howson-Jan, K., Shore, T.B., Walker, I.R., Browett, P., Messner, H.A., et al. (2002). A randomized multicenter comparison of bone marrow and peripheral blood in recipients of matched sibling allogeneic transplants for myeloid malignancies. *Blood* *100*, 1525–1531.
- Craig, W., Kay, R., Cutler, R.L., and Lansdorp, P.M. (1993). Expression of Thy-1 on human hematopoietic progenitor cells. *J Exp Med* *177*, 1331–1342.
- Csaszar, E., Kirouac, D.C., Yu, M., Wang, W., Qiao, W., Cooke, M.P., Boitano, A.E., Ito, C., and Zandstra, P.W. (2012). Rapid expansion of human hematopoietic stem cells by automated control of inhibitory feedback signaling. *Cell Stem Cell* *10*, 218–229.
- Cutler, C., Giri, S., Jeyapalan, S., Paniagua, D., Viswanathan, A., and Antin, J.H. (2001). Acute and chronic graft-versus-host disease after allogeneic peripheral-blood stem-cell and bone marrow transplantation: a meta-analysis. *JCO* *19*, 3685–3691.
- Cutler, C., Multani, P., Robbins, D., Kim, H.T., Le, T., Hoggatt, J., Pelus, L.M., Despons, C., Chen, Y.-B., Rezner, B., et al. (2013). Prostaglandin-modulated umbilical cord blood hematopoietic stem cell transplantation. *Blood* *122*, 3074–3081.
- Danner, R., Chaudhari, S.N., Rosenberger, J., Surls, J., Richie, T.L., Brumeanu, T.-D., and Casares, S. (2011). Expression of HLA Class II Molecules in Humanized NOD.Rag1KO.IL2RgcKO Mice Is Critical for Development and Function of Human T and B Cells. *PLoS ONE* *6*, e19826.
- Delaney, C., Heimfeld, S., Brashem-Stein, C., Voorhies, H., Manger, R.L., and Bernstein, I.D. (2010). Notch-mediated expansion of human cord blood progenitor cells capable of rapid myeloid reconstitution. *Nature Medicine* *16*, 232–236.
- Devine, S.M., Flomenberg, N., Vesole, D.H., Liesveld, J., Weisdorf, D., Badel, K., Calandra, G., and DiPersio, J.F. (2004). Rapid mobilization of CD34+ cells following administration of the CXCR4 antagonist AMD3100 to patients with multiple myeloma and non-hodgkin's lymphoma. *JCO* *22*, 1095–1102.
- Devine, S.M., Vij, R., Rettig, M., Todt, L., McGlauchlen, K., Fisher, N., Devine, H., Link, D.C., Calandra, G., Bridger, G., et al. (2008). Rapid mobilization of functional donor hematopoietic cells without G-CSF using AMD3100, an antagonist of the CXCR4/SDF-1 interaction. *Blood* *112*, 990–998.
- DiPersio, J.F., Stadtmauer, E.A., Nademanee, A., Micallef, I.N.M., Stiff, P.J., Kaufman, J.L., Maziarz, R.T., Hosing, C., Früehauf, S., Horwitz, M., et al. (2009a). Plerixafor and G-CSF versus placebo and G-CSF to mobilize hematopoietic stem cells for autologous stem cell transplantation in patients with multiple myeloma. *Blood* *113*, 5720–5726.
- DiPersio, J.F., Micallef, I.N., Stiff, P.J., Bolwell, B.J., Maziarz, R.T., Jacobsen, E., Nademanee, A., McCarty, J., Bridger, G., and Calandra, G. (2009b). Phase III prospective randomized double-blind placebo-controlled trial of plerixafor plus granulocyte colony-stimulating factor

compared with placebo plus granulocyte colony-stimulating factor for autologous stem-cell mobilization and transplantation for patients with non-hodgkin's lymphoma. *JCO* 27, 4767–4773.

Doerschuk, C.M., Beyers, N., Coxson, H.O., Wiggs, B., and Hogg, J.C. (1993). Comparison of neutrophil and capillary diameters and their relation to neutrophil sequestration in the lung. *Journal of Applied Physiology* 74, 3040–3045.

Donzella, G.A., Schols, D., Lin, S.W., Esté, J.A., Nagashima, K.A., Maddon, P.J., Allaway, G.P., Sakmar, T.P., Henson, G., DeClercq, E., et al. (1998). AMD3100, a small molecule inhibitor of HIV-1 entry via the CXCR4 co-receptor. *Nat Med* 4, 72–77.

Doulatov, S., Notta, F., Laurenti, E., and Dick, J.E. (2012). Hematopoiesis: A Human Perspective. *Cell Stem Cell* 10, 120–136.

Dykstra, B., Kent, D., Bowie, M., McCaffrey, L., Hamilton, M., Lyons, K., Lee, S.-J., Brinkman, R., and Eaves, C. (2007). Long-term propagation of distinct hematopoietic differentiation programs in vivo. *Cell Stem Cell* 1, 218–229.

Dykstra, B., Olthof, S., Schreuder, J., Ritsema, M., and de Haan, G. (2011). Clonal analysis reveals multiple functional defects of aged murine hematopoietic stem cells. *Journal of Experimental Medicine* 208, 2691–2703.

Eaves, C., Glimm, H., Eisterer, W., Audet, J., Maguer-Satta, V., and Pireta, J. (2001). Characterization of human hematopoietic cells with short-lived in vivo repopulating activity. *Annals of the New York Academy of Sciences* 938, 63–71.

Ema, H., Morita, Y., and Suda, T. (2014). Heterogeneity and hierarchy of hematopoietic stem cells. *Experimental Hematology* 42, 74–82.e2.

Engelhardt, M. (2001). Hematopoietic recovery of ex vivo perfusion culture expanded bone marrow and unexpanded peripheral blood progenitors after myeloablative chemotherapy. *Bone Marrow Transplantation* 27.

Fares, I., Chagraoui, J., Gareau, Y., Gingras, S., Ruel, R., Mayotte, N., Csaszar, E., Knapp, D.J.H.F., Miller, P., Ngom, M., et al. (2014). Pyrimidoindole derivatives are agonists of human hematopoietic stem cell self-renewal. *Science* 345, 1509–1512.

Flomenberg, N., Devine, S.M., DiPersio, J.F., Liesveld, J.L., McCarty, J.M., Rowley, S.D., Vesole, D.H., Badel, K., and Calandra, G. (2005). The use of AMD3100 plus G-CSF for autologous hematopoietic progenitor cell mobilization is superior to G-CSF alone. *Blood* 106, 1867–1874.

Flomenberg, N., Comenzo, R.L., Badel, K., and Calandra, G. (2010). Plerixafor (Mozobil®) alone to mobilize hematopoietic stem cells from multiple myeloma patients for autologous transplantation. *Biology of Blood and Marrow Transplantation* 16, 695–700.

Gan, O.I., Murdoch, B., Larochelle, A., and Dick, J.E. (1997). Differential maintenance of primitive human SCID-repopulating cells, clonogenic progenitors, and long-term culture-initiating cells after incubation on human bone marrow stromal cells. *Blood* *90*, 641–650.

Gilliland, D.G., and Griffin, J.D. (2002). The roles of FLT3 in hematopoiesis and leukemia. *Blood* *100*, 1532–1542.

Glimm, H., Eisterer, W., Lee, K., Cashman, J., Holyoake, T.L., Nicolini, F., Shultz, L.D., von Kalle, C., and Eaves, C.J. (2001). Previously undetected human hematopoietic cell populations with short-term repopulating activity selectively engraft NOD/SCID- β 2 microglobulin-null mice. *Journal of Clinical Investigation* *107*, 199–206.

Glimm, H., Schmidt, M., Fischer, M., Schwarzwaelder, K., Wissler, M., Klingenberg, S., Prinz, C., Waller, C.F., Lange, W., Eaves, C.J., et al. (2005). Efficient marking of human cells with rapid but transient repopulating activity in autografted recipients. *Blood* *106*, 893–898.

Gluckman, E., Ruggeri, A., Volt, F., Cunha, R., Boudjedir, K., and Rocha, V. (2011). Milestones in umbilical cord blood transplantation. *British Journal of Haematology* *154*, 441–447.

Goessling, W., Allen, R.S., Guan, X., Jin, P., Uchida, N., Dovey, M., Harris, J.M., Metzger, M.E., Bonifacio, A.C., Stroncek, D., et al. (2011). Prostaglandin E2 enhances human cord blood stem cell xenotransplants and shows long-term safety in preclinical nonhuman primate transplant models. *Cell Stem Cell* *8*, 445–458.

Goldman, J.M., Gale, R.P., Horowitz, M.M., Biggs, J.C., Champlin, R.E., Gluckman, E., Hoffman, R.G., Jacobsen, S.J., Marmont, A.M., McGlave, P.B., et al. (1988). Bone marrow transplantation for chronic myelogenous leukemia in chronic phase. *Annals of Internal Medicine* *108*, 806.

Gratwohl, A., Pasquini, M.C., Aljurf, M., Atsuta, Y., Baldomero, H., Foeken, L., Gratwohl, M., Bouzas, L.F., Confer, D., Frauendorfer, K., et al. (2015). One million haemopoietic stem-cell transplants: a retrospective observational study. *The Lancet Haematology*.

Guenechea, G., Segovia, J.C., Albella, B., Lamana, M., Ramirez, M., Regidor, C., Fernandez, M.N., and Bueren, J.A. (1999). Delayed engraftment of nonobese diabetic/severe combined immunodeficient mice transplanted with ex vivo-expanded human CD34+ cord blood cells. *Blood* *93*, 1097–1105.

Guenechea, G., Gan, O.I., Dorrell, C., and Dick, J.E. (2001). Distinct classes of human stem cells that differ in proliferative and self-renewal potential. *Nature Immunology* *2*, 75–82.

Haas, R., Mohle, R., Fruhauf, S., Goldschmidt, H., Witt, B., Flentje, M., Wannemacher, M., and Hunstein, W. (1994). Patient characteristics associated with successful mobilizing and autografting of peripheral blood progenitor cells in malignant lymphoma. *Blood* *83*, 3787–3794.

Hess, D.A., Bonde, J., Craft, T.C., Wirthlin, L., Hohm, S., Lahey, R., Todt, L.M., Dipersio, J.F., Devine, S.M., and Nolta, J.A. (2007). Human progenitor cells rapidly mobilized by AMD3100

repopulate NOD/SCID mice with increased frequency in comparison to cells from the same donor mobilized by granulocyte colony stimulating factor. *Biology of Blood and Marrow Transplantation* 13, 398–411.

Hodgson, G.S., and Bradley, T.R. (1979). Properties of haematopoietic stem cells surviving 5-fluorouracil treatment: evidence for a pre-CFU-S cell? *Nature* 281, 381–382.

Hogan, C.J., Shpall, E.J., and Keller, G. (2002). Differential long-term and multilineage engraftment potential from subfractions of human CD34+ cord blood cells transplanted into NOD/SCID mice. *Proceedings of the National Academy of Sciences of the United States of America* 99, 413–418.

Hoggatt, J., Singh, P., Sampath, J., and Pelus, L.M. (2009). Prostaglandin E2 enhances hematopoietic stem cell homing, survival, and proliferation. *Blood* 113, 5444–5455.

Hogge, D.E., Lansdorp, P.M., Reid, D., Gerhard, B., and Eaves, C.J. (1996). Enhanced detection, maintenance, and differentiation of primitive human hematopoietic cells in cultures containing murine fibroblasts engineered to produce human steel factor, interleukin-3, and granulocyte colony-stimulating factor. *Blood* 88, 3765–3773.

Holmes, T., Yan, F., Ko, K.-H., Nordon, R., Song, E., O'Brien, T.A., and Dolnikov, A. (2012). Ex vivo expansion of cord blood progenitors impairs their short-term and long-term repopulating activity associated with transcriptional dysregulation of signalling networks. *Cell Proliferation* 45, 266–278.

Holyoake, T.L., Freshney, M.G., McNair, L., Parker, A.N., McKay, P.J., Steward, W.P., Fitzsimons, E., Graham, G.J., and Pragnell, I.B. (1996). Ex vivo expansion with stem cell factor and interleukin-11 augments both short-term recovery posttransplant and the ability to serially transplant marrow. *Blood* 87, 4589–4595.

Holyoake, T.L., Alcorn, M.J., Richmond, L., Farrell, E., Pearson, C., Green, R., Dunlop, D.J., Fitzsimons, E., Pragnell, I.B., and Franklin, I.M. (1997). CD34 positive PBPC expanded ex vivo may not provide durable engraftment following myeloablative chemoradiotherapy regimens. *Bone Marrow Transplant.* 19, 1095–1101.

Holyoake, T.L., Nicolini, F.E., and Eaves, C.J. (1999). Functional differences between transplantable human hematopoietic stem cells from fetal liver, cord blood, and adult marrow. *Experimental Hematology* 27, 1418–1427.

Hu, Y., and Smyth, G.K. (2009). ELDA: extreme limiting dilution analysis for comparing depleted and enriched populations in stem cell and other assays. *Journal of Immunological Methods* 347, 70–78.

Hu, Z., and Yang, Y.-G. (2012). Full reconstitution of human platelets in humanized mice after macrophage depletion. *Blood* 120, 1713–1716.

- Hughes, P.E., and Pfaff, M. (1998). Integrin affinity modulation. *Trends in Cell Biology* 8, 359–364.
- Jones, R.J., Wagner, J.E., Celano, P., Zicha, M.S., and Sharkis, S.J. (1990). Separation of pluripotent haematopoietic stem cells from spleen colony-forming cells. *Nature* 347, 188–189.
- Kallinikou, K., Anjos-Afonso, F., Blundell, M.P., Ings, S.J., Watts, M.J., Thrasher, A.J., Linch, D.C., Bonnet, D., and Yong, K.L. (2012). Engraftment defect of cytokine-cultured adult human mobilized CD34+ cells is related to reduced adhesion to bone marrow niche elements. *British Journal of Haematology* 158, 778–787.
- Kamel-Reid, S., and Dick, J.E. (1988). Engraftment of immune-deficient mice with human hematopoietic stem cells. *Science* 242, 1706–1709.
- Kawabata, K., Ujikawa, M., Egawa, T., Kawamoto, H., Tachibana, K., Iizasa, H., Katsura, Y., Kishimoto, T., and Nagasawa, T. (1999). A cell-autonomous requirement for CXCR4 in long-term lymphoid and myeloid reconstitution. *PNAS* 96, 5663–5667.
- Keeney, M., Chin-Yee, I., Weir, K., Popma, J., Nayar, R., and Sutherland, D.R. (1998). Single platform flow cytometric absolute CD34+ cell counts based on the ISHAGE guidelines. *Cytometry* 34, 61–70.
- Kent, D.G., Dykstra, B.J., Cheyne, J., Ma, E., and Eaves, C.J. (2008). Steel factor coordinately regulates the molecular signature and biologic function of hematopoietic stem cells. *Blood* 112, 560–567.
- Kent, D.G., Copley, M.R., Benz, C., Wohrer, S., Dykstra, B.J., Ma, E., Cheyne, J., Zhao, Y., Bowie, M.B., Zhao, Y., et al. (2009). Prospective isolation and molecular characterization of hematopoietic stem cells with durable self-renewal potential. *Blood* 113, 6342–6350.
- Kittler, E.L.W., Peters, S.O., Crittenden, R.B., Debatis, M.E., Ramshaw, H.S., Stewart, F.M., and Quesenberry, P.J. (1997). Cytokine-facilitated transduction leads to low-level engraftment in nonablated hosts. *Blood* 90, 865–872.
- Kovach, N.L., Lin, N., Yednock, T., Harlan, J.M., and Broudy, V.C. (1995). Stem cell factor modulates avidity of alpha 4 beta 1 and alpha 5 beta 1 integrins expressed on hematopoietic cell lines. *Blood* 85, 159–167.
- Lapidot, T., and Petit, I. (2002). Current understanding of stem cell mobilization: the roles of chemokines, proteolytic enzymes, adhesion molecules, cytokines, and stromal cells. *Experimental Hematology* 30, 973–981.
- Larochelle, A., Gillette, J.M., Desmond, R., Ichwan, B., Cantilena, A., Cerf, A., Barrett, A.J., Wayne, A.S., Lippincott-Schwartz, J., and Dunbar, C.E. (2012). Bone marrow homing and engraftment of human hematopoietic stem and progenitor cells is mediated by a polarized membrane domain. *Blood* 119, 1848–1855.

- Lemischka, I.R. (1991). Clonal, in vivo behavior of the totipotent hematopoietic stem cell. *Semin. Immunol.* 3, 349–355.
- Lévesque, J.-P., Leavesley, D.I., Niutta, S., Vadas, M., and Simmons, P.J. (1995). Cytokines increase human hemopoietic cell adhesiveness by activation of very late antigen (VLA)-4 and VLA-5 integrins. *The Journal of Experimental Medicine* 181, 1805–1815.
- Lévesque, J.-P., Takamatsu, Y., Nilsson, S.K., Haylock, D.N., and Simmons, P.J. (2001). Vascular cell adhesion molecule-1 (CD106) is cleaved by neutrophil proteases in the bone marrow following hematopoietic progenitor cell mobilization by granulocyte colony-stimulating factor. *Blood* 98, 1289–1297.
- Liles, W.C., Broxmeyer, H.E., Rodger, E., Wood, B., Hübel, K., Cooper, S., Hangoc, G., Bridger, G.J., Henson, G.W., Calandra, G., et al. (2003). Mobilization of hematopoietic progenitor cells in healthy volunteers by AMD3100, a CXCR4 antagonist. *Blood* 102, 2728–2730.
- Liles, W.C., Rodger, E., Broxmeyer, H.E., Dehner, C., Badel, K., Calandra, G., Christensen, J., Wood, B., Price, T.H., and Dale, D.C. (2005). Augmented mobilization and collection of CD34+ hematopoietic cells from normal human volunteers stimulated with granulocyte–colony-stimulating factor by single-dose administration of AMD3100, a CXCR4 antagonist. *Transfusion* 45, 295–300.
- Liu, F., Poursine-Laurent, J., and Link, D.C. (2000). Expression of the G-CSF receptor on hematopoietic progenitor cells is not required for their mobilization by G-CSF. *Blood* 95, 3025–3031.
- Loo, J. van der, and Ploemacher, R.E. (1995). Marrow- and spleen-seeding efficiencies of all murine hematopoietic stem cell subsets are decreased by preincubation with hematopoietic growth factors. *Blood* 85, 2598–2606.
- Magli, M.C., Iscove, N.N., and Odartchenko, N. (1982). Transient nature of early haematopoietic spleen colonies. *Nature* 295, 527–529.
- Majeti, R., Park, C.Y., and Weissman, I.L. (2007). Identification of a hierarchy of multipotent hematopoietic progenitors in human cord blood. *Cell Stem Cell* 1, 635–645.
- Mathioudakis, G., Storb, R., McSweeney, P.A., Torok-Storb, B., Lansdorp, P.M., Brümmendorf, T.H., Gass, M.J., Bryant, E.M., Storek, J., Flowers, M.E.D., et al. (2000). Polyclonal hematopoiesis with variable telomere shortening in human long-term allogeneic marrow graft recipients. *Blood* 96, 3991–3994.
- Mazurier, F., Gan, O.I., McKenzie, J.L., Doedens, M., and Dick, J.E. (2004). Lentivector-mediated clonal tracking reveals intrinsic heterogeneity in the human hematopoietic stem cell compartment and culture-induced stem cell impairment. *Blood* 103, 545–552.

McKenzie, J.L., Gan, O.I., Doedens, M., and Dick, J.E. (2005). Human short-term repopulating stem cells are efficiently detected following intrafemoral transplantation into NOD/SCID recipients depleted of CD122+ cells. *Blood* *106*, 1259–1261.

McNiece, I.K., Stewart, F.M., Deacon, D.M., and Quesenberry, P.J. (1988). Synergistic interactions between hematopoietic growth factors as detected by in vitro mouse bone marrow colony formation. *Experimental Hematology* *16*, 383–388.

Metcalf, D. (2008). Hematopoietic cytokines. *Blood* *111*, 485–491.

Metcalf, D., and Nicola, N.A. (1992). The clonal proliferation of normal mouse hematopoietic cells: enhancement and suppression by colony-stimulating factor combinations. *Blood* *79*, 2861–2866.

Miller, C.L., and Eaves, C.J. (1997). Expansion in vitro of adult murine hematopoietic stem cells with transplantable lympho-myeloid reconstituting ability. *PNAS* *94*, 13648–13653.

Miller, P.H., Cheung, A.M.S., Beer, P.A., Knapp, D.J.H.F., Dhillon, K., Rabu, G., Rostamirad, S., Humphries, R.K., and Eaves, C.J. (2013a). Enhanced normal short-term human myelopoiesis in mice engineered to express human-specific myeloid growth factors. *Blood* *121*, e1–e4.

Miller, P.H., Knapp, D.J.H.F., and Eaves, C.J. (2013b). Heterogeneity in hematopoietic stem cell populations: implications for transplantation. *Current Opinion in Hematology* *20*, 257–264.

Morita, Y., Ema, H., and Nakauchi, H. (2010). Heterogeneity and hierarchy within the most primitive hematopoietic stem cell compartment. *J Exp Med* *207*, 1173–1182.

Morrison, S.J., Wright, D.E., and Weissman, I.L. (1997). Cyclophosphamide/granulocyte colony-stimulating factor induces hematopoietic stem cells to proliferate prior to mobilization. *Proc Natl Acad Sci U S A* *94*, 1908–1913.

Morton, J., Hutchins, C., and Durrant, S. (2001). Granulocyte–colony-stimulating factor (G-CSF)–primed allogeneic bone marrow: significantly less graft-versus-host disease and comparable engraftment to G-CSF–mobilized peripheral blood stem cells. *Blood* *98*, 3186–3191.

Muench, M.O., Schneider, J.G., and Moore, M.A. (1992). Interactions among colony-stimulating factors, IL-1 beta, IL-6, and kit-ligand in the regulation of primitive murine hematopoietic cells. *Exp. Hematol.* *20*, 339–349.

Muench, M.O., Firpo, M.T., and Moore, M.A. (1993). Bone marrow transplantation with interleukin-1 plus kit-ligand ex vivo expanded bone marrow accelerates hematopoietic reconstitution in mice without the loss of stem cell lineage and proliferative potential. *Blood* *81*, 3463–3473.

Muller-Sieburg, C.E., Sieburg, H.B., Bernitz, J.M., and Cattarossi, G. (2012). Stem cell heterogeneity: implications for aging and regenerative medicine. *Blood* *119*, 3900–3907.

- Nademanee, A.P., DiPersio, J.F., Maziarz, R.T., Stadtmauer, E.A., Micallef, I.N., Stiff, P.J., Hsu, F.J., Bridger, G., and Bolwell, B.J. (2012). Plerixafor plus granulocyte colony-stimulating factor versus placebo plus granulocyte colony-stimulating factor for mobilization of CD34+ hematopoietic stem cells in patients with multiple myeloma and low peripheral blood CD34+ cell count: results of a subset analysis of a randomized trial. *Biology of Blood and Marrow Transplantation* 18, 1564–1572.
- Nakahata, T., and Ogawa, M. (1982). Hemopoietic colony-forming cells in umbilical cord blood with extensive capability to generate mono- and multipotential hemopoietic progenitors. *J Clin Invest* 70, 1324–1328.
- Nicolini, F.E., Cashman, J.D., Hogge, D.E., Humphries, R.K., and Eaves, C.J. (2004). NOD/SCID mice engineered to express human IL-3, GM-CSF and Steel factor constitutively mobilize engrafted human progenitors and compromise human stem cell regeneration. *Leukemia* 18, 341–347.
- North, T.E., Goessling, W., Walkley, C.R., Lengerke, C., Kopani, K.R., Lord, A.M., Weber, G.J., Bowman, T.V., Jang, I.-H., Grosser, T., et al. (2007). Prostaglandin E2 regulates vertebrate haematopoietic stem cell homeostasis. *Nature* 447, 1007–1011.
- Notta, F., Doulatov, S., Laurenti, E., Poeppl, A., Jurisica, I., and Dick, J.E. (2011). Isolation of single human hematopoietic stem cells capable of long-term multilineage engraftment. *Science* 333, 218–221.
- Okano, A., Suzuki, C., Takatsuki, F., Akiyama, Y., Koike, K., Ozawa, K., Hirano, T., Kishimoto, T., Nakahata, T., and Asano, S. (1989). In vitro expansion of the murine pluripotent hemopoietic stem cell population in response to interleukin 3 and interleukin 6. Application to bone marrow transplantation. *Transplantation* 48, 495–498.
- Pang, W.W., Price, E.A., Sahoo, D., Beerman, I., Maloney, W.J., Rossi, D.J., Schrier, S.L., and Weissman, I.L. (2011). Human bone marrow hematopoietic stem cells are increased in frequency and myeloid-biased with age. *Proceedings of the National Academy of Sciences* 108, 20012–20017.
- Papayannopoulou, T., and Nakamoto, B. (1993). Peripheralization of hemopoietic progenitors in primates treated with anti-VLA4 integrin. *PNAS* 90, 9374–9378.
- Payen, E., and Leboulch, P. (2012). Advances in stem cell transplantation and gene therapy in the β -hemoglobinopathies. *ASH Education Program Book 2012*, 276–283.
- Peled, A., Petit, I., Kollet, O., Magid, M., Ponomaryov, T., Byk, T., Nagler, A., Ben-Hur, H., Many, A., Shultz, L., et al. (1999). Dependence of human stem cell engraftment and repopulation of NOD/SCID mice on CXCR4. *Science* 283, 845–848.
- Perez, L.E., Rinder, H.M., Wang, C., Tracey, J.B., Maun, N., and Krause, D.S. (2001). Xenotransplantation of immunodeficient mice with mobilized human blood CD34+ cells

provides an in vivo model for human megakaryocytopoiesis and platelet production. *Blood* 97, 1635–1643.

Peters, S.O., Kittler, E.L., Ramshaw, H.S., and Quesenberry, P.J. (1995). Murine marrow cells expanded in culture with IL-3, IL-6, IL-11, and SCF acquire an engraftment defect in normal hosts. *Exp. Hematol.* 23, 461–469.

Peters, S.O., Kittler, E.L., Ramshaw, H.S., and Quesenberry, P.J. (1996). Ex vivo expansion of murine marrow cells with interleukin-3 (IL-3), IL-6, IL-11, and stem cell factor leads to impaired engraftment in irradiated hosts. *Blood* 87, 30–37.

Petzer, A.L., Zandstra, P.W., Piret, J.M., and Eaves, C.J. (1996a). Differential cytokine effects on primitive (CD34+ CD38-) human hematopoietic cells: novel responses to Flt3-ligand and thrombopoietin. *The Journal of Experimental Medicine* 183, 2551–2558.

Petzer, A.L., Hogge, D.E., Landsdorp, P.M., Reid, D.S., and Eaves, C.J. (1996b). Self-renewal of primitive human hematopoietic cells (long-term-culture-initiating cells) in vitro and their expansion in defined medium. *Proceedings of the National Academy of Sciences* 93, 1470–1474.

Pluznik, D.H., and Sachs, L. (1965). The cloning of normal “Mast” cells in tissue culture. *J. Cell. Comp. Physiol.* 66, 319–324.

Powles, R., Mehta, J., Kulkarni, S., Treleaven, J., Millar, B., Marsden, J., Shepherd, V., Rowland, A., Sirohi, B., Tait, D., et al. (2000). Allogeneic blood and bone-marrow stem-cell transplantation in haematological malignant diseases: a randomised trial. *The Lancet* 355, 1231–1237.

Ramírez, M., Segovia, J.C., Benet, I., Arbona, C., Güenechea, G., Blaya, C., García-Conde, J., Bueren, J.A., and Prosper, F. (2001). Ex vivo expansion of umbilical cord blood (UCB) CD34+ cells alters the expression and function of $\alpha 4\beta 1$ and $\alpha 5\beta 1$ integrins. *British Journal of Haematology* 115, 213–221.

Rocha, V., and Gluckman, E. (2006). Clinical use of umbilical cord blood hematopoietic stem cells. *Biology of Blood and Marrow Transplantation* 12, 34–41.

Rocha, V., Labopin, M., Sanz, G., Arcese, W., Schwerdtfeger, R., Bosi, A., Jacobsen, N., Ruutu, T., de Lima, M., Finke, J., et al. (2004). Transplants of umbilical-cord blood or bone marrow from unrelated donors in adults with acute leukemia. *New England Journal of Medicine* 351, 2276–2285.

Rongvaux, A., Willinger, T., Takizawa, H., Rathinam, C., Auerbach, W., Murphy, A.J., Valenzuela, D.M., Yancopoulos, G.D., Eynon, E.E., Stevens, S., et al. (2011). Human thrombopoietin knockin mice efficiently support human hematopoiesis in vivo. *Proceedings of the National Academy of Sciences* 108, 2378–2383.

Rongvaux, A., Willinger, T., Martinek, J., Strowig, T., Gearty, S.V., Teichmann, L.L., Saito, Y., Marches, F., Halene, S., Palucka, A.K., et al. (2014). Development and function of human innate immune cells in a humanized mouse model. *Nat Biotech advance online publication*.

Rönstrand, L. (2004). Signal transduction via the stem cell factor receptor/c-Kit. *CMLS, Cell. Mol. Life Sci.* 61, 2535–2548.

Rubinstein, P., Carrier, C., Scaradavou, A., Kurtzberg, J., Adamson, J., Migliaccio, A.R., Berkowitz, R.L., Cabbad, M., Dobrila, N.L., and Taylor, P.E. (1998). Outcomes among 562 recipients of placental-blood transplants from unrelated donors. *New England Journal of Medicine* 339, 1565–1577.

Sauvageau, G., Lansdorp, P.M., Eaves, C.J., Hogge, D.E., Dragowska, W.H., Reid, D.S., Largman, C., Lawrence, H.J., and Humphries, R.K. (1994). Differential expression of homeobox genes in functionally distinct CD34+ subpopulations of human bone marrow cells. *Proceedings of the National Academy of Sciences* 91, 12223–12227.

Schindler, C.W. (2002). JAK-STAT signaling in human disease. *J Clin Invest* 109, 1133–1137.

Schmitz, N., Dreger, P., Suttorp, M., Rohwedder, E.B., Haferlach, T., Loffler, H., Hunter, A., and Russell, N.H. (1995). Primary transplantation of allogeneic peripheral blood progenitor cells mobilized by filgrastim (granulocyte colony-stimulating factor). *Blood* 85, 1666–1672.

Sekulovic, S., Gasparetto, M., Lecault, V., Hoesli, C.A., Kent, D.G., Rosten, P., Wan, A., Brookes, C., Hansen, C.L., Piret, J.M., et al. (2011). Ontogeny stage-independent and high-level clonal expansion in vitro of mouse hematopoietic stem cells stimulated by an engineered NUP98-HOX fusion transcription factor. *Blood* 118, 4366–4376.

Senn, J.S., McCulloch, E.A., and Till, J.E. (1967). Comparison of colony-forming ability of normal and leukæmic human marrow in cell culture. *The Lancet* 290, 597–598.

Sheridan, W.P., Fox, R.M., Begley, C.G., Maher, D., McGrath, K.M., Begley, C.G., Juttner, C.A., To, L.B., Szer, J., and Mostyn, G. (1992). Effect of peripheral-blood progenitor cells mobilised by filgrastim (G-CSF) on platelet recovery after high-dose chemotherapy. *The Lancet* 339, 640–644.

Shier, L.R., Schultz, K.R., Imren, S., Regan, J., Issekutz, A., Sadek, I., Gilman, A., Luo, Z., Panzarella, T., Eaves, C.J., et al. (2004). Differential effects of granulocyte colony-stimulating factor on marrow- and blood-derived hematopoietic and immune cell populations in healthy human donors. *Biology of Blood and Marrow Transplant* 10, 624–634.

Shpall, E.J., Quinones, R., Giller, R., Zeng, C., Baron, A.E., Jones, R.B., Bearman, S.I., Nieto, Y., Freed, B., Madinger, N., et al. (2002). Transplantation of ex vivo expanded cord blood. *Biology of Blood and Marrow Transplantation* 8, 368–376.

Shultz, L.D., Banuelos, S.J., Leif, J., Appel, M.C., Cunningham, M., Ballen, K., Burzenski, L., and Greiner, D.L. (2003). Regulation of human short-term repopulating cell (STRC) engraftment in NOD/SCID mice by host CD122+ cells. *Experimental Hematology* 31, 551–558.

Shultz, L.D., Lyons, B.L., Burzenski, L.M., Gott, B., Chen, X., Chaleff, S., Kotb, M., Gillies, S.D., King, M., and Mangada, J. (2005). Human lymphoid and myeloid cell development in NOD/LtSz-scid IL2R γ null mice engrafted with mobilized human hemopoietic stem cells. *The Journal of Immunology* 174, 6477–6489.

Shultz, L.D., Ishikawa, F., and Greiner, D.L. (2007). Humanized mice in translational biomedical research. *Nature Reviews Immunology* 7, 118–130.

Shultz, L.D., Brehm, M.A., Garcia-Martinez, J.V., and Greiner, D.L. (2012). Humanized mice for immune system investigation: progress, promise and challenges. *Nature Reviews Immunology* 12, 786–798.

Sloma, I., Imren, S., Beer, P.A., Zhao, Y., Lecault, V., Leung, D., Raghuram, K., Brimacombe, C., Lambie, K., Piret, J., et al. (2012). Ex vivo expansion of normal and chronic myeloid leukemic stem cells without functional alteration using a NUP98HOXA10homeodomain fusion gene. *Leukemia* 27, 159–169.

Smith, T.J., Hillner, B.E., Schmitz, N., Linch, D.C., Dreger, P., Goldstone, A.H., Boogaerts, M.A., Ferrant, A., Link, H., Zander, A., et al. (1997). Economic analysis of a randomized clinical trial to compare filgrastim-mobilized peripheral-blood progenitor-cell transplantation and autologous bone marrow transplantation in patients with Hodgkin's and non-Hodgkin's lymphoma. *JCO* 15, 5–10.

Sponcer, E., and Dexter, T.M. (1983). Transplantation of long-term cultured bone marrow cells. *Transplantation* 35, 624–627.

Sun, J., Ramos, A., Chapman, B., Johnnidis, J.B., Le, L., Ho, Y.-J., Klein, A., Hofmann, O., and Camargo, F.D. (2014). Clonal dynamics of native haematopoiesis. *Nature* 514, 322–327.

Sutherland, H.J., Eaves, C.J., Eaves, A.C., Dragowska, W., and Lansdorp, P.M. (1989). Characterization and partial purification of human marrow cells capable of initiating long-term hematopoiesis in vitro. *Blood* 74, 1563–1570.

Sutherland, H.J., Lansdorp, P.M., Henkelman, D.H., Eaves, A.C., and Eaves, C.J. (1990). Functional characterization of individual human hematopoietic stem cells cultured at limiting dilution on supportive marrow stromal layers. *PNAS* 87, 3584–3588.

Suwa, T., Hogg, J.C., Klut, M.E., Hards, J., and van Eeden, S.F. (2001). Interleukin-6 changes deformability of neutrophils and induces their sequestration in the lung. *American Journal of Respiratory and Critical Care Medicine* 163, 970–976.

- Szilvassy, S.J., Bass, M.J., Zant, G.V., and Grimes, B. (1999). Organ-selective homing defines engraftment kinetics of murine hematopoietic stem cells and is compromised by ex vivo expansion. *Blood* 93, 1557–1566.
- Szilvassy, S.J., Meyerrose, T.E., Ragland, P.L., and Grimes, B. (2001). Homing and engraftment defects in ex vivo expanded murine hematopoietic cells are associated with downregulation of $\beta 1$ integrin. *Experimental Hematology* 29, 1494–1502.
- Takahashi, K., Tanabe, K., Ohnuki, M., Narita, M., Ichisaka, T., Tomoda, K., and Yamanaka, S. (2007). Induction of Pluripotent Stem Cells from Adult Human Fibroblasts by Defined Factors. *Cell* 131, 861–872.
- Takatoku, M., Sellers, S., Agricola, B.A., Metzger, M.E., Kato, I., Donahue, R.E., and Dunbar, C.E. (2001). Avoidance of stimulation improves engraftment of cultured and retrovirally transduced hematopoietic cells in primates. *Journal of Clinical Investigation* 108, 447–455.
- Takenaka, K., Prasolava, T.K., Wang, J.C.Y., Mortin-Toth, S.M., Khalouei, S., Gan, O.I., Dick, J.E., and Danska, J.S. (2007). Polymorphism in Sirpa modulates engraftment of human hematopoietic stem cells. *Nature Immunology* 8, 1313–1323.
- Tenenbaum, J.B., Silva, V. de, and Langford, J.C. (2000). A global geometric framework for nonlinear dimensionality reduction. *Science* 290, 2319–2323.
- Tisdale, J.F., Hanazono, Y., Sellers, S.E., Metzger, M.E., Donahue, R.E., and Dunbar, C.E. (1998). Ex vivo expansion of genetically marked rhesus peripheral blood progenitor cells results in diminished long-term repopulating ability. *Blood* 92, 1131–1141.
- To, L.B., Roberts, M.M., Haylock, D.N., Dyson, P.G., Branford, A.L., Thorp, D., Ho, J.Q., Dart, G.W., Horvath, N., and Davy, M.L. (1992). Comparison of haematological recovery times and supportive care requirements of autologous recovery phase peripheral blood stem cell transplants, autologous bone marrow transplants and allogeneic bone marrow transplants. *Bone Marrow Transplant*. 9, 277–284.
- To, L.B., Levesque, J.-P., and Herbert, K.E. (2011). How I treat patients who mobilize hematopoietic stem cells poorly. *Blood* 118, 4530–4540.
- Udomsakdi, C., Lansdorp, P.M., Hogge, D.E., Reid, D.S., Eaves, A.C., and Eaves, C.J. (1992). Characterization of primitive hematopoietic cells in normal human peripheral blood. *Blood* 80, 2513–2521.
- Vallera, D.A., and Blazar, B.R. (1988). Depressed leukocyte reconstitution and engraftment in murine recipients of T cell-depleted histoincompatible marrow pretreated with interleukin 3. *Transplantation* 46, 616–620.
- Wang, J., Guan, E., Roderiquez, G., Calvert, V., Alvarez, R., and Norcross, M.A. (2001). Role of tyrosine phosphorylation in ligand-independent sequestration of CXCR4 in human primary monocytes-macrophages. *J. Biol. Chem.* 276, 49236–49243.

- Wang, J.C., Doedens, M., and Dick, J.E. (1997). Primitive human hematopoietic cells are enriched in cord blood compared with adult bone marrow or mobilized peripheral blood as measured by the quantitative in vivo SCID-repopulating cell assay. *Blood* 89, 3919–3924.
- Watts, K.L., Zhang, X., Beard, B.C., Chiu, S.Y., Trobridge, G.D., Humphries, R.K., and Kiem, H.-P. (2011). Differential effects of HOXB4 and NUP98-HOXA10hd on hematopoietic repopulating cells in a nonhuman primate model. *Human Gene Therapy* 22, 1475–1482.
- Wilson, A., Laurenti, E., Oser, G., van der Wath, R.C., Blanco-Bose, W., Jaworski, M., Offner, S., Dunant, C.F., Eshkind, L., Bockamp, E., et al. (2008). Hematopoietic stem cells reversibly switch from dormancy to self-renewal during homeostasis and repair. *Cell* 135, 1118–1129.
- Wohrer, S., Knapp, D.J.H.F., Copley, M.R., Benz, C., Kent, D.G., Rowe, K., Babovic, S., Mader, H., Oostendorp, R.A.J., and Eaves, C.J. (2014). Distinct stromal cell factor combinations can separately control hematopoietic stem cell survival, proliferation, and self-renewal. *Cell Reports* 7, 1956–1967.
- Wunderlich, M., Chou, F.-S., Link, K. a, Mizukawa, B., Perry, R.L., Carroll, M., and Mulloy, J.C. (2010). AML xenograft efficiency is significantly improved in NOD/SCID-IL2RG mice constitutively expressing human SCF, GM-CSF and IL-3. *Leukemia* 24, 1785–1788.
- Wyss, B.K., Donnelly, A.F.W., Zhou, D., Sinn, A.L., Pollok, K.E., and Goebel, W.S. (2009). Enhanced homing and engraftment of fresh but not ex vivo cultured murine marrow cells in submyeloablated hosts following CD26 inhibition by Diprotin A. *Experimental Hematology* 37, 814–823.
- Yamamura, K., Ohishi, K., Masuya, M., Miyata, E., Sugimoto, Y., Nakamura, S., Fujieda, A., Araki, H., and Katayama, N. (2008). Ex vivo culture of human cord blood hematopoietic stem/progenitor cells adversely influences their distribution to other bone marrow compartments after intra-bone marrow transplantation. *Stem Cells* 26, 543–549.
- Yeager, A.M., Kaizer, H., Santos, G.W., Saral, R., Colvin, O.M., Stuart, R.K., Braine, H.G., Burke, P.J., Ambinder, R.F., and Burns, W.H. (1986). Autologous bone marrow transplantation in patients with acute nonlymphocytic leukemia, using ex vivo marrow treatment with 4-hydroperoxycyclophosphamide. *The New England Journal of Medicine* 315, 141–147.
- Zandstra, Conneally, Piret, and Eaves (1998). Ontogeny-associated changes in the cytokine responses of primitive human haemopoietic cells. *British Journal of Haematology* 101, 770–778.
- Zandstra, P.W., Conneally, E., Petzer, A.L., Piret, J.M., and Eaves, C.J. (1997). Cytokine manipulation of primitive human hematopoietic cell self-renewal. *Proceedings of the National Academy of Sciences* 94, 4698–4703.
- Zhang, X.-B., Beard, B.C., Beebe, K., Storer, B., Humphries, R.K., and Kiem, H.-P. (2006). Differential effects of HOXB4 on nonhuman primate short- and long-term repopulating cells. *PLoS Med* 3, e173.