THE ROLE OF THE *IN VIVO* MICROENVIRONMENT

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HUMAN STEM CELL FATE DECISIONS

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BY

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Abstract

Years of research in the field of stem cell biology have resulted in only modest gains in our ability to purify human stem cells and manipulate their function *ex vivo,* suggesting that stem cell fate decisions are highly dependent upon non-cell-autonomous parameters in their physiologic *in vivo* setting. A number of non-human model systems have now revealed that stem cell function appears to be regulated by a specific *in vivo* microenvironment, also known as the niche. Prior to the work undertaken in this thesis, the role of the niche in the function of normal and transformed human stem cells had not been investigated, existing only as a theoretical concept originally proposed by the biologist Raymond Schofield. We therefore hypothesized that *the in vivo microenvironment is an essential regulator of human stem cell fate decisions, and that the niche is a determinant of both functional heterogeneity in the human stem cell compartment as well as the process of transformation.*

Initially, we postulated that if human stem cell fate decisions are dependent upon an *in vivo* niche, then we could identify novel molecular regulators of human stem cell fate decisions within the context of the *in vivo* microenvironment, and show that these regulators function uniquely within this setting. Our findings revealed the Bcl-2 family member MCL-1 as a novel molecular regulator of human hematopoietic stem cell (hHSC) self-renewal *in vivo*, and showed that the role of MCL-1 is unique to the *in vivo* setting as opposed to *in vitro* culture systems.

Subsequently, we sought to characterize the anatomical and molecular parameters that define the human stem cell microenvironment and regulate stem cell function *in vivo*.

We identified a specific *in vivo* niche in the trabecular bone region of the marrow, which regulates hHSC fate decisions through a Notch/Notch-ligand axis. We further showed that *in vivo* niche propensity underlies functional heterogeneity in the hHSC compartment, and that this niche propensity could be exploited to prospectively isolate hHSCs that are enhanced for *in vivo* regenerative function.

Finally, we investigated whether human cancer stem cells (CSCs) are dependent upon the same niches as their normal tissue counterparts *in vivo* as part of the transformation process. We found that transformed leukemic stem cells (LSCs) dynamically compete with normal human HSCs for niche occupancy *in vivo* to support their stem cell self-renewal function, and further by replacing a transformed LSC with a normal human HSC in the *in vivo* niche, we could eradicate self-renewing LSCs and reduce the leukemic burden *in vivo*.

Overall, this thesis has demonstrated that normal and transformed human stem cell fate decisions are controlled in a non-cell-autonomous manner by their *in vivo* microenvironment. The insights presented here support the theory originally proposed by Raymond Schofield that stem cells are not independent functional entities, but rather function as fixed tissue cells *in vivo*. These findings warrant a novel approach in the field to both stem cell-based therapies in regenerative medicine, and to targeting human malignancies at the stem cell level.

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And finally, I would like to thank my mother, Sandra Campbell, who has dedicated her entire life to the well-being and success of her children, and who has always whole-heartedly and unconditionally supported my ideas, my goals and my aspirations. She has been my greatest role model over the years.

Forward

I'm not sure if the "war on cancer" was ever really a war. It seems more like a defensive position, where over time we are slowly gaining the ability to hold off these ancient and merciless diseases for just a little while longer. In my experience, those who succumb to a cancer do not lose any "battle". Rather, their disease relentlessly tortures them, both physically and psychologically, until in their inevitable death occurs. Those who survive their cancer should not be deemed "winners" (as the often are), as this denigrates those who do not survive as being weak or somehow at fault. The survivors are merely lucky and fortunate enough to live in an age where science and technology has significant influence in medicine. However, today I can say with cautious optimism that there appears to be new hope for some significant progress against this onslaught. Behind all of the myth, media hype and politics that surround the stem cell field, I truly believe that there are some tangible, novel paradigms that may tip the balance in our favour against some cancers. If there is any small contribution that I can make to science and medicine during my time here, it will be toward the eradication of one or more cancers utilizing therapies derived from our understanding of human stem cell biology. Besides a clear focus and brilliant thinking, this will only be achieved through a high degree of collaboration, without which this work would not have been possible.

This work is dedicated to my father,

Clinton Lorne Campbell

1954-1997

I want to thank you for everything you've done for me.

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List of Abbreviations

Abbreviation	Long form
ALL	Acute lymphoblastic leukemia
AML	Acute myelogenous leukemia
Ang1	Angiopoietin 1
BFU	Blast forming unit
BFU-E	Blast forming unit erythroid
BM	Bone marrow
BMP	Bone morphogenic protein
CB	(Umbilical) cord blood
CFU	Colony forming unit
CFU-E	Erythroid colony forming unit
CFU-GEMM	Granulocyte, erythrocyte, macrophage, megakaryocyte colony forming unit
CFU-GM	Granulocyte, macrophage colony forming unit
CFU-S	Spleen colony forming unit
CML	Chronic myelogenous leukemia
CSC	Cancer stem cell
EN	Endosteal niche
FACS	Fluorescence activating cell sorting
FGF	Fibroblast growth factor
FLT3L	FMS-like tyrosine kinase 3 ligand
FZD	Frizzled
GLI	Glioblastoma

Abbreviation	Long form
GSC	Gonadal stem cell
GSK-3	Glycogen synthase kinase 3
hdF	hESC-derived fibroblast-like cell
hESC	Human embryonic stem cell
hGSC	Human gonadal stem cell
Hh	Hedgehog
hHPC	Human hematopoietic progenitor cell
hHSC	Human hematopoietic stem cell
hHSPC	Human hematopoietic stem and progenitor cell
HLA	Human leukocyte antigen
hMSC	Human mesenchymal stem cell
HPC	Hematopoietic progenitor cell
HSC	Hematopoietic stem cell
HSPC	Hematopoietic stem and progenitor cell
hSSC	Human somatic stem cell
IF	Intrafemoral
IGF	Insulin-like growth factor
IHC	Immunohistochemistry
IN	Interniche
IV	Intravenous
LBA	Long bone area
Lin-	Lineage negative

Abbreviation	Long form
LKS	Lineage negative c-Kit positive Sca-1 positive
LSC	Leukemic stem cell
LTC-IC	Long-term culture initiating cell
MDS	Myelodysplastic syndrome
MEF	Mouse embryonic fibroblast
mHSC	Mouse hematopoietic stem cell
MPB	Mobilized peripheral blood
MPD	Myeloproliferative disorder
MSC	Mesenchymal stem cell
NS	NOD/SCID mouse
NSB	NOD/SCID/β2m^{null} mouse
NSG	NOD/SCID/γ_c^{null} mouse
PB	Peripheral blood
PSC	Pluripotent stem cell
PTC	Patched
PTHr	Parathyroid hormone receptor
RAR	Retinoic acid receptor
Rb	Retinoblastoma
SCF	Stem cell factor
SDF-1	Stromal-derived factor 1
SLAM	Signaling lymphocyte activation molecule
SL-IC	SCID leukemia initiating cell

Abbreviation	Long form
SMO	Smoothened
SRC	SCID repopulating cell
TBA	Trabecular bone area
TGF-β	Transforming growth factor beta
TIC	Tumour initiating cell
TPO	Thrombopoietin
VE	Vascular endothelium
VEGFR	Vascular endothelial growth factor receptor
VN	Vascular niche
WNT	Wint

Chapter 1

Introduction[1]

1.1 Overview

Stem cells are defined by their ability to self-renew, and by their robust multilineage differentiation capacity (TILL et al., 1964) . Hematopoietic stem cells (HSCs) were the first stem cells to be defined and studied experimentally (BECKER et al., 1963; Jacobson, 1950; MCCULLOCH and TILL, 1960; NOWELL et al., 1956; TILL and McCULLOCH, 1961), and to date are the only type of stem cell to have been reproducibly used in clinical applications (Smith et al., 2006). A significant limitation to the clinical use of human HSCs (hHSCs) has been the inability to acquire sufficient numbers of donor stem cells to successfully reconstitute recipient hematopoiesis (Anasetti et al., 2001; Brown and Boussiotis, 2008; Little and Storb, 2002; Tse et al., 2008). Consequently, the field of stem cell biology has focused on understanding the molecular regulation of stem cell fate decisions such as survival, self-renewal and differentiation, in the hope of expanding hHSC numbers *ex vivo* and improving transplant efficiency. This work has been met with limited success, as human somatic stem cells (hSSCs) such as HSCs have proven extremely difficult to purify and manipulate *ex vivo* (Dahlberg et al., 2011). These experimental challenges have led to a recent renewed interest in the role of the *in vivo* microenvironment, also called the stem cell niche (Carlesso and Cardoso, 2010; Jones and

[1] Parts of this introduction were adapted from the following published review article: **Campbell C,** Risueno RM, Salati S, Guezguez B and Bhatia M. **Signal control of hematopoietic stem cell fate: Wnt, Notch and Hedgehog as the usual suspects.** *Curr. Op. Hem.* (2008) **15(4)** 319-325. Text has been reproduced with permission from Lippincott, Williams and Wilkins. I wrote the abstract, introduction, and section entitled "Intracellular signaling and hematopoietic stem cell survival", and assembled and edited the manuscript. B Guezguez, R Risueno and S Salati wrote the remainder of the review. S Salati also assembled the figure. M Bhatia provided intellectual input into the manuscript.

Wagers, 2008; Kiel and Morrison, 2008; Nilsson and Simmons, 2004; Oh and Kwon, 2010; Ohlstein et al., 2004; Raaijmakers and Scadden, 2008b), in the function of human stem cells. A large number of studies, originating in the fruit fly *drosophila* (Song et al., 2002; Xie and Spradling, 2000) but more recently using the mouse as a model system (Calvi et al., 2003; Chan et al., 2009; Lo Celso et al., 2009; Naveiras et al., 2009; Xie et al., 2009; Zhang et al., 2003), have provided overwhelming evidence that stem cell fate decisions are controlled by non-cell autonomous signals originating in their *in vivo* microenvironment. Furthermore, these studies also suggest that when stem cells are removed from this unique *in vivo* setting they do not retain their characteristic properties (Calvi et al., 2003; Dahlberg et al., 2011; Kiel and Morrison, 2008; Spradling et al., 2001). To date, however, the role of the *in vivo* microenvironment in human stem cell function has not been explored, and it is becoming evident that understanding how human stem cells function within a specific and dynamic *in vivo* context will be essential towards realizing their therapeutic potential. In addition, a large body of evidence now suggests that many human cancers are initiated and maintained by rare cells that have undergone transformation in human patients, and possess stem cell characteristics such as vigorous self-renewal and proliferative capacities (Clevers, 2011). These cells are known synonymously as tumour initiating cells (TICs) or cancer stem cells (CSCs), and analogous to their normal tissue counterparts, recent studies have provided evidence that CSCs may also require a specific *in vivo* niche for their function, and that niche occupancy by CSCs may play a role in neoplasia and transformation (Carlesso and Cardoso, 2010; Lane et al., 2009). As we enter the sixth decade of human stem cell

research, we see the concepts of human stem cell function, transformation, and the *in vivo* microenvironment not only emerging at the forefront of the field, but also as being contiguous rather than independent. Clearly, an understanding of how the *in vivo* microenvironment regulates human stem cell fate decisions such as self-renewal, differentiation and transformation will be essential to moving forward in the field and unlocking their long-touted therapeutic potential.

1.2 Stem cells

Stem cells are defined by two properties (figure 1A): 1) the ability to self-renew, giving rise to one or more identical daughter stem cells during cell division (TILL et al., 1964) and 2) the ability to proliferate robustly and differentiate into multiple cellular lineages (figure 1B) (Schnerch et al., 2010; TILL et al., 1964). In theory, a stem cell self-renewal division may be asymmetrical, generating an identical daughter stem cell as well as a daughter cell destined to differentiate; or symmetrical, wherein two identical daughter stem cells are generated (figure 1A)((TILL et al., 1964)). The balance between self-renewal and other stem cell fate decisions such differentiation and death contributes to maintenance or expansion of the stem cell pool (figure 1A) (Blank et al., 2008), both during embryonic development and in adult tissue homeostasis, as discussed further below. Stem cells can be classified broadly as 1) pluripotent stem cells (PSCs), such as human embryonic stem cells (hESCs), which have the ability to generate all of the embryonic germ layers (ectoderm, mesoderm, endoderm) (Hipp and Atala, 2008); 2) multipotent stem cells, such as hHSCs, which have the ability to generate all cells of a

given tissue and (Hipp and Atala, 2008); and 3) unipotent stem cells, such as human gonadal stem cells, which have the ability to generate cells of a single lineage (Cinalli et al., 2008). A common theme throughout biology is hierarchical organization (Sales-Pardo et al., 2007), and human stem cells can be organized into both ontogenetic and tissue-specific hierarchies based upon differentiation potential (figures 1B and 2). At the apex of the ontogenetic hierarchy sits the fertilized human embryo, which is defined as a totipotent cell in that it can give rise to both embryonic and extra-embryonic (placental) tissues, thereby possessing the ability to generate an entire organism (figure 2) (Rossant, 2008). Although the totipotent cell is the only cell with this capability (Rossant, 2008), it is generally not considered a stem cell by definition, as it is a transient cell that does not exhibit long-term self-renewal capacity[2] (Levi and Morrison, 2008). Totipotent cells are followed ontogenetically by PSCs such as hESCs (Levi and Morrison, 2008) (Rossant, 2008), which are *in vitro* cell lines derived from the inner cell mass (ICM) of the human blastocyst following embryonic cleavage, usually at day 6 post-fertilization (Stewart et al., 2008). hESCs are defined by 1) their ability to self-renew on serial passage *in vitro* (Stewart et al., 2008); and 2) pluripotency, or the ability to differentiate into all three embryonic tissue germ layers both *in vitro* and *in vivo* (Stewart et al., 2008). hESCs serve as an *in vitro* model system for studying stem cell pluripotency, as well as in theory a potential source of other types of human tissues through directed differentiation (Cerdan and Bhatia, 2010). Recent work has also revealed the ability to reprogram terminally

[2] The totipotent cell does generate progeny with totipotent potential during early embryonic cleavage, but this is only a transient occurrence. Levi, B., and Morrison, S. (2008). Stem cells use distinct self-renewal programs at different ages. Cold Spring Harbor Symposia on Quantitative Biology *73*, 539.

differentiated adult cells to an induced pluripotent state (iPS) by overexpressing several key developmental transcription factors (Takahashi et al., 2007). iPS cells display similarity to hESCs in both phenotype and function (Plath and Lowry, 2011), and like hESCs represent an experimental model for studying stem cell pluripotency and human tissue development (Plath and Lowry, 2011).

hESCs are followed ontogenetically by human multipotent stem cells (hMSCs), (Slack, 2008), also known as hSSCs, as they reside in somatic (or non-reproductive) adult human tissues[3]. hSSCs are defined by 1) the property of long-term self-renewal *in vivo* (Orkin and Zon, 2008); and 2) multipotency, or the ability to differentiate into all cell types of a given tissue (Orkin and Zon, 2008). Therefore, compared to hESCs, hSSCs are more restricted in their differentiation capacity, and sit at the top of a tissue-specific hierarchy followed by a series of progenitor cells with increasingly limited self-renewal and differentiation capacity, and finally by terminally differentiated adult tissue cells (figures 1B, 2 and 3)(Chao et al., 2008). Adult tissues are thought to possess a finite pool of long-lived SSCs at birth, which function to clonally maintain tissue homeostasis throughout life (Beerman et al., 2010) (Smith et al., 1991). In comparison with their more differentiated progenitor cell progeny, hSSCs also exhibit relative cell cycle quiescence in their physiologic *in vivo* settings (Arai et al., 2004; Fleming et al., 2008; Li and Bhatia, 2011; Sugiyama et al., 2006), a property which may decrease the probability of acquiring genetic damage and help to maintain long-term stem cell function (Li and Bhatia, 2011).

[3] The term "adult" here denotes any point following birth.

The gonadal stem cell (GSC) represents an additional broad class of stem cell. GSCs are derived from primordial germ cells during gastrulation (germ layer formation), and reside in the gonads, or reproductive organs throughout life (Cinalli et al., 2008). hGSCs are capable of self-renewing, but are restricted to generating the germ cell lineages (sperm and ova), and therefore have been described as unipotent stem cells (Cinalli et al., 2008). However, GSCs are unique from other types of stem cells in that they maintain the ability to contribute to the totipotent cell at fertilization (Cinalli et al., 2008), and as such may represent a potential source of both totipotent and pluripotent stem cells (Cinalli et al., 2008).

To date putative hSSCs have been isolated from many human tissues, including neural (Breunig et al., 2011), intestinal (Umar, 2010), epithelial (Majo et al., 2008), hematopoietic (Bryder et al., 2006) and mesenchymal (Salem and Thiemermann, 2010). Unlike hESCs however, hSSCs have proven extremely difficult to isolate and maintain *in vitro*, and phenotypically homogenous populations of putative mammalian "SSC" display a great deal of functionally heterogeneity (Graf and Stadtfeld, 2008; Raaijmakers and Scadden, 2008a). SSC also tend to rapidly lose their self-renewal and differentiation capacities when removed from their physiologic *in vivo* setting (Dahlberg et al., 2011). Consequently, as discussed in detail below, hSSCs must be defined rigorously by functional assays as opposed to phenotype, demonstrating the ability to both self-renew and to regenerate their tissue of origin *in vivo*.

1.2.1 The functional implications of a stem cell hierarchy

Evidence in both mice (Nijnik et al., 2007; Rossi et al., 2007) and humans (Rossi et al., 2008; Sahin and Depinho, 2010) suggest that the functional properties of stem cells, such as self-renewal and proliferation, decline with aging (Morrison and Spradling, 2008). This may occur due to stem cell "exhaustion" after a limited number of self-renewing divisions, or to genetic mutations acquired during cell division, in both cases leading to either stem cell senescence or programmed cell death (apoptosis) (Rossi et al., 2008; Sahin and Depinho, 2010). Therefore, a hierarchical organization would serve to limit the number divisions that a stem cell must undergo, decreasing the probability of mitotic errors and preserving the stem cell pool (Chao et al., 2008). Indeed, the bulk of tissue regeneration is carried out by lineage-restricted progenitor cells, which are relatively mitotically active and short-lived compared to SSCs (figure 1B) (Chao et al., 2008; Rossi et al., 2008). One can therefore appreciate that in theory, a transforming mutation would be much less deleterious when occurring in a short-lived progenitor cell compared to a long-lived SSC.

1.3 Hematopoietic stem cells

HSCs are the best-studied type of mammalian stem cell, and act as a model system for the study of SSC biology in general (Bryder et al., 2006). This is related to both the ease with which HSCs can be attained and studied, and also to a great deal of historic and seminal work done in the fields of hematology and immunology that has defined the hematopoietic tissue hierarchy (Chao et al., 2008). HSCs sit at the apex of a

hematopoietic hierarchy (figure 3), and are followed by a series of hematopoietic progenitor cells (HPCs) with increasingly limited differentiation and self-renewal capacities, and finally by terminally differentiated adult blood cells of the myeloerythroid lineage (granulocytes, monocytes, megakaryocytes, erythrocytes), and lymphoid lineage (B and T lymphocytes, natural killer cells, dendritic cells) (figure 3) (Chao et al., 2008; Lévesque and Winkler, 2011; Quesenberry, 2006). HSCs reside within the bone marrow (BM) in healthy individuals, and function to maintain and regenerate the adult blood system throughout life (Orkin and Zon, 2008).

1.3.1 The dynamic origins of the hematopoietic system

The principles of early hematopoietic development are largely conserved among vertebrates (Tavian, 2010). Early hematopoietic development is a dynamic process, moving through several different anatomical locations that represent unique microenvironments throughout development (Tavian 2005, Dzierzak 2003). The hematopoietic system is of mesodermal origin (Mikkola and Orkin, 2006), and the earliest human hematopoietic cells are thought to originate during the third week of human development within the in the embryonic yolk sac, in regions known as blood islands (Tavian et al., 1999). These early blood precursors consist largely of nucleated erythrocytes and primitive myeloid progenitors known as BFU-E (figure 3), and are devoid of long term repopulating HSCs (Tavian and Péault, 2005). This stage of hematopoietic development is sometimes known as “primitive”, “yolk-sac” or “extra-embryonic” hematopoiesis (Tavian and Péault, 2005). Following the onset of circulation

at the end of the third week (Tavian and Péault, 2005), a decline in hematopoietic progenitors occurs in the yolk sac, concomitant with an increase in hHPCs in the liver, which continues through sixth week of development (Migliaccio et al., 1986; Tavian and Péault, 2005). This change is thought to be due to a dynamic migration of early hematopoietic cells from the yolk sac to the liver microenvironment (Tavian and Péault, 2005), and these migrating precursors represent transient, short-term HPCs (Cumano et al., 2001; Tavian et al., 2001). At the same time, during the fourth week of development, a second population of hematopoieitic cells appears to originate on the endothelium of the dorsal aorta, which in humans has been dubbed the hemogenic endothelium (Cumano et al., 2001; Dzierzak, 2005; Tavian et al., 2001). This second wave of blood genesis is known as "definitive hematopoiesis", and the vascular endothelium may provide an early supportive niche for hematopoietic cells (Dzierzak, 2005). These primitive intra-embryonic hematopoieitic cells also colonize the liver beginning in the forth week of development, and at this time hematopoietic cells expressing CD34, a common marker of hHSCs, are first detected in the fetal liver (Tavian et al., 1996). It is this second wave of hematopoiesis originating from the intra-embryonic vascular endothelium that is thought to give rise to the long-term self-renewing HSCs that sustain the human blood system throughout life (Cumano et al., 2001; Dzierzak, 2005; Dzierzak and Speck, 2008; Tavian et al., 2001). By the sixth week of development, hematopoiesis takes place entirely in the fetal liver (Tavian and Péault, 2005), and beginning at the tenth week of development through birth HSCs leave the liver to begin colonizing the BM microenvironment (Tavian and Péault, 2005). While there is still much to be unraveled regarding the early origins of

hHSCs, it is clear the hHSCs dynamically move between various independent anatomical locations that represent unique supportive microenvironments throughout the antenatal period. Interestingly, these extramedullary sites retain some capacity to support hematopoiesis throughout life, as is evident in adult disease states such leukemia and myeloproliferative disorders, as discussed further below (O'malley, 2007).

1.3.2 *In vitro* assays of hematopoietic stem and progenitor cell function

The properties that define stem cells imply that they are functional entities, and as such their biology is studied through the use of assays that measure these stem cell properties. This also implies that in the absence of phenotypic markers that can identify stem cells at the single-cell level, they can only be defined in a retrospective manner based upon their self-renewal and regenerative capacities (van Os et al., 2004). This has posed significant challenges both in the study of hSSCs, as well as in the prospective isolation of hSSCs for clinical applications (Dahlberg et al., 2011). The best-characterized type of hSSC in terms of both phenotypic and function are hHSCs, and as such hHSCs have served as a model system for hSSC research in general (Bryder et al., 2006). The first assays used to measure putative hHSC function were *in vitro* colony forming assays such as the colony forming unit (CFU) assay (figure 4A) (Pike and Robinson, 1970). The CFU assay measures the frequency of primitive hematopoieitic cells that initiate clonal myeloerythroid colonies *in vitro* in the presence of hematopoietic growth factors (cytokines), as well as the short-term self-renewal capacity of clones to reinitiate colonies on serial passage (Ash et al., 1981). Therefore, the CFU serves as an operational

definition of hematopoietic stem and progenitor cells (HSPCs), providing a retrospective analysis of HSPC frequency, myeloerythroid differentiation (granulocytes, monocytes, megakaryocytes, erythrocytes, see figure 3), and short-term self-renewal capacity (Pike and Robinson, 1970). *In vivo* clonal assays have also been used to assess putative HSC function in the mouse, where cells known spleen-colony forming units (CFU-S) were originally defined as HSCs based upon their ability to generate myeloerythroid colonies on the spleens of irradiated mice (MCCULLOCH and TILL, 1960; TILL and McCULLOCH, 1961; TILL et al., 1964). CFU-S also exhibited limited self-renewal capacity, as measured by their ability for reinitiate colonies on serial passage into irradiated secondary recipients (MCCULLOCH and TILL, 1960; TILL and McCULLOCH, 1961; TILL et al., 1964). Other assays have been developed based upon *in vitro* culture conditions that support cells known as long-term culture initiation cells (LTC-ICs), which are capable of initiating myelopoiesis on supporting marrow stromal cells (Fraser et al., 1992; Hao et al., 1995). However, in regards to assaying for HSC function, the *in vitro* and *in vivo* CFU and LTC-IC assays have several important limitations, namely 1) they do not measure the ability to regenerate a true multilineage hematopoiesis, as these assays are a poor surrogate for lymphopoiesis (B and T lymphocytes, natural killer cells, dendritic cells, see figure 3)(Ash et al., 1981); and 2) these assays do not discriminate between HPCs that possess limited self-renewal and differentiation capacity, and true HSCs that exhibit long-term self-renewal and multilineage differentiation capacities (Bhatia et al., 1997).

1.3.3 *In vivo* assays of hematopoietic stem cell function

Limitations in CFU assays led to the development of more rigorous *in vivo* repopulating assays, where hHSCs were defined based upon their ability to competitively regenerate long-term multilineage hematopoiesis (erythroid, myeloid, and lymphoid blood cells, see figure 3) in irradiated recipient mice (Purton and Scadden, 2007). As hHSCs cannot be experimentally studied *in situ*, xenograft[4] assays were developed in which primitive human hematopoietic cells could be studied in mice with severe immune deficiency, which would tolerate the human graft (figure 4B) (Dick et al., 1997). The NOD/SCID (NS) mouse strain has attenuated DNA repair mechanisms and immune function (Shultz et al., 1995)(table 1). Consequently, it has been called the "gold-standard" assay for hHSC function, and is the model in which hHSCs are most commonly studied. As hHSCs are functionally characterized and quantified based upon their ability to engraft NS mice as opposed to human recipients, they are defined operationally as SCID repopulating cells (SRCs) rather than being labeled true human HSCs (Bhatia et al., 1997). Experimentally, the SRC represents the functional definition of a human HSC, and SRCs are defined by their ability to initiate and maintain multilineage human hematopoiesis and self-renew upon serial passage *in vivo* (Bhatia et al., 1997) (Dick et al., 1997) (figure 4B). Human chimerism in this assay is commonly assessed using flow cytometry[5]. Although both B-

[4] Xenograft denotes establishing a tissue graft in one species that is derived from another species, for example, a human hematopoietic graft in a mouse.

[5] Flow cytometry is a quantitative technique based upon detection of flurorecence-emission and light-scatter properties of small particles. It is commonly used in the study of hematopoietic cells. Cells may be stained with fluorescent antibodies that recognize specific cell-surface proteins, and then passed through a light source such as a laser beam, which both excites fluorochromes and becomes scattered as it passes through cells. Scattered and fluorescent light is subsequently picked up by a detector. Flow cytometry provides quantitative information on multiple cellular parameters such as surface phenotype, size, internal

lymphoid and myeloid cells are present in SRC-generated grafts in NS mice (Hogan et al., 1997; Larochelle et al., 1996), these grafts do not represent true human hematopoiesis, as they do not contain fully differentiated functional human leukocytes (white blood cells) (Larochelle et al., 1996) (Hogan et al., 1997; Manz and Santo, 2009), are devoid of the T-cell lineage (Manz and Santo, 2009) and are deficient in erythropoiesis (Hogan et al., 1997). While this decreases the utility of xenograft models to study human blood development and immunological function, it makes these mice the ideal model to study human HSC function, precluding any potential graft-versus-host effect. To assay for stem cell self-renewal function, grafts from primary recipients are serially passaged into secondary recipient mice (figure 4B). This step is required due to the short lifespan of NS mice (Shultz et al., 1995), which have a high incidence of thymic lymphomas after 12 weeks (Shultz et al., 1995), and is the standard measure of HSC self-renewal within the field (Dick et al., 1997). Human SRCs are most commonly delivered to the blood of recipient mice using intravenous (IV) injection (Mazurier et al., 2003), as well as by direct injection into the femurs of recipient mice (intrafemoral or IF) (Mazurier et al., 2003). While IF delivery appears to improve sensitivity of the assay (Levac et al., 2005; Mazurier et al., 2003), it also bypasses the physiologic homing of HSCs from circulation into the BM microenvironment, as occurs in clinical human transplants (Copelan, 2006). Overall, regardless of the delivery method, this rigorous *in vivo* assay therefore measures both the differentiation and self-renewal properties that define human HSCs. A number

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complexity, and viability, and can additionally be used to sort cells based upon fluorescence (fluorescence activated cell sorting, FACS). For further information on this technique, see Herzenberg, L. A., Parks, D., Sahaf, B., Perez, O., Roederer, M., and Herzenberg, L. A. (2002). The history and future of the fluorescence activated cell sorter and flow cytometry: a view from Stanford. Clinical Chemistry *48*, 1819-1827.

of other mouse models have subsequently been developed from the NS strain with further immunological defects that render them more permissive to human xenografts (table 1) (Ito et al., 2002; Kollet et al., 2000; Manz and Santo, 2009). Although these models allow for advantages in terms of more robust levels of engraftment, it should be kept in mind that SRCs, and their corresponding frequencies in various human hematopoietic sources (table 2), have been defined using the NS strain (Bhatia et al., 1997).

1.3.4 Assaying other types of human stem cells

PSCs such as hESCs are also characterized by well-defined functional assays. hESCs are cell lines which are maintained and primarily studied *in vitro*, being quantified and functionally defined by their ability to initiate colonies upon serial passage *in vitro* that consist all three tissue germ layers, thereby demonstrating both self-renewal and pluripotency (reviewed in (Rossant, 2008; Stewart et al., 2008)). Further stringency is added to the functional study of hESCs in that they must also demonstrate the ability to form subcutaneous teratomas in NS mice, which are non-malignant embryonic tumours consisting of the three tissue germ layers (reviewed in (Stewart et al., 2008)).

Apart from hESCs and hHSCs, other putative types of human stem cells have not been defined by such rigorous functional assays. Both human neural and prostate SSCs are defined using *in vitro* assays where stem cell function is measured by the ability to form structures known as spheres (Pastrana et al., 2011) and to self-renew by passage in culture. BM mesenchymal stem cells (MSCs), which give rise to cells in the osteocytic, adipocytic and fibroblastic lineages (Bianco et al., 2001), have also been characterized

using *in vitro* and *in vivo* clonal assays (Salem and Thiemermann, 2010). Whether these cells, and other more poorly defined putative hSSCs, represent true hSSCs remains to be established as *in vivo* assays and phenotypic markers delineating populations enriched for these cell types are better developed.

1.3.5 Cell surface markers that enrich for hematopoietic stem cells

Efforts to purify and prospectively isolate mammalian SSCs at the single-cell level have not been realized. In many tissues, cell-surface markers that identify populations of cells enriched for SSCs have described, although these populations are functionally heterogeneous and contain both progenitor and stem cell fractions (Raaijmakers and Scadden, 2008a). The hematopoietic system has been extensively interrogated in both mice and humans for surface markers which prospectively identify HSCs. Early BM transplant experiments demonstrated that lethally irradiated mice could be rescued by infusion of BM cells isolated from non-irradiated syngeneic[6] donor mice (Lorenz et al., 1951). It was subsequently demonstrated that rare cells within the mouse BM could reproducibly reconstitute long-term multilineage hematopoiesis in irradiated recipient mice and self-renew on serial passage (Abramson et al., 1977). These cells represented the first true mammalian HSCs, and investigation of their surface phenotype using flow cytometry revealed that mHSCs were highly enriched in the primitive population of BM cells devoid of any lineage-specific markers (lin-), and expressing the surface receptor tyrosine kinase c-kit and the antigen Sca-1 (lin-ckit+Sca1+ or LKS) (table 3) (Purton and

[6] Genetically identical.

Scadden, 2007; Spangrude et al., 1988). However, although enriched for mHSCs, the LKS population displayed considerable functional heterogeneity, consisting of both HSCs as well as more restricted HPCs (Purton and Scadden, 2007). More recent studies revealed that mHSCs can be purified to a greater extent using the signaling lymphocyte activation molecule (SLAM) surfaces markers in the lin- population (lin-CD41-CD48-CD150+ or lin-SLAM+) (table 3) at a frequency of approximately 50% (Kiel et al., 2005). The SLAM population represents the hematopoietic fraction most enriched for mHSCs, and like all other types of SSCs, functionally identical mouse HSCs have not been isolated to phenotypic purity (Kiel et al., 2005; Purton and Scadden, 2007).

Attempts to study and purify hHSCs developed relatively later than the mouse (Civin et al., 1984), largely because of the inability to study human stem cells experimentally *in situ*. The most common sources of hHSCs are BM, mobilized peripheral blood (MPB)[7] and umbilical cord blood (CB) (table 2) (Copelan, 2006). Early xenograft studies revealed that hHSCs reside in the rare CD34+ hematopoietic fraction, which normally represents less than 1% of hematopoietic cells (Berenson et al., 1988). Further functional studies in the NS mouse have shown that the human hematopoietic hierarchy can be phenotypically constructed on the basis of expression of both CD34 and CD38 (figure 3), with human HSCs (or SRCs, as defined in the NS assay) highly enriched in the lin-CD34+CD38- hematopoietic population (approximately 1 in 600 in CB, table 2)

[7]Circulating HSCs in peripheral blood of healthy individuals are rare. Treatment of HSC donors with hematopoieitic growth factors (cytokines) induces HSCs to leave the BM and enter circulation prior to harvest (mobilization), allowing for a minimally invasive procedure for HSC collection. Bensinger, W. I., Weaver, C. H., Appelbaum, F. R., Rowley, S., Demirer, T., Sanders, J., Storb, R., and Buckner, C. D. (1995). Transplantation of allogeneic peripheral blood stem cells mobilized by recombinant human granulocyte colony-stimulating factor. Blood *85*, 1655-1658..

(Bhatia et al., 1997). Other studies have made further refinements to the phenotype of the SRC-enriched population, such as addition of the marker CD90 to slightly enhance purity (table 2) (Majeti et al., 2007). A recent report suggests that human HSCs can be further purified to a frequency of up to 28% based upon expression of integrinα6 (CD49f) and rhodamine dye exclusion, in the lin-CD34+CD38-CD90+rhodamine^{low}CD49f+ fraction using single-cell IF transplantation in the NOD/SCIDγc null (NSG) strain (Notta et al., 2011a). However, a previous study revealed as that few as 5 lin-CD34+CD38- cells were sufficient to regenerate multilineage hematopoiesis by IF injection into NOD/SCIDβ2 microglobulin null (NSB) mice (Levac et al., 2005), suggesting the lin-CD34+CD38- phenotype alone is sufficient for nearly clonal repopulation using the IF transplantation approach. Currently, the lin-CD34+CD38- population is the most robust and reproducible phenotype enriched for human HSCs, and similar to the mouse LKS population, the human lin-CD34+CD38- population is functionally heterogeneous (Bhatia et al., 1997).

1.3.6 Hematopoietic stem cells in clinical applications

hHSCs not only serve as a model system for human stem cell biology in general, but also are the only type of human stem cell that have been reproducibly used in human clinical applications (Smith et al., 2006). The first BM transplant was conducted over 60 years ago (Little and Storb, 2002), and since then, transplantation of hHSCs from BM, MPB and CB has revolutionized human regenerative medicine, having applications in many hematological malignancies, inherited conditions and autoimmune diseases (Copelan,

2006). Although HSC transplantation can be used to treat and sometimes cure many of these conditions, the procedure nonetheless remains limited by the availability of human leukocyte antigen (HLA)-matched donors[8] (Brown and Boussiotis, 2008; Copelan, 2006; Tse et al., 2008). This is related both to the inability to maintain and expand hSSCs *in vitro* (Dahlberg et al., 2011), and he lack of surface markers which reliably identify hSSCs such as hHSCs (Cerdan and Bhatia, 2010). Consequently, the field of human stem cell biology has focused much research effort on understanding the cell intrinsic and extrinsic parameters that regulate stem cell fate decisions and define heterogeneity the HSC compartment (Dahlberg et al., 2011), in the hopes of expanding HSC populations for regenerative medicine applications.

1.4 Stem cell fate decisions

A stem cell may undergo one of several possible cell fate decisions, including self-renewal, differentiation or death (figure 1) (Blank et al., 2008) (Taichman, 2005). The appropriate balance between stem cell fate decisions maintains tissue homeostasis and the stem cell pool (Bryder et al., 2006), and prevents both under and over-proliferation of a tissue (Wicha et al., 2006). The precise molecular signals that control a stem cell's decision to self-renew, differentiate, or die have been the subject of intense investigation in the field of stem cell biology, with the goal of modulating stem cell fate decisions by interrogating these pathways. As hSSCs rapidly undergo apoptosis and lose their self-

[8] Human leukocyte antigens (HLA) or major histocompatibility antigens (MHC) are used by the adaptive immune system to recognize and distinguish between foreign and self antigens. A high degree of HLA matching is correlated with better engraftment and long-term outcomes in human HSC transplanation Hamadani, M., Awan, F. T., and Copelan, E. A. (2008). Hematopoietic stem cell transplantation in adults with acute myeloid leukemia. Biol Blood Marrow Transplant *14*, 556-567.

renewal capacity *in vitro* (Dahlberg et al., 2011), understanding the pathways that control these critical cell fate decisions would in theory allow for *in vitro* or *in vivo* expansion of stem cell numbers by suppressing apoptosis and differentiation while enhancing self-renewal (Sauvageau et al., 2004). Although to date efforts to significantly expand hSSC numbers *in vitro* have been not been successful (Dahlberg et al., 2011), a number of key molecular pathways regulating human stem cell fate decisions *in vitro* have been identified, again largely through studies done in the hematopoietic system.

1.4.1 Cytokines and morphogens as regulators of human stem cell fate decisions

The unique self-renewal properties of HSCs appear to be closely related to the cell cycle (Trowbridge et al., 2006b), and HSCs are thought to reside in a quiescent state in their normal physiologic setting *in vivo* that supports the long-term maintenance of their self-renewal capacity (Arai et al., 2004; Fleming et al., 2008; Li and Bhatia, 2011; Sugiyama et al., 2006). Prolonged entrance into the cell cycle correlates with the loss of HSC self-renewal function, and eventual exhaustion of the stem cell pool *in vivo* (Trowbridge et al., 2006b). Therefore, the field has focused on defining culture conditions which promote the survival and expansion of hHSCs while maintaining long-term quiescence, towards augmenting hHSC numbers for therapeutic applications (Dahlberg et al., 2011). Hematopoietic cytokines[9] were known to be abundant in serum and play a central role in the function of hematopoietic cells (Blank et al., 2008), and as such were candidate regulators of hHSC fate decisions. A large number of cytokines which influence both

[9] Cytokines are intracellular signaling proteins (or hormones) that have roles in many tissues, most notably the hematopoietic system.

hHSC survival and differentiation *in vitro* have been identified (Zhang and Lodish, 2008), although only three cytokines, stem cell factor (SCF), FMS-like tyrosine kinase-3 ligand (FLT3L) and thrombopoietin (TPO) were found to be minimally necessary to maintain numbers of self-renewing hHSCs by promoting survival *in vitro* for short periods of time (Murdoch et al., 2002). Despite these findings however, approaches to modulate HSC fate decisions with cytokines have been limited by 1) their inability to support HSC maintenance for extended periods of time *in vitro* (Ogawa, 1993); 2) the fact that they tend to stimulate hHSC to enter the cell cycle, thereby leading to the eventual loss of their self-renewal capacity (Ogawa, 1993; van der Loo and Ploemacher, 1995; vand der Sluijs et al., 1993); and 3) their inability to expand HSC numbers to clinically significant extent (Bhardwaj et al., 2001; Karanu et al., 2000).

In the context of these findings, subsequent efforts to identify molecular regulators of SSC fate decisions shifted focus, and turned to morphogenic[10] signaling pathways known to control lineage-fate specification in early development (Bhardwaj et al., 2001; Bhatia et al., 1999; Chadwick et al., 2005; Karanu et al., 2000; Murdoch et al., 2003). The WNT (pronounced "wint"), Notch, Hedgehog (Hh) and TGF-ß morphogenic pathways were known to be highly conserved regulators of tissue patterning, organogenesis and cell-fate specification throughout evolution, from *drosophila* to zebrafish to mammals (as reviewed in (Campbell et al., 2008)). These morphogenic pathways share a conserved elegant functional arrangement, where inhibition of an intracellular signaling component is relieved by the binding of a cell-surface ligand, leading to the nuclear translocation of a

[10] A morphogen is a signaling protein that is involved in the formation and patterning of tissues during embryonic development via a concentration gradient.

signal-transducing component and subsequent transcriptional activation of target genes involved in regulating cell fate decisions (Campbell et al., 2008; Cerdan and Bhatia, 2010). The WNT pathway consists of two distinct signaling networks, known as canonical and non-canonical WNT signaling, respectively (Clevers, 2006). The canonical pathway is currently better understood at the molecular level (Cerdan and Bhatia, 2010), wherein a soluble WNT ligand binds a cell-surface receptor known Frizzled (FZD), leading to the stabilization and nuclear translocation of the transcription factor β-catenin, and subsequent activation of pathway target genes, many of which are involved in cell cycling and proliferation (Clevers, 2006). Similarly, in the Hh pathway a soluble Hh ligand such as Sonic Hedgehog (SHh), Desert Hedgehog (DHh) or Indian Hedgehog (IHh) in mammals (Cerdan and Bhatia, 2010) binds a transmembrane protein known as Patched (PTC), relieving its inhibition of another transmembrane protein Smoothened (SMO), leading to transcriptional activation of Hh target genes through the Glioblastoma (GLI) family of transcriptional factors (Jiang and Hui, 2008). Notch signaling likewise follows this general pattern, where a ligand of the Jagged and Delta families engage a transmembrane Notch receptor, resulting in the cleavage of the Notch receptor intracellular domain (NICD), and its subsequent localization to the nucleus and activation of Notch pathway target genes (Kopan and Ilagan, 2009), many of which play a central role in development of the T-cell lineage (Rothenberg et al., 2008). Not surprisingly, these pathways known to regulate cell fate decisions such as proliferation and differentiation have well characterized roles in a variety of human cancers, such as colon

(Clevers, 2006), neural and skin (Jiang and Hui, 2008) (Epstein, 2008) and leukemia (Aster et al., 2008; Klinakis et al., 2011).

Subsequent studies in both mouse and human ESCs (Cerdan and Bhatia, 2010), SSCs such as HSCs (Bhardwaj et al., 2001; Bhatia et al., 1999; Campbell et al., 2008; Chadwick et al., 2005; Karanu et al., 2000; Murdoch et al., 2003; Trowbridge et al., 2010) as well as transformed CSCs (Reya and Clevers, 2005; Rubin and De Sauvage, 2006) went on to reveal these developmental morphogenic pathways as apparent master regulators of normal and transformed stem cell self-renewal and differentiation. Paradoxically however, similar to hematopoietic cytokines, efforts to expand HSC numbers through interrogation of these pathways *in vitro* have led to only modest increases as measured rigorously by *in vivo* assays (Bhardwaj et al., 2001; Bhatia et al., 1999; Chadwick et al., 2005; Dahlberg et al., 2011; Delaney et al., 2010; Karanu et al., 2000; Murdoch et al., 2003). Similarly, attempts to generate hHSCs from hESCs by directed differentiation via modulation of WNT, Notch and Hh signaling *in vitro* have to date not yielded cells with robust multilineage *in vivo* regenerative capacity (Cerdan and Bhatia, 2010). Furthermore, although modulation of WNT, Notch and Hh signaling *in vitro* can enhance hHSC self-renewal both *in vitro* and *in vivo*, mouse knockout models targeting the WNT, Notch and Hh pathways have provided conflicting evidence (Hofmann et al., 2009; Maillard et al., 2008; Orkin and Zon, 2008), revealing that these pathways may enhance but are not necessarily required for HSC function *in vivo*. While it has been proposed that this is due to functional redundancy of ligands to these pathways *in vivo* (Hofmann et al., 2009), it appears that there are unique parameters governing

these putative stem cell regulatory pathways within in the *in vivo* setting, and consequently their role in stem cell fate decisions may only be fully realized within the niche, as outlined below.

1.4.2 The Bcl-2 family and stem cell fate decisions

Many hematopoietic cytokines function to promote cell survival (Lotem and Sachs, 1999) through intracellular signaling pathways such as the JAK/STAT, MAP kinase (MAPK)/ERK and PI3K/AKT pathways (reviewed in (Huang et al., 2000; Jee et al., 2002; Wei et al., 2001)), which confer these non-cell autonomous signals to the cell by modulating the activity and expression of the Bcl-2 family of proteins (Lotem and Sachs, 1999). The Bcl-2 protein family have a well-established role in human cell lines as intracellular regulators of the first committed step to programmed cell death (apoptosis), which is loss of outer mitochondrial membrane integrity (reviewed in (Leber et al., 2007)). The Bcl-2 family regulates apoptosis through a complex interplay of pro-survival members such as BCL-2, BCL-X and MCL-1, and pro-death members such as BAX and BAK (Adams and Cory, 2007). A number of studies have also revealed that in addition to controlling cell survival, the Bcl-2 family may have unique roles beyond survival in both development and in primitive cell populations *in vivo*. BCL-2 is the prototypical pro-survival Bcl-2 family member (Youle and Strasser, 2008), and overexpression of BCL-2 in mHSCs leads to increased survival and radiation resistance, as well as enhanced HSC regenerative function (Domen et al., 2000). In hHSCs, overexpression of BCL-2 leads to enhanced self-renewal capacity without affecting survival, whereas loss of p53

decreases hHSC self-renewal again without affecting survival, implicating a Bcl-2/p53 axis in the regulation of stem cell self-renewal *in vivo* (Milyavsky et al., 2010) . The BCL-2 homologue MCL-1 is a relatively short-lived, highly regulated protein whose expression and activity are rapidly modulated in response to non-cell autonomous signals like hematopoietic cytokines, especially during periods of critical change such cellular differentiation (Yang et al., 1996). Conditional knockout of Mcl-1 in mouse hematopoietic cells demonstrated that Mcl-1 was essential for maintenance of mouse hematopoiesis and mHPC survival *in vivo* (Opferman et al., 2005b). Interestingly however, Mcl-1 knockout mouse embryos show developmental arrest at the blastocyst stage of development, without affecting cell survival (Rinkenberger et al., 2000). This premise is again evident in developing and mature mouse lymphocytes *in vivo*, where loss of MCL-1 led to a significant decrease in the number of more mature T-cell and B-cell precursors, but only a moderate reduction in the number of precursors undergoing apoptosis (Opferman et al., 2003). In addition, mouse embryonic stem cells (mESC) up-regulate MCL-1 expression during differentiation (H et al., 1998), suggesting that MCL-1 may have a critical role beyond regulating survival in primitive cell compartments.

There are also some potential links between the WNT, Notch and Hh pathways, and Bcl-2 family protein expression and activity. Recently, it was demonstrated that *in vivo* inhibition of glycogen synthase kinase 3 (GSK-3), which is involved in Notch, Hh and Wnt signaling (Foltz et al., 2002; Jia et al., 2002; Yost et al., 1996), leads to increased hHSC regenerative function (Trowbridge et al., 2006c). GSK-3 inhibition increased HSC regenerative function by enhancing self-renewal and augmenting progenitor output of

hHSCs through modification of genes targeted in the Notch, Hh and WNT pathways. Another study provided evidence that upon cytokine withdrawal in a human cell line, GSK-3 phosphorylates MCL-1 leading to its degradation (Maurer et al., 2006b), providing a potential link between MCL-1 function and intracellular signaling mechanisms that have been shown to promote self-renewal in hHSC (Opferman et al., 2005a; Trowbridge et al., 2006a). In addition, HSC self-renewal could be induced by WNT pathways ligands in mice transgenic for Bcl-2 (Willert et al., 2003), again raising the notion that the Bcl-2 pathway could be an intracellular convergence point for signals influencing HSC self-renewal. Bcl-2 has also been shown to be regulated at the transcriptional level by the Gli transcription factors via the Hh signaling pathway in some types of human cells (Bigelow et al., 2004). Furthermore, Notch signaling was found to regulate the survival of primitive neural precursor cells (NPCs) by upregulating expression of Bcl-2 and Mcl-1 (Oishi et al., 2004), and has also been implicated in the survival of epithelial stem cells (Felszeghy et al., 2010). Taken together, evidence to date suggests 1) that Bcl-2 family members may have novel roles beyond survival *in vivo* versus *in vitro* in primitive cell populations; and 2) that the Bcl-2 family member MCL-1 appears to have a particularly important role in periods of cell fate changes such as self-renewal and differentiation.

1.5 The stem cell niche

Collectively, the aforementioned observations elicit several salient points highly relevant to further exploration of molecular pathways regulating human stem cell fate decisions,

namely 1) pathways shown to be sufficient to control stem cell fate decisions *in vitro* may not be necessary for stem cell function *in vivo*; 2) *in vitro* studies do not appear to reliably predict the *in vivo* biology of stem cells; and 3) stem cell fate decisions seem to be uniquely regulated *in vivo*, and will only be fully understood by studying their regulation within this physiologic *in vivo* context.

The significant challenges presented in purifying functionally homogeneous populations of human stem cells and manipulating their cell fate decisions *ex vivo* has led to a recent renewed in interest regarding the role of the *in vivo* microenvironment in stem cell fate decisions (Spradling et al., 2001). The stem cell microenvironment is not new concept in the field, and is generally credited to the biologist Raymond Schofield, who in 1978 presented it as a hypothesis (figure 5)(Schofield, 1978). Schofield proposed that 1) stem cells do not functionally exist on their own, rather, they only exist as stem cells when they reside within a specific anatomical microenvironment, called a niche, and therefore are fixed tissue cells (Schofield, 1978); 2) the niche controls stem cell fate decisions such as survival, self-renewal and differentiation (lineage specification), and is required for maintenance of these stem cell properties (Schofield, 1978); 3) the niche is composed of defined cell types or other extracellular components that produce the signals which control these stem cell fate decisions (Schofield, 1978). This hypothesis was based on the observation that CFU-S, which were considered HSCs at the time (Schofield, 1978), demonstrated unique behaviour depending on the *in vivo* location from which they were isolated, and lost their long-term self-renewal function when removed from the marrow (Gidáli et al., 1974; Micklem et al., 1975; Schofield, 1978). Subsequent mouse

transplantation studies examining the clonal composition of the stem cell compartment demonstrated that regardless of the transplanted dose of mHSCs, the size of the stem cell pool remained constant (Jordan and Lemischka, 1990; Smith et al., 1991), suggesting that stem cell numbers are limited by extrinsic parameters in their microenvironment. These studies further revealed a clonal fluctuation of stem cells during long-term steady state hematopoiesis (Jordan and Lemischka, 1990; Smith et al., 1991), and subsequent work showed that HSCs constantly recirculate in and out of the BM (Bhattacharya et al., ; Wright et al., 2001), which together supported the stem cell niche hypothesis (Schofield, 1978). Although these studies rendered the niche as an attractive theory to explain a number of important observations mHSC biology, they did not provide direct functional validation of the niche as regulator of stem cell fate decisions.

1.5.1 Direct evidence for a stem cell niche in *drosophila*

The first direct experimental evidence confirming the existence of a stem cell niche was provided relatively recently in the fruit fly *drosophila*. Studies of *drosophila* germline or gonadal stem cells (GSCs), which have been defined at the single cell level (Losick et al., 2011; Margolis and Spradling, 1995), have revealed that these cells are maintained at specific anatomical locations in the gonads, composed of defined cell types that regulate their stem cell function, known as cap and hub cells for ovaries and testes respectively (Losick et al., 2011). As predicted by Schofield (Schofield, 1978), these niches function regulate GSC fate decisions by maintaining stem cell quiescence and regulating self-renewal and differentiation (Xie and Spradling, 2000). The *drosophila* GSC are held in

their niches through adherins junctions mediated by cadherin proteins (Song et al., 2002), and polar asymmetrical division is attained by specifically orienting dividing cells so that the daughter stem cell is retained in the niche, and the daughter cell destined for differentiation exits the niche (Losick et al., 2011; Spradling et al., 2001; Xie and Spradling, 2000). Studies in drosophila further revealed that 1) GSC function is only supported when a stem cell is residing within a niche (Xie and Spradling, 2000) (Kiel and Morrison, 2008); and 2) filling a vacant niche with a displaced GSC reinitiates niche support for GSC function (Xie and Spradling, 2000) (Kiel and Morrison, 2008). These findings not only support the premise that stem cells function as fixed tissue cells, but also that a vacant niche retains its ability to support stem cell function when occupied by another stem cell, a finding that has important implications for stem cell-driven tumours, as detailed below (figure 5). Additionally, signals known to be conserved regulators of stem cell fate decisions through *ex vivo* studies, such as Hh, bone morphogenic protein (BMP), Notch and WNT[11] function to regulate GSCs in their niches (Losick et al., 2011). Overall, these elegant invertebrate studies encompass both genetic and imaging techniques, and undeniably show the existence of a defined *in vivo* microenvironment that regulates stem cell fate decisions through specific molecular signals.

1.5.2 The mammalian stem cell niche

Direct studies of the mammalian stem cell niche have proven more challenging, due both to the inability to visualize mammalian SSCs at the single cell level (Kiel and Morrison,

[11] Wingless is the WNT orthologue in drosophila.

2008; Morrison and Spradling, 2008), and also to the relative size of the tissues coupled with the extreme rarity of SSCs in mammals (Kiel and Morrison, 2008; Morrison and Spradling, 2008). Again the mouse hematopoietic system has served as a model, and the best characterized mammalian stem cell niche is that of the mHSCs. It has been known for many years that HSCs normally reside in the BM in a non-diseased state, where hematopoiesis in maintained and regenerated (Weissman and Shizuru, 2008). At the macroscopic level, the constituents of bone include a calcified bone matrix consisting of hydroxyapatite crystals deposited on type I collagen protein fibers (Clarke, 2008), and a soft tissue interior (marrow) consisting of arterioles, venoles, venous sinusoids and both hematopoietic and non-hematopoietic cells (Clarke, 2008). The bony matrix can be divided into cancelous trabecular bone, consisting of hollow areas interspersed with fine spongy trabeculae (Clarke, 2008), and compact cortical bone, which is comparatively more dense and contains small blood vessels and nerve fibers in tubes known as haversian canals (Clarke, 2008). At the microscopic level, apart from hematopoietic cells, the BM consists of many additional cell types, including bone-forming osteoblasts and bone-resorbing osteoclasts (Clarke, 2008) which line the inner surface of bone forming a layer called the endosteum; vascular endothelial (VE) cells which form the blood vessels (Clarke, 2008); and interstitial or stromal cells, which include fibroblasts, adipocytes and other mesenchymal cells (Clarke, 2008). All of these cell types are potential constituents of the HSC niche.

The first direct evidence of a mHSC niche *in vivo* arose from a series of seminal genetic studies, which revealed some of the cell types that constitute mHSC niches *in vivo*

(Calvi et al., 2003; Yoshimoto et al., 2003; Zhang et al., 2003). Increasing osteoblast numbers either by overexpression the parathyroid hormone receptor (PTHr) or deletion of the BMP1a receptor led to increased HSC numbers (Calvi et al., 2003; Zhang et al., 2003), implicating osteoblasts as constituents of the mHSC niche *in vivo*. Similarly, loss of the vascular endothelial growth factor 2 receptor (VEGFR2) functioning in the VE led to the loss of long-term mHSC self-renewal *in vivo* (Hooper et al., 2009), indicating the VE cells were also constituents of the mHSC niche. These genetic studies were supporting by imaging studies of primitive mouse LKS and SLAM+ HPCs *in situ,* which were found to be in contact with both osteoblasts and sinusoidal VE cells, primarily in the trabecular regions of the bone (Kiel et al., 2005; Lo Celso et al., 2009; Xie et al., 2009). Together these findings defined cellular and anatomic components of the mHSC niche *in vivo*, but also suggested the existence of at least two types of niches in the BM, namely 1) an endosteal niche comprised of osteoblasts at the surface of the bone (Kiel and Morrison, 2008; Morrison and Spradling, 2008) and 2) a vascular niche comprised of VE cells that form sinusoidal vessels (Kiel and Morrison, 2008; Morrison and Spradling, 2008). Furthermore, these findings indicated that niches were anatomically located in the trabecular bone region (Calvi et al., 2003; Kiel et al., 2005; Lo Celso et al., 2009; Xie et al., 2009; Yoshimoto et al., 2003; Zhang et al., 2003). mHSCs were also found in regions between the endosteum and venous sinusoids (Kiel et al., 2005), as well as in contact with both of these areas (Kiel et al., 2005). Some have proposed these to be additional types of niches (Kiel and Morrison, 2008; Morrison and Spradling, 2008), although this currently remains unresolved. Other BM cells such as adipocytes (Naveiras et al., 2009),

osteoclasts (Wilson and Trumpp, 2006), megakaryoctyes (Kacena et al., 2006), mesenchymal cells (Wilson and Trumpp, 2006) (Méndez-Ferrer et al., 2010) and extracellular matrix components such as osteopontin (OPN) (Stier et al., 2005) have been implicated in regulating mHSC homeostasis *in vivo*, revealing the potential complexity of the *in vivo* niche in mammals.

Several additional types of putative mouse stem cell niches have also been identified, such as that of bulge stem cells in the hair follicle (Blanpain and Fuchs, 2006) and epithelial cells in the small intestine (Barker et al., 2007), but have not yet been rigorously characterized in the manner of mHSCs (Morrison and Spradling, 2008). Regardless, the studies to date have provided clear evidence of niches in the mouse BM that regulate stem cell fate decisions, and indicated that stem cell niche heterogeneity can exist within a given mammalian tissue (Jones and Wagers, 2008; Kiel and Morrison, 2008; Morrison and Spradling, 2008). The functional implication of distinct *in vivo* niches has not yet been examined, although it provides a potential mechanism underlying heterogeneity in the stem cell compartment.

1.5.3 Molecular interactions and signaling in the niche

Efforts to unravel the molecular pathways and signals that control mHSC function in the niche have implicated several pathways known to regulate stem cell function. The Notch ligand Jagged-1 was upregulated in osteoblasts when PTHr was overexpressed (Calvi et al., 2003), implicating the Notch pathway in maintenance of mHSC in the niche, a finding that is in agreement with previous studies in *drosophila* (Losick et al., 2011). *In vivo*

studies have also shown BMP and WNT signaling to be utilized by the niche to regulate HSC function (Morrison and Spradling, 2008). However, as discussed earlier, Notch and WNT signaling seem to be dispensable for HSC homeostasis *in vivo* (Hofmann et al., 2009; Maillard et al., 2008; Orkin and Zon, 2008), and furthermore although one study (Zhang et al., 2003) found that mHSCs homed to N-cadherin+ osteoblasts on the BM endosteum using immunohistochemistry (IHC), N-cadherin deficient mice do not display defective HSC homing or hematopoiesis (Kiel et al., 2009). These seemingly disparate findings may be explained by redundancy of signaling and multiple types of niches *in vivo*, but again underscore that the unique signaling which regulates stem cells within the niche can only be assessed through *in vivo* functional studies. Interactions between the receptor tyrosine kinase Tie2 on HSCs and its ligand angiopoietin 1 (Ang1) expressed by osteoblasts and VE cells are important for mHSC function *in vivo* (Arai et al., 2004), and Nestin-positive mesenchymal cells were shown to control HSC numbers and retention in the BM through β-adrenergic signaling *in vivo* (Méndez-Ferrer et al., 2010), implicating these pathways in niche regulation of HSC fate decisions. Another pathway linked to mHSC function in the niche is that of the chemokine CXCL12 (stromal derived factor 1, or SDF-1) and its receptor CXCR4 (Kollet et al., 2001; Otsuru et al., 2008; Sugiyama et al., 2006; Tavor et al., 2004). CXCR4 is expressed on putative mHSCs and hHSCs, and signaling between SDF-1 and CXCR4 has been shown to be critical for HSC homing to and retention in the BM (Kollet et al., 2001; Otsuru et al., 2008; Sugiyama et al., 2006; Tavor et al., 2004), and has also been implicated in LSC function (Tavor et al., 2004) (Tavor et al., 2008). SDF-1 is expressed by osteoblasts and VE cells in the BM

(Sugiyama et al., 2006), and SDF-1/CXCR4 signaling has been shown to be required for the retention of mHSCs in the VE niche through SDF-1 mediated homing and subsequent VCAM-1 and VLA-4 interactions between HSCs and niche cells (Sugiyama et al., 2006). In both humans and mice, the CXCR4 antagonist AMD3100 can mobilize HSCs out of the BM (Broxmeyer et al., 2005), providing further evidence of a conserved role for this pathway in the regulation of HSCs by the *in vivo* microenvironment.

Although cell-autonomous stimuli such as DNA damage can regulate Bcl-2 family expression and activity (Youle and Strasser, 2008), as discussed above this pathway is highly responsive to non-cell-autonomous signals such as hematopoietic cytokines, many of which have also been implicated in signaling within the HSC niche (Dellatore et al., 2008). The cytokines granulocyte macrophage colony-stimulating factor (GM-CSF) and SCF are involved in signaling HSCs to egress from their niches as well as maintenance of the stem cell compartment *in vivo* (Maeda et al., 2006), and both of these cytokines control MCL-1 expression (Huang et al., 2000; Lotem and Sachs, 2002). Loss of caspase-3 expression in mHSCs, which is key converging point in the Bcl-2 family regulatory pathway (Youle and Strasser, 2008), resulted in increased sensitivity to cytokine signaling in the microenvironment, leading to increased HSC proliferation while not affecting HSC survival (Janzen et al., 2008). In addition, bulge stem cells (BSCs) within hair follicles respond to DNA damage in their niche by upregulating Bcl-2 expression (Sotiropoulou et al., 2010). Furthermore, as discussed above, WNT and Notch signaling have been directly linked to Bcl-2 family member expression and activity (Oishi et al., 2004; Willert et al., 2003), and both of these pathways have been shown to regulate HSC fate decisions within

the niche. These findings implicate the Bcl-2 regulatory pathway in regulating stem cell fate decisions through non-cell-autonomous signaling within the niche, and again imply that there may be novel roles for Bcl-2 family members in the *in vivo* regulation of stem cell function.

Clearly, there is still a great deal to be learned regarding the signals that regulate stem cell fate decisions in the HSC niche, and signaling within any type of human stem cell niche has yet to be described. Nonetheless, several conserved pathways implicated as regulators of stem cell fates such as WNT, Notch, and CXCR4 are emerging as candidate regulators of human stem cell fate decisions in the niche, and pathways unexplored with respect to human stem cell function such as the Bcl-2 regulatory pathway are potential novel regulators of human stem cell fate decisions by their microenvironment.

1.5.4 Moving to the human stem cell niche

To date, the human stem cell niche has not been characterized *in vivo*. The only type of human stem cell niche to be characterized is that of hESCs (Bendall et al., 2007; Stewart et al., 2008). hESCs require support from conditioned media which contains factors secreted by feeder cells known as mouse embryonic fibroblasts (MEFs) to maintain pluripotency (Stewart et al., 2008), thereby acting as a surrogate *in vitro* support system (Stewart et al., 2008). However, in the absence of being grown on MEFs, hESCs differentiate into fibroblast-like cells (hdFs), which act as a supportive niche which maintains hESC function through an interplay of insulin-like growth factor II (IGF-II) and basic fibroblast growth factor (bFGF) signaling (Bendall et al., 2007). This work

therefore revealed that 1) hESCs exhibit niche dependency *in vitro* to maintain their stem cell properties (Bendall et al., 2007); and 2) in the absence of a supportive niche, hESCs will differentiate into cell types that constitute their niche (Bendall et al., 2007). Although hESCs are only a transient cell *in vivo* that exist within the blastocyst (Stewart et al., 2008), these *in vitro* findings provided the first experimental evidence of a human stem cell niche, and like the work done in the mouse hematopoietic system, suggest that human stem cells may exhibit a similar niche dependency *in vivo*.

1.6 Cancer, stem cells and the niche

1.6.1 The cancer stem cell hypothesis

Cancer is a disease characterized by aberrant clonal cell proliferation due to genetic and epigenetic changes, resulting tumour formation and subsequent invasion of other tissues by tumour cells (metastasis) (Hahn and Weinberg, 2002; Hanahan and Weinberg, 2000). During the process of transformation from a normal cell to an abnormal neoplastic cell, a series of mutations are successively acquired over time resulting in the development several well-described attributes of neoplasia (Fearon and Vogelstein, 1990) (Hahn and Weinberg, 2002; Hanahan and Weinberg, 2000). In the traditional model of cancer, individual clones within a tumour acquire mutations that allow for a growth advantage, and function to drive tumour growth and metastasis until another clone acquires additional mutations that allow it dominate tumour growth (Hanahan and Weinberg, 2000). In this "clonal evolution" model, although a tumour is genetically heterogeneous (Hanahan and Weinberg, 2000; Nowell, 1986), each clone in the tumour is theoretically

capable of driving tumour growth (Hanahan and Weinberg, 2000; Nowell, 1986). This model has been challenged in recent years due to the finding that many human cancers seem to be organized into functional hierarchies, and are initiated and maintained by rare cells that possess stem cell-like properties (Dick, 2008; Lapidot et al., 1994)(figure 6). These cells are known as tumour-initiating cells (TICs) or CSCs, and according the CSC cell hypothesis 1) the functional heterogeneity observed in some tumours reflects a hierarchical organization (Dick, 2008); 2) at the top of the hierarchy sit rare cells that self-renew and are capable of robust proliferation, thereby functioning to initiate and maintain tumour growth (Dick, 2008); 3) these rare self-renewing cells give rise to the cells that comprise the bulk of the tumour, which are not capable of long-term self-renewal and tumour initiation (Dick, 2008). These properties bear remarkable similarity to the stem cell hierarchy of normal healthy tissues (Weissman and Shizuru, 2008), and therefore in this model tumours represent an aberrant stem cell hierarchy that mirrors the organization of their normal tissue counterparts (figure 6) (Clevers, 2011). Furthermore, similar to normal hSSCs, CSCs are thought to be relatively quiescent (Clevers, 2011; Ishikawa et al., 2007), which may render them resistant to current chemotherapeutic regimens which target rapidly dividing cells in the bulk tumour population, but often fail to completely eradicate tumour growth as evidenced by the phenomenon of cancer relapse (Dick, 2008).

The paradigm model of CSCs is acute myelogenous leukemia (AML), an aggressive hematological malignancy that results in the accumulation of immature hematopoietic precursors known as blast cells in the BM and peripheral blood (PB) of affected patients (Buzzai and Licht, 2008; Estey and Döhner, 2006; Liesner and

Goldstone, 1997). In a series of seminal studies (Hope et al., 2004; Lapidot et al., 1994) (Bonnet and Dick, 1997), Johns Dick's group in Toronto showed that cells present the BM and PB of some AML patients were capable of serially transplanting human leukemia in NS mice. Leukemic grafts were initiated and maintained in these mice by rare self-renewing cells termed leukemic stem cells (LSCs), which resided in the CD34+CD38- fraction SRC fraction but not the CD34+CD38+ progenitor fraction (Bonnet and Dick, 1997; Hope et al., 2004; Lapidot et al., 1994), reflecting a functional stem cell-like hierarchy similar to that seen in normal hematopoietic tissues. Similar to normal HSCs, AML LSCs are defined in the NS model as SCID leukemia initiating cells (SL-ICs) (Lapidot et al., 1994), and SL-ICs are subject to the same stringent *in vivo* functional interrogations as normal SRCs (Dick, 2008) (figure 4B). Since these initial discoveries using AML, a considerable number of studies in both mouse and human systems have indicated that many other cancers, from other blood malignancies to solid tissue tumours (Jordan et al., 2006), are organized in a hierarchical manner similar to AML.

Despite the significant amount of evidence now supporting the CSC hypothesis, there has been a great deal of contention surrounding CSCs due to recent evidence indicating that some cancers seem to be organized according to the traditional model (Ishizawa et al., 2010; Kelly et al., 2007; Quintana et al., 2008) where each cell in the tumour is capable of re-initiating tumour growth. Similarly, other recent studies have revealed that CSCs are present in both stem cell and progenitor fractions of some AMLs and other cancers (Goardon et al., 2011; Majeti and Weissman, 2011), raising questions

regarding the cell of origin in the CSC model, and how it relates to normal stem cells and progenitors. Additional criticisms of this theory have alleged that CSCs are a phenomenon of xenograft mouse models (Kelly et al., 2007); however, recent studies have provided preliminary evidence that the CSC clones which initiate and maintain leukemia in NS mice may drive neoplasia in some human patients (Anderson et al., 2010; Notta et al., 2011b). Although there is still much to be learned regarding the actual physiologic relevance and origin of CSCs in human patients, clearly the CSC hypothesis has shifted the field of human stem cell biology. Furthermore, as discussed below, there is now evidence indicating that similar to normal hSSCs, CSCs may also be dependent upon a specific *in vivo* niche to support their unique self-renewal and proliferative capacities, a finding which could have tremendous implications on how we understand and treat human cancer.

1.6.2 The cancer stem cell niche

The large body of evidence supporting both the role of the niche in normal stem cell fate decisions has led to speculation that the niche may be similarly required for CSC cell function (Borovski et al., 2011; Ho and Wagner, 2006; Lane et al., 2009). This idea has also been influenced by the observation that despite years of research characterizing the cell-autonomous molecular biology of cancer cells (Hanahan and Weinberg, 2000; Luo et al., 2009), with some notable exceptions (Aggarwal, 2010), many cancers continue to present a poor clinical prognosis (Jemal et al., 2009), suggesting that non cell-autonomous factors may have a crucial role in transformation. Clinical observations in

many types of hematological malignancies have provided circumstantial evidence supporting a role for the niche in cancer pathogenesis. Acute leukemias such as AML, myelodysplastic syndromes (MDS) and myeloproliferative disorders (MPDs) are thought to be initiated and maintained at the stem cell level (Dick, 2008; Tan et al., 2006). In patients with these conditions, normal BM hematopoiesis is often suppressed by poorly understood mechanisms, and similar to early hematopoietic development (Tavian et al., 2010; Tavian and Péault, 2005), hematopoiesis moves to extramedullary sites such as the spleen and liver in some patients (O'malley, 2007). At the same time, neoplastic, dysplastic or hyperplastic cells accumulate in the BM, where HSCs normally reside in their niches (Lane et al., 2009). This would suggest 1) that normal hHSCs have lost their supportive BM niches; 2) the dysplastic or neoplastic process somehow causes normal HSCs to be displaced from, or egress from, their niches; and 3) these niches are able to support the function of dysplastic or neoplastic cells, and therefore are somehow involved in disease pathophysiology. While some have suggested that neoplasia represents an acquisition of niche independency by CSCs (Seoane, 2010; Vermeulen et al., 2008), evidence to date supports the opposite conclusion, namely, that cancer cells exhibit some kind of microenvironmental dependence (Colmone et al., 2008; Ishikawa et al., 2007; Lane et al., 2009; Sipkins et al., 2005), and in some cases, CSCs may compete with normal stem cells for niche occupancy as part of the malignant process (Jin et al., 2008; Schuettpelz and Link, 2011; Shiozawa et al., 2011b; Yamashita, 2008). The notion that stem cells can compete for niche occupancy has been suggested in the context of the clinical observation that when CB cells from two donors are transplanted into the same

patient, cells from one donor always dominate the graft (Eldjerou et al., 2010b; Haspel and Ballen, 2006; Majhail et al., 2006). Direct evidence of niche competition again comes from the fruit fly *drosophila*, where *in vivo* imaging studies at single cell resolution has shown not only that normal GSCs can compete for niche occupancy (Jin et al., 2008; Yamashita, 2008), but also that GSCs which have acquired mutations that render them differentiation-defective (dysplastic) are able to out-compete their normal counterparts for niche occupancy (Jin et al., 2008; Yamashita, 2008). Several phenotypic studies have provided some experimental evidence supporting a role for niche competition in the human transformation process (Shiozawa et al., 2011b) (Colmone et al., 2008; Ishikawa et al., 2007; Sipkins et al., 2005). A recent study suggests that prostate cancer cells may compete for the BM niche normally occupied by normal hHSCs (Shiozawa et al., 2011b). Studies using an acute lymphoblastic leukemia (ALL) cell line called Nalm-6 have shown that ALL cells home to VE niches in the marrow through SDF-1/CXCR4 signaling, and that these ALL cells can create aberrant niches that recruit and sequester CD34+ hHPCs (Colmone et al., 2008; Sipkins et al., 2005). Another study has shown that CD34+ AML cells home to the same regions of the BM marrow as CD34+CD38- HPCs, namely the BM endosteum and vascular endothelium in the trabecular region, where they reside in a quiescent state and resist chemotherapy (Ishikawa et al., 2007). The Bcl-2 family member MCL-1 has been implicated as a mediator of transformation and chemotherapy resistence in a number of hematological malignancies (Gandhi et al., 2008; Kaufmann et al., 1998). In some patients, MCL-1 is found to be upregulated at the time of leukemic relapse (Kaufmann et al., 1998), a process which may be mediated by CSCs which evade

chemotherapy-mediated apoptosis by occupying BM niches (Ho and Wagner, 2006; Lane et al., 2009). Therefore, like with normal hHSCs, MCL-1 is an attractive candidate regulator of CSC fates decisions within the BM niche *in vivo*. Although these studies collectively support a role for niche occupancy in the leukemic process, none of them assessed the relationship of these observations to hHSCs or CSCs at the functional level, and therefore their physiologic relevance remains unknown.

Competitive processes are not the only mechanism that may underlie the role of the niche in malignancy. Additional studies using mouse knockout models of retinoblastoma (Rb), the retinoic acid receptor (RAR) or the E3 ubquitin ligase Mib1, which regulates Notch expression (Kim et al., 2008), have shown that loss of these pathways leads to MPDs in normal HSCs transplanted into mutant mice (Kim et al., 2008; Walkley et al., 2007a; Walkley et al., 2007b). These findings indicate that at least in the mouse system, an abnormal microenvironment can induce a potentially pre-malignant condition in normal BM stem cells. Similarly, CB HSCs that have undergone transformation due to overexpression of the oncogene *MLL-AF9,* which is a translocation found in human AML and acute lymphoblastic leukemia (ALL) (Pui and Evans, 2006; Pui et al., 2008), produced a leukemic phenotypic that was dependent upon recipient mouse strain used (Wei et al., 2008). Similarly, our group has shown that transplantation of LSCs from the same human patients into both NS and NSG mice demonstrates a similar phenomenon, where the recipient mouse microenvironment dictated a T-lymphocytic versus myeloid leukemia phenotype *in vivo* (Risueño et al., 2011).

Therefore, findings to date suggest that alterations in both niche occupancy, and the niche itself may be involved in the neoplastic process and regulation of CSC fate decisions. It has been proposed that leukemic relapse occurs because rare LSCs are not targeted by current chemotherapeutic regimens (Dick, 2008). If CSCs do indeed depend on niche occupancy, the niche would be the ideal sanctuary for CSCs to reside and maintained in a quiescent state, protected from cytotoxic therapies that target dividing cancer cells. Establishing 1) whether human CSCs depend on *in vivo* niches for their function, and 2) whether CSCs compete for the same niches as normal stem cells as part of the neoplastic process will therefore be essential towards fully understanding the relationship between human stem cell fate decisions, the niche and transformation.

1.7 Summary of intent

A large number of studies utilizing multiple tissue types in both vertebrate and invertebrate systems have revealed that stem cell fate decisions such as self-renewal, survival and differentiation are a function of cell-extrinsic signals within the local *in vivo* microenvironment, as oppose to processes driven solely by cell-autonomous mechanisms (Kiel and Morrison, 2008; Morrison and Spradling, 2008). Although several molecular pathways have been identified that regulate human stem cell fate decisions *in vitro*, the *in vivo* relevance of these pathways remains controversial (Hofmann et al., 2009; Maillard et al., 2008; Orkin and Zon, 2008; Zhang et al., 2003a). Efforts to maintain and expand populations of cells enriched for hSCC *in vitro* have been largely unsuccessful to date (Dahlberg et al., 2011), as a great deal of functional heterogeneity exists within phenotypically homogeneous populations of cells enriched for human stem cells (Graf and Stadtfeld, 2008; Raaijmakers and Scadden, 2008a), and conditions to support these heterogeneous subsets have been difficult to define. This suggests that the *in vivo* microenvironment from which these cells are derived plays an indispensable role in normal human stem cell function, and that the microenvironment may be a key determinant of functional heterogeneity in the human stem cell compartment. Furthermore, in the context of the emerging role of the niche in normal stem cell function, it has been postulated that CSCs may also be dependent upon an *in vivo* niche, and that the niche may play a role in transformation (Carlesso and Cardoso, 2010; Ho and Wagner, 2006; Lane et al., 2009; Schuettpelz and Link, 2011). To date however, the *in vivo* human stem cell niche has not been characterized, and most putative molecular

signals and pathways regulating human stem cell function have been identified in *ex vivo* systems as opposed to a physiologic, *in vivo* setting.

Therefore, several key questions are implied regarding characterization of the *in vivo* human stem cell microenvironment:

1) *Can we identify novel molecular regulators of human stem cell fate decisions exclusively within the in vivo microenvironment, and do they exhibit unique function in response to non-cell-autonomous parameters within this physiologic setting?*

2) *What specific anatomical locations, cell types and molecular interactions define a human stem cell niche in vivo?*

3) Can we use this information to better understand heterogeneity in the human stem cell compartment, and prospectively isolate human stem cells for regenerative medicine?

4) *Do transformed human CSCs depend on an in vivo niche for their function, and can niche occupancy by CSCs be targeted as a therapy to eradicate human malignancies at the stem cell level?*

Understanding the anatomic and molecular characteristics of the *in vivo* stem cell microenvironment, and in turn how the microenvironment functions to regulate human

stem cell fate decisions, will be a key step toward developing protocols to modulate these fate decisions in both normal and transformed human stem cells.

Based upon our current knowledge, **I hypothesize that the *in vivo* microenvironment is an essential regulator of human stem cell fate decisions, and that the niche is a critical determinant of both functional heterogeneity in the human stem cell compartment and of neoplastic transformation.**

If this idea is true, then understanding the structure of the human stem cell niche *in vivo* and identifying pathways which uniquely regulate human stem cell function within this *in vivo* context will be required to unlock the full potential of human stem cells in regenerative medicine. Furthermore, characterizing the human stem cell niche will be a critical step towards therapeutically targeting human neoplasms at the stem cell level. To address my hypothesis, I defined the following specific objectives:

1) Investigate the role of the Bcl-2 family members as molecular regulators of normal and transformed human stem cell fate decisions within the context of the *in vivo* microenvironment, and show that these regulators function uniquely in the context of non-cell autonomous parameters within the *in vivo* microenvironment.

2) Characterize the anatomical parameters and molecular interactions that define the human stem cell niche *in vivo*, and show that these parameters can be used to both

understand heterogeneity in the human stem cell compartment, and to prospectively isolate human stem cells on the basis of their ability to interact with their niche *in vivo.*

3) Investigate whether human CSCs are dependent upon the same niches as their normal tissue counterparts *in vivo* as part of the neoplastic process, and if competition for niche occupancy can be targeted to eradicate human malignancies at the stem cell level.

Using hHSCs and transformed LSCs as a model system, we investigated the role of the traditional pro-survival Bcl-2 family members in normal and transformed human stem cell fate decisions within the *in vivo* microenvironment **(Chapter 1)**. *In vivo* pharmacological inhibition of the BCL-2 family members indicated that one or more members were essential for both HSC and LSC self-renewal function within the *in vivo* microenvironment, while survival was not significantly affected **(Chapter 1).** Unlike previous data utilizing human cell lines *in vitro*, knockdown of the BCL-2 homologue MCL-1 in hHSCs did not lead to an increase in cell death within the lin-CD34+CD38- HSC compartment *in vivo.* However, Mcl-1-targeted hHSCs lost their ability to regenerate human hematopoiesis, and serial passage indicated this was due to a complete loss of self-renewal capacity specifically within the CD34+CD38- HSC compartment, while the CD34+CD38+ progenitor compartment was unaffected **(Chapter 1).** These findings 1) implicated the Bcl-2 regulatory pathway a having a key role in human stem cell fate decisions; 2) identified MCL-1 as a novel regulator of human stem cells fate decisions, and showed that MCL-1 functionally defines the human stem cell hierarchy; 3)

demonstrated that molecular regulators of human stem cell function could be identified exclusively within the physiologic *in vivo* microenvironment; and 4) showed that MCL-1 function in human stem cells was uniquely regulated by non-cell-autonomous parameters within the physiologic *in vivo* setting.

In the context of these findings highlighting the importance of non-cell autonomous factors on pathways regulating stem cell fate decisions, further investigation was initiated to better characterize the anatomical and molecular parameters that define the hHSC niche *in vivo* **(Chapter 2).** We identified a unique population of hHSCs that resided in the trabecular region of the bone marrow *in vivo* and exhibited enhanced *in vivo* regenerative and self-renewal function **(Chapter 2).** Trabecular region HSCs were defined by a distinct gene expression signature that included Notch pathway target genes **(Chapter 2).** IHC staining revealed that trabecular region HSCs interacted with osteoblasts expressing the Notch-ligand Jagged-1. Notch-ligand binding assays subsequently allowed for prospective isolation of hHSCs enhanced for *in vivo* regenerative capacity. Collectively, these findings 1) provided the first evidence of the anatomical and molecular parameters that define a human stem cell niche *in vivo*; 2) showed that *in vivo* niche propensity is an essential determinant of functional heterogeneity in the human stem cell compartment, and linked this heterogeneity to both anatomical location and molecular signature; 3) show that molecular regulation of a stem cell by its niche *in vivo* can be exploited to prospectively isolate human stem cell populations enhanced for *in vivo* regenerative function.

Following investigation of the *in vivo* microenvironment in normal human stem cell function, we went on to further investigate whether transformed human LSCs are dependent upon an *in vivo* niche for their function, and whether LSCs compete for niche occupancy as part of the neoplastic process **(Chapter 3).** Co-transplantation of highly purified hHSCs with patient-derived LSCs indicated that transformed LSCs dynamically compete with normal human HSCs for occupancy of BM niches *in vivo* to initiate the leukemogenic process **(Chapter 3).** Titration of increasing doses of normal human HSCs revealed a dose-dependent reduction in leukemic engraftment, and serial passage of co-transplanted grafts demonstrated a similar dose-dependent eradication of self-renewing LSCs using purified human HSCs **(Chapter 3).** Similar to clinical observations (Corey et al., 2007; Estey and Döhner, 2006), the leukemic graft altered normal myelopoiesis *in vivo*, but eradication of LSCs through niche competition restored the balance toward normal hematopoiesis **(Chapter 3).** These findings demonstrated that 1) transformed LSCs dynamically compete with normal human HSCs for niche occupancy *in vivo*; 2) similar to their normal counterparts, transformed LSCs depend upon niche occupancy to support stem cell self-renewal function; 3) replacing a transformed LSC with a normal human HSC in the *in vivo* niche can be used to eradicate self-renewing LSCs and reduce the leukemic burden *in vivo*.

Overall, this thesis has advanced the field of human stem cell biology by providing novel insight into the role of the *in vivo* human stem cell microenvironment in both normal physiologic and malignant settings. We have identified a novel molecular regulator of human stem cell fate decisions that has a unique and previously unidentified

role within the *in vivo* microenvironment as oppose to *in vitro* **(Chapters 1)**; provided the first evidence of the structural and molecular parameters of the human HSC niche **(Chapter 2);** and shown that the niche is a key determinant of heterogeneity in the human stem cell compartment **(Chapter 2)**. We have further demonstrated that *in vivo* niche competition is a requisite step in the leukemogenic process in our model system, and that this step can be targeted as a therapeutic intervention **(Chapter 3)**. These findings have revealed that a better understanding of the *in vivo* human stem cell niche will be essential towards fully releasing the therapeutic potential of human stem cells.

Figure 1. Stem cell properties and fate decisions

A. Stem cells are defined by their ability to self-renew by asymmetrical cell division, producing both an identical daughter cell, as well as a more differentiated daughter cell. Apart from an asymmetrical self-renewal division, stem cells may undergo one of several additional cell fate decisions. These include a symmetrical self-renewal division, producing two identical daughter stem cells; differentiation into a specific cellular lineage; or death, for example, by programmed cell death (apoptosis).

B. Self-renewal by asymmetrical division is a hallmark property of stem cells. As daughter cells become increasingly differentiated into progenitor and mature tissue cells, long-term self-renewal capacity is lost.

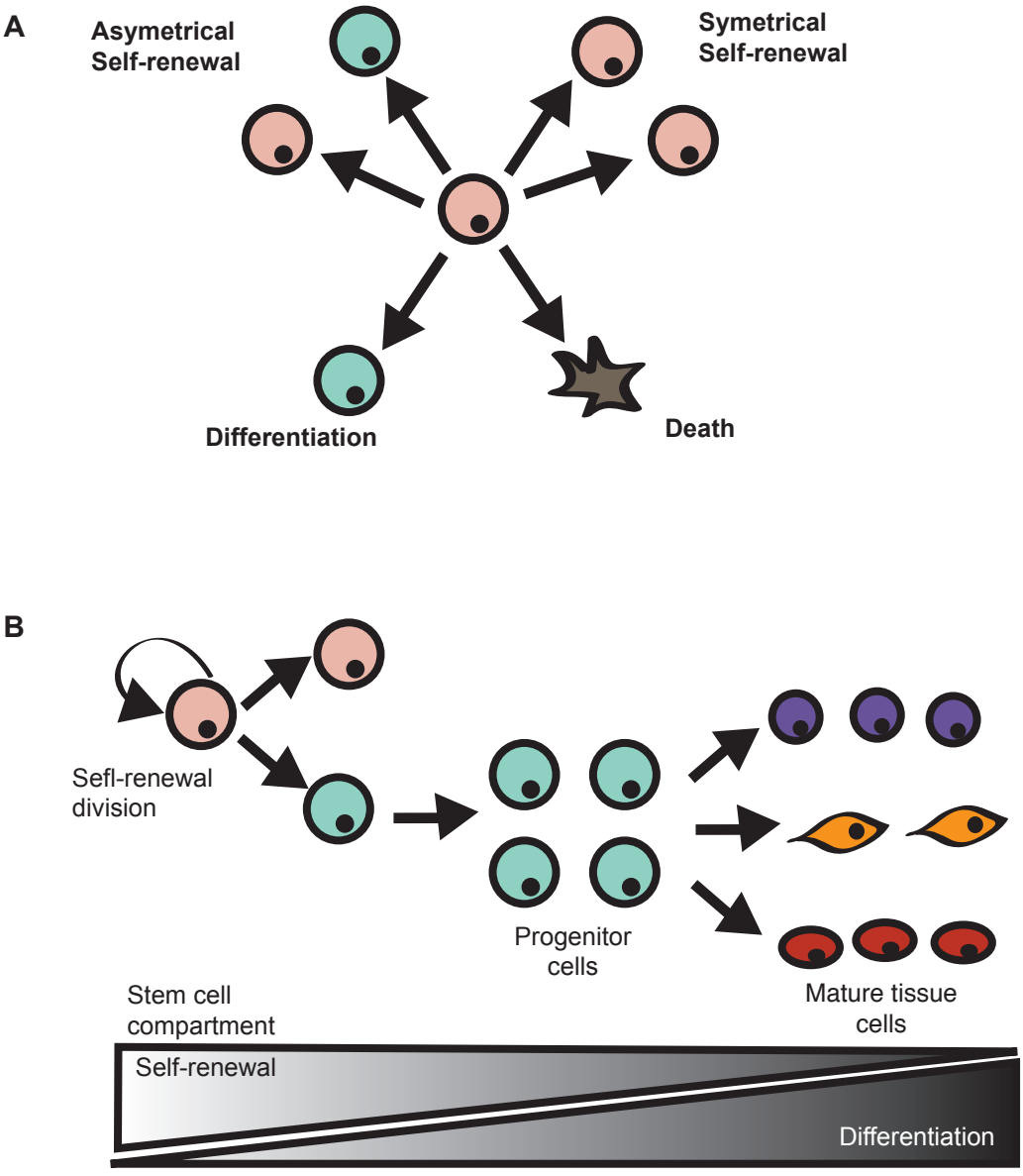

A
Asymetrical
Self-renewal
Symetrical
Self-renewal
Differentiation
Death
B
Sefl-renewal
division
Progenitor
cells
Mature tissue
cells
Stem cell
compartment
Self-renewal
Differentiation

Figure 2. Ontogenetic and tissue-specific stem cell hierarchies

Mammalian stem cells can be organized into functional hierarchies both in ontogeny and within a given tissue. The fertilized embryo has the ability to generate the entire organism, and is therefore known as a totipotent cell. The totipotent cell is by strict definition not a stem cell, as it is not capable of self-renewal. The totipotent cell is followed ontogenetically by the blastocyst, from which pluripotent stem cells (PSCs) are derived by isolating cells from the inner cell mass (ICM). PSCs such as human embryonic stem cells (hESCs) are capable of both self-renewal, and differentiation into the three embryonic germ layers, therefore in theory giving rise to all the tissues within the embryo. PSCs are followed in ontogeny by multipotent stem cells, usually called somatic stem cells (SCCs). SCCs are capable of giving rise to all of the cell lineages in their resident tissue, and sit at the apex of a tissue-specific hierarchy based upon self-renewal and differentiation capacities.

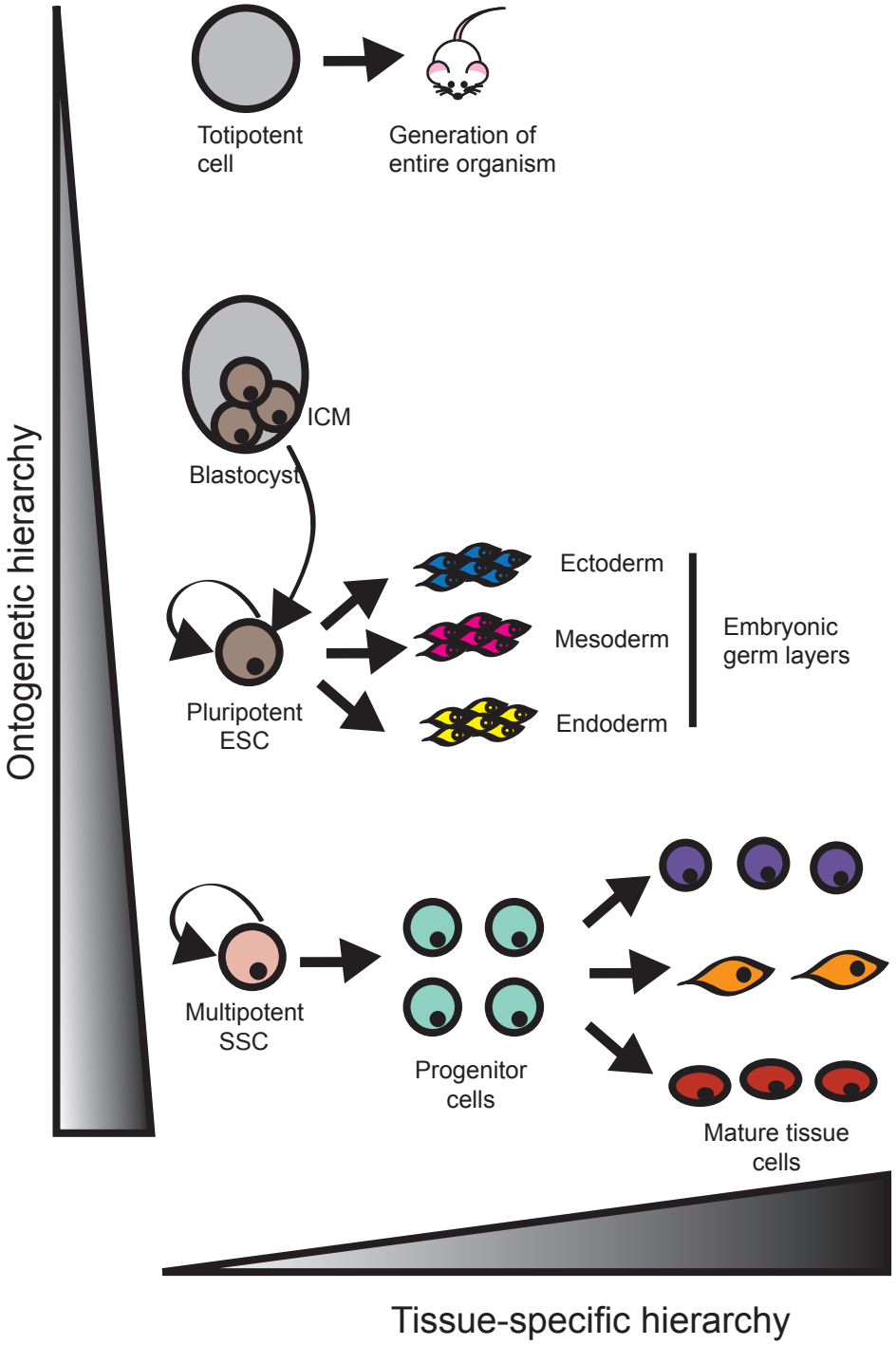

Totipotent cell
Generation of entire organism
ICM
Blastocyst
Pluripotent ESC
Ectoderm
Mesoderm
Endoderm
Embryonic germ layers
Multipotent SSC
Progenitor cells
Mature tissue cells
Ontogenetic hierarchy
Tissue-specific hierarchy

Figure 3. The functional human hematopoietic hierarchy

Like all tissues, the human hematopoietic system is organized into a functional hierarchy based upon self-renewal and differentiation capacities. At the apex of the hierarchy sit human hematopoietic stem cells (hHSCs), which demonstrate long-term self-renewal* and robust multilineage differentiation capacities. hHSCs are followed by multipotent progenitor cells (MPPs), also called long-term culture initiating cells (LTC-ICs), which demonstrate multilineage differentiation capacity, but exhibit limited self-renewal capacity. These are followed by a series of progenitor cells with increasing lineage commitment and limited self-renewal capacities, and finally by mature, fully differentiated hematopoietic cells of a given lineage. Note that many of the human progenitor cells are defined functionally as colony-forming units (CFU) or blast-forming units (BFU), as they are not well characterized at the phenotypic level. The corresponding broad phenotype of each hematopoietic compartment in terms of CD34 and CD38 expression are indicated on the right. Note also that an arrow from one cell to another cell does not necessary represent a direct transition, in many cases there are several transitional progenitor cells types in between. This is a simplified schematic of the hierarchy.

*T and B-lymphocytes demonstrate self-renewal by clonal expansion, but are not capable of multilineage differentiation.

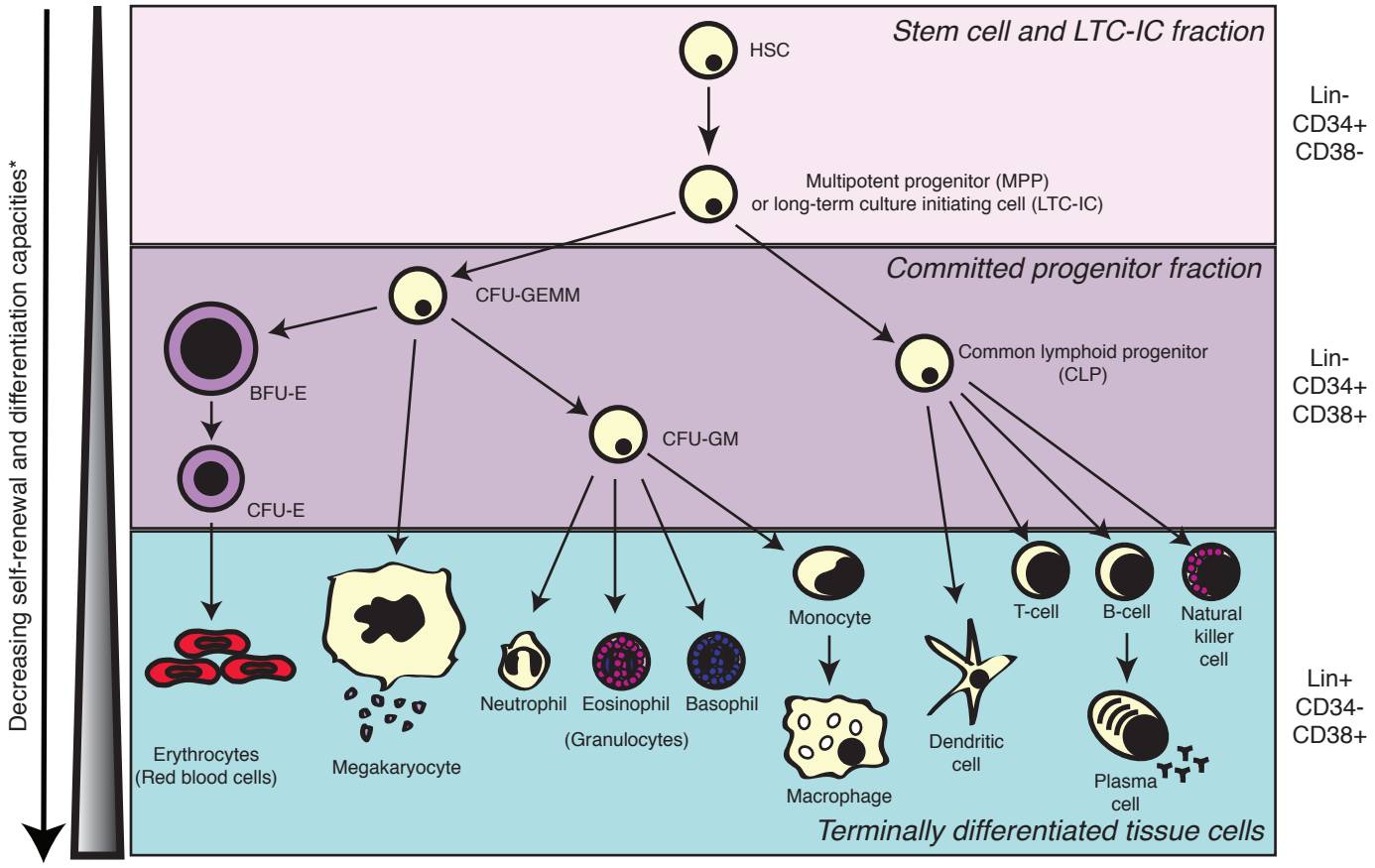
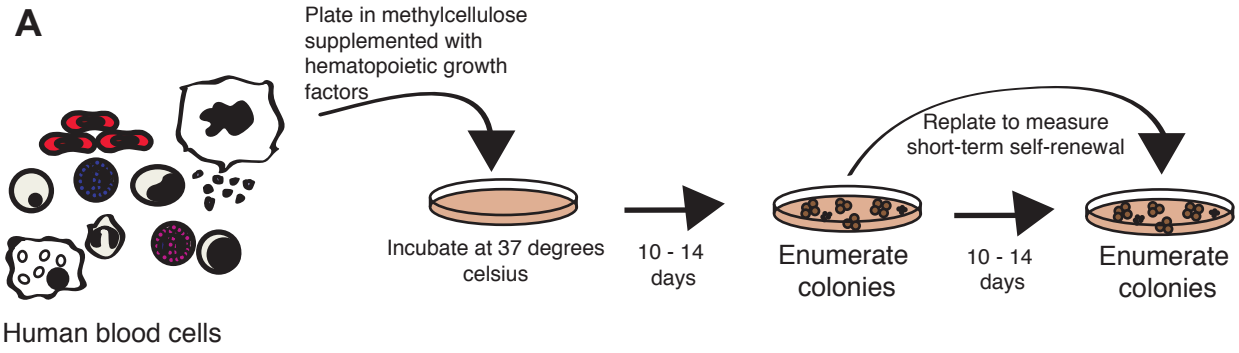

Stem cell and LTC-IC fraction
HSC
Multipotent progenitor (MPP)
or long-term culture initiating cell (LTC-IC)
Committed progenitor fraction
CFU-GEMM
BFU-E
CFU-E
CFU-GM
Common lymphoid progenitor
(CLP)
Terminally differentiated tissue cells
Erythrocytes
(Red blood cells)
Megakaryocyte
Neutrophil
Eosinophil
Basophil
(Granulocytes)
Monocyte
Macrophage
Dendritic
cell
T-cell
B-cell
Natural
killer
cell
Plasma
cell
Lin-
CD34+
CD38-
Lin-
CD34+
CD38+
Lin+
CD34-
CD38+
Decreasing self-renewal and differentiation capacities*

Figure 4. Assays of human hematopoietic stem and progenitor cell function

A. Human hematopoietic progenitor cells (hHPCs) are assayed *in vitro* by the ability to differentiate into clonal myeloerythroid colonies in the colony-forming unit (CFU) assay. Human blood cells, which include hHPCs, are plated in a methylcellulose medium, which after an incubation period is screened for colonies, allowing for determination of the frequency of various types of human hHPCs. In this assay, hHPCs are defined in retrospect by their function as CFUs or blast-forming units (BFUs).

B. Human hematopoietic stem cells (hHSCs) are assayed *in vivo* by the ability to initiate multilineage human hematopoiesis in immune-deficient mice. Human blood cells, which include hHSCs are most commonly delivered intravenously into mice, and after an incubation period, mouse hematopoietic tissues are analyzed for multilineage human chimerism by flow cytometry. Due to the short lifespan of immune-deficient mice, hHSC self-renewal is measured by serial transplantation of human grafts into secondary recipients, and similar analysis for multilineage human chimerism by flow cytometry.


A
Plate in methylcellulose supplemented with hematopoietic growth factors
Incubate at 37 degrees celsius
10 - 14 days
Enumerate colonies
Replate to measure short-term self-renewal
10 - 14 days
Enumerate colonies
Human blood cells
Sources:
Umbilical cord blood (CB)
Mobilized peripheral blood (MPB)
Bone marrow (BM)
CFU types:
Granulocyte, erythroid, macrophage, megakaryocyte (CFU-GEMM)
Granulocyte, macrophage (CFU-GM)
Erythroid (CFU-E)
Burst-forming unit erythroid (BFU-E)

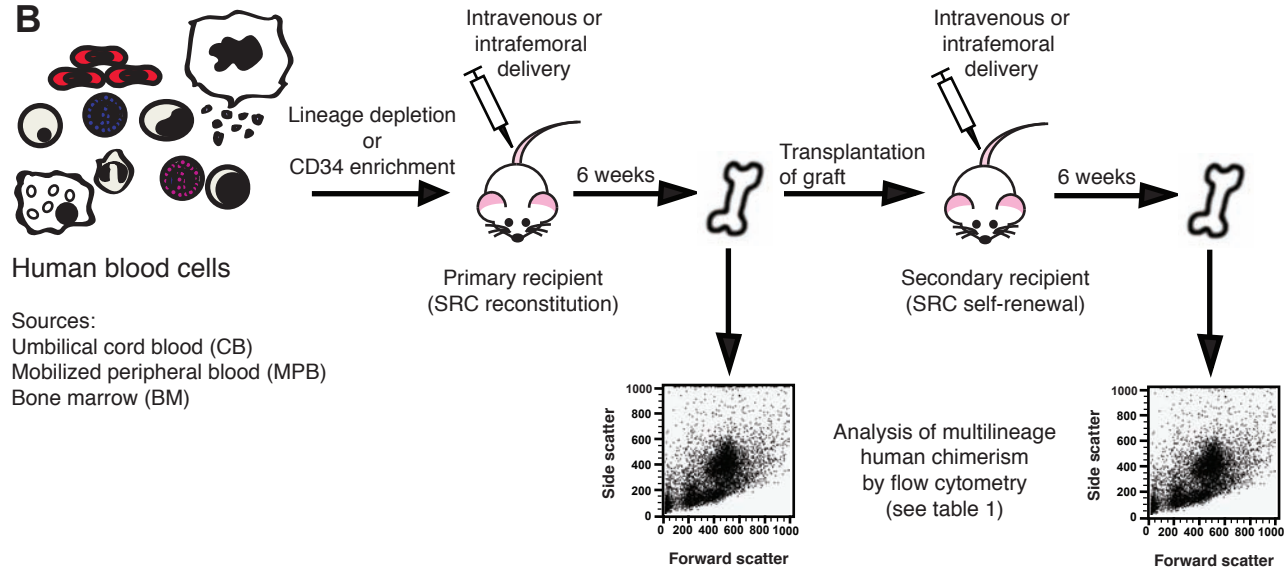

B
Lineage depletion or CD34 enrichment
Intravenous or intrafemoral delivery
6 weeks
Primary recipient (SRC reconstitution)
Transplantation of graft
Intravenous or intrafemoral delivery
6 weeks
Secondary recipient (SRC self-renewal)
Side scatter
Forward scatter
Analysis of multilineage human chimerism by flow cytometry (see table 1)
Human blood cells
Sources:
Umbilical cord blood (CB)
Mobilized peripheral blood (MPB)
Bone marrow (BM)

Figure 5. The stem cell niche hypothesis

There are several salient points underlying the concept of stem cells niche, as proposed by Raymond Schofield Schofield, R. (1978). The relationship between the spleen colony-forming cell and the haemopoietic stem cell. Blood Cells *4*, 7-25.

A. Stem cells are do not functionally exist in isolation, rather, they exist as functional stem cells only when residing in a specific microenvironment or niche.

B. The niche functions to regulate stem cell fate decisions, and maintains long-term self-renewal function in these cells.

C. Niche regulation of stem cell function occurs through both 1) direct contact with niche components such as extracellular matrix (ECM) proteins or cell surface proteins of niche constituent cells and 2) secreted factors available in the niche.

D. Stem cells are capable of exiting the niche, and a vacant niche retains the ability to support stem cell function should it be occupied again.

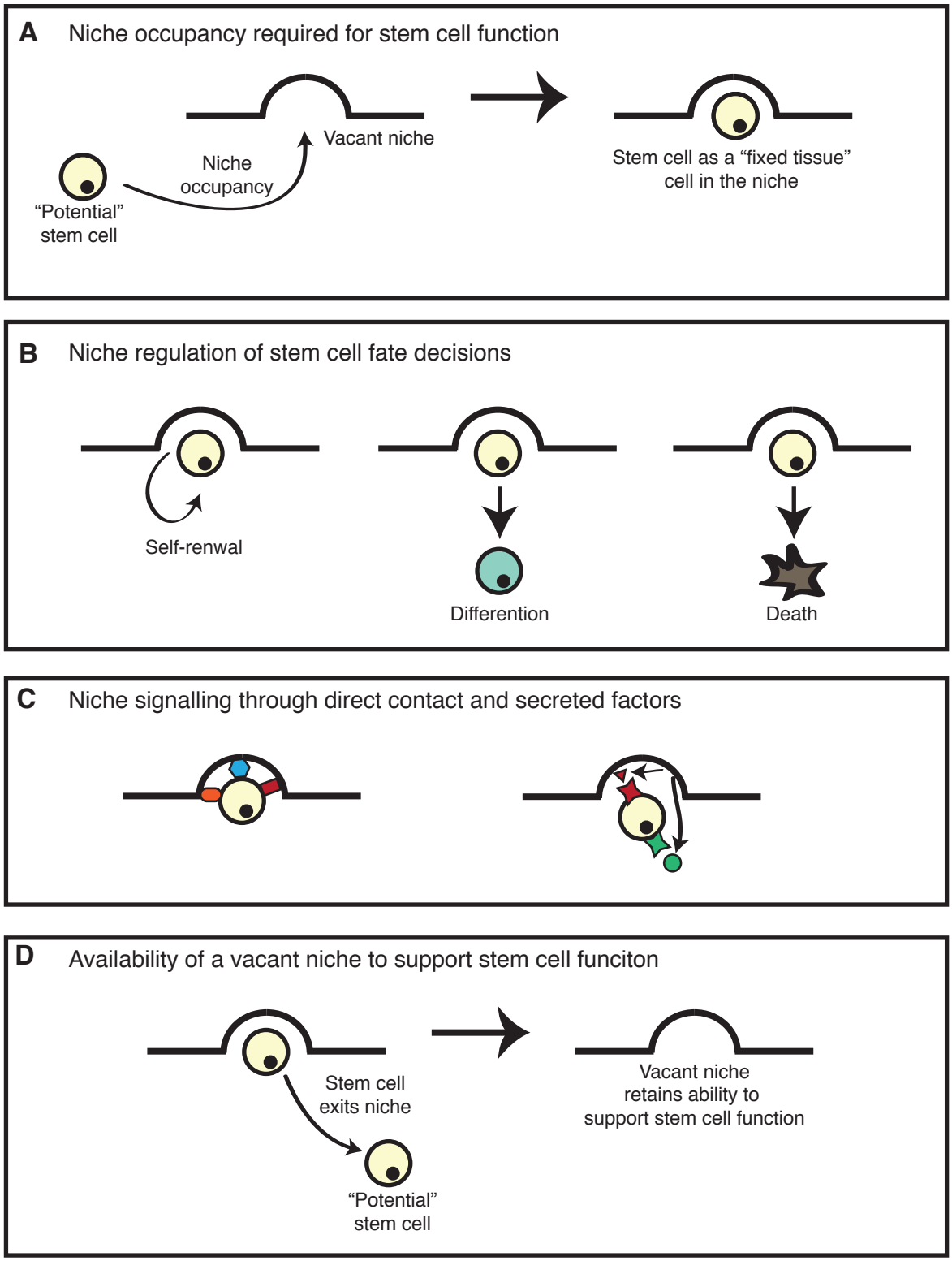
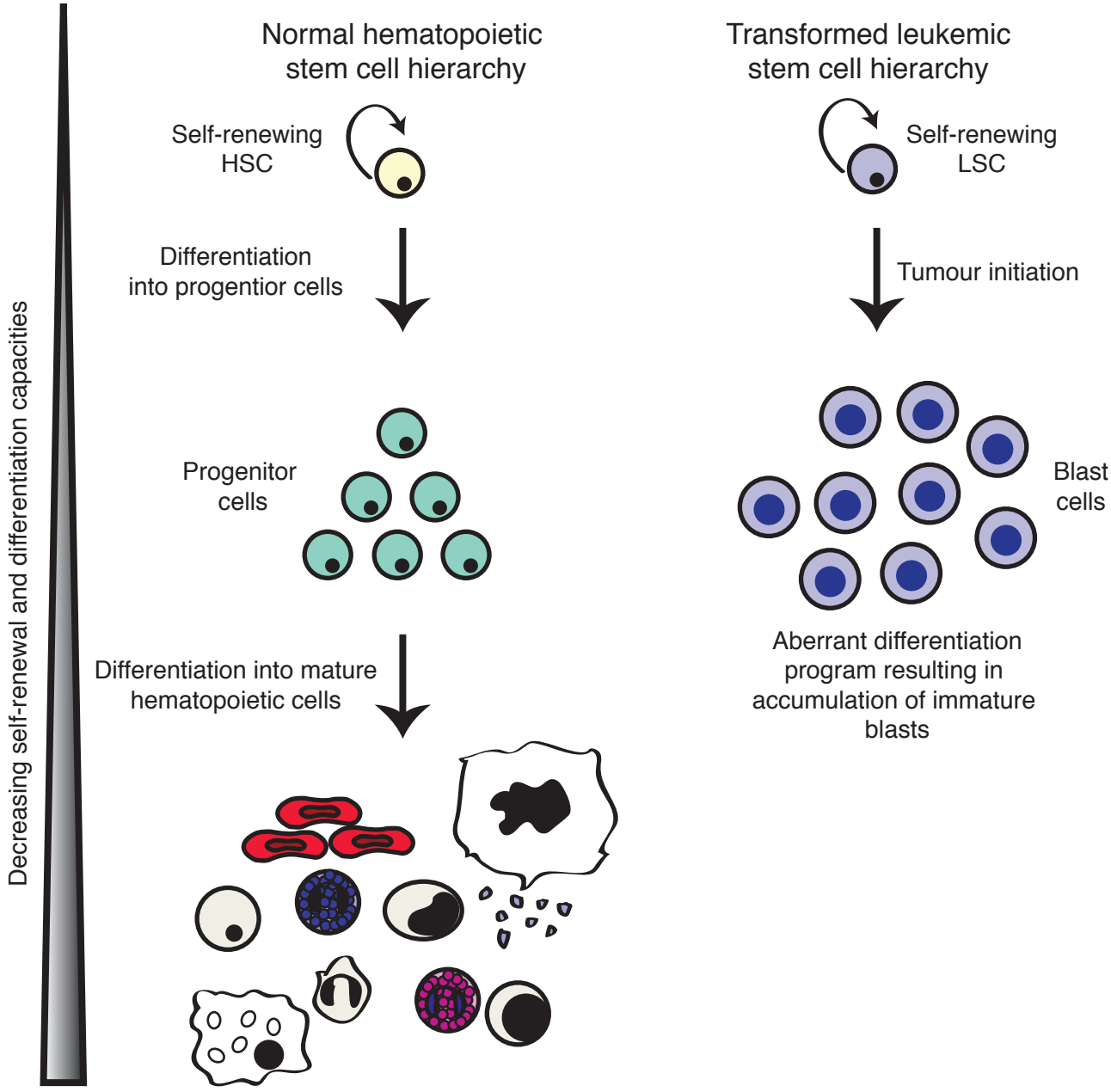

A
Niche occupancy required for stem cell function
Vacant niche
Niche occupancy
"Potential" stem cell
Stem cell as a "fixed tissue" cell in the niche
B
Niche regulation of stem cell fate decisions
Self-renwal
Differention
Death
C
Niche signalling through direct contact and secreted factors
D
Availability of a vacant niche to support stem cell funciton
Stem cell exits niche
"Potential" stem cell
Vacant niche retains ability to support stem cell function

Figure 6. The human stem cell hierarchy in normal and transformed states

In the normal human hematopoietic stem cell (hHSC) hierarchy, self-renewing hHSCs sit at the apex, giving rise to cells with increasingly limited self-renewal and differentiation capacities, and finally mature hematopoietic cells. In the transformed leukemic stem cell (LSC) hierarchy, self-renewing LSCs sit at the apex, giving rise to immature leukemic blast cells that accumulate in the blood, bone marrow and eventually other tissues. Importantly, in the cancer stem cell hypothesis, the functional heterogeneity observed in some tumours reflects a hierarchical organization where rare self-renewing LSCs give rise to the blast cells that comprise the bulk of the tumour and are not capable of long-term self-renewal and tumour initiation. These properties bear remarkable similarity to the stem cell hierarchy of normal healthy tissues, and therefore in this model tumours represent an aberrant stem cell hierarchy that mirrors the organization of their normal tissue counterparts. Dick, J. E. (2008). Stem cell concepts renew cancer research. Blood *112*, 4793-4807.


Normal hematopoietic stem cell hierarchy
Self-renewing HSC
Differentiation into progentior cells
Progenitor cells
Differentiation into mature hematopoietic cells
Transformed leukemic stem cell hierarchy
Self-renewing LSC
Tumour initiation
Blast cells
Aberrant differentiation program resulting in accumulation of immature blasts
Decreasing self-renewal and differentiation capacities

Mouse strain	Relevant mutations	Phenotype	Reference
NOD/SCID	Non-obese diabetic (NOD) Prkdc^{scid}	Defective lymphopoiesis (No T-cell development, deficient B-cell maturation) Defective myelopoiesis Lack of complement activity Low levels of natural killer (NK) cell activity	Shultz *et al. J. Immunol* (1995) **154:**180-191
NOD/SCID/β2m^{null}	Non-obese diabetic (NOD) Prkdc^{scid} β2 microglobulin null	Defective lymphopoiesis (No T-cell development, deficient B-cell maturation) Defective myelopoiesis Lack of complement activity Lack of (NK) cell activity Loss of MHC class I expression	Christianson *et al. J. Immunol* (1997) **158:**3578-3586
NOD/SCID/γ_c^{null}	Non-obese diabetic (NOD) Prkdc^{scid} Interleukin-2 receptor gamma chain null	Defective lymphopoiesis (No T-cell development, deficient B-cell maturation) Defective myelopoiesis Lack of complement activity Lack of (NK) cell activity Deficient cytokine signaling	Ito *et al. Blood* (2002) **100:**3175-3182

Table 1: An overview of mouse strains commonly used in xenotransplant models of human stem cell biology.

Tissue source	Phenotype	SRC frequency	Reference
Umbilical cord blood	CD34+	1 in 9.3 x 10^5	Wang *et al. Blood* (1997) **89:**3919-3924
	Lin-CD34+CD38-	1 in 617	Bhatia *et al. PNAS* (1997) **94:**5320-5325
	Lin-CD34+CD38-CD90+CD45RA-	1 in 10	Mejeti *et al. Cell Stem Cell* (2007) **1:**635 -645
	Lin-CD34+CD38-CD90+CD45RA-CD49f+	1 in 3	Notta *et al. Science* (2011) **333:**218-221
	Lin-CD34+CD38+	0	Bhatia *et al. PNAS* (1997) **94:**5320-5325
	Lin-CD34-CD38+	0	Bhatia *et al. PNAS* (1997) **94:**5320-5325
	Lin+	0	Bhatia *et al. PNAS* (1997) **94:**5320-5325
Mobilized peripheral blood	CD34+	1 in 8 x 10^6	Wang *et al. Blood* (1997) **89:**3919-3924
Bone marrow	CD34+	1 in 3 x 10^6	Wang *et al. Blood* (1997) **89:**3919-3924

Table 2: Frequencies of human hematopoietic stem cells in various hematopoietic tissue sources.

Cell type	Phenotype	Reference
Hematopoietic stem cell	Lin-CD34+CD38-	Bhatia *et al. PNAS* (1997) **94:**5320-5325
	Lin-CD34+CD38-CD90+CD45RA-	Mejeti *et al. Cell Stem Cell* (2007) **1:**635-645
	Lin-CD34+CD38-CD90+CD45RA-CD49f+	Notta *et al. Science* (2011) **333:**218-221
Multipotent progenitor cell	Lin-CD34+CD38-	Bhatia *et al. PNAS* (1997) **94:**5320-5325
	Lin-CD34+CD38-CD90-CD45RA-	Mejeti *et al. Cell Stem Cell* (2007) **1:**635-645
Restricted progenitor cell	Lin-CD34+CD38+	Bhatia *et al. PNAS* (1997) **94:**5320-5325
		Mejeti *et al. Cell Stem Cell* (2007) **1:**635-645
Differentiated mature hematopoietic cell	Lin+CD34-CD38+	Bhatia *et al. PNAS* (1997) **94:**5320-5325

Table 3: Cell surface phenotypes of human hematopoietic stem and progenitor cells.

Chapter 2

The human stem cell hierarchy is defined by a functional dependence on Mcl-1 for self-renewal capacity

Preamble

This chapter is an original published article. It is presented in its published format.

*"This research was originally published in Blood. **Campbell CJ**, Lee JB, Levadoux-Martin M, Wynder T, Xenocostas A, Leber B and Bhatia M. The human stem cell hierarchy is defined by a functional dependence on Mcl-1 for self-renewal capacity. Blood. 2010;**116**:1433-1442. © the American Society of Hematology."*

I performed all hHSC and CSC purifications and experiments, generated all of the figures, and wrote the initial draft of the manuscript. Dr. Mickie Bhatia and myself analyzed hHSC and CSC data, and made revisions to the manuscript. hHSC experiments were designed by Dr. Mickie Bhatia and myself, with significant intellectual input from Dr. Brian Leber. Dr. Jung Bok Lee performed hESC experiments and analyzed hESC data. hESC experiments were designed by Dr. Jung Bok Lee and Dr. Mickie Bhatia, with intellectual input from myself. Dr. Marilyne Levadoux-Martin provided technical assistance with cell sorting. Dr. Tracy Wynder provided technical assistance with *in vivo* experiments. Dr. Anargyros Xenocostas provided vital reseach reagents and intellectual input. Dr. Brian Leber also provided vital research reagents.

To date, a number of molecular pathways have been identified as regulators of hHSC fate decisions based upon *in vitro* studies (Campbell et al., 2008). In many cases, the relevance of these pathways to the *in vivo* function of hHSCs has not been assessed

(Campbell et al., 2008), or in cases where *in vivo* studies have been performed, they do not always correlate with *in vitro* findings (Hofmann et al., 2009; Maillard et al., 2008; Orkin and Zon, 2008; Zhang et al., 2003a). This suggests firstly, that molecular regulators of hSCC fate decisions should be identified via *in vivo* functional approaches; and secondly, that these pathways may function uniquely in response to non-cell-autonomous parameters within the *in vivo* microenvironment. We therefore sought to directly establish that pathways critical for hHSC fate decisions could be identified via an *in vivo* functional gene-knockdown approach, and furthermore, that these pathways demonstrated unique function within the *in vivo* microenvironment as opposed to the *in vitro* setting. The BCL-2 protein family has a well established and evolutionarily conserved role in regulating critical cell fate decisions, most notably apoptosis, in many cell types in response to both cell autonomous and non-cell autonomous signals (Adams and Cory, 2007). The role of the BCL-2 protein family members in human stem cell function was unexplored in any type of human stem cell prior to this work[1], and as such we interrogated the functional role of the BCL-2 family in hHSCs within the context of the *in vivo* microenvironment. Using pharmacological and genetic approaches, we identified the BCL-2 homologue MCL-1 as an essential regulator of hHSC self-renewal *in vivo*, and showed that a functional dependence on MCL-1 *in vivo* hierarchically delineated true hHSCs from primitive HPCs. Identification of this unique and previously unappreciated role for MCL-1 as a regulator of human stem cell self-renewal was entirely

[1] Concomitant with our study, another group published regarding the role of the Bcl-2/p53 axis in human stem cell self-renewal *in vivo*. Milyavsky, M., Gan, O. I., Trottier, M., Komosa, M., Tabach, O., Notta, F., Lechman, E., Hermans, K. G., Eppert, K., Konovalova, Z., *et al.* (2010). A Distinctive DNA Damage Response in Human Hematopoietic Stem Cells Reveals an Apoptosis-Independent Role for p53 in Self-Renewal. Cell Stem Cell 7, 186-197.

dependent upon implementing an *in vivo* approach, and our concomitant *in vitro* findings showed that MCL-1 functions uniquely in the context of non-cell-autonomous parameters *in vivo.* Our work therefore provided direct evidence that human stem cell fate decisions are distinctly regulated within the *in vivo* microenvironment, and suggested that investigations into the molecular regulation of hSCC function should be done within this unique *in vivo* context.

The human stem cell hierarchy is defined by a functional dependence on Mcl-1 for self-renewal capacity

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the Ontario Institute for Cancer Research (OICR) to M.B., and graduate research scholarships from OGS and NSERC to C.J.V.C.

Abstract

The molecular basis for the unique proliferative and self-renewal properties that hierarchically distinguish human stem cells from progenitors and terminally differentiated cells remains largely unknown. We report a role for the Bcl-2 family member myeloid cell leukemia -1 (Mcl-1) as an indispensable regulator of self-renewal in human stem cells, and show that a functional dependence on Mcl-1 defines the human stem cell hierarchy. *In vivo* pharmacological targeting of the Bcl-2 family members in human hematopoietic stem cells (HSCs) and human leukemic stem cells (LSCs) reduced stem cell regenerative and self-renewal function. Subsequent protein expression studies revealed that among the Bcl-2 family members, only Mcl-1 was upregulated exclusively in the human HSC fraction upon *in vivo* regeneration of hematopoiesis. shRNA-knockdown of Mcl-1 in human cord blood cells did not affect survival in the HSC or hematopoietic progenitor cell (HPC) fractions *in vitro*, but specifically reduced the *in vivo* self-renewal function of human HSCs. Moreover, knockdown of Mcl-1 in ontogenetically primitive human pluripotent stem cells (hPSCs) resulted in almost complete ablation of stem cell self-renewal function. Our findings reveal that Mcl-1 is an essential regulator of stem cell self-renewal in humans, and therefore represents an axis for therapeutic interventions.

Keywords: Bcl-2 protein family / human stem cells / leukemia / Mcl-1 / self-renewal / stem cell hierarchy

Introduction

Human stem cells are defined by their vast self-renewal and multilineage differentiation capacities, which place them at the top of a cellular hierarchy within a given tissue. Self-renewing multipotent HSCs are at the apex of the hematopoietic hierarchy based upon their ability to give rise to all cell types of the hematopoietic system (Mazurier et al., 2004). HSCs are followed by a series of HPCs with increasingly limited differentiation and self-renewal potential, and finally by terminally differentiated mature hematopoietic cells. The hierarchical arrangement found in normal hematopoiesis is conserved in human hematopoietic malignancies such as acute myelogenous leukemia (AML), where rare self-renewing LSCs capable of initiating leukemogenesis can be detected in the bone marrow and peripheral blood of some patients(Hope et al., 2003; Warner et al., 2004).

Independent of their tissue of origin, human stem cells can be more broadly organized into a hierarchy based upon their ontogenetic origin. hPSCs isolated from the inner cell mass of a human blastocyst can be cultured *in vitro*, and are characterized by their ability to expand robustly while maintaining pluripotency, or the ability to give rise to all three tissue germ layers (Stewart et al., 2006). This property places hPSCs at the top of the human stem cell hierarchy, followed by multipotent adult stem cells such as HSCs that possess more limited tissue-specific differentiation potential. hPSCs therefore not only represent a model system for normal human pluripotent stem cell function, but also provide a means to study how complex processes such as survival, self-renewal and differentiation are fundamentally regulated in the most primitive human stem cells.

One of the foremost challenges in human regenerative medicine has been to delineate the molecular basis for the unique self-renewal and differentiation properties that functionally define the human stem cell hierarchy. Human HSC transplants have been used clinically for over 40 years (Jansen, 2005); however, the engraftment efficiency of these transplants is limited by the numbers of donor HSCs in the graft that correlate with hematopoietic reconstitution in the recipient (Goldstein et al., 2006). In an attempt to improve HSC transplant efficiency, human regenerative medicine has focused on selectively increasing the number of donor HSCs via *ex vivo* expansion by augmenting self-renewal while suppressing differentiation (Bhardwaj et al., 2001a; Bhatia et al., 1999; Karanu et al., 2001; Masuya et al., 2002; Murdoch et al., 2003a; Peled et al., 1999; Trowbridge et al., 2006c). This has led to the identification of hematopoietic cytokines such as FLT-3L, SCF and TPO, as well as the Hedgehog, Notch and Wnt morphogenic signaling pathways as key regulators of self-renewal and differentiation in human HSCs (Campbell et al., 2008). Nonetheless, efforts to maintain and expand human HSCs in *ex vivo* culture have been met with limited success (Karanu et al., 2001; Karanu et al., 2003; Murdoch et al., 2003a; Murdoch et al., 2002), and the search for molecular pathways that govern the unique functional properties of human stem cells remains central to the field of regenerative medicine. Although it has been known for almost 20 years that Bcl-2 family members are regulators of critical cell fate decisions such as survival(Leber et al., 2007) and transformation(Reed, 2008) in human cell lines, little is known about the roles of individual Bcl-2 family proteins in human normal or cancer stem cells (CSCs) (Leber et al., 2007; Yang et al., 1996b). Previous studies in the mouse hematopoietic system have

shown that overexpression of Bcl-2 can augment mouse HSC survival and regenerative capacity (Domen et al., 2000; Domen et al., 1998; Domen and Weissman, 2000), and that the Bcl-2 homologue Mcl-1 is an essential regulator of survival in the primitive lineage-depleted (lin-) c-Kit+ Sca-1+ (LSK) population, which includes both mouse HSCs and HPCs (Opferman et al., 2005a). While these studies positioned the Bcl-2 family members as attractive candidate regulators of survival in the human stem cell fraction, the biological functions of individual Bcl-2 family proteins have yet to be defined in primary human stem cells.

Accordingly, we examined the role of the Bcl-2 family members as regulators of survival, self-renewal and differentiation in human stem cells using available *in vivo* and *in vitro* assays for stem cell and progenitor function. We describe a role for the Bcl-2 family member Mcl-1 as an indispensable regulator of self-renewal in both human HSCs and hPSCs. Furthermore, in contrast to the mouse system, Mcl-1 dependence was specific to the human stem cell fraction, as the human HPC fraction did not require Mcl-1 for survival or differentiation. Our findings demonstrate a tissue – specific and ontogenetic hierarchical dependence of primary human stem cells on Mcl-1 for their self-renewal capacity, indicating that a functional dependence on Mcl-1 for self-renewal is a defining characteristic of human stem cells.

Materials and Methods

Mice

For mouse HSC experiments, B6;129-*MCL1*^{tm3Sjk}/J *Mcl-1* homozygous floxed (f/f) mice (Opferman et al., 2005a; Opferman et al., 2003) (The Jackson Laboratory) and C57BL/6 wild type mice (The Jackson laboratory) were used. For human SRC and SL-IC experiments, we used NOD/Prkdc^{scid} (NOD/SCID)(Larochelle et al., 1996) and β2 microglobulin knockout (NOD/SCID/B2^{null}) (van der Loo et al., 1998) mice. Mice were bred and maintained in the human Stem Cell and Cancer Research Institute (SCC-RI) animal barrier facility at McMaster University. All animal procedures received the approval of the animal ethics board at McMaster University.

Obatoclax

The small molecule BH3-mimetic obatoclax (GX15-070MS) (Nguyen et al., 2007) was obtained in clinical trial and *in vitro* formulations from GeminX Pharmaceuticals. For *in vivo* administration, obatoclax was prepared and delivered at 4 mg / kg, as described previously (Nguyen et al., 2007; Trudel et al., 2007).

Purification of primitive mouse hematopoietic cells

Mouse bone marrow cells were isolated from the iliac crests, tibiae and femurs of wild type and *Mcl-1*$^{f/f}$ mice. Lin- cells were purified using a StemSep^{Tm} negative selection mouse hematopoietic progenitor enrichment kit (StemCell Technologies).

Purification of primitive human hematopoietic cells

Human umbilical cord blood (CB) mononuclear cells (MNC) were isolated as described previously(Bhatia et al., 1999). Lin- cells were purified from whole MNC populations by negative selection using a custom antibody cocktail kit (StemCell Technologies), while CD45+CD34+ cells were prepared by magnetic bead enrichment using either positive or negative selection (StemCell Technologies). MNCs were harvested from the peripheral blood of adult AML patients (AML-PB) as described (Bhatia et al., 1999), using centrifugation on Ficoll-Paque. All patient samples were obtained with the approval of local human subject research ethics boards at McMaster University and the University of Western Ontario.

Flow cytometry and cell sorting

Flow cytometry was performed on a FACScalibur flow cytometer (Becton Dickenson). Cell sorting was performed using a FACSaria cell sorter (Becton Dickenson).

Quantitative intracellular protein staining

Human MNC and lin- cells were fixed and permeabilized using the Fix and Perm Kit for intracellular flow cytometry (Invitrogen). Cells were first stained for human CD34, CD38, or CD45 for human grafts, and then stained with either mouse anti-Bcl-2 FITC (Becton Dickenson, product # 340575), rabbit anti-Bcl-x_L Alexafluor488 (Cell Signaling Technology, product # 2767) or unconjugated rabbit anti-Mcl-1 (Abcam product # ab32087). For isotype controls, cells were stained with either mouse IgG FITC (Becton

Dickenson), rabbit IgG Alexafluor 488 (Cell Signaling Technology) or unconjugated rabbit IgG (Abcam). Cells stained with either anti-Mcl-1 or rabbit IgG antibodies were subsequently stained with an anti-rabbit secondary antibody conjugated to either FITC (Jackson Immunoresearch) or Alexafluor647 (Molecular Probes, Invitrogen). Protein expression was assessed quantitatively by using the mean fluorescence intensity (MFI) of the protein signal relative to that of the relevant isotype control (van Stijn et al., 2003b).

Quantitative real-time and genomic PCR

For quantitative real-time PCR (qRT-PCR), total RNA was isolated using an RNeasy RNA micro isolation kit (Qiagen). Human lin- cells transduced with lentivirus were sorted to isolate the GFP+ fraction prior to RNA extraction. First-strand cDNA synthesis was performed using a SuperScript III cDNA synthesis kit (Invitrogen). qRT-PCR was performed using the SYBR Green qPCR detection system (Invitrogen) in conjunction with an Mx4000P light cycler (Stratagene). Quantification of transcripts was assessed using the housekeeping gene *Gapdh*. For genomic PCR, genomic DNA was isolated from sorted GFP+ wild type and *Mcl-1*$^{f/f}$ mouse lin- bone marrow cells using a DNeasy Blood and Tissue Kit (Qiagen). Amplified product sizes were verified on a 1-3% (w/v) agarose gel stained with ethidium bromide. A complete list of all primer sequences used (Lefever et al., 2009) can be found in Supplementary Table 1.

Lentiviral vectors, production and infection

We targeted Mcl-1 in human lin- cells using lenti-lox 3.7 (pLL3.7) (Rubinson et al., 2003). pLL3.7 was obtained from Addgene. The shRNA sequence used to target human Mcl-1 has been described previously (van Delft et al., 2006), and corresponds to the following sequence on the human Mcl-1 transcript, NCBI accession number NM_021960: 5'-GCAAGAGGATTATGGCTAA-3'. For knockout of Mcl-1, mouse lin- cells were targeted with pLV-CIG (Pfeifer et al., 2001). Lentiviral particles were produced using the Virapower packaging system (Invitrogen) with the 293FT packaging cell line (Invitrogen), and high-titer lentiviral stocks were prepared using ultracentrifugation. All lentiviral transductions were performed at a multiplicity of infection of 10 – 100 as described previously(Mazurier et al., 2004). Mouse lin- cells were transduced for 24 h in StemPro 34 medium supplemented with 2 mM L-glutamine, 1% BSA, 10 ng / mL mouse SCF, and 100 ng / mL mouse TPO. For hPSC cultures, cell populations were transduced starting at day 2 after passage for 48 h, in mouse embryonic fibroblast conditioned medium (MEF-CM). Gene transfer efficiency was assessed in all cases by flow cytometry.

***In vivo* hematopoietic repopulation assays**

Human and mouse lin- cells and human AML MNC were transplanted into sublethally irradiated (350 – 365 cGy, ^{137}Cs) NOD/SCID or NOD/SCID/B2^{null} recipient mice by intravenous tail vein injection and analyzed at 6 – 8 weeks post –transplant for human or mouse donor engraftment as described previously(Trowbridge et al., 2006c). In all

secondary transplants performed, at least 5000 CD34+CD38- cells isolated from primary recipients were injected.

Cell culture

hPSC cultures were maintained on matrigel in MEF-CM as previously described (Stewart et al., 2006), using the hPSC lines H1 ad H9. Cell cultures were passaged every 5-7 days, and cultures were analyzed at each passage for total viable, GFP+ and primitive SSEA-3+ cells using flow cytometry. CIC assays were performed on sorted GFP+ cells as previously described following passage 1 (Stewart et al., 2006). Human lin- CB blood cells were cultured in serum-free medium (X-VIVO 10) supplemented with hematopoietic cytokines as described (Hess et al., 2003).

Cell viability

Cell viability was assessed using the viability dye 7AAD or annexin V (Becton Dickenson).

Hematopoietic progenitor assays

Clonogenic hematopoietic progenitor assays for mouse or human HPCs were performed using Methocult H3434 or H4434 (StemCell Technologies) as described previously(Trowbridge et al., 2006c).

Image acquisition, data and statistical analysis

Images were acquired with a CoolSNAP digital camera and an Olympus 1X51 microscope. Images were analyzed using the software Image-Pro Plus 6.0. Flow cytometry data were analyzed using the software Flowjo (Tree Star). qRT-PCR data were analyzed using the MxPro software (Stratagene) and the ΔΔCt method. The significance of any differences between groups was assessed using the Student's t-test, and $p < 0.05$ was considered to be statistically significant.

Results

In vivo pharmacological inhibition of the Bcl-2 family members leads to decreased human hematopoietic and leukemic stem cell regenerative function

The immune-deficient NOD/SCID mouse provides an *in vivo* model for the study of both normal and transformed human stem cells. Human HSCs are defined functionally in this xenograft model as Scid Repopulating Cells or SRCs(Larochelle et al., 1996), which represent extremely rare cells that uniquely possess the ability to reconstitute human hematopoiesis in NOD/SCID recipients. Using the same xenograft model, rare cells that have undergone neoplastic transformation in AML patients can initiate leukemic grafts, and are functionally defined as Scid Leukemia Initiating Cells or SL-ICs(Lapidot et al., 1994). We utilized an *in vivo* strategy to examine whether survival and self-renewal are regulated in human SRCs and SL-ICs by the Bcl-2 family members (Figure 1A). Bcl-2, Bcl-X_L and Mcl-1 were targeted using a small-molecule inhibitor of Bcl-2 family proteins known as obatoclax (Nguyen et al., 2007; Trudel et al., 2007). Obatoclax can inhibit the

ability of anti-apoptotic Bcl-2 family members to bind and sequester pro-apoptotic Bcl-2 family members, causing increased cell death through apoptosis (Konopleva et al., 2008; Nguyen et al., 2007). Obatoclax is unique to other Bcl-2 family inhibitor compounds in that it is capable of antagonizing Mcl-1 in addition to Bcl-2 and Bcl-X_L (Nguyen et al., 2007). In mice reconstituted with human SRCs, administration of obatoclax did not affect total human engraftment (Figure 1B and C), the frequency of lymphoid or myeloid lineage differentiation within the human graft (Figure 1B and F) and did not detectably affect viability or the frequency of pre-apoptotic cells within the human graft (Figure 1D and 1E). Additionally, administration of obatoclax did not affect the body weight, health or survival of the recipients compared to vehicle control. We did however observe that recipient mice administered obatoclax demonstrated a reduced frequency of primitive human CD34+ hematopoietic cells compared to vehicle control (Figure 1G). Upon examination of the different populations within the human HSC hierarchy, it was evident that loss of the primitive CD34+ phenotype corresponded to a reduced frequency of CD34+CD38- cells, a population that is enriched for human SRCs (Bhatia et al., 1997b) (18 mice transplanted with SRC isolated from 3 independent human donors) (Figure 1H). In contrast, the frequency of cells in the CD34+CD38+ and CD34-CD38+ populations, which are enriched for progenitors and mature cells (Bhatia et al., 1997b), was unaffected (Figure 1H). In order to relate this phenotypic observation to SRC function, we performed secondary SRC transplants as a functional measure of self-renewal, and found that administration of obatoclax to primary NOD/SCID recipients caused a greater than 6-fold reduction in the ability of human SRCs to generate secondary hematopoietic grafts

(Figure 1I). Similarly, when mice reconstituted by human SL-ICs were administered obatoclax, we observed a reduction in the frequency of the primitive SL-IC -enriched CD34+ hematopoietic cell population within the leukemic graft (Lapidot et al., 1994), while no change in total leukemic engraftment was observed (18 mice transplanted with SL-ICs isolated from 3 independent AML patients representing FAB subtypes M2 and M4) (Figure 2A, B, C and D). This decrease in primitive phenotype translated into an approximately 3-fold reduction in the ability of SL-ICs to generate secondary leukemic grafts **(**Figure 2E**)**. Together, these findings identified the Bcl-2 family members as candidate regulators of survival and self-renewal in both normal SRCs and transformed SL-ICs.

Mcl-1 expression is uniquely upregulated in the hematopoietic stem cell fraction upon in vivo regeneration of human hematopoiesis

To characterize which Bcl-2 family members may be required for the *in vivo* function of human SRCs, we used quantitative intracellular (IC) staining (Dewson et al., 1999; Moulding et al., 2001; van Stijn et al., 2003a; van Stijn et al., 2003b) to examine expression of Bcl-2, Bcl-X_L and Mcl-1 at the protein level in human cord blood (CB) hematopoietic cells (Figure 3A). Using *de novo* isolated CB hematopoietic cells, we found that Bcl-2 and Bcl-x_L were expressed at low to undetectable levels, whereas Mcl-1 was highly expressed in all populations comprising the human hematopoietic hierarchy (Figure 3B). Furthermore, almost all human primitive CD34+ cells also expressed Mcl-1, as opposed to Bcl-X_L and Bcl-2 (Figure 3C and supplemental figure 1B). Upon *in vivo*

reconstitution of human hematopoietic cells, only Mcl-1 was upregulated exclusively in the SRC-enriched CD34+CD38- fraction, but not in the HPC or mature hematopoietic cell fractions (Figure 3B). Consistent with these findings, Mcl-1 was also upregulated at the transcriptional level in the primitive CD45+CD34+ fraction of reconstituted human CB cells (Supplemental figure 1C). This indicated a unique role for Mcl-1 specifically in the HSC fraction of the human hematopoietic hierarchy during *in vivo* regeneration of hematopoiesis.

Mcl – 1 is required for maintaining the primitive lin-CD34+CD38- SRC – enriched phenotype in vitro

A previous study of the mouse hematopoietic system identified Mcl-1 as an important regulator of primitive mouse HPCs(Opferman et al., 2005a). In order to directly assess whether Mcl-1 is required for mouse HSC repopulating function, we utilized a cre-excision system in conjunction with lineage-depleted (lin-) *Mcl-1* floxed (*Mcl-1*$^{f/f}$) bone marrow (BM) cells (Supplemental figure 2A and 2B). Loss of *Mcl-1* caused a reduction in the viability and frequency of primitive LSK bone marrow cells (Supplemental figure 2C, 2D and 2E), a reduced frequency of hematopoietic progenitors (Supplementary figure 2F), and resulted in a nearly 6-fold reduction in HSC regenerative capacity (Supplemental figure 2G).

Similar to the mouse LSK compartment, the lin- human hematopoietic compartment is heterogeneous, consisting of both a self-renewing CD34+CD38- multipotent stem cell fraction and a developmentally restricted CD34+CD38+ progenitor

fraction (Bhatia et al., 1997b). To examine the role of Mcl-1 in human HSC and HPC function, we targeted Mcl-1 with a lentiviral vector(Rubinson et al., 2003) expressing a short-hairpin RNA (Figure 4A). In contrast to the rapid increase in cell death observed in the mouse HPC fraction less than 24 h following loss of Mcl-1 expression (Opferman et al., 2005a) (Supplemental figure 2C) shRNA - knockdown of Mcl-1 in the human lin- CD34+ CB cells (Figure 4B, 4C and supplemental figure 3) did not significantly affect viability, even after 1 week of *ex vivo* culture (Figure 4D), although there was a trend toward decreased viability (Figure 4D). Similarly, the percentage of pre-apoptotic (viable annexin V+) human lin- CD34+ CB cells was not affected by Mcl-1 knockdown (Figure 4E). Although knockdown of Mcl-1 did not affect survival in primitive human hematopoietic cells, it nonetheless caused a rapid reduction in the frequency of lin- CD34+CD38- cells, a population that is highly enriched for human SRCs **(**Figure 4F, left panel, 7 independent cord blood donors and Figure 4G). Comparatively, the frequency of lin-CD34+CD38+ cells, which are enriched for progenitors and largely devoid of SRCs (Bhatia et al., 1997b), was unaffected by Mcl-1 knockdown (Figure 4F, right panel and Figure 4G). In agreement with this observation, knockdown of Mcl-1 did not significantly affect the frequency of human hematopoietic progenitors as measured by the CFU assay (Trowbridge et al., 2006c) (Figure 4H). Collectively, these findings indicated that cells found exclusively within the rare the SRC-enriched CD34+CD38- fraction of human CB require Mcl-1 for their *in vitro* function, and that Mcl-1 may have a role is these cells that is unique from regulation of cell survival.

A functional dependence on Mcl-1 for self-renewal capacity defines the human stem cell hierarchy

To examine whether the rapid loss of lin-CD34+CD38- cells following Mcl-1 knockdown translated into a reduction in human SRC regenerative capacity, we implemented our Mcl-1 loss-of-function strategy in human CB SRCs (Figure 5A). In agreement with our phenotypic observations in the SRC-enriched lin-CD34+CD38- fraction (Figure 4F), knockdown of Mcl-1 led to an 8-fold reduction in the hematopoietic regenerative capacity of human SRCs, demonstrating that Mcl-1 is a critical regulator of human SRC regenerative function *in vivo* (42 mice transplanted using 7 independent cord blood donors) (Figure 5B and 5C). To verify the specificity of our knockdown, we targeted Mcl-1 with a second independent shRNA sequence, and observed a similar reduction in human SRC regenerative capacity (Supplemental figure 4). Consistent with our *in vitro* findings (Figure 4D), knockdown of Mcl-1 did not affect viability within the human CD34+CD38- SRC fraction or human CD34+CD38+ progenitor fraction *in vivo* (Figure 5D). However, again parallel to our *in vitro* findings (Figure 4F), knockdown of Mcl-1 caused a 10-fold reduction in the frequency of human cells with the CD34+CD38- SRC-enriched phenotype within the human graft, indicating that Mcl-1 is required for maintaining the most primitive phenotype of the human hematopoietic hierarchy *in vivo* (Figure 5E and 5F). Additionally, Mcl-1 knockdown did not affect the frequency of lymphoid, myeloid or total primitive CD34+ cells within the human graft (Figure 5G), indicating that knockdown of Mcl-1 did not affect the ability of primitive human HPCs to differentiate *in vivo*. Self-renewal is rigorously assessed in human SRCs by their ability

to reinitiate hematopoietic grafts following serial passage into secondary NOD/SCID recipients. To directly assay whether the loss of the CD34+CD38- phenotype following Mcl-1 knockdown and the reduced hematopoietic regenerative capacity of Mcl-1 -deficient SRCs was due to a decreased capacity for stem cell self -renewal, we performed secondary transplants as a functional measure of self-renewal. Using equal numbers of transplanted cells isolated from primary recipient mice, we found that knockdown of Mcl-1 in human SRCs resulted in the complete ablation of their secondary hematopoietic regenerative capacity, demonstrating that Mcl-1 is essential for the self-renewal capacity of human SRCs (Figure 5H, cells isolated from primary grafts representing 3 independent cord blood donors) and revealing a role for Mcl-1 as a regulator of self-renewal in the human stem cell compartment.

To assess whether the role of Mcl-1 as a regulator of self-renewal function was ontogenetically conserved throughout the human stem cell hierarchy, we implemented our loss-of-function strategy in purified hPSCs in conjunction with established *in vitro* assays for hPSC function (Stewart et al., 2006) (Figure 6A). While knockdown of Mcl-1 (data not shown) did not affect viability in the primitive self-renewing SSEA-3+ clonogenic fraction of hPSC cultures over three passages (Figure 6B), it almost completely abrogated stem cell self-renewal (Figure 6C and 6D, and Supplemental Figure 5), indicating that the stem cell fraction in hPSC cultures is highly dependent on Mcl-1 for self-renewal capacity. The ability to reinitiate colonies and reestablish hPSC cultures provides the most rigorous functional measurement of hPSC self-renewal. Using the established hPSC colony initiating cell (CIC) assay (Stewart et al., 2006), we found that

knockdown of Mcl-1 had an extremely potent effect on hPSC self-renewal capacity, resulting in near total loss of the ability of sorted GFP+SSEA-3+ hPSCs to reinitiate colonies following the first passage (Figure 6A and E). Together, these observations established Mcl-1 expression as an absolute requirement for self-renewal in the ontogenetically primitive hPSC fraction, and revealed a mechanistic conservation of Mcl-1 function in the human stem cell compartment.

Discussion

The molecular regulators that control the complex processes of survival, self-renewal and differentiation that define the human stem cell compartment remain largely unknown. Our study reveals a hierarchical dependence of human stem cells on the Bcl-2 family member Mcl-1 for their self-renewal capacity, and demonstrates that Mcl-1 is a defining molecular regulator of human stem cell function. Further investigation into the mechanistic regulation of stem cell self-renewal by Mcl-1 as well as the individual roles of other Bcl-2 family members will likely provide additional insights into how survival, self-renewal and differentiation are regulated in human stem cells. Specifically, our findings using the Bcl-2 family inhibitor obatoclax (Figures 1 and 2) in combination with our Bcl-2 family expression data (Figure 3) suggest that the other Bcl-2 family members may have important roles in regulating human stem cell function. Particularly, it is interesting to note that expression of Bcl-2 was also highly upregulated in the CD34+CD38- SRC – enriched and CD34+CD38+ progenitor fractions upon *in vivo* regeneration of human hematopoiesis (Figure 3).

One of the central challenges to studying survival in human stem cells is that they are not defined phenotypically, but rather are defined using functional assays such as the NOD/SCID xenotransplantation assay. Therefore, if a stem cell has undergone apoptosis it is not possible to directly detect this retrospectively *per se*, but rather it can only be characterized as a function of the available endpoint readouts in the assay. Although we did not observe any change in the viability of Mcl-1 – targeted primitive lin- CB cells *in vitro* or of human grafts where Mcl-1 was targeted *in vivo* (Figures 1, 4 and 5), it is possible that extremely rare cells within the CD34+CD38- fraction may have undergone apoptosis *in vivo* following loss of Mcl-1. Mechanistically this decreased survival may have translated into a decreased SRC regenerative function and self-renewal capacity. We were not able to achieve levels of Mcl-1 protein knockdown in primary human lin- CD34+ cells beyond 36% despite testing three shRNA sequences against Mcl-1 (Figure 5C and supplemental figure 3), and as there are few studies examining gene knockdown in primary human stem cells, it is difficult to characterize the relative efficacy of this knockdown. It may be possible that achieving higher levels of Mcl-1 knockdown could translate into a survival versus a self-renewal effect in the primitive SRC fraction. However, our observation that the human stem cell compartment requires Mcl-1 for self-renewal was not limited to the hematopoietic system, as demonstrated by the potent effect on self-renewal in hPSCs following loss of Mcl-1 function (Figure 6). These analogous findings in more than one type of human stem cell support a conservation of Mcl-1 function as a regulator of self-renewal in the human stem cell compartment. Interestingly, our finding that Mcl-1 has a functional role beyond regulation of cell survival is not

without precedent, as a previous study using Mcl-1 null mice showed that loss of Mcl-1 function resulted in developmental arrest at the blastocyst stage, but did not lead to an increase in cell death (Rinkenberger et al., 2000). We found that primitive hPSCs, which are derived from human blastocysts, absolutely required Mcl-1 for self-renewal versus survival (Figure 6). This raises the possibility that Mcl-1 may have a broader, conserved regulatory role in early mammalian ontogeny apart from regulating cell survival.

From a clinical perspective, our findings suggest that therapeutic interventions targeting Mcl-1 or its upstream regulators such as Mule (Mcl-1 ubiquitin ligase E3) (Zhong et al., 2005) can be developed to improve the efficiency of HSC transplants by increasing HSC numbers through increased self-renewal capacity. Intriguingly, Mcl-1 has been shown to be targeted for proteasomal degradation by glycogen synthase kinase 3 (GSK-3) phosphorylation in several human cell lines (Maurer et al., 2006b). We have previously established that GSK-3 is another indispensable regulator of primary human stem cell function (Trowbridge et al., 2006c), and inhibition of GSK-3 has shown positive results in augmenting the efficiency of human HSC transplants in recent clinical trials (Martinez, 2008). Further work could be conducted to assess whether GSK-3 – dependent regulation of HSC function may involve control of Mcl-1 stability. Additionally, Mcl-1 has specifically been implicated as a regulator of survival and transformation in scores of human blood cancers and solid tumors (Akgul, 2008). Human malignancies such as AML have been shown to be initiated in a hierarchical manner by rare LSCs analogous to normal HSCs (Ward and Dirks, 2007). We found that the leukemic stem cell hierarchy is also defined by a functional dependence on one or more

Bcl-2 family members, for the first time providing direct experimental evidence that the Bcl-2 regulatory pathway is of key importance in the survival or self-renewal of primary human CSCs. Further work into the roles of individual Bcl-2 family proteins including Mcl-1 in primary CSCs may therefore be an important factor in designing treatment strategies that specifically target CSCs while leaving other cell populations intact.

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Figure 1. In vivo pharmacological inhibition of the Bcl-2 family members leads to decreased human hematopoietic stem cell regenerative and self-renewal function

(A) Experimental strategy used to examine the effect of obatoclax on primitive human hematopoietic cell phenotype and SRC self-renewal capacity.

(B) Representative examples of flow cytometry analyses used to assess the effects of obatoclax on human hematopoietic engraftment relative to vehicle control. The inlay plots represent the isotype staining control for each antibody.

(C) Analysis of the effect of obatoclax on human SRC regenerative capacity. The average frequency of human hematopoietic engraftment (CD45+ cells) in the bone marrow of primary recipient mice administered obatoclax or vehicle control. Error bars represent the mean +/- SEM of three independent experiments, each with 3 mice per group.

(D) Analysis of the effect of obatoclax on the viability of engrafted human hematopoietic cells. The average frequency of dead cells within the human hematopoietic graft (CD45+ 7AAD+) in the bone marrow of primary recipient mice administered obatoclax or vehicle control. Error bars represent the mean +/- SEM of three independent experiments, each with 3 mice per group.

(E) Analysis of the effect of obatoclax on apoptosis in engrafted human hematopoietic cells. Average frequency of viable (7AAD-) annexin V+ (pre-apoptotic) cells within the human hematopoietic graft (CD45+ 7AAD+) in the bone marrow of primary recipient mice administered obatoclax or vehicle control. Error bars represent the mean +/- the SEM (n = 2- 3 mice per group).

(F) Analysis of the effect of obatoclax on multilineage human hematopoietic engraftment. The average frequency of lymphoid (CD19+) and myeloid (CD33+) cells within the human hematopoietic graft (gated CD45+ cells) in the bone marrow of primary recipient mice administered obatoclax or vehicle control. Error bars represent the mean +/- SEM of three independent experiments, each with 3 mice per group.

(G) Analysis of the effect of obatoclax on primitive (CD34+) human hematopoietic engraftment. The average frequency of CD34+ cells within the human hematopoietic graft (gated CD45+ cells) in primary recipient mice administered obatoclax is expressed relative to mice administered vehicle control. Error bars represent the mean +/- SEM of three independent experiments, each with 3 mice per group. $*P < 0.05$.

(H) Analysis of the effect of obatoclax on each population within the human hematopoietic hierarchy. The average frequency of cells in each population within human hematopoietic graft (gated CD45+ cells) in primary recipient mice administered obatoclax

is expressed relative to mice administered vehicle control. Error bars represent the mean +/- SEM of three independent experiments, each with 3 mice per group. $**P < 0.01$.

(I) Analysis of the effect of obatoclax on human SRC self-renewal capacity. The average frequency of human hematopoietic engraftment (CD45+ cells) in the bone marrow of secondary recipient mice transplanted with human-engrafted bone marrow isolated from primary recipient mice administered obatoclax or vehicle control. Error bars represent the mean +/- SEM of three independent experiments, each with 3 mice per group. $*P < 0.05$.

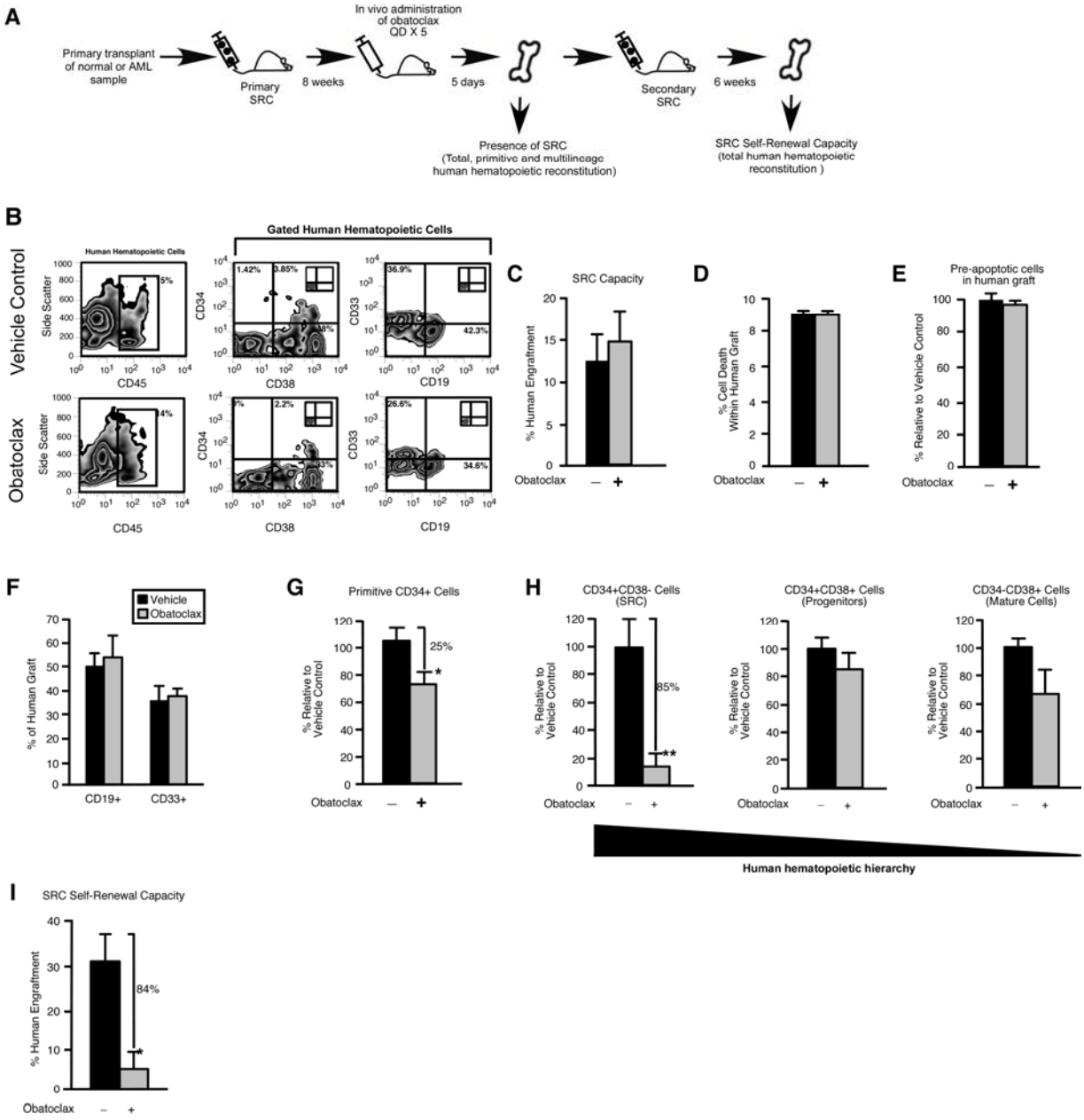

A
Primary transplant of normal or AML sample
Primary SRC
8 weeks
In vivo administration of obatoclax QD X 5
5 days
Secondary SRC
6 weeks
Presence of SRC (Total, primitive and multilineage human hematopoietic reconstitution)
SRC Self-Renewal Capacity (total human hematopoietic reconstitution)
B
Gated Human Hematopoietic Cells
Human Hematopoietic Cells
Vehicle Control
Obatoclax
Side Scatter
CD45
CD34
CD38
CD33
CD19
C
SRC Capacity
% Human Engraftment
Obatoclax
D
% Cell Death Within Human Graft
E
Pre-apoptotic cells in human graft
% Relative to Vehicle Control
F
Vehicle
Obatoclax
% of Human Graft
CD19+
CD33+
G
Primitive CD34+ Cells
% Relative to Vehicle Control
25%
H
CD34+CD38- Cells (SRC)
CD34+CD38+ Cells (Progenitors)
CD34-CD38+ Cells (Mature Cells)
85%
Human hematopoietic hierarchy
I
SRC Self-Renewal Capacity
% Human Engraftment
84%

Figure 2. In vivo pharmacological inhibition of the Bcl-2 family members leads to decreased human leukemic stem cell regenerative and self-renewal function

(A) Experimental strategy used to examine the effects of obatoclax on primitive human leukemic cell phenotype, and SL-IC self-renewal capacity.

(B) Representative examples of flow cytometry analyses used to assess the effects of obatoclax on human leukemic engraftment relative to vehicle control. The bone marrow of recipient mice that were transplanted with human SL-IC and subsequently administered obatoclax or vehicle control was analyzed for total (CD45 expression), myeloid (CD33 expression) and primitive (CD34 expression) human leukemic engraftment.

(C) Analysis of the effect of obatoclax on human SL-IC capacity. The average frequency of human leukemic engraftment (CD45+ cells) in the bone marrow of primary recipient mice administered obatoclax is expressed relative to mice administered vehicle control. Error bars represent the mean +/- SEM of three independent experiments, each with 3 mice per group.

(D) Analysis of the effect of obatoclax on primitive (CD34+) human leukemic engraftment. The average frequency of CD34+ cells within the human leukemic graft (gated CD45+ cells) in the bone marrow of primary recipient mice administered

obatoclax is expressed relative to mice administered vehicle control. Error bars represent the mean +/- SEM of three independent experiments, each with 3 mice per group. $**P < 0.01$.

(E) Analysis of the effect of obatoclax on human SL-IC self-renewal capacity. The average frequency of human leukemic engraftment (CD45+ cells) in the bone marrow of secondary recipient mice transplanted with human leukemia-engrafted bone marrow isolated from primary recipient mice administered obatoclax is expressed relative to vehicle control. Error bars represent the mean +/- SEM of three independent experiments, each with 3 mice per group. $*P < 0.05$.

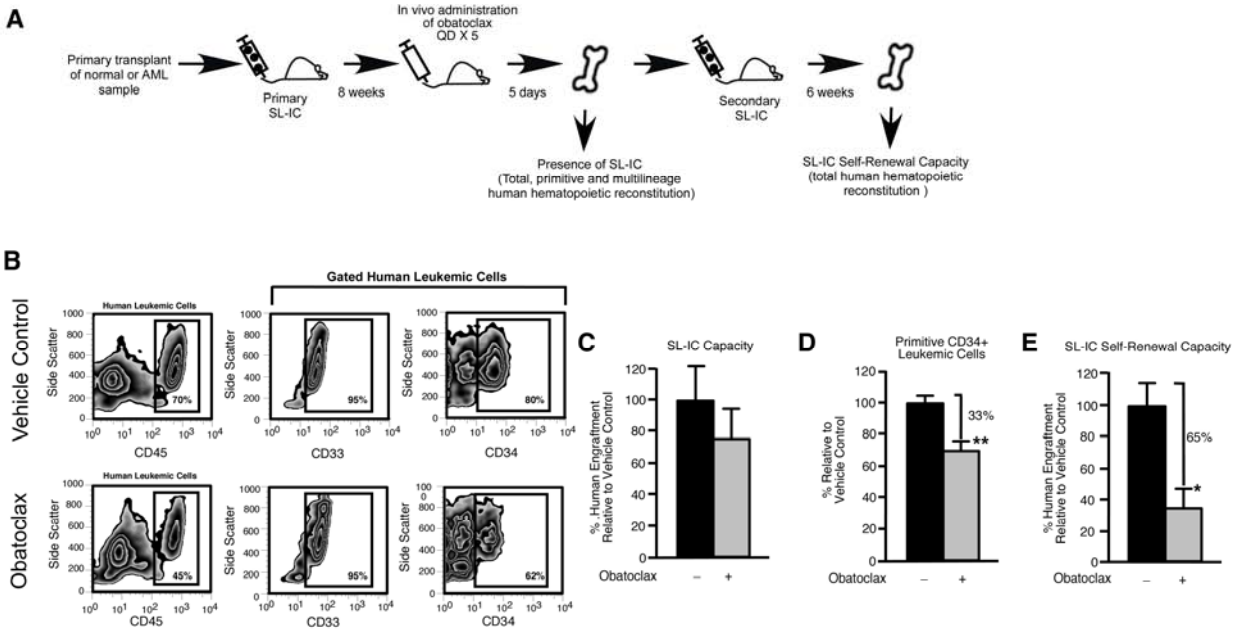

A
Primary transplant of normal or AML sample
Primary SL-IC
8 weeks
In vivo administration of obatoclax QD X 5
5 days
Secondary SL-IC
6 weeks
Presence of SL-IC (Total, primitive and multilineage human hematopoietic reconstitution)
SL-IC Self-Renewal Capacity (total human hematopoietic reconstitution)
B
Gated Human Leukemic Cells
Vehicle Control
Obatoclax
Human Leukemic Cells
Side Scatter
CD45
CD33
CD34
70%
95%
80%
45%
95%
62%
C
SL-IC Capacity
% Human Engraftment Relative to Vehicle Control
Obatoclax
D
Primitive CD34+ Leukemic Cells
% Relative to Vehicle Control
33%
**
E
SL-IC Self-Renewal Capacity
65%
*

Figure 3. Mcl-1 is uniquely upregulated in the HSC fraction of reconstituted human hematopoietic cells

(A) Experimental strategy used to examine the protein expression of Bcl-2 family members in *de novo* isolated and reconstituted human hematopoietic cells.

(B) Quantitative flow cytometric analysis of Bcl-2 family member protein expression in each population within the human hematopoietic hierarchy in both *de novo* isolated and *in vivo* reconstituted human hematopoietic cells. The average protein expression of Bcl-2, Bcl-x_L and Mcl-1 in each population of the hematopoietic hierarchy relative to isotype control is shown. Protein expression is the mean fluorescence intensity (MFI) relative to the isotype control. Error bars represent the mean +/- SEM of three independent experiments. *$P < 0.05$, **$P < 0.01$.

(C) Representative examples of flow cytometry analysis used to assess the frequency of primitive CD34+ human hematopoietic cells that express Bcl-2, Bcl-x_L and Mcl-1. Frequencies represent the percent of cells positive for the respective protein and CD34 expression, +/- the SEM. Plots are representative of at least 3 independent experiments.

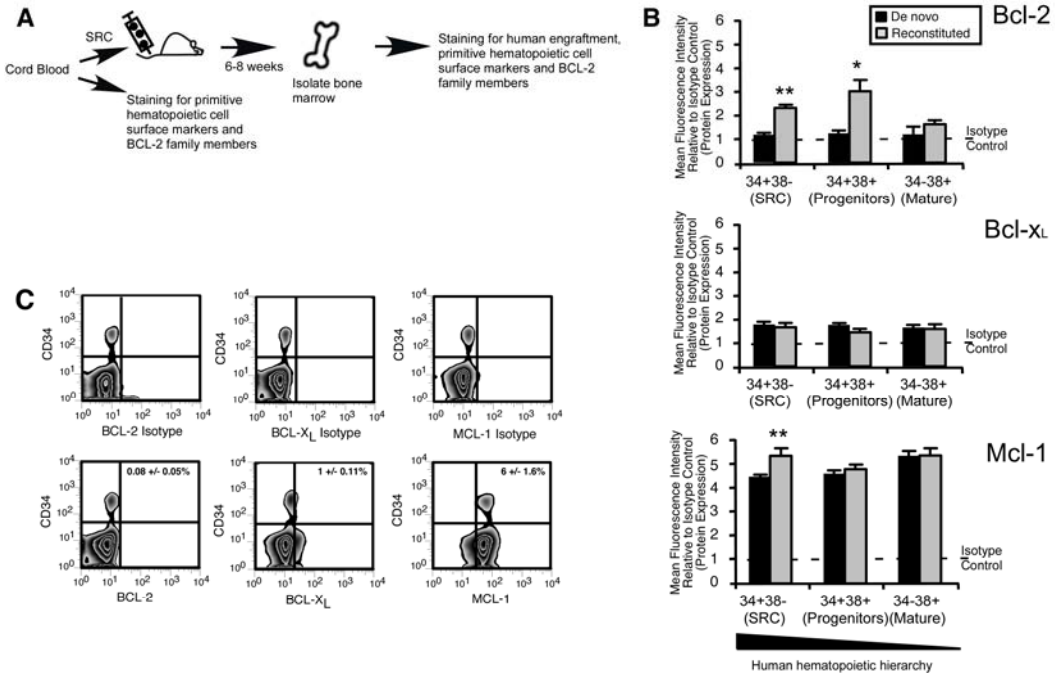

A
SRC
Cord Blood
6-8 weeks
Isolate bone marrow
Staining for human engraftment, primitive hematopoietic cell surface markers and BCL-2 family members
Staining for primitive hematopoietic cell surface markers and BCL-2 family members
B
De novo
Reconstituted
Bcl-2
Bcl-xL
Mcl-1
Mean Fluorescence Intensity Relative to Isotype Control (Protein Expression)
Isotype Control
34+38- (SRC)
34+38+ (Progenitors)
34-38+ (Mature)
Human hematopoietic hierarchy
C
CD34
BCL-2 Isotype
BCL-XL Isotype
MCL-1 Isotype
0.08 +/- 0.05%
1 +/- 0.11%
6 +/- 1.6%
BCL-2
BCL-XL
MCL-1

Figure 4. Mcl-1 is required for maintaining the primitive lin- CD34+CD38- SRC-enriched phenotype *in vitro*

(A) Experimental strategy used to examine the role of Mcl-1 in the viability and function of primitive human hematopoietic progenitor cells *in vitro*

(B) Knockdown of the human Mcl-1 transcript in lin- cord blood hematopoietic cells. Error bars represent the mean +/- SEM of three independent experiments.

(C) Knockdown of human Mcl-1 protein expression in lin- cord blood hematopoietic cells. Protein expression is the mean fluorescence intensity (MFI) relative to the isotype control. Error bars represent the mean +/- SEM of three independent experiments.

(D) Analysis of the effect of Mcl-1 knockdown on the viability of human lin- CD34+CD38- and CD34+CD38+ cord blood hematopoietic cells at 24 h post-transduction and following 1 week of *ex vivo* culture post-transduction. Error bars represent the mean +/- SEM of three independent experiments.

(E) Average frequency of viable (7AAD-) annexin V+ human lin- hematopoietic cells 24 h post-transduction with the empty vector or Mcl-1 shRNA-expressing vector. Error bars represent the SEM (n = 3).

(F) Analysis of the effect of Mcl-1 knockdown on primitive cell phenotypes within the human hematopoietic stem cell hierarchy. Analysis was performed on transduced lin- cells isolated from 7 independent cord blood donors.

(G) Representative examples of flow cytometry analyses used to assess the effects of Mcl-1 knockdown on the primitive human hematopoietic phenotypes.

(H) Analysis of the effect of Mcl-1 knockdown on the frequency of human hematopoietic progenitors. Progenitor frequency is expressed as the number of hematopoietic colonies scored after 12-14 days per 1000 cells plated. Error bars represent the mean +/- SEM of four independent experiments.

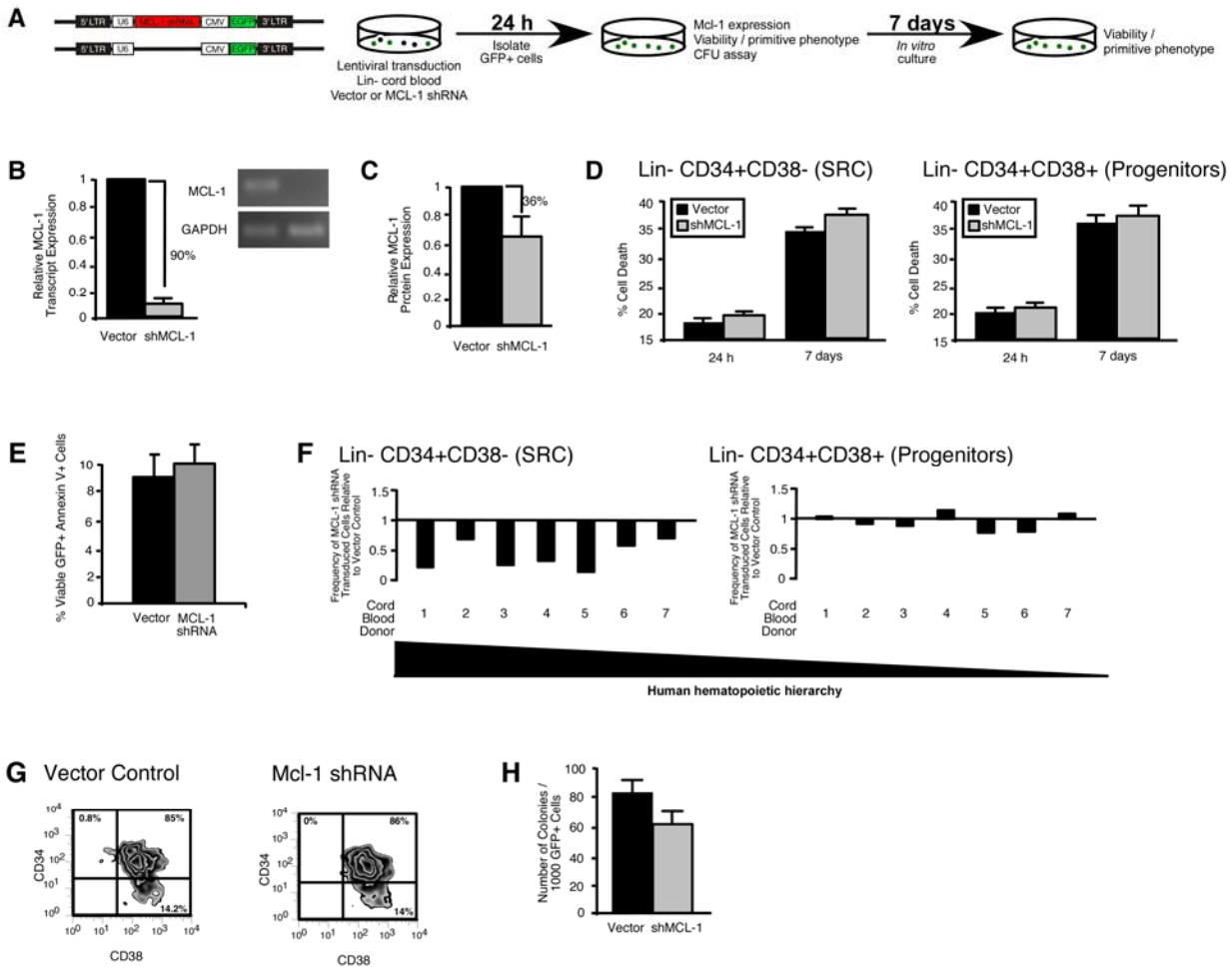

A
5'LTR
U6
MCL-1 shRNA
CMV
GFP
3'LTR
Lentiviral transduction
Lin- cord blood
Vector or MCL-1 shRNA
24 h
Isolate
GFP+ cells
Mcl-1 expression
Viability / primitive phenotype
CFU assay
7 days
In vitro
culture
Viability /
primitive phenotype
B
Relative MCL-1
Transcript Expression
MCL-1
GAPDH
90%
Vector shMCL-1
C
Relative MCL-1
Protein Expression
36%
Vector shMCL-1
D
Lin- CD34+CD38- (SRC)
Lin- CD34+CD38+ (Progenitors)
% Cell Death
Vector
shMCL-1
24 h
7 days
E
% Viable GFP+ Annexin V+ Cells
Vector MCL-1
shRNA
F
Lin- CD34+CD38- (SRC)
Lin- CD34+CD38+ (Progenitors)
Frequency of MCL-1 shRNA
Transduced Cells Relative
to Vector Control
Cord
Blood
Donor
Human hematopoietic hierarchy
G
Vector Control
Mcl-1 shRNA
CD34
CD38
0.8%
85%
14.2%
0%
86%
14%
H
Number of Colonies /
1000 GFP+ Cells
Vector shMCL-1

Figure 5. A functional dependence on Mcl-1 for self-renewal capacity hierarchically distinguishes human hematopoietic stem cells from hematopoietic progenitor cells

(A) Experimental strategy used to examine the role of Mcl-1 in human SRC regenerative function and self-renewal *in vivo*.

(B) Representative examples of flow cytometry analyses used to examine the effect of Mcl-1 knockdown on human SRC regenerative capacity *in vivo*.

(C) Effect of Mcl-1 knockdown on human SRC regenerative capacity *in vivo*. Each dot represents the frequency of CD45+GFP+ human hematopoietic cells in the bone marrow of one primary recipient mouse transplanted with Mcl-1 shRNA or vector control transduced SRCs. The frequencies represent the average frequency of GFP+ human hematopoietic cells in the bone marrow of recipient mice. Bars represent the mean of seven independent experiments, each with 3 mice per group. $^{**}P < 0.01$.

(D) Analysis of the effect of Mcl-1 knockdown on the viability of engrafted human hematopoietic cells. The average frequency of dead cells in the CD34+CD38- or CD34+CD38+ fraction of the transduced human hematopoietic graft (GFP+7AAD+) in the bone marrow of primary recipient mice transplanted with Mcl-1 shRNA or vector

control transduced SRCs. Error bars represent the mean +/- SEM of three independent experiments, each with 3 mice per group.

(E) Analysis of the effect of Mcl-1 knockdown on the CD34+CD38- stem cell and CD34+CD38+ progenitor compartments *in vivo*. The average frequency of CD34+CD38- and CD34+CD38+ cells within the GFP+ human hematopoietic graft in the bone marrow of primary recipient mice reconstituted with Mcl-1 shRNA transduced SRCs is expressed relative to vector control. Error bars represent the mean +/- SEM. $*P < 0.05$.

(F) Representative examples of flow cytometry analyses used to examine the effect of Mcl-1 knockdown on the human CD34+CD38- SRC – enriched fraction of the human hematopoietic graft *in vivo*.

(G) Analysis of the effect of Mcl-1 knockdown on multilineage and primitive human hematopoietic engraftment. The average frequency of lymphoid (CD19+), myeloid (CD33+) and primitive (CD34+) cells within the GFP+ human hematopoietic graft (gated CD45+GFP+ cells) in the bone marrow of primary recipient mice reconstituted with vector or Mcl-1 shRNA transduced SRCs. Error bars represent the mean +/- SEM of at least three independent experiments.

(H) Effect of Mcl-1 knockdown on human SRC self-renewal capacity *in vivo*. Each dot represents the frequency of CD45+GFP+ human hematopoietic cells in the bone

marrow of one secondary recipient mouse transplanted with human-engrafted bone marrow isolated from a primary recipient mouse that was transplanted with Mcl-1 shRNA or vector control transduced SRCs. The frequencies represent the average frequency of GFP+ human hematopoietic cells in the bone marrow of secondary recipient mice. Equal numbers of GFP+CD45+CD34+ cells isolated from primary grafts representing 3 independent cord blood donors were used.

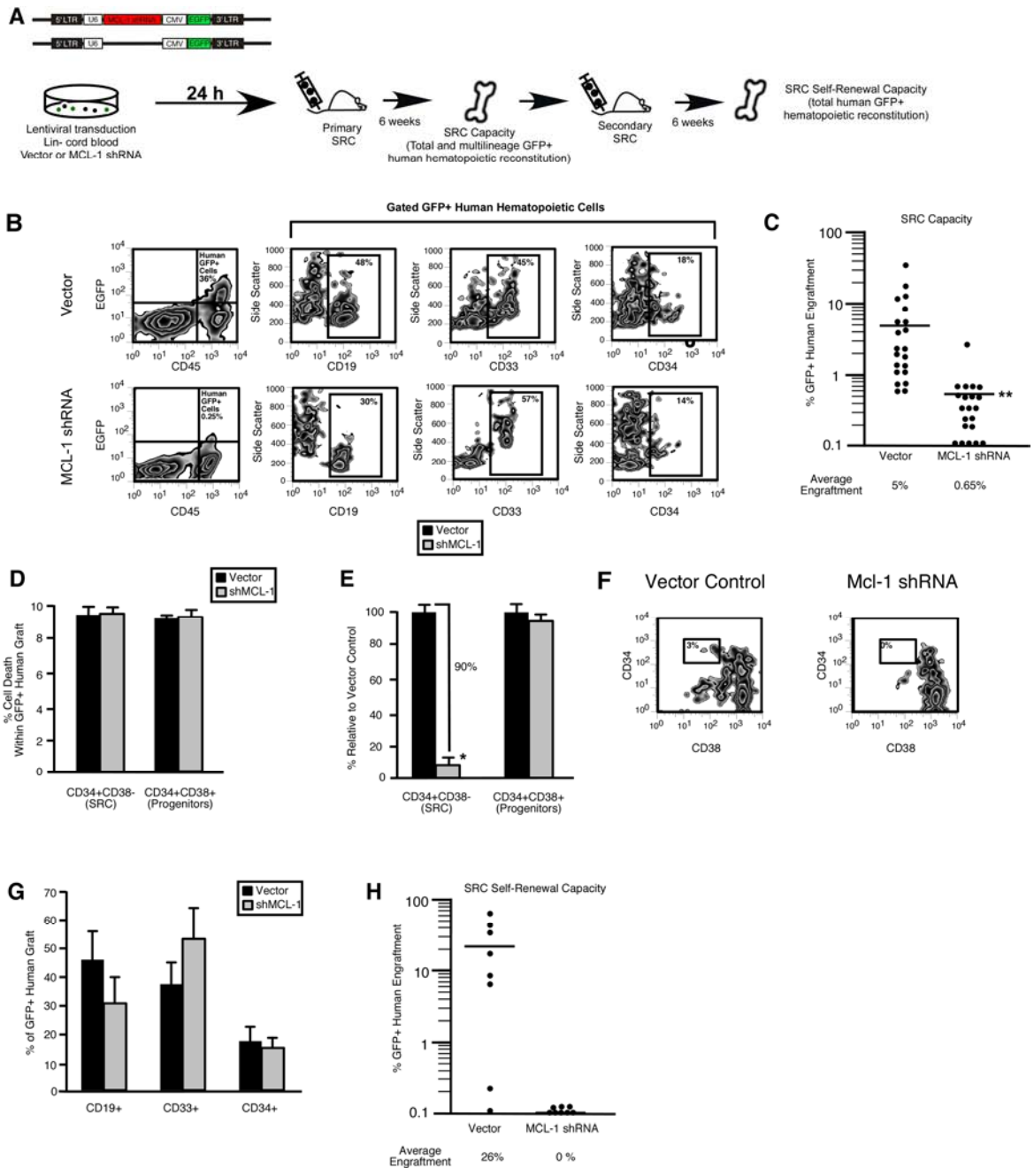

A
5'LTR
U6
MCL-1 shRNA
CMV
EGFP
3'LTR
Lentiviral transduction
Lin- cord blood
Vector or MCL-1 shRNA
24 h
Primary SRC
6 weeks
SRC Capacity
(Total and multilineage GFP+ human hematopoietic reconstitution)
Secondary SRC
6 weeks
SRC Self-Renewal Capacity
(total human GFP+ hematopoietic reconstitution)
B
Gated GFP+ Human Hematopoietic Cells
Vector
MCL-1 shRNA
EGFP
Side Scatter
CD45
CD19
CD33
CD34
Human GFP+ Cells 36%
48%
45%
18%
Human GFP+ Cells 0.25%
30%
57%
14%
Vector
shMCL-1
C
SRC Capacity
% GFP+ Human Engraftment
Vector
MCL-1 shRNA
**
Average Engraftment
5%
0.65%
D
Vector
shMCL-1
% Cell Death Within GFP+ Human Graft
CD34+CD38- (SRC)
CD34+CD38+ (Progenitors)
E
% Relative to Vector Control
90%
*
CD34+CD38- (SRC)
CD34+CD38+ (Progenitors)
F
Vector Control
Mcl-1 shRNA
CD34
CD38
3%
3%
G
Vector
shMCL-1
% of GFP+ Human Graft
CD19+
CD33+
CD34+
H
SRC Self-Renewal Capacity
% GFP+ Human Engraftment
Vector
MCL-1 shRNA
Average Engraftment
26%
0 %

Figure 6. Knockdown of Mcl-1 in human pluripotent stem cells reveals a mechanistic conservation of Mcl-1 function as a regulator of self-renewal in human stem cells

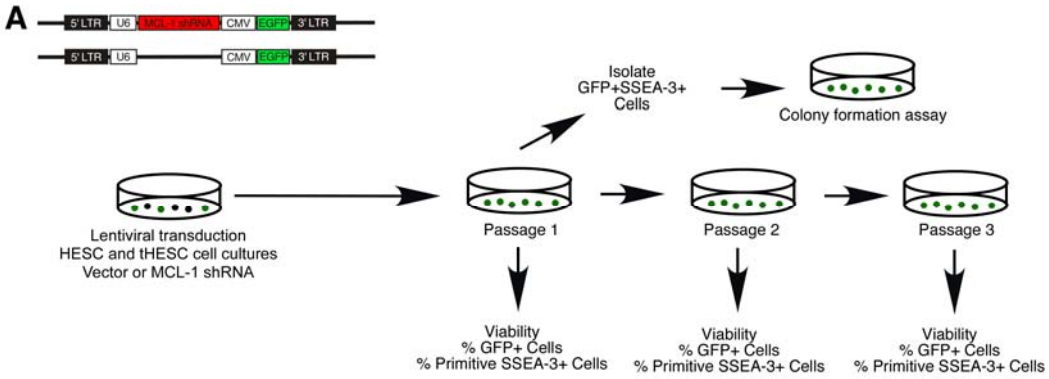
(A) Experimental strategy used to examine whether Mcl-1 is required for survival and self-renewal in hPSC cultures.

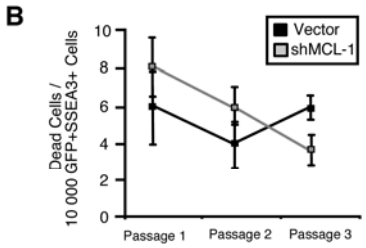
(B) Analysis of the effect of Mcl-1 knockdown on cell viability in the primitive self-renewing SSEA-3+ population of hPSC cultures. hPSC cultures were transduced with the empty vector or Mcl-1 shRNA-expressing vector, and analyzed for the frequency of dead cells in the GFP+SSEA-3+ population at each passage. Error bars represent the mean +/- SEM of three independent experiments.

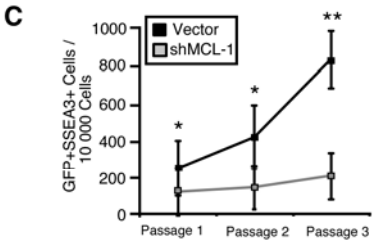
(C) Analysis of the effect of Mcl-1 knockdown on clonogenic self-renewal capacity in the primitive fraction of hPSC cultures. hPSC cultures were transduced with the empty vector or Mcl-1 shRNA-expressing vector, and analyzed for the GFP expression in the viable primitive (SSEA-3+) fraction at each passage. The primitive clonogenic fraction is expressed as the frequency of GFP+SSEA-3+ cells per 10 000 cells. Error bars represent the mean +/- SEM of three independent experiments. $*P < 0.05$, $**P < 0.01$.

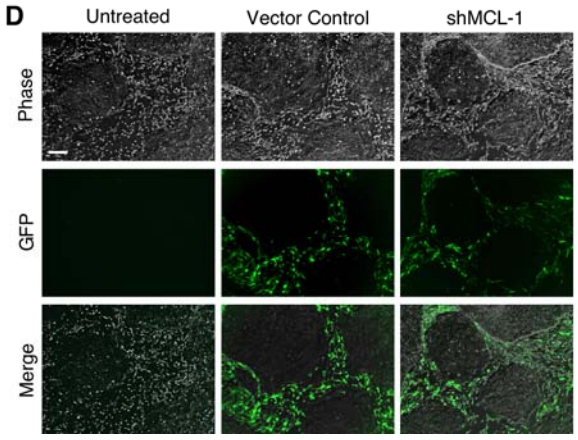
(D) Representative examples of hPSC cultures transduced with the empty vector, Mcl-1 shRNA expressing vector or untreated. Scale bar represents 50 μM.

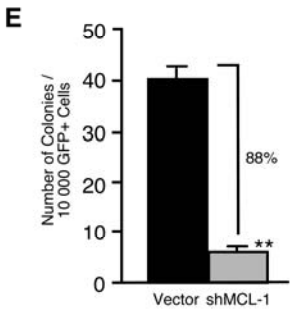
(E) Effect of Mcl-1 knockdown on the frequency of hPSC colony initiating cells. hPSC cultures were transduced with the empty vector or Mcl-1 shRNA expressing vector, and the GFP+ SSEA-3+ fraction was analyzed for the frequency of hPSC colony initiating cells after the first passage. Error bars represent the mean +/- SEM of three independent experiments. $^{**}P < 0.01$.


A
5' LTR
U6
MCL-1 shRNA
CMV
EGFP
3' LTR
5' LTR
U6
CMV
EGFP
3' LTR
Isolate
GFP+SSEA-3+
Cells
Colony formation assay
Lentiviral transduction
HESC and tHESC cell cultures
Vector or MCL-1 shRNA
Passage 1
Passage 2
Passage 3
Viability
% GFP+ Cells
% Primitive SSEA-3+ Cells
Viability
% GFP+ Cells
% Primitive SSEA-3+ Cells
Viability
% GFP+ Cells
% Primitive SSEA-3+ Cells


B
Vector
shMCL-1
Dead Cells /
10 000 GFP+SSEA3+ Cells
10
8
6
4
2
0
Passage 1
Passage 2
Passage 3


C
Vector
shMCL-1
GFP+SSEA3+ Cells /
10 000 Cells
1000
800
600
400
200
0
*
*
**
Passage 1
Passage 2
Passage 3


D
Untreated
Vector Control
shMCL-1
Phase
GFP
Merge

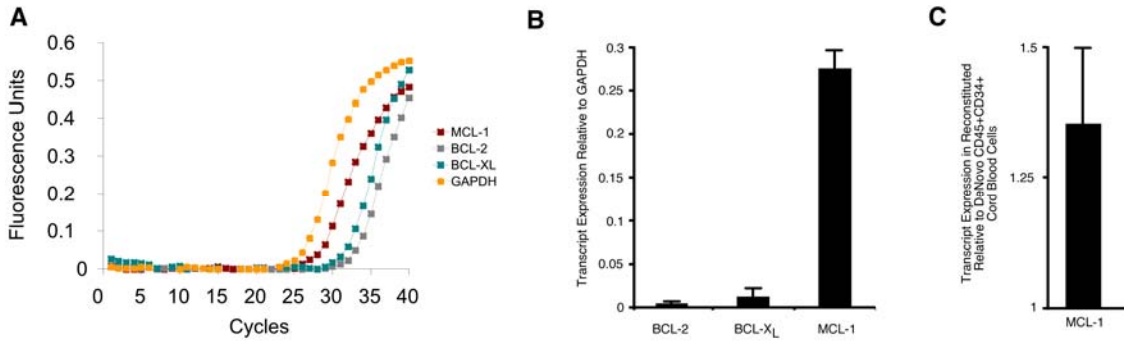

E
Number of Colonies /
10 000 GFP+ Cells
50
40
30
20
10
0
88%
**
Vector
shMCL-1

Supplementary figure 1. Representative qRT-PCR plots

(A) Representative amplification plots for qRT-PCR analysis of Mcl-1, Bcl-2, Bcl-x_L and Gapdh expression in human cord blood MNC.

(B) qRT-PCR analysis of Mcl-1, Bcl-2 and Bcl-x_L expression in human cord blood hematopoietic cells. Expression was analyzed relative to the housekeeping gene Gapdh. Error bars represent the mean +/-SEM of two independent experiments.

(C) qRT-PCR analysis of Mcl-1 expression in reconstituted relative to *de novo* purified CD45+CD34+ human cord blood hematopoietic cells. Error bars represent the mean +/-SEM of three independent cord blood samples analyzed both pre and post transplantation.


A
Fluorescence Units
0.6
0.5
0.4
0.3
0.2
0.1
0
0 5 10 15 20 25 30 35 40
Cycles
MCL-1
BCL-2
BCL-XL
GAPDH
B
Transcript Expression Relative to GAPDH
0.3
0.25
0.2
0.15
0.1
0.05
0
BCL-2
BCL-XL
MCL-1
C
Transcript Expression in Reconstituted Relative to DsNovo CD45+CD34+ Cord Blood Cells
1.5
1.25
1
MCL-1

Supplementary figure 2. Knockout of *Mcl1* in mouse bone marrow cells

(A) Experimental strategy use to examine the whether Mcl-1 is required for mouse HSC regenerative capacity *in vivo*.

(B) Analysis of the efficiency of cre-mediated deletion of the *Mcl-1*$^{f/f}$ allele in primitive mouse bone marrow cells. Genomic DNA from transduced (lentiviral transduction +) or control non-transduced (lentiviral transduction -) lin- wild type or *Mcl-1*$^{f/f}$ bone marrow cells was analyzed by PCR for the presence of the *Mcl-1* floxed allele, *Mcl-1* wild type allele, and *Mcl-1* deleted allele as well as the CMV promoter present on the lentiviral vector.

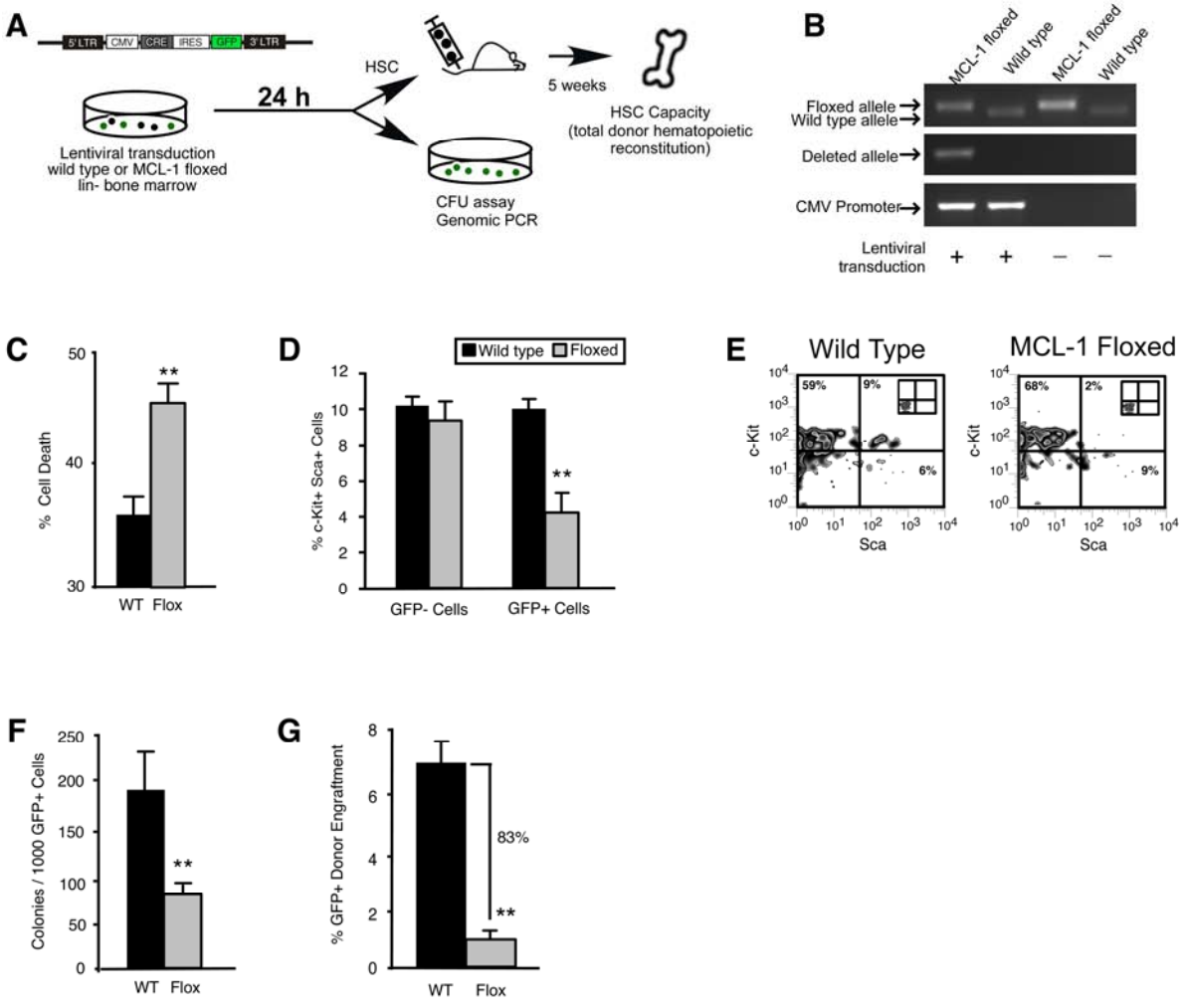
(C) Loss of *Mcl-1* in primitive mouse bone marrow cells leads to an increase in cell death. Average frequency of dead cells in the cre-transduced population (GFP+7AAD+) of control wild type or *Mcl-1*$^{f/f}$ lin- bone marrow cells. Error bars represent the mean +/- SEM. ***P < 0.01*.

(D) *Mcl-1* knockout in primitive mouse bone marrow cells leads to loss of cells with the lin- Sca-1+ c-Kit+ (LSK) phenotype. Average frequency of Sca-1+ c-Kit+ cells in the cre-transduced population (GFP+) and non-transduced population (GFP-) of control wild type or *Mcl-1*$^{f/f}$ lin- bone marrow cells. Error bars represent the mean +/- SEM ***P < 0.01*.

(E) Representative examples of flow cytometry analyses used to assess the effects of *Mcl-1* knockout on the primitive mouse LSK phenotype. The inlay plots represent the isotype staining control for each antibody.

(F) Analysis of the effect of *Mcl-1* knockout on the frequency of mouse hematopoietic progenitors. Average frequency of wild type or *Mcl-1*$^{f/f}$ bone marrow hematopoietic progenitors following transduction with a lentiviral vector expressing cre-recombinase. Progenitor frequency is expressed as the number of hematopoietic colonies scored after 10-12 days per 1000 cells plated. Error bars represent the mean +/- SEM of three independent experiments. $**P < 0.01$.

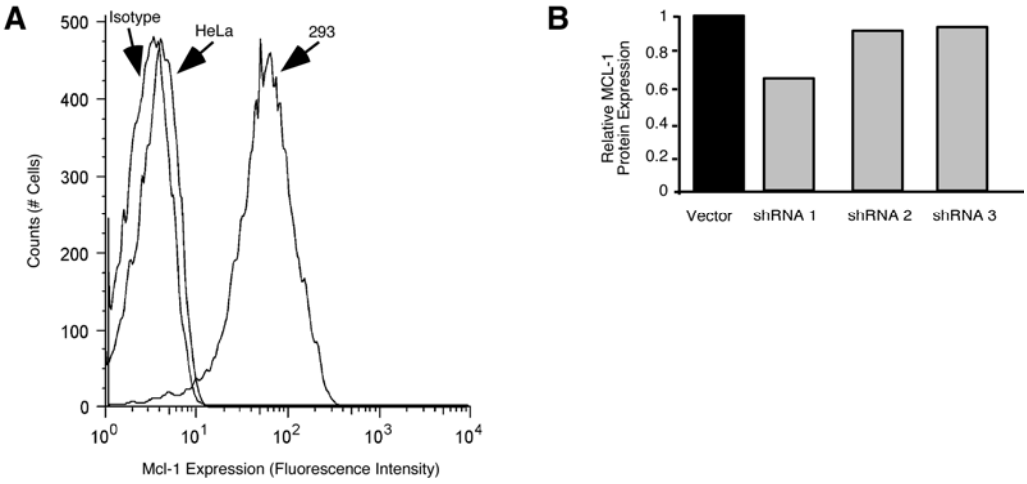
(G) Analysis of the effect of *Mcl-1* knockout on mouse HSC regenerative capacity *in vivo*. Average frequency of GFP+ donor hematopoietic engraftment in recipient mice reconstituted with cre-transduced control wild type or *Mcl-1*$^{f/f}$ HSC. Error bars represent the mean +/- SEM. $**P < 0.01$.


A
5' LTR
CMV
CRE
IRES
GFP
3' LTR
24 h
HSC
5 weeks
HSC Capacity
(total donor hematopoietic
reconstitution)
Lentiviral transduction
wild type or MCL-1 floxed
lin- bone marrow
CFU assay
Genomic PCR
B
MCL-1 floxed
Wild type
MCL-1 floxed
Wild type
Floxed allele
Wild type allele
Deleted allele
CMV Promoter
Lentiviral
transduction
+ + – –
C
% Cell Death
WT Flox
D
Wild type
Floxed
% c-Kit+ Sca+ Cells
GFP- Cells
GFP+ Cells
E
Wild Type
MCL-1 Floxed
c-Kit
Sca
59%
9%
6%
68%
2%
9%
F
Colonies / 1000 GFP+ Cells
WT Flox
G
% GFP+ Donor Engraftment
83%
WT Flox

Supplementary figure 3. Flow cytometry analysis of MCL-1 protein expression

(A) Flow cytometry analysis of Mcl-1 protein expression in the human HeLa and 293FT cell lines. Mcl-1 expression is measured as the mean fluorescence intensity of staining compared to the isotype control.

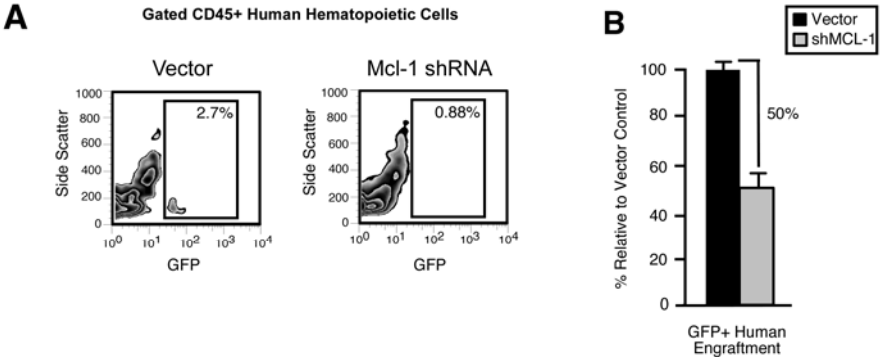
(B) Knockdown of human Mcl-1 protein expression in human lin-CD34+CD38- cord blood hematopoietic cells using three independent shRNAs targeting the Mcl-1 transcript. Protein expression is the mean fluorescence intensity relative to the isotype control.


A
Isotype
HeLa
293
Counts (# Cells)
Mcl-1 Expression (Fluorescence Intensity)
B
Relative MCL-1 Protein Expression
Vector
shRNA 1
shRNA 2
shRNA 3

Supplementary figure 4. MCL-1 knockdown using a second shRNA sequence

(A) Representative examples of flow cytometry analyses used to examine the effect of Mcl-1 knockdown on human SRC regenerative capacity *in vivo* using a second shRNA sequence.

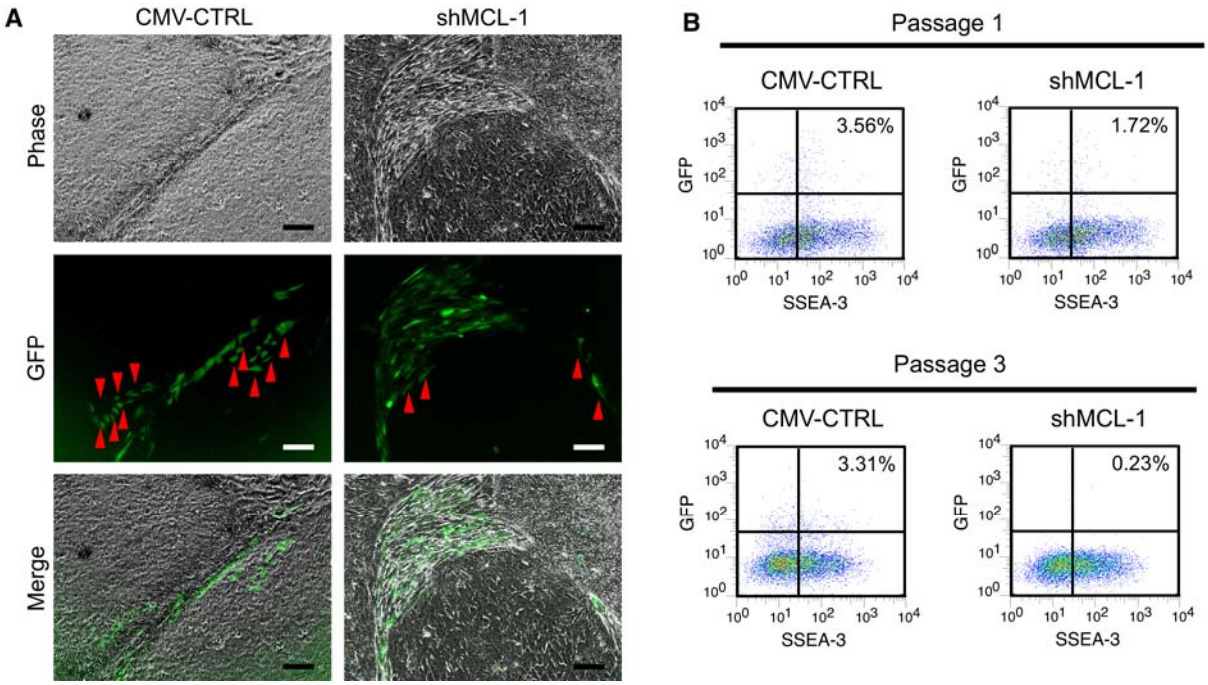
(B) Effect of Mcl-1 knockdown on human SRC regenerative capacity *in vivo*. The average frequencies of GFP+ human hematopoietic cells in the bone marrow of recipient mice. Error bars represent the mean +/- SEM (n = 4 mice per group).


A
Gated CD45+ Human Hematopoietic Cells
Vector
Mcl-1 shRNA
Side Scatter
GFP
2.7%
0.88%
B
Vector
shMCL-1
% Relative to Vector Control
50%
GFP+ Human Engraftment

Supplementary figure 5. MCL-1 knockdown in hPSC cultures

(A) Representative examples of hPSC cultures transduced with the empty vector or Mcl-1 shRNA-expressing vector. Red arrows (GFP, middle panel) indicate GFP+ cells inside of undifferentiated hPSC colonies. Scale bars 50 μM.

(B) Representative flow cytometric analysis at passage 1 and passage 3 of the GFP+ fraction in the primitive self-renewing SSEA-3+ population of hPSC cultures transduced with the empty vector or Mcl-1 shRNA-expressing vector.


A
CMV-CTRL
shMCL-1
Phase
GFP
Merge
B
Passage 1
CMV-CTRL
shMCL-1
3.56%
1.72%
GFP
SSEA-3
Passage 3
CMV-CTRL
shMCL-1
3.31%
0.23%
GFP
SSEA-3

Chapter 3

In vivo bone marrow niche propensity reveals functional heterogeneity of the human hematopoietic stem cell compartment

Preamble

A version of this chapter is currently under review at *Nature Immunology*. The authorship is as follows: Guezguez B*, **Campbell CJ***, Karanu F, Schnerch A, Smith MJ, Levadoux-Martin M and Bhatia M.

*B Guezguez and CJ Campbell contributed equally to this work.

Dr. Borhane Guezguez and myself conceived and co-designed all experiments presented figures 1-4, S1 and S2, and both contributed significant intellectual input to this work. Dr. Francis Karanu and Dr. Mickie Bhatia designed experiments presented in figure 6. Dr. Borhane Guezguez and Dr. Mickie Bhatia designed affymetrix experiments presented in figure 5. Angelique Schnerch analyzed affymetrix data presented in figures 5 and S2. Dr. Borhane Guezguez and myself co-performed all *in vivo* experiments and co-analyzed data presented in figures 1-3, 4b and figure S1. Dr. Borhane Guezguez analysed IHC experiments, performed gene expression PCR studies, performed and analyzed *in vitro* CFU experiments presented in figure 4e and 4g, assembled LDA data in figure 4j, assembled the figures, and wrote the initial draft of the manuscript. I co-wrote subsequent drafts of the manuscript, assisted with figure design, performed irradiated versus non-irradiated host experiments in supplemental figure 1, provided CFU pictures in figure 4f,

and contributed to revisions with Dr. Borhane Guezguez and Dr. Mickie Bhatia. Mary-Jo Smith performed IHC staining. Dr. Marilyne Levadoux-Martin provided technical assistance with flow cytometry and cell sorting. Dr. Mickie Bhatia contributed significant intellectual input and support.

Although multiple model systems have characterized the *in vivo* stem cell niche (Bhattacharya et al., 2006; Calvi et al., 2003; Kiel et al., 2005; Lo Celso et al., 2009; Xie et al., 2009; Zhang et al., 2003a), prior to this work, the human stem cell niche was a hypothesis. Drawing from studies in the mouse hematopoietic system, we sought to characterize anatomical and molecular parameters that define a human stem cell niche *in vivo*. Our initial strategy was to acquire functional evidence of specific non-cell-autonomous parameters that influence stem cell fate decisions, thereby demonstrating the existence of a true hHSC niche by definition. Using a basic anatomical approach as a starting point, we identified SRCs with enhanced *in vivo* regenerative capacity in the trabecular bone area (TBA) versus the long bone area (LBA) of the BM. Imaging studies then revealed that primitive hHPCs resided in close approximation to osteoblasts on the endosteal surface of the bone, as well as to endothelial cells on the vascular endothelium. We also found an increased frequency of hHPCs interacting with Jagged-1 positive osteoblasts in the TBA. This led us to investigate whether there was a unique gene expression signature that defined SRCs in the TBA. We identified a number of differentially expressed genes in the TBA versus the LBA, including Notch receptors and Notch target genes such as Hes-1, which have been previously implicated in hHSC self-renewal. Using this unique molecular signature, SRCs with enhanced *in vivo* regenerative

capacity were prospectively isolated based upon Notch-ligand binding affinity. Our work for the first time revealed the structure of a human stem cell niche *in vivo,* a structure that showed remarkable similarity to previous findings in the mouse system, being defined by anatomical, cellular and molecular parameters. Our findings further revealed that both *in vivo* anatomical propensity and specific molecular signature based upon niche localization could be used to prospectively isolate stem cells enhanced for regenerative function. Collectively our study has for the first time characterized a hHSC niche *in vivo*, and demonstrated that niche localization is a key non-cell-autonomous determinant of functional heterogeneity in the hHSC compartment.

In vivo bone marrow niche propensity reveals functional heterogeneity of the human hematopoietic stem cell compartment

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Abstract

In vivo transplantation studies of prospectively isolated cells have uncovered significant functional heterogeneity within the compartment of reconstituting human hematopoietic stem cells (HSCs). Despite the dependence of the recipient bone marrow microenvironment on *in vivo* reconstitution, HSC heterogeneity has yet to be examined *in situ*. Here using an *in vivo* approach, we reveal that human NOD/SCID Repopulating Cells (SRCs) are heterogeneous for functional reconstituting ability. SRC in the trabecular bone area (TBA) of recipient immune deficient mice possessed superior self-renewal and regenerative capacity to those in long bone area (LBA). Molecular signatures of purified TBA-SRC uniquely indicated distinct Notch pathway regulation in contrast to SRC found in the LBA, and were physically identified to be exclusively associated with TBA bone endosteum expressing the Notch ligand Jagged-1. This *in vivo* niche affinity allowed independent *de novo* enrichment of human SRC based on direct Notch-ligand binding. Our study demonstrates that heterogeneity of human HSCs can be functionally defined by the combination of specific BM niche occupancy and active *in situ* molecular interactions that define *in vivo* HSC capacity.

Introduction

A fundamental understanding of HSC biology was initiated more than 40 years ago from murine studies that allowed reconstitution of the entire hematopoietic system using *in vivo* transplantation [1-4]. Further refinement of transplantation studies led to the development of limiting dilution analysis, as well as serial transplantation assays to achieve quantitative functional characterization of mouse HSCs [5,6] that eventually led to evaluation and purification of mouse HSCs at nearly single cell resolution [5,7,8]. In the human system, xenograft assays using immune deficient recipient mice are considered the "gold standard" for the measurement of human HSC regenerative and self-renewal function *in vivo* [9-11]. Putative human HSCs have been identified and enriched within the lineage depleted (lin^-) $CD34^+CD38^-$ hematopoietic fraction and are termed SCID repopulating cells (SRCs) based upon their ability to reconstitute long-term multilineage hematopoiesis in NOD/SCID mice [12,13]. However, unlike the mouse system, studies using the human SRC assay have revealed significant functional heterogeneity in purified fractions enriched for reconstituting function and self-renewal capacity ($lin^-CD34^+CD38^-$) [14-16] or at the single cell level [17-20]. This has limited the ability to prospectively isolate and predict HSC capacity for experimental and clinical applications [21,22]. Therefore, an increased understanding and dissection of the functional heterogeneity in the human HSC compartment will be central for developing novel methods to increase donor HSC yields through prospective isolation techniques and optimized *ex vivo* expansion methods [23].

The concept of the HSC niche was proposed to encompass the molecular pathways that regulate the interactions between individual HSCs and their bone marrow

(BM) microenvironment to maintain steady-state hematopoiesis [24,25]. Conceptually, this unique microenvironment functions to support steady-state hematopoiesis by regulating HSC fate decisions such as quiescence, apoptosis and self-renewal [26,27]. These studies showed that mouse HSCs within the heterogeneous lin$^-$ Sca-1$^+$c-Kit$^+$ (LSK) fraction reside in specific *in vivo* niches that are at least partially composed of osteoblasts at the endosteal surface of trabecular bone [24,25]. These niches regulate HSC function through well-established signaling pathways that affect HSC fate decisions in both mice and humans, such as Wnt [28,29], BMP [25,30] and the Notch pathways [24,31]. Additionally, subsequent studies showed that HSCs within the significantly more homogenous lin-CD150$^+$CD244$^-$CD48$^-$ (lin-SLAM+) fraction were associated with the sinusoidal vascular endothelium in the BM [32-34]. These seminal observations provide evidence that understanding functional heterogeneity within the HSC fraction goes beyond the intrinsic phenotypic characteristics of these cells, and is indeed also a function of unique molecular interactions that occur within the context of a defined microenvironment. Using the human SRC assay as a surrogate human reconstitution model that recapitulates interactions between human HSCs and their BM microenvironment *in vivo*, we reveal a distinct SRC subset localized in the trabecular bone area (TBA) of the epiphysial region that possessed enhanced self-renewal potential and regenerative capacity. Global gene expression and immunohistological analysis revealed that TBA-derived SRCs are regulated by a Notch-Notch ligand axis that enabled *de novo* enrichment of human SRCs. Our study demonstrates that heterogeneity of human HSCs can be functionally defined by specific *in vivo* BM anatomical location that uniquely depends on endosteal niche

propensity and Notch-Notch ligand interactions.

Results

Primitive human hematopoietic cells distribute into spatially distinct bone marrow region in xenografted recipients

Analysis of human SRC function [9,35,36] has yet to be examined in relation to the host xenograft microenvironment *in vivo*. Consequently, the potential role of the *in vivo* niche in human SRC function is unknown. Using recipient immune deficient mice transplanted with purified SRC to reconstitute human multilineage hematopoiesis (Fig.1a-b), we investigated the spatial organization of individual human BM engrafting cells *in situ*. Examination of cryosectioned engrafted femur specimens using immunohistochemistry (IHC) (Nilsson et al., 2001) to identify cells (DAPI+) that were either endothelial (PECAM1/CD31+) representing vascular niche, endosteal bone (Osteopontin-OPN+) representing endosteal niche, or equidistant from these niches, defined as inter-niche. These experiments revealed that cells which stained positive for human CD34, a common marker for human HSCs and progenitors, was localized to the three anatomical areas in transplanted recipient BM; near endosteal niche (Fig.1c), close to blood vessels of the vascular niche (Fig.1d) and inter-niche (Fig.1e). These observations were rigorously analyzed quantitatively, including 15 sections for 10 recipients of human SRC. These data revealed that primitive human $CD34^+$ cells were distributed at different frequencies among the three recipient BM niche microenvironments (Fig.1f and Table 1) and were primarily located next to the endosteal niche (46%) rather than to the vascular niche

(15%) but were found at similar levels alongside the inter-niche (33%). These results indicate that the microcellular features harboring engraftment of primitive human HSCs *in vivo* are predominately osteoblasts (endosteal niche) within the BM of xenograft recipients.

Previous anatomical studies in the mouse system have show that the BM cavity is subdivided into middle long bone area (LBA) terminated in both ends by the cancellous/trabecular bone area (TBA) [24,25,32,33]. The TBA of the femoral bone is defined as both epiphysis and metaphysis, and the LBA as the diaphysis for subdivision as shown in Fig.2a. To investigate whether these anatomical regions support human HSCs and provide a better indicator of human SRC niche than micro-domains of endosteal and vascular cells residing within the BM, we quantitatively analyzed the presence of engrafted human cells among the three types of BM niche further subdivided between TBA and LBA of recipient mice (Fig.2b and Table 1). Remarkably, 36.4% within the 46% of osteoblast-associated (endosteal niche) primitive human HSCs were found in the TBA, in contrast to 9.6% in the LBA (Fig.2c). In contrast, all sinusoidal vessels (vascular niche) in close contact with human HSCs were detected at similar frequencies between TBA vs. LBA (Fig.2d). Interestingly, 33% of engrafting human HSCs adjacent to the inter-niche showed comparable distribution to the endosteal niche in TBA (28%) versus LBA (5%) (Fig.2e). Collectively, these observations provide the first evidence that microenvironmental structural components, combined with their anatomical BM location, outlines the existence of variable niche frameworks (macro-niche based on anatomical location versus micro-niche based on cellular localization) associated with human HSCs.

To explore the influence of these observations of macro-niche location of HSCs *in vivo*, we quantitatively analyzed the frequency of primitive engrafted human cells in both the TBA and LBA of recipient mice (Fig.3a). Total human hematopoietic engraftment, as assessed by expression of the human-specific hematopoietic marker CD45, was 3.5-fold greater in the TBA versus the LBA (Fig.3b,c). This difference was further enhanced for the engrafted human $CD34^+CD38^-$ SRC-enriched fraction, which was 4-fold more prevalent in the TBA versus LBA (Fig.3e,f). In contrast, $CD34^+CD38^+$ subsets enriched for hematopoietic progenitors [9-11] were similar between the TBA and LBA of recipients (Fig.3d), indicating that $CD34^+CD38^-$ cells enriched for SRC preferentially localized to the TBA. These observations were consistent among 40 individually transplanted mice (Fig.3c,d,f), even under varying experimental conditions including the number of injected cells (Fig.3g), bone type (tibia or humerus), NOD/SCID sub-strain, or irradiated versus non-irradiated recipients (Supplemental Fig.1). Collectively, these results indicate that the specific to anatomical locations of TBA vs. LBA may represent an overlooked critical determinant of the heterogeneity of functioning human HSCs *in vivo*.

Human SRC located in trabecular region possess superior self-renewal and hematopoietic regenerative capacity

To determine if *in situ* location of human engrafting cells was related to functional differences between primitive human hematopoietic cells in the TBA versus LBA regions, we examined *in vitro* progenitor capacity of primitive human hematopoietic cells residing in these sites by direct explant from primary recipients (illustrated in Fig.4a). The

ratio of the myeloid and lymphoid lineages of the human graft was distinct between these bone regions, where the frequency of myeloid cells was higher in TBA, and this ratio was reversed in the LBA (Fig.4b-d). Total myeloerythroid progenitor output as assessed by the hematopoietic colony-forming unit (CFU) assay, was significantly higher from TBA-derived cells (2.2-3 fold) than cells isolated from the LBA (Fig.3e). Although both primary engrafting TBA and LBA-SRC could give rise to all myeloerythroid colony subtypes (Fig.4f), consistent with enhanced myeloid phenotype in the TBA (Fig.3b), TBA-SRC exhibited higher CFU frequencies (CFU-GM, -G, -M) in comparison to LBA-SRC (Fig.4g). These observations suggest that observed niche composition differences in TBA versus LBA regions govern unique differentiation programs of HSCs regulated by anatomical location in the recipient BM.

Human SRCs are defined functionally by their ability to regenerate hematopoiesis upon transplantation into recipient mice [9-11]. To investigate the *in vivo* regenerative and self-renewal capacity of SRCs engrafting TBA and LBA regions of recipients, human $CD34^{+}CD38^{-}$ subsets were purified from the TBA vs. the LBA of primary recipients, and were subsequently transplanted at equal doses in secondary mice (Fig.4h). Consistent with the observed human cell phenotype (Fig.3c,f), a 3-fold increase in the level of engraftment in mice receiving primary SRCs isolated from the TBA (SRC-TBA) versus the LBA (SRC-LBA) was detected, indicating that functionally distinct population of human SRCs with increased repopulating capacity resides in the TBA region of the recipients (Fig.4i). To further assess whether this increased repopulating function was due to enhanced SRC self-renewal versus increased SRC number, we performed limiting

dilution analysis[12] (LDA) of engrafted human SRCs isolated from the TBA and the LBA. Quantitatively, the SRC frequency was higher at intermediate and higher injected cell doses (10,000 – 50,000), where more than 90% of mice receiving SRC-TBA were engrafted with a higher level of human cells compared to those receiving SRC-LBA (Fig.4j). Importantly, our analysis revealed that human SRCs isolated from the TBA were enhanced for *in vivo* repopulating function compared to those isolated from the LBA (2.3 fold-change) (Fig.4k). Multilineage analysis revealed an unbalanced contribution of the myeloid and lymphoid lineages between secondary-engrafted TBA versus LBA (supplemental Fig.2a) indicating the TBA and LBA-SRC explanted from primary hosts retain the distinct myeloid-biased programming (Fig.4d and g). Collectively these data reveal that subset of human SRCs with enhanced self-renewal capacity reside in the trabecular BM region of recipients, suggesting that distinct *in vivo* niches may exist in these anatomical regions that attribute functional capacity and heterogeneity to human HSC compartment.

Molecular signature and binding pattern along the Notch-ligand axis defines with distinct anatomical locations of human HSC niche

To investigate the potential molecular mechanisms underlying the differential SRC capacity derived from TBA vs. LBA (Fig.5a), global gene expression analysis was conducted on purified $CD34^{+}CD38^{-}$ populations enriched for human TBA-SRC and LBA-SRC from recipient mice (Fig.5b). Strikingly, 598 and 593 genes were found exclusively expressed in SRC-LBA and SRC-TBA respectively, whereas 155 genes were

found differentially expressed with an absolute fold change ≥ 2 among 53,383 genes commonly expressed by the two subsets (Fig.5c and Table 2 and 3). Selected candidates were validated by real time PCR (Fig.5d). Overall, the majority of the genes showing exclusive or high differential expression between the TBA and LBA-SRC subsets were previously associated in the processes of HSC self-renewal, myeloid differentiation and BM niche retention (Fig.5e). Genes implicated in HSC mobilization and homing (N-cadherin, Casr, Gnas, Rac1, Rac2, Cxcr4, CD49D, Egr1) [38-41] did not show significant changes (supplemental 2b). However, genes associated with both HSC quiescence and survival (including N-Myc, Cdc42, PTEN, ALDH, Prostaglandin E2) [42-45] as well as genes involved in erythromyeloid lineage commitment (Meis-1, C/EBP-alpha) [46,47] were found to be increased or exclusively expressed in SRC-TBA (Fig.4e).

One of the most prevalent genes expressed TBA-SRC was Hes-1, a Notch pathway gene-target involved in the preservation of long-term human HSC function [48,49] (Fig.5e and Table 3). Consistent with Notch activity, activator components of the Notch pathway (Presenilin, Mastermind-like, and Nicastrin) were found exclusively in TBA-SRC as opposed to LBA-SRC (Fig.5f). Furthermore, as determined by quantitative-PCR, targets of Notch pathway activity, Notch-1, Notch-2, Presenilin, Mastermind-like, Nicastrin and Hes-1 were exclusively upregulated in highly purified SRC-TBA (Fig.5g). To biologically validate these gene signature findings, we enumerated the number of human $CD34^+$ cells associated with BM osteoblasts expressing Jagged-1 in the TBA and LBA regions *in vivo* (Fig.5h). In agreement with these findings, human primitive hematopoietic cells were associated with a subset of osteoblasts that specifically

expressed Jagged-1 in the TBA versus the LBA of recipient mice (Fig.5i). These observations were consistent with the spatial distribution analysis described for the osteoblastic niche within the trabecular region (Fig.2c and 5j). Our study illustrates that human TBA-SRC have a unique molecular profile that is in part affected by a Notch-receptor / Notch-ligand mediated axis, related to both BM niche type and anatomical location *in vivo*.

Human HSCs with enhanced regenerative capacity can be prospectively isolated using the Notch/Notch-ligand axis

The Notch pathway involves the interaction between Notch receptor genes (mainly Notch-1 and -2) and two Notch-ligand gene families (Delta and Jagged), as well as several effector and regulatory proteins (Gordon et al., 2008). While some progress has been made in characterizing of specific Notch-ligand/receptor pairing in human hematopoiesis (Yuan et al., 2010), it has been reported that Notch receptors are able to bind with equal affinity to all Delta and Jagged families (Radtke et al., 2010). Based on the functional and molecular evidence of human SRC-niche dependent capacity involving interactions with Notch cell surface components, we examined whether niche occupancy of trabecular osteoblasts expressing notch ligands could provide an independent approach to *de novo* enrichment of putative human HSCs. Capitalizing on Notch-ligand binding affinities (Fig.6a), in combination with CD34 expression, lineage depleted (lin^-) human UCB cells were found to be heterogeneous with respect to binding of the notch ligand Delta-4, thereby identifying two distinct cell populations: $CD34^+$ lin^- $Notch^{binding+}$ and

$CD34^+$ lin^- $Notch^{binding\text{-}lo}$, respectively, hereafter referred to as $Notch^+$ and $Notch^-$ cells (Fig.6b). The specificity of the Notch ligand interaction was verified by using a Delta-4 conjugated FLAG competitive binding assay based on previous studies [53,54]. We observed a dose dependent inhibition of Delta-4-FLAG when competed with Delta-4-IgG in lin^- $CD34^+$ cells peaking at 80% (Fig.6c,d). As binding of Notch ligands depends on the presence of Notch receptors, and can be modulated by expression of the Fringe family of glycosyltransferases [55,56], we analyzed the expression level of Notch receptors and fringe modifiers in both $Notch^+$ and $Notch^-$ subfractions of human $CD34^+Lin^-$ cells. $Notch^+$ cells showed 15-Fold increase in the expression of Notch-1 and a 3-fold increase in the expression of Notch-2 compared to $Notch^-$ cells (Fig.6e), whereas Fringe was expressed equally in both cell fractions (supplemental Fig.2b). These data demonstrated the previously unappreciated existence of a unique level of heterogeneity within the primitive human hematopoietic compartment, based on functional binding affinity to Notch pathway receptor-ligand binding.

Primitive human $Notch^+$ and $Notch^-$ hematopoietic cells were further examined for Notch activity responsiveness *in vitro*. Using direct treatment with Notch ligands, highly purified cells were treated with 10μg/ml human IgG Fc (control) or one of the chimeric ligand proteins: Delta-4 IgG, Delta-1 IgG or Jagged-1 IgG. In contrast to $Notch^+$ cells, the $Notch^-$ compartment showed no difference in CFU capacity and total progenitor expansion in all culture conditions, suggesting that this human primitive fraction was unresponsive or refractory to Notch-ligand treatment (Fig.6f). The plating efficiency (ratio of number of CFU: number of input cells plated) of $Notch^+$ cells remained higher

(range 1:56±11 to 1:126±69) compared to Notch$^-$ cells (range 1:144±108 to 1:740±444) (Table 3). However, for treated Notch$^+$ cells, total progenitor expansion was significantly increased (2.5 to 3 fold) in ligand-treated cells over control treatment, indicating that this subset was responsive to Notch-ligand, consistent with it basis of isolation (Fig.6f). Moreover, the plating efficiency (number CFU per number input cells) of Notch$^+$ cells remained higher (range 1:56±11 to 1:126±69) compared to similarly cultured Notch$^-$ cells (range 1:144±108 to 1:740±444) (Table 4). The response of Notch$^+$ cells to Delta-4, Delta-1, or Jagged-1 treatment was not ligand-specific, since all three ligands promoted similar cell proliferation and CFU expansion (Fig.6f). Importantly, both Notch$^+$ and Notch$^-$ fractions showed equivalent CFU capacity under control conditions, suggesting that Notch ligand-receptor engagement is required *in vitro* to induce a response, and showing that the selection method does not prevent cells from responding to Notch ligand. Qualitatively, CFU composition between ligand and control treated cells for either subset was similar, and both erythrocyte and granulocyte/macrophage lineages were present at similar ratios, indicating that Notch ligand stimulation does not restrict developmental potential of generated progenitors. Taken together, these results validate the correlation between Notch ligand responsiveness and progenitor expansion among human primitive hematopoietic compartment.

To assess the *in vivo* hematopoietic regenerative potential of purified Notch$^+$ and Notch$^-$ fractions, recipient NOD/SCID mice were injected with different cell doses of each subset for limiting-dilution analysis. SRC regenerative capacity was significantly higher in mice receiving Notch$^+$ cells compared to Notch$^-$ cells (Fig.6g), and resulted in

both myeloid and lymphoid human cell engraftment, with an increase in myeloid reconstitution from of Notch$^+$ transplanted fraction, similar to TBA-SRC (supplemental Fig.2c). This difference was greater at intermediate cell doses (5,000-10,000 cells), where 75-80 % of mice receiving Notch$^+$ cells were engrafted, while no SRC were detected (0%) in mice transplanted with Notch$^-$ cells (Fig.6g), and required up to 30,000 injected cells to detect any repopulation capacity. Using Poisson statistics, the frequency of SRCs was 10-fold enriched in the Notch$^+$ fraction (1 in 5,253) compared to the Notch$^-$ fraction (1 in 44,667)(table 5). In the absence of pre-treatment, these data show that Notch$^+$ are highly enriched for SRC cells, suggesting the presence of different SRC classes based on their cell surface expression of the Notch receptor. Interestingly, the human SRC frequency of Notch$^+$ cells injected intravenously was similar to lin$^-$CD34$^+$CD38$^-$ injected by more permissive intrafemorally delivery [57,58] attesting to the quality of the SRCs isolated by the Notch ligand binding and the correlation to niche affinity of these SRC *in vivo*. Collectively, these data demonstrate that specific anatomical location and Notch-ligand axis for human HSC revealed *in vivo* provides unique insights into heterogeneity of the human HSC compartment, leading to the successful enrichment of human SRC subclasses.

Discussion

Our study demonstrates that human SRCs displaying enhanced hematopoietic regenerative capacity and self-renewal ability are exclusively located in the TBA of recipients. This functional propensity for anatomical niche occupancy in the TBA reflects

a previously unappreciated heterogeneity among purified human HSCs that surpasses cell surface phenotype. Furthermore, our data reveals that human SRCs are found in close interaction with osteoblasts presenting Jagged-1, and interaction that is selective to TBA osteoblasts vs LBA, indicating that specific organization and physical interaction based on anatomical location govern stem cell function in vivo. Accordingly, these results constitute the first evidence linking human SRC function and BM niche positioning, that allow for prospective characterization of human HSCs using *in vitro* or *in vivo* readout assays [59-61], and define the niche framework as a spatial bone area instead of micro-structure alone. Supporting this hypothesis, recent findings using transgenic mice showed significant increases in HSC numbers in conjunction with elevated numbers of trabecular osteoblasts [24,62-64], suggesting a functional conservation of the HSC interaction with the BM anatomy that regulates HSC properties among species.

Early studies have established the fundamental importance of Notch signaling in lymphoid development and embryonic hematopoiesis [65-67,68,69], but its direct implication in HSC maintenance in the BM niche is still debated [24,70,71]. Our findings provide further insight into the functional HSC heterogeneity related to BM niche location. TBA-SRC exhibited a singular gene expression profile with heightened Notch pathway activity *in vivo*. In addition to up-regulation of Notch pathway activators, we found that Hes-1, a Notch-signaling target gene, was highly expressed in the TBA-SRC, and correlates with enhanced functional repopulation activity and HSC self-renewal. This is in accordance with previous observations where overexpression of Hes-1 or the activated cytoplasmic domain of the NOTCH-1 receptor (NICD) in BM progenitors resulted in increased HSC

numbers and enhanced self-renewal *in vitro* [48,72]. Additionally, we found that PTEN and cdc42, two important cytoplasmic signaling coordinators of HSC quiescence and niche interaction in the BM, were also up-regulated in TBA-SRC, revealing an environment that preserves highly potent SRCs. Although most recent studies have produced results that may conflict with earlier interpretations into the actual role of the Notch pathway [74-78], a better understanding will be needed to identify the physiologically relevant combination of Notch receptors and ligands implicated in the regulation of HSC behavior *in vivo*. Collectively, these findings indicate the importance of maintaining tight control on both the level and duration of Notch activity in HSC fate control.

Isolation of high numbers of human HSCs is constrained by both the limited ability to expand and maintain these cells *ex vivo*, and by the difficulty in obtaining purified HSCs due to the significant phenotypic and functional heterogeneity in cell populations enriched for human adult stem cells. In this study, we have been able to prospectively isolate primary human hematopoietic cells with enhanced SRC repopulation activity based on their ability to engage Notch ligands. Functional analysis of these $Notch^+$ and $Notch^-$ subfractions revealed that intravenous injection of $Notch^+$ cells are enriched in SRC, and allowed purification to levels of SRC similar to direct intrafemoral injection of phenotypically purified subsets of human SRC. A better understanding of the cellular and molecular and micro-environmental mechanisms that influence HSC fate decisions to home and reconstitute the most favorable BM niche of recipient patients should yield enhanced engraftment capacity of transplanted cells, and thus may help to alleviate the need for high numbers of HSCs. The clinical value of these

approaches to purify or transplant HSC with niche modulation *in vivo* will need to be evaluated in human trials.

Materials and methods

Mice

We used NOD/Prkdc^{scid} (NOD/SCID)[10] and β2 microglobulin knockout (NOD/SCID/B2^{null}) (Christianson et al., 1997) mice. Mice were bred and maintained in the human Stem Cell and Cancer Research Institute (SCC-RI) animal barrier facility at McMaster University. All animal procedures received the approval of the animal ethics board at McMaster University.

Purification of primitive human hematopoietic cells

All patient samples were obtained with the approval of local human subject research ethics boards at McMaster University. Human umbilical cord blood (UCB) mononuclear cells (MNC) were isolated as described previously [12]. Briefly, mononuclear cells were collected by centrifugation on Ficoll-Hypaque (Pharmacia) and incubated with a cocktail of lineage specific antibodies (CD2, CD3, CD14, CD16, CD19, CD24, CD41, CD56, CD66b, and Glycophorin A) for negative selection of Lin- cells using an immunomagnetic column (StemSep; StemCell Technologies Inc, Vancouver, BC, Canada). For notch subfractions isolation, Lin- cells were preincubated with human IgG1 Fc fragment (100ug/ml) and donkey serum (20ug/ml) to block non-specific Fc binding. Cells were washed once and added to a trimeric ligand-antibody complex that was

prepared by incubating Delta-4-FLAG chimeric protein (10-15ug/ml) with rabbit anti-FLAG antibody (5ug/ml) (Sigma, St Louis, MO) and donkey anti-rabbit-PE (Phycoerythrin) conjugated antibody (1:50) (Jackson ImmunoResearch Laboratories, West Grove, PA) in FACS binding buffer (PBS containing 1% BSA and 25ug/ml $CaCl_2$). Cells were incubated in the dark for 30 min at 4^{o}C. When indicated, antibodies to other surface markers including CD34-APC and CD38-FITC (Becton Dickinson, San Jose, CA) were included in the final incubation step. Cells were washed once in binding buffer for analysis on a FACSCalibur or for sorting on a FACSAria SE (BD, Becton Dickinson, San diego, CA). Appropriate isotype matched control antibodies were used to define background staining and establish sorting gates. The purity of sorted populations was routinely >95 % (data not shown). Data acquisition and analysis was performed using Flowjo software (Treestar**).**

***In vivo* NOD/SCID transplantation assays**

Mice were sublethally irradiated at 350 cGy using a 137Cs γ -irradiator prior to intravenous injection (tail vein) of purified human cells. Injected mice were analyzed for human hematopoietic engraftment at 9-10 weeks post transplantation. Mouse bones (femurs, tibiae, humeri) were measured and cut with a razor to separate TBA and LBA regions. Bones were then crushed with a pestle and mortar, cells were isolated by filtration, RBC lysis was performed using ammonium chloride (StemCell Technologies Inc.), and cells were counted and immediately transplanted into secondary recipients or used for clonogenic progenitor assays and analyzed by flow cytometry. Secondary

transplantation assays were performed as described in the text. Bone marrow (BM) cells harvested from transplanted mice were stained with fluorochrome-conjugated antibodies specific to human CD45, CD34 and CD38 (Becton Dickinson, San Jose, CA) and sorted using FACSAria II cell sorter (BD Biosciences). For multilineage analysis, BM cells from engrafted animals were stained with CD45 FITC and gated to analyze human cells in combination with CD33 APC and CD15 PE (myeloid cells), CD19 APC and CD20 PE (B lymphoid cells), or CD34 FITC and CD38 PE (primitive cells) (all antibodies from Becton Dickinson, BD Pharmingen). Cell viability was assessed using the viability dye 7AAD (Beckman Coulter). All flow cytometry analysis were performed using BD FACSCalibur (BD Biosciences).

Limiting dilution analysis

Preconditioned mice received an intravenous transplant of 1000, 5000, 10.000, or 50.000 $Lin^{-}CD34^{+}$ cells and 1000, 5000, 10.000, or 30.000 $Lin^{-}CD34^{+}Notch^{+}$ cells or $Lin^{-}CD34^{+}Notch^{-}$ cells therefore were killed after 10 to 12 weeks. Recipients were considered being engrafted if the femur contained more than 0.5% $CD45^{+}$ human cells by flow cytometry or was positive by Southern blot analysis using a human-specific probe as described[83]. Data from 4 limiting dilution experiments (n = 65 mice) were pooled and analyzed using L-Calc software (Stem Cell Technologies).

Immunohistochemistry and bone image analysis

Mouse femur and tibiae were fixed for 24-48 h in 4% paraformaldehyde in PBS at 4^0C. Bones were then washed in PBS and decalcified, infiltrated with sucrose, embedded in OCT compound (Sakura, Tokyo, Japan) and frozen in liquid nitrogen. Cyrosections of decalcified bone were obtained using a cryostat microtome (CM3050S, Leica, Germany) and stored at -80^0C until staining. For immunohistochemistry, sections were stained with anti-mouse PECAM-1/CD31 hamster monoclonal antibody 2H8 (1:50 dilution) (Abcam), anti-human CD34 chicken polyclonal antibody (1:50) (Thermo Scientific Pierce Antibodies), anti-osteopontin rabbit polyclonal antibody O-17 (1:50), and anti-Jagged-1 rabbit polyclonal antibody H-114 (1:100) (both Santa Cruz Biotechnology, Inc.), then revealed by immunofluoresecence by a goat anti-hamster Cy3, goat anti-rabbit Cy5 (both Jackson Immunoresearch) and goat anti-chicken Alexa Fluor 488 (Invitrogen). All slides are mounted using prolong Gold anti-fade reagent with DAPI. Alternatively, slides were counterstained with Mayer's haematoxylin and eosin. Slides were examined and images captured using an TCS SP5 Leica confocal microscope and a 63x/1.2 numeric aperture (NA) c-apochromat objective lens (Leica, Germany). Images were transferred and modified on Adobe Photoshop CS3 (Adobe systems, San Jose, CA).

Spatial distribution of human hematopoietic cells

The location of cells was designated as either endosteal (within 12 cells of the endosteum) or central (>12 cells of the endosteum) as described previously (Nilsson et al., 2001). To accurately assess the proportion of endosteal area, the diameter of the diaphyseal shaft

was evaluated by counting the number of cells from one side of the endosteum to the other along the line perpendicular to the longitudinal axis of the bone. CD34+ human hematopoietic cells present in the diaphyseal shaft were counted under light microscopy with assistance of a Olympus 1X81.

Notch ligand binding competition assays

Preparation of soluble Notch ligand chimeric proteins has been previously described [53,54]. For competitive binding assays, Lin- cells were pre-incubated with different concentrations of Delta-4 IgG, Delta-1 IgG, Jagged-1 IgG, or IgG Fc (control). To determine Delta-4 receptor occupancy, pre-incubated cells were subsequently incubated with the Delta-4 FLAG trimeric complex. Cells were washed once after the final incubation and analyzed on a FACSCalibur to determine the degree of inhibition of Delta-4 FLAG binding.

Quantitative RT-PCR analysis

Total cellular RNA was isolated from purified Lin-CD34+Delta-4binder and Lin-CD34+Delta-4non-binder cells using the RNeasy Mini RNA isolation kit according to the manufacturer's instructions (Qiagen, Mississauga, ON). Real-time quantitative PCR (Q-PCR) was done with the MX4000 Q-PCR System (Stratagene, CA) using SYBR green detection (Applied Biosystems, CA). Reactions included 2ul cDNA, 2mM MgCl2, 0.4mM DNTP, 8% Glycerol, 3% DMSO, 150nM of each primer, 30nM reference Dye, 0.5XSYBR green dye and 2.5 U SureStart Taq DNA polymerase (Stratagene). Reaction

conditions were: primary denaturation at 95^0C for 1 min, 40 cycles of 95^0C for 10 seconds, 600C for 1min, and 720C for 30 sec. A dissociation curve was performed for analyzing the final PCR product (n=5). The endpoint used in the QPCR quantification, Ct, is defined as the PCR cycle number that crosses an arbitrarily placed signal threshold. Gene expression is presented using a modification of the 2-ΔΔCt 2 method as previously described (Livak and Schmittgen, 2001). The expression of each housekeeping gene was presented as 2-ΔCt, where ΔCt (CtTime X - CtTime 0) and time 0 represents the 1 X expression of each gene. The primer sets used for the PCR reactions are listed in supplementary table 1. For expression of the Notch regulatory gene Fringe, mRNA was isolated from cells using the QuickPrep Micro RNA purification kit (Amersham Pharmacia Biotech, Buckinghamshire, England), and reverse transcribed into first strand cDNA using First Strand cDNA Synthesis Kit as above. The PCR products were sequenced to verify specificity of amplified cDNA sequences.

Liquid serum free culture of purified cells

Purified $Lin^-CD34^+Notch^+$ and $Lin^-CD34^+Notch^-$ cell populations were cultured in serum-free media containing hematopoietic cytokines using fibronectin-coated plates (n=10). Briefly, cultures contained 9500 BIT media (Stem Cell Technologies Inc, Vancouver, Canada) supplemented with 10-4 M b-mercaptoethanol and 2mM L-glutamine. Growth factors were used at final concentrations of 300ng/ml recombinant human Flt-3 ligand (rhu-Flt-3-l) (R&D Systems, Minneapolis, MN) and rhu-stem cell factor (rhu-SCF, Amgen, Thousand Oaks, CA), 50 ng/mL rhu-G-CSF (Amgen, Thousand Oaks, CA), and

10 ng/mL rhu-IL-3 (Norvatis Pharmaceuticals, Dorval, Quebec) and rhu-IL-6 (R&D Systems, Minneapolis, MN). Cells were cultured for 6 or 9 days at 37^0C and 5% CO_2 with 10ug/mL human IgG Fc (control), hDelta-4 IgG1, hDelta-1 IgG1 or hJagged-1-IgG1. Initial experiments with human IgG1 Fc protein added to control for non-specific effects of IgG1 in the chimeric protein showed no effect on cells in culture. Cells were harvested, counted and aliquots were used for CFU analysis or stained for phenotypic analysis.

Clonogenic progenitor assays

Human clonogenic progenitor assays were done by plating freshly isolated or cultured cells in semi-solid methylcellulose medium (Methocult H4230, Stem Cell Technologies, Vancouver, BC) containing 30% fetal bovine serum, 50ng/ml rhu-SCF, 10ng/ml rhu-GM-CSF (Amgen, Thousand Oaks, CA) and rhu-IL-3, and 3 units/ml rhu-Erythropoietin (Amgen, Thousand Oaks, CA). Differential colony counts were done following incubation for 10-14 days at 37^0C and 5% CO_2 in a humidified atmosphere.

Global gene expression profiling

Total RNA was extracted using Qiagen RNAeasy kit (Qiagen) and was amplified using Message Amp aRNA kit (Ambion) [84]. Fifteen micrograms of fragmented antisense RNA (aRNA) was used for hybridizing human HG-U133AB arrays (Affymetrix) at the London Regional Genomic Center (Ontario, Canada). GeneSpring 6.0 was used for data analysis. Genes that were flag passed in at least one of the populations and significantly ($p < 0.05$,

different by 2.5-fold) upregulated in SRC-TBA, in comparison to SRC-LBA or vice-versa, are shown.

Statistical analysis

The significance of any differences between groups was assessed using the Student's T-test, and $p < 0.05$ was considered to be statistically significant.

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Figure 1. Spatial distribution of engrafted human HSCs within the mouse bone marrow recipient

(a) Experimental design to study human HSC distribution in recipient mice. **(b)** Representative FACS histogram of recipient engrafted bone marrow, denoting frequency of human chimerism (CD45) and it composition with primitive hematopoietic fraction (CD34 and CD38), myeloid cells (CD33 and CD15) and lymphoid cells (CD19 and CD20). **(c-e)** Serial bone marrow sections of mouse xenografted femurs. Scale bars = 10um. **(c)** Human $CD34^+$ cells (arrowheads) were found associated to the endosteum (OPN)**, (d)** attached to the vascular cells (CD31) or **(e)** located at equal distance between endosteum and blood vessels**. (f)** Statistical summary of human $CD34^+$ distribution within BM niche (n=10).

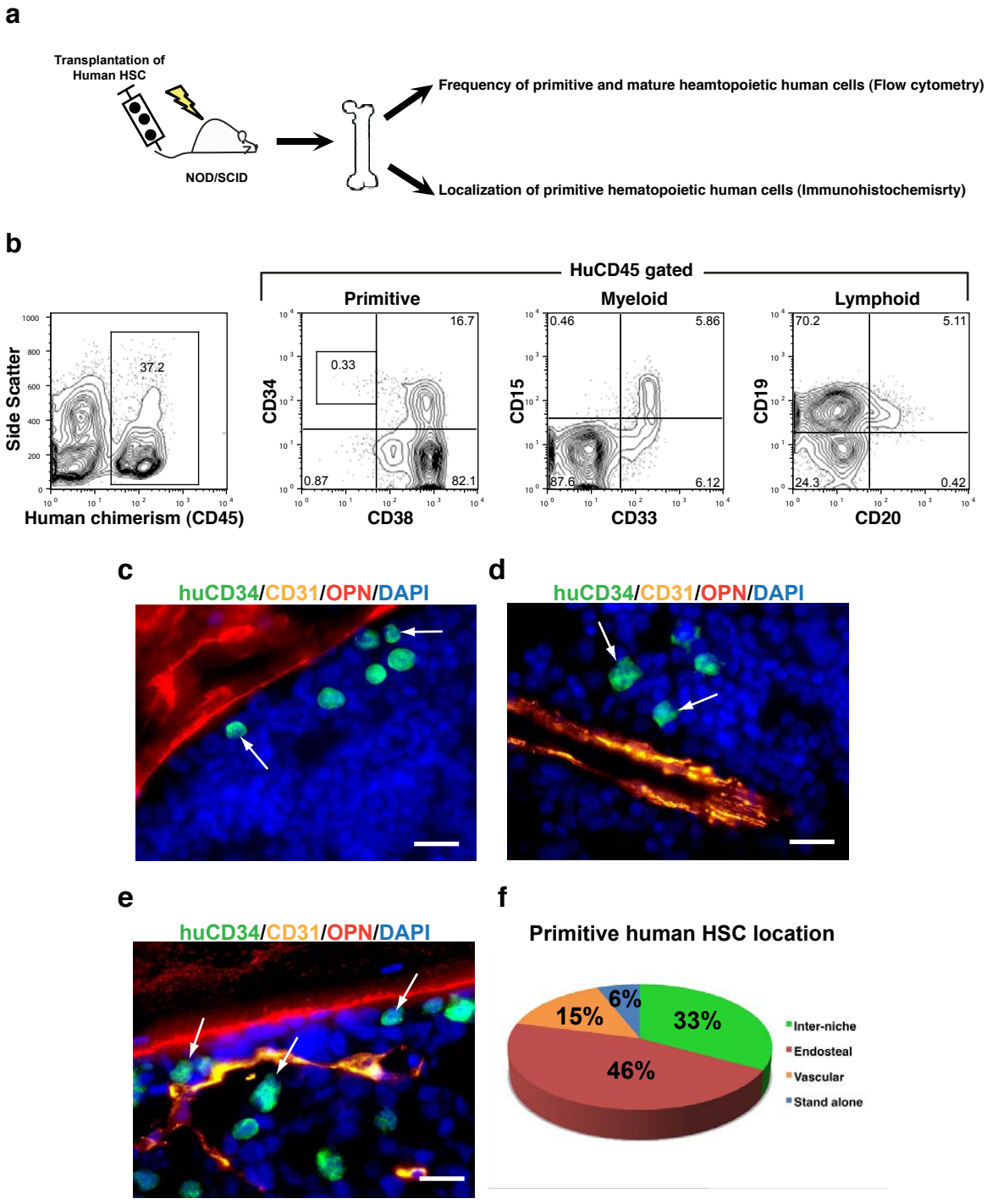

a
Transplantation of Human HSC
NOD/SCID
Frequency of primitive and mature heamtopoietic human cells (Flow cytometry)
Localization of primitive hematopoietic human cells (Immunohistochemisrty)
b
HuCD45 gated
Primitive
Myeloid
Lymphoid
Side Scatter
Human chimerism (CD45)
37.2
CD34
CD38
0.33
16.7
0.87
82.1
CD15
CD33
0.46
5.86
87.6
6.12
CD19
CD20
70.2
5.11
24.3
0.42
c
huCD34/CD31/OPN/DAPI
d
huCD34/CD31/OPN/DAPI
e
huCD34/CD31/OPN/DAPI
f
Primitive human HSC location
6%
33%
15%
46%
Inter-niche
Endosteal
Vascular
Stand alone

Figure 2. Location of engrafted human HSCs associated with bone marrow niches between long (LBA) and trabecular bone area (TBA)

(a) Equivalence between histology and schematic representation of LBA and TBA in the mouse femoral bone. **(b)** Experimental design to study engrafted human hematopoietic cells location within LBA and TBA. **(c-e)** Spatial distribution along with statistical summary of human $CD34^+$ cells between LBA and TBA, associated with either **(c)** endosteal niche, **(d)** vascular niche or **(e)** interniche (n=10 independent mice, 15 sections per mouse).

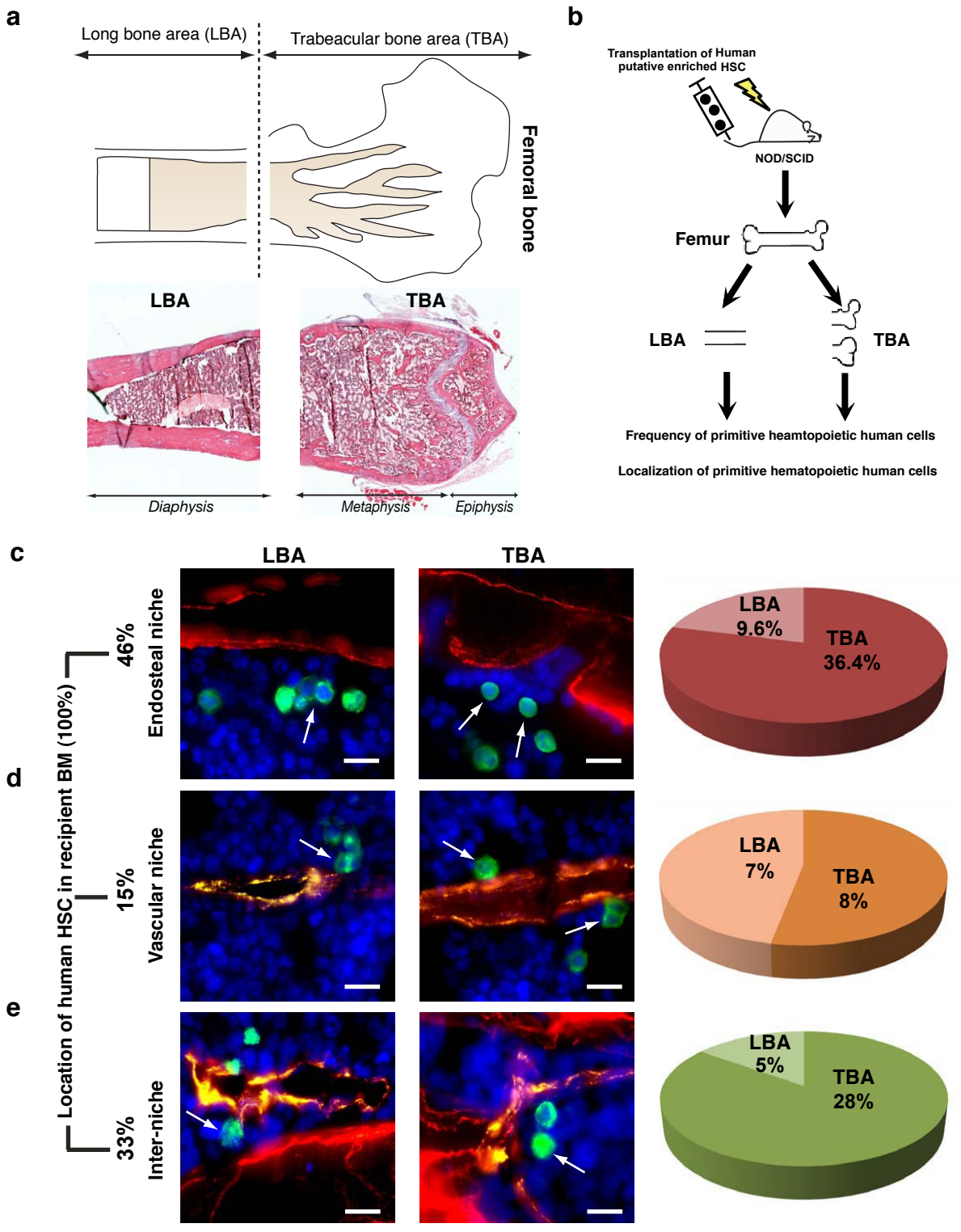

a
Long bone area (LBA)
Trabeacular bone area (TBA)
Femoral bone
LBA
TBA
Diaphysis
Metaphysis
Epiphysis
b
Transplantation of Human putative enriched HSC
NOD/SCID
Femur
LBA
TBA
Frequency of primitive heamtopoietic human cells
Localization of primitive hematopoietic human cells
c
d
e
LBA
TBA
Location of human HSC in recipient BM (100%)
46%
Endosteal niche
15%
Vascular niche
33%
Inter-niche
LBA 9.6%
TBA 36.4%
LBA 7%
TBA 8%
LBA 5%
TBA 28%

Figure 3. Total human hematopoietic engraftment along with HSC number is higher in trabecular bone

(a) Experimental design to study human hematopoietic engraftment based on bone marrow location. **(b)** Representative FACS histogram of recipient engrafted bone marrow, denoting frequency of human chimerism (CD45) in LBA and TBA. **(c)** Composition of engrafted primitive hematopoietic cell compartment in TBA and LBA at 10 wk post-transplantation. **(d)** Level of bone marrow engraftment in LBA and TBA of total human hematopoietic cells, **(e)** primitive stem cells **(f)** and progenitor cells. Each circle represents a mouse, and bars represent average level of engraftment per cell type (n=45). **(g)** Linear correlation analysis between total human engraftment and the TBA/LBA ratio of engrafted HSCs. Each circle represents one mouse (n=35).

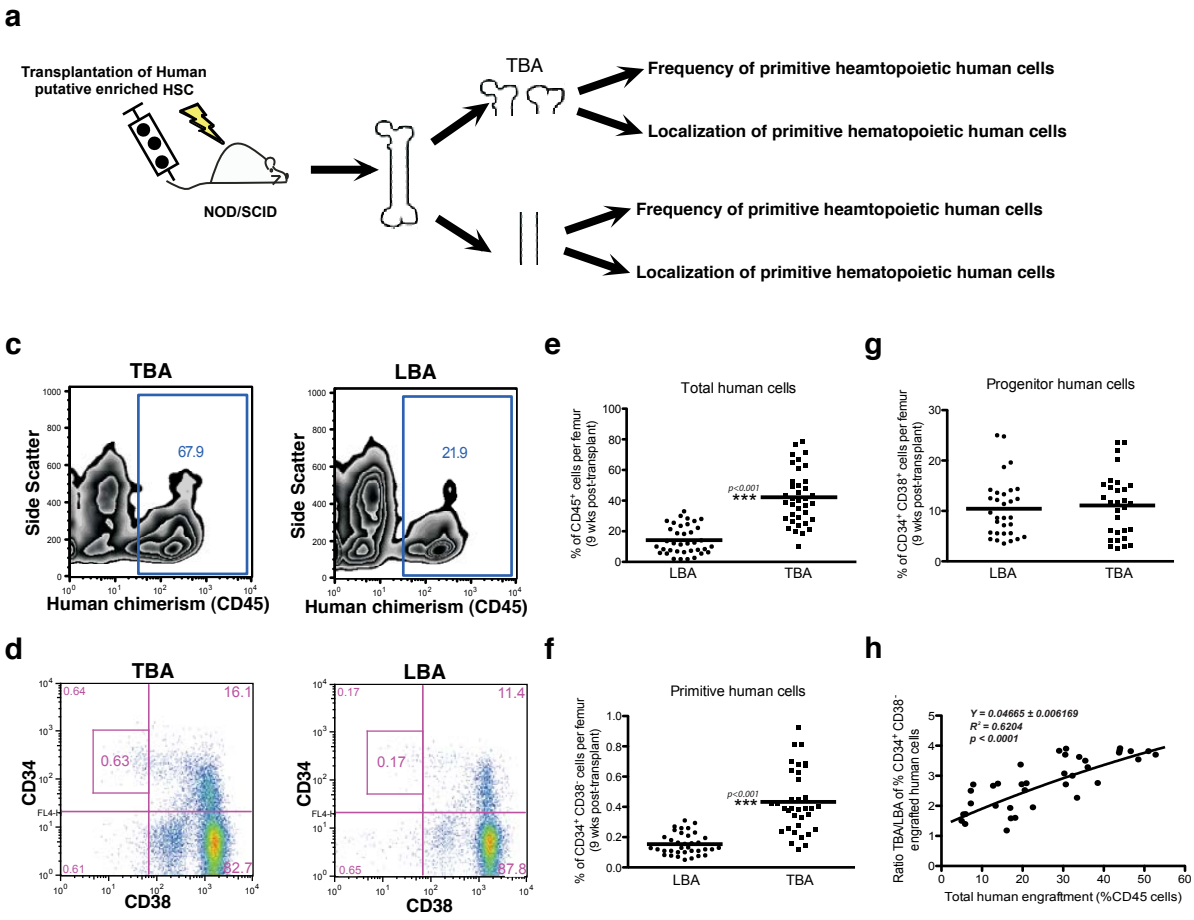

a
Transplantation of Human putative enriched HSC
NOD/SCID
TBA
Frequency of primitive heamtopoietic human cells
Localization of primitive hematopoietic human cells
Frequency of primitive heamtopoietic human cells
Localization of primitive hematopoietic human cells
c
TBA
LBA
Side Scatter
67.9
21.9
Human chimerism (CD45)
Human chimerism (CD45)
e
Total human cells
% of CD45+ cells per femur (9 wks post-transplant)
p<0.001
***
LBA
TBA
g
Progenitor human cells
% of CD34+ CD38+ cells per femur (9 wks post-transplant)
LBA
TBA
d
TBA
LBA
CD34
0.64
16.1
0.63
0.61
82.7
0.17
11.4
0.17
0.65
87.8
CD38
CD38
f
Primitive human cells
% of CD34+ CD38- cells per femur (9 wks post-transplant)
p<0.001
***
LBA
TBA
h
Y = 0.04665 ± 0.006169
R2 = 0.6204
p < 0.0001
Ratio TBA/LBA of % CD34+ CD38- engrafted human cells
Total human engraftment (%CD45 cells)

Figure 4. Human SRC purified from mouse TBA display better hematopoietic regenerative capacity

(a) Experimental design to study *in vitro* and *in vivo* multilineage repopulating capacity of engrafted human SRCs in LBA and TBA. (**b)** and **(c)** Representative FACS histogram of human lymphoid and myeloid cells generated in primary transplants. **(d)** Frequency of human lymphoid and myeloid cells in primary bone marrow recipient (n=10), *p<0.05. **(e)** Total number of SRC-CFU per 1000 produced by $CD34^+$ $CD38^-$ cells purified from LBA and TBA. Values shown are mean ± SEM (n=5-10), *p<0.05. **(f)** Representative GM-CFC colonies generated by SRCs, scale bar 25um (inset). **(g)** Frequency of colony subtypes generated by SRC-LBA and SRC-TBA (n=5), *p<0.05, **p<0.01. **(h)** Experimental design to study *in vivo* self-renewal capacity of engrafted human SRCs from LBA and TBA in secondary transplants. **(i)** BM engraftment levels of secondary recipients of transplanted SRC-LBA and SRC-TBA purified cells (n=20) **p<0.01. **(j)** Limiting dilution of secondary mice transplanted with freshly purified SRC-LBA and SRC-TBA subsets for the range of injected cell doses (n=4). Horizontal bars indicate the average level of human chimerism for each cell dose. The frequency of engrafted mice is shown below as the % positive mice (n=12)**. (k)** Frequency of secondary human primitive hematopoietic cells generated by SRC-LBA and SRC-TBA (n=15), **p<0.01**.**

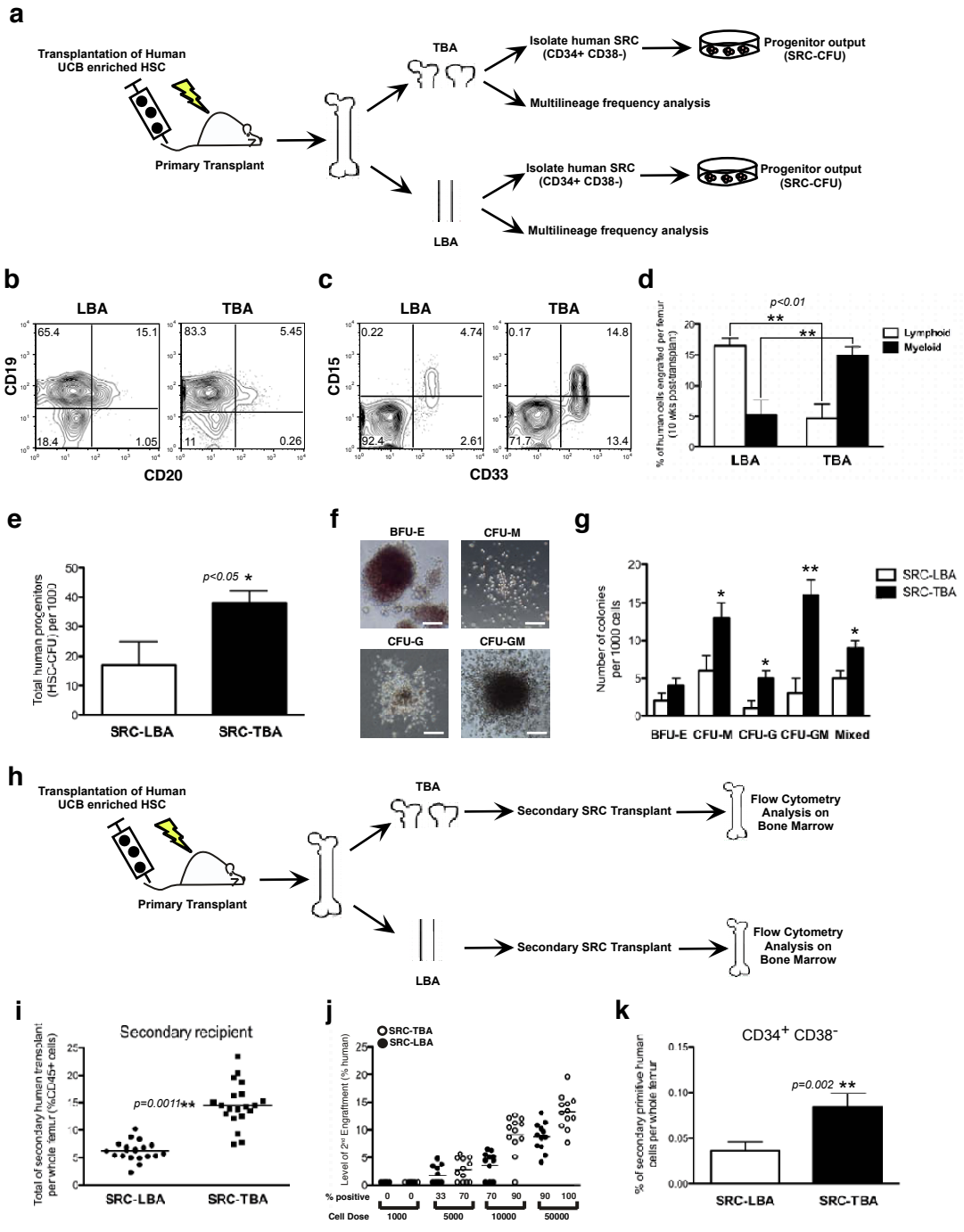

a
Transplantation of Human UCB enriched HSC
Primary Transplant
TBA
LBA
Isolate human SRC (CD34+ CD38-)
Progenitor output (SRC-CFU)
Multilineage frequency analysis
b
LBA
TBA
CD19
CD20
65.4
15.1
18.4
1.05
83.3
5.45
11
0.26
c
CD15
CD33
0.22
4.74
92.4
2.61
0.17
14.8
71.7
13.4
d
p<0.01
Lymphoid
Myeloid
e
Total human progenitors (HSC-CFU) per 1000
p<0.05 *
SRC-LBA
SRC-TBA
f
BFU-E
CFU-M
CFU-G
CFU-GM
g
Number of colonies per 1000 cells
SRC-LBA
SRC-TBA
BFU-E CFU-M CFU-G CFU-GM Mixed
h
Transplantation of Human UCB enriched HSC
Primary Transplant
Secondary SRC Transplant
Flow Cytometry Analysis on Bone Marrow
i
Secondary recipient
Total of secondary human transplant per whole femur (%CD45+ cells)
p=0.0011**
SRC-LBA
SRC-TBA
j
SRC-TBA
SRC-LBA
Level of 2nd Engraftment (% human)
% positive
Cell Dose
k
CD34+ CD38-
% of secondary primitive human cells per whole femur
p=0.002 **
SRC-LBA
SRC-TBA

Figure 5. Different anatomical location of human SRC correlates with distinct molecular signature

(a) Experimental design to study global gene expression and osteblastic niche location of engrafted SRC-LBA and SRC-TBA from primary recipients. **(b)** Hierarchical cluster analysis using global gene expression. Tree branch lengths are calculated based on Pearson's correlation coefficients. HSC-LBA: N=2, HPC-LBA: N=3, HSC-TBA: N=2 and HPC-TBA: N=2 biological replicates. **(c)** Schematic representation of HU133 2.0 Affymetrix analysis conducted on SRC-LBA vs. SRC-TBA. **(d)** QPCR validation of selected genes at least 4-fold up-regulated or present only in SRC-LBA or SRC-TBA (n=3). **(e)** Overview of genes differentially expressed in both SRC-LBA and SRC-TBA. **(f)** Selected expression of Notch pathway components that are up-regulated in SRC-TBA. (g) QPCR validation of selected Notch pathway components in SRC-LBA and SRC-TBA relative to de novo $CD34^+$ $CD38^-$ cells (n=5), *p<0.05, **p<0.01. **(h)** Spatial position of human $CD34^+$ cells (arrowheads) towards jagged-1^+ osteoblastic endosteum in LBA and TBA of bone marrow recipients (n=10). **(i)** Frequency of Jagged-1+ cells, human CD34+ cells, human CD34+ cells associated to Jagged-1+ cells in LBA vs. TBA (n=10), **p<0.01.

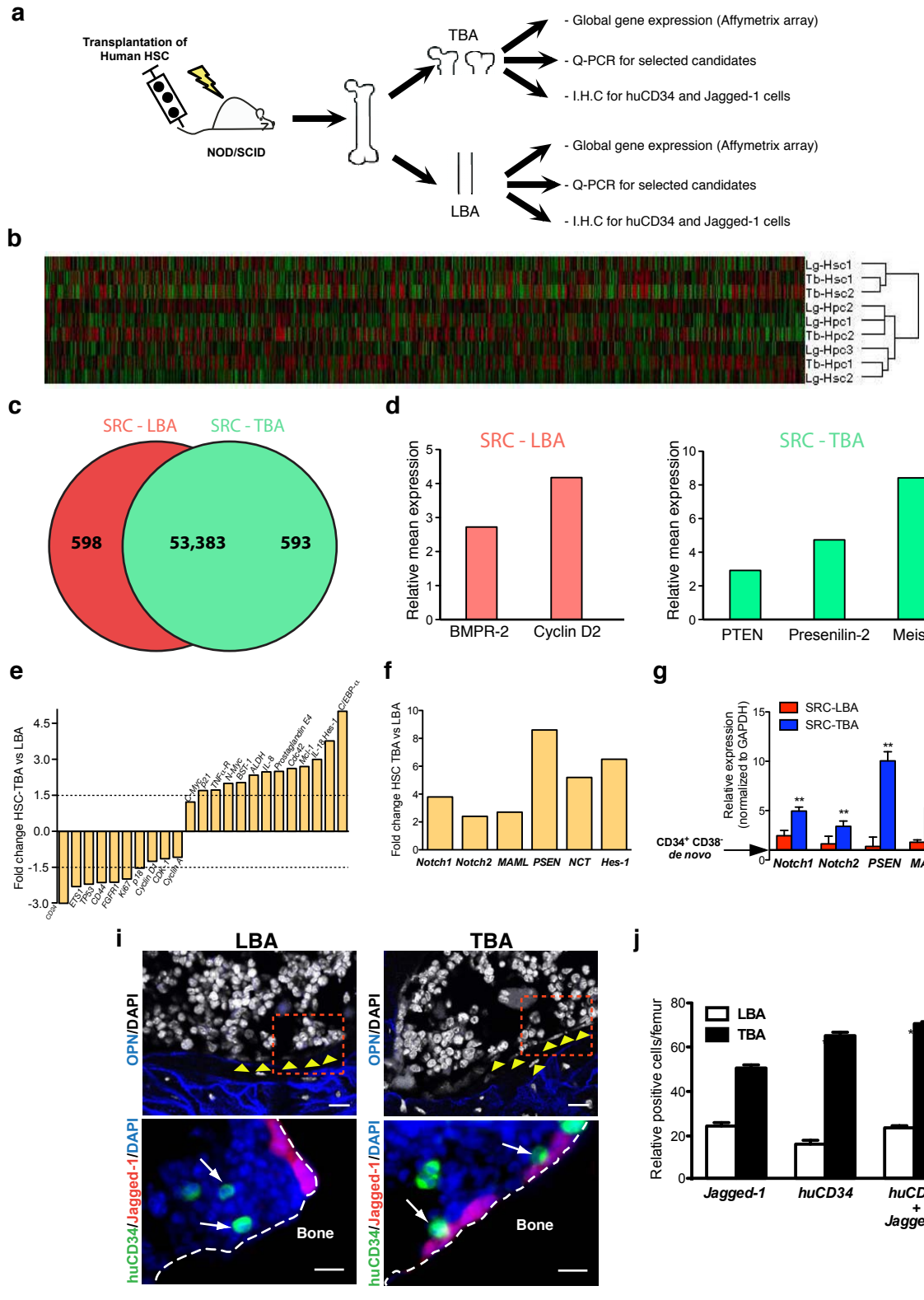
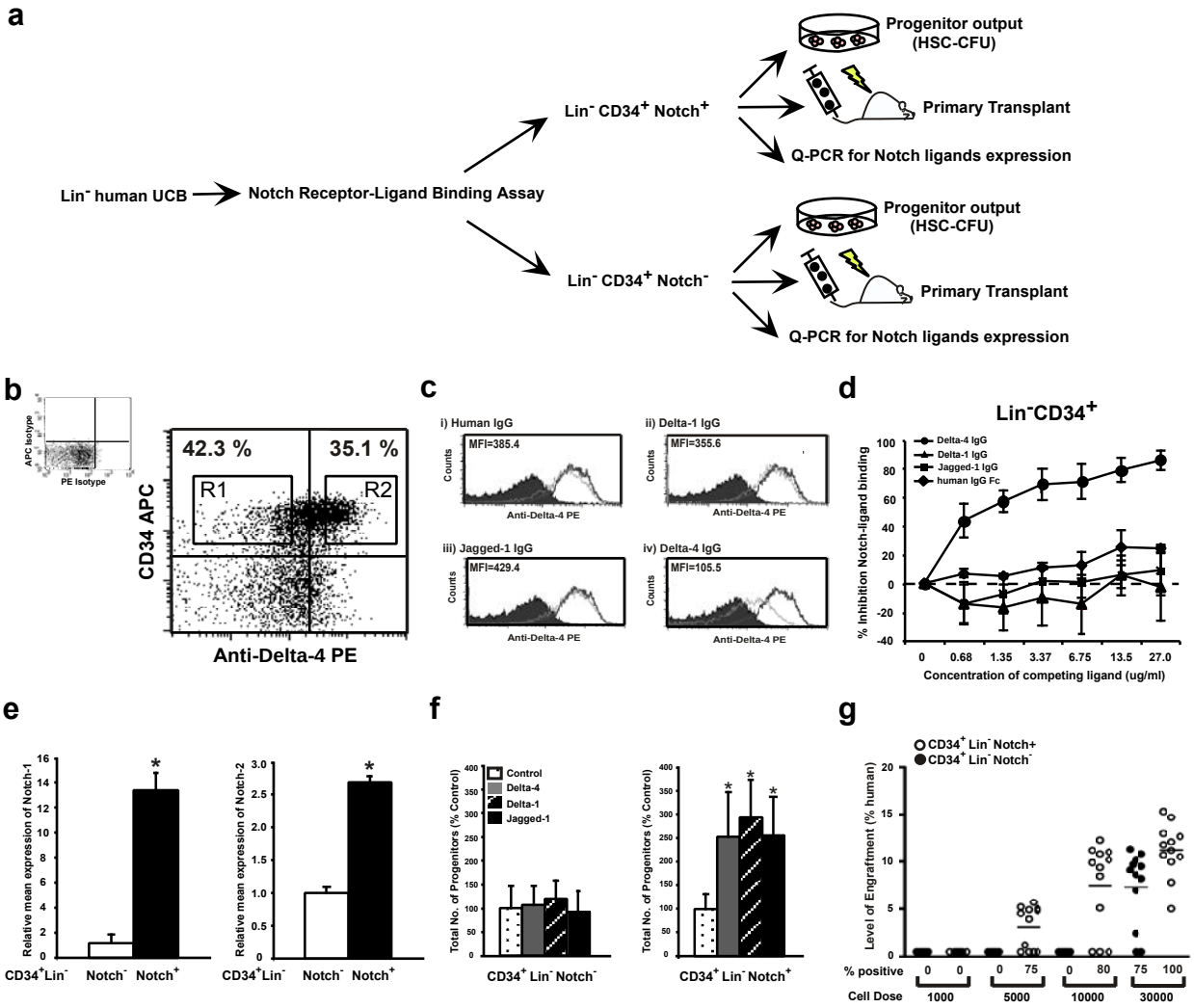

a
Transplantation of Human HSC
NOD/SCID
TBA
LBA
- Global gene expression (Affymetrix array)
- Q-PCR for selected candidates
- I.H.C for huCD34 and Jagged-1 cells
- Global gene expression (Affymetrix array)
- Q-PCR for selected candidates
- I.H.C for huCD34 and Jagged-1 cells
b
Lg-Hsc1
Tb-Hsc1
Tb-Hsc2
Lg-Hpc2
Lg-Hpc1
Tb-Hpc2
Lg-Hpc3
Tb-Hpc1
Lg-Hsc2
c
SRC - LBA
SRC - TBA
598
53,383
593
d
SRC - LBA
Relative mean expression
BMPR-2
Cyclin D2
SRC - TBA
Relative mean expression
PTEN
Presenilin-2
Meis-1
e
Fold change HSC-TBA vs LBA
f
Fold change HSC TBA vs LBA
Notch1 Notch2 MAML PSEN NCT Hes-1
g
SRC-LBA
SRC-TBA
Relative expression (normalized to GAPDH)
CD34+ CD38- de novo
Notch1 Notch2 PSEN MAML Hes-1
i
LBA
TBA
OPN/DAPI
huCD34/Jagged-1/DAPI
Bone
j
Relative positive cells/femur
LBA
TBA
Jagged-1
huCD34
huCD34 + Jagged-1

Figure 6. Prospective isolation of human primitive hematopoietic cell subsets with enhanced repopulation capacity using Notch-ligand binding assays

(a) Experimental design to test *in vitro* and *in vivo* hematopoietic capacity of human Notch binding and non-binding cells. **(b)** Cord blood Lin^- cells were stained for Delta-4 binding affinity combined with CD34 APC as described in Materials and Methods. Regions R1 and R2 are used to isolate respectively $Lin^-CD34^+Notch^-$ cells and $Lin^-CD34^+Notch^+$ cells. The purity of each sorted population was routinely greater than 95% (data not shown). **(c)** For competitive Notch ligand binding assays, Lin^-CD34^+ cord blood cells were pre-incubated with increasing concentrations of IgG chimeric Notch ligands and then Delta-4-FLAG. Median fluorescence intensity (MFI) was used to determine the degree of Delta-4 binding inhibition by comparing Delta-4 FLAG binding in the presence and absence of competing ligand. Representative histogram plots are shown of live cells pre-incubated with (c i) human IgG (c ii) Delta-1 IgG (c iii) Jagged-1 IgG and (c iv) Delta-4 IgG. The shaded area indicates background (isotype control) staining (MFI=26.7), the thick line indicates Delta-4 binding in absence of competing ligand (MFI=474.6), and the thin line indicates Delta-4 binding subsequent to incubation with the indicated ligand. Representative MFI for competed Delta-4 binding is indicated on each histogram. **(d)** Summary of Delta-4 binding inhibition for increasing concentrations of competing ligand. The horizontal line at y=0 indicates no inhibition of Delta-4 binding. (n=4-5, data points are mean ± SEM). **(e)** Quantitative expression of Notch-1 and Notch-2 in $Lin^-CD34^+Notch^+$ cells relative to $Lin^-CD34^+Notch^-$ cells (n=5), *$p<0.05$. **(f)**

Progenitors output for Notch-ligand treated cells after 9 days is expressed relative to the number of colonies in control treated cells (100%). Values shown are mean ± SEM (n=3-5), *$p<0.05$. **(g)** Limiting dilution of mice transplanted with freshly purified Notch subsets for the range of injected cell doses. Horizontal bars indicate the average level of human chimerism for each cell dose. The frequency of engrafted mice is shown below as the % positive mice (n=12)**.**


a
Lin- human UCB
Notch Receptor-Ligand Binding Assay
Lin- CD34+ Notch+
Progenitor output (HSC-CFU)
Primary Transplant
Q-PCR for Notch ligands expression
Lin- CD34+ Notch-
Progenitor output (HSC-CFU)
Primary Transplant
Q-PCR for Notch ligands expression
b
APC Isotype
PE Isotype
42.3 %
35.1 %
R1
R2
CD34 APC
Anti-Delta-4 PE
c
i) Human IgG
MFI=385.4
ii) Delta-1 IgG
MFI=355.6
iii) Jagged-1 IgG
MFI=429.4
iv) Delta-4 IgG
MFI=105.5
Counts
Anti-Delta-4 PE
d
Lin-CD34+
Delta-4 IgG
Delta-1 IgG
Jagged-1 IgG
human IgG Fc
% Inhibition Notch-ligand binding
Concentration of competing ligand (ug/ml)
e
Relative mean expression of Notch-1
Relative mean expression of Notch-2
CD34+Lin-
Notch- Notch+
f
Control
Delta-4
Delta-1
Jagged-1
Total No. of Progenitors (% Control)
CD34+ Lin- Notch-
CD34+ Lin- Notch+
g
CD34+ Lin- Notch+
CD34+ Lin- Notch-
Level of Engraftment (% human)
% positive
Cell Dose
1000
5000
10000
30000

Spatial distribution of human hematopoietic stem/progenitor cells in the murine BM compartment at 10 weeks of highly reconstituted mice with UCB Lin$^-$ CD34$^+$

Total no. cells counted	Total no. cells in the endosteal region	Cells in the Endosteal region/slide %	Total no. cells in the vascular region	Cells in the vascular region/slide %	Total no. cells in the inter-niche region	Cells in the Inter-niche region/slide %
2514	1162	46.2 ± 17.3	390	15.5 ± 4.6	827	32.9 ± 5.7

Fifteen slides from at least 10 different mice were examined to count each cell type. Slides containing 134 ± 12 cells in the diaphyseal cavity were chosen for this analysis. Because the endosteal region was arbitrarily decided within 12 cells of both endosteum, the endosteal area comprised approximately 21.4% ± 3.8% of the BM cavity in this study. The proportion of cells located in each area was calculated for each slide and expressed as the means ± SD.

Spatial distribution of interacting human hematopoietic stem/progenitor cells with endosteal vs. vascular niche within BM cavity

Total no. cells in the endosteal region	No and % cells in the endosteal region within Long bone area	No and % cells in the endosteal region within Trabecular bone area	Total no. cells in the vascular region	% cells in the vascular region within Long bone area	% cells in the vascular region within trabecular bone area
1238	259 (21.3 ± 7.9)	978 (79.4 ± 6.2)	437	205 (53.5 ± 3.2)	231 (47.9 ± 8.4)

Table 1: Spatial distribution of primitive hematopoietic cells within the niche

Gene Symbol	Gene Description	Affymetrix Probe ID
MEIS1	Meis1, myeloid ecotropic viral integration site 1 homolog (mouse)	1559477_s_at
ANGPTL2	Angiopoietin-like 2	213004_at
PSEN2	Presenilin-2	211373_s_at
CDH2	cadherin 2, type 1, N-cadherin (neuronal)	237305_at
PTEN	phosphatase and tensin homolog	204054_at
DAAM2	Dishevelled associated activator of morphogenesis 2	241442_at
ARHGEF9	Cdc42 guanine nucleotide exchange factor (GEF) 9	203264_s_at
FAS	Fas (TNF receptor superfamily, member 6)	204781_s_at
PTGER3	Prostaglandin E receptor 3 (subtype EP3)	210375_at
TSPAN2	Tetraspanin 2	227233_at
TNFRSF19	Tumor necrosis factor receptor superfamily, member 19	227812_at
CD48	CD48 antigen (B-cell membrane protein)	237759_at
PARD3	Par-3 partitioning defective 3 homolog (C. elegans)	237782_at
CUL4A	Cullin 4A	240971_x_at
CTNNB1	catenin (cadherin-associated protein), beta 1, 88kDa	1554411_at
SNAI3	Snail homolog 3 (Drosophila)	1560228_at
IL12RB2	Interleukin 12 receptor, beta 2	1560999_a_at
ICAM4	Intercellular adhesion molecule 4 (Landsteiner-Wiener blood group)	207194_s_at
CXCL3	chemokine (C-X-C motif) ligand 3	207850_s_at
AGTR1	angiotensin II receptor, type 1	208016_s_at
MYCN	v-myc myelocytomatosis viral related oncogene, neuroblastoma derived (avian)	209757_s_at

Table 2: Most representative genes present in SRC-TBA but absent in SRC-LBA

Gene Symbol	Gene Description	Affymetrix Probe ID
RARB	Retinoic acid receptor, beta	205080_at
CXCL14	Chemokine (C-C motif) ligand 14	205392_s_at
BMP5	Bone morphogenetic protein 5	205431_s_at
CDH2	cadherin 2, type 1, N-cadherin (neuronal)	237305_at
MSTR1	Macrophage stimulating 1 receptor (c-met-related tyrosine kinase)	205455_at
IL2RA	Interleukin 2 receptor, alpha	206341_at
CCR6	chemokine (C-C motif) receptor 6	206983_at
CD84	CD84 antigen (leukocyte antigen)	2111909_x_at
ITGB1	Integrin, beta 1 (fibronectin receptor, beta polypeptide, antigen CD29 includes MDF2, MSK12)	215879_at
GSK3B	Glycogen synthase kinase 3 beta	219285_s_at
CX3CL1	chemokine (C-X3-C motif) ligand 1	823_at
CLDN1	Claudin 1	222549_at
LIFR	Leukemia inhibitory factor receptor alpha	225571_at
ANGPT2	Angiopoietin 2	230562_at
BMPR2	Bone morphogenetic protein receptor, type II (serine/threonine kinase)	238393_at
ITGA9	Integrin, alpha 9	240962_at
BCL2	BCL2-like 11 (apoptosis facilitator)	1555372_at
CCND2	Cyclin D2	207194_s_at
RAB35	RAB35, member RAS oncogene family	225620_at
RAB18	RAB18, member RAS oncogene family	233024_at
RAB3GAP2	RAB3 GTPase activating protein subunit 2 (non-catalytic)	240234_at

Table 3: Most representative genes present in SRC-LBA but absent in SRC-TBA

Cell Type	Treatment	Type of Colony (% ± SEM)					Plating Efficiency (PE) ± SEM
		BFU-E	CFU-M	CFU-G	CFU-GM	CFU-GEMM	
Lin$^-$CD34$^+$ Notch$^-$	Control	39±11	13±4	43±11	5±4	0±0	1:155±82
	hDelta-4	40±15	8±3	50±17	2±1	0±0	1:144±108
	hDelta-1	31±14	10±5	58±18	1±1	0±0	1:370±222
	hJagged-1	29±12	10±5	58±17	3±2	0±0	1:740±444
Lin$^-$CD34$^+$ Notch$^+$	Control	29±5	16±7	49±7	6±2	0±0	1:99±58
	hDelta-4	35±4	14±4	50±5	2±1	0±0	1:119±42
	hDelta-1	26±6	27±4	44±5	3±1	0.5±0.5	1:56±11
	hJagged-1	25±7	30±7	42±8	4±2	0±0	1:126±69

Table 4. Composition of clonogenic progenitors after culture in the absence (control) or presence of Notch ligands. Lin-CD34+Notch- and Lin-CD34+Notch+ cells were purified, cultured for 9 days in the presence or absence of Notch ligands, and progenitor content was measured by enumerating the colony forming units (CFU) inmethylcellulose cultures. Burst Forming Unit-Erythroid (BFU-E), CFU-Macrophage (CFU-M), CFU-Granulocyte(CFU-G), CFU-Granulocyte-Macrophage (CFU-GM), and CFU-Granulocytic, Erythroid, Macrophage,Megakaryocytic (CFU-GEMM) were scored according to standard criteria. The mean percentage of each colonytype and the mean plating efficiency for each treatment is shown (n=4-5 independent cord blood samples).

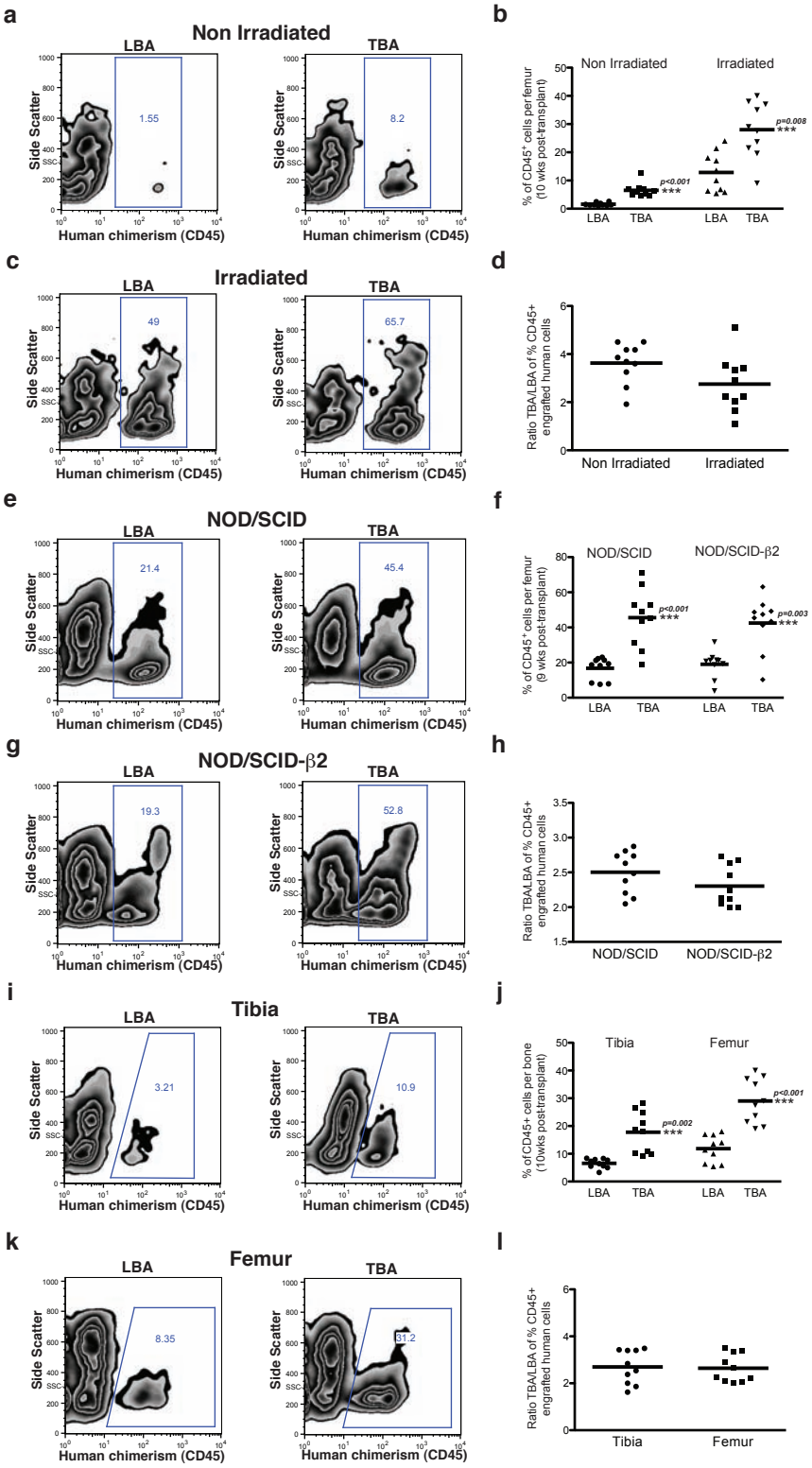
Table 5: SRC frequency determined by limiting-dilution analysis

Number of injected cells per mouse	Number of mice engrafted with ≥0.5% $CD45^+$ cells per total number of mice	
	$Lin^- CD34^+ CD38^-$ from LBA	**$Lin^- CD34^+ CD38^-$ from TBA**
1,000	0/12 (0%)	0/12 (0%)
5,000	3/12 (25%)	8/12 (66.6%)
10,000	8/12 (66.6%)	11/12 (91.6%)
50,000	11/12 (91.6%)	12/12 (100%)
SRC frequency (95% CI)	**1 in 15,280 (1 in 9,165 - 1 in 25,474)**	**1 in 4,598 (1 in 2,853 - 1 in 7,412)**
No. of SRCs per 1×10^6 starting cells	65.4	217.5
	$Lin^- CD34^+ Notch^-$ from CB	**$Lin^- CD34^+ Notch^+$ from CB**
1,000	0/12 (0%)	0/12 (0%)
5,000	0/12 (0%)	9/12 (75%)
10,000	1/12 (0.83%)	10/12 (83.3%)
30,000	9/12 (75%)	12/12 (100%)
SRC frequency (95% CI)	**1 in 39,486 (1 in 21,555 - 1 in 72,332)**	**1 in 5,253 (1 in 3,302 - 1 in 8,356)**
No. of SRCs per 1×10^6 starting cells	25.3	190.4

All mice were analyzed for long-term engraftment after 10-12 weeks post-transplant. Data from 4 limiting dilution experiments (n = 48 mice) were pooled and analyzed using L-Calc software (Stem Cell Technologies). CI indicates confidence interval.

Supplementary figure 1. TBA displayed higher human engraftment even under varying experimental conditions

Representative FACS histogram, statistical frequency and TBA/LBA ratio of recipient engrafted bone marrow, denoting percentage of human chimerism (CD45) in LBA and TBA under different **(a-d)** irradiation conditions, **(e-h)** NOD/SCID substrain and **(i-l)** bone type. Each circle represents a mouse, and bars represent average level of engraftment per cell type (n=45), **p<0.01.


a
Non Irradiated
LBA
TBA
1.55
8.2
Side Scatter
SSC
Human chimerism (CD45)
b
Non Irradiated
Irradiated
% of CD45+ cells per femur (10 wks post-transplant)
p=0.008
***
p<0.001
***
LBA
TBA
LBA
TBA
c
Irradiated
LBA
TBA
49
65.7
Side Scatter
SSC
Human chimerism (CD45)
d
Ratio TBA/LBA of % CD45+ engrafted human cells
Non Irradiated
Irradiated
e
NOD/SCID
LBA
TBA
21.4
45.4
Side Scatter
SSC
Human chimerism (CD45)
f
NOD/SCID
NOD/SCID-β2
% of CD45+ cells per femur (9 wks post-transplant)
p<0.001
***
p=0.003
***
LBA
TBA
LBA
TBA
g
NOD/SCID-β2
LBA
TBA
19.3
52.8
Side Scatter
SSC
Human chimerism (CD45)
h
Ratio TBA/LBA of % CD45+ engrafted human cells
NOD/SCID
NOD/SCID-β2
i
Tibia
LBA
TBA
3.21
10.9
Side Scatter
SSC
Human chimerism (CD45)
j
Tibia
Femur
% of CD45+ cells per bone (10wks post-transplant)
p=0.002
***
p<0.001
***
LBA
TBA
LBA
TBA
k
Femur
LBA
TBA
8.35
31.2
Side Scatter
SSC
Human chimerism (CD45)
l
Ratio TBA/LBA of % CD45+ engrafted human cells
Tibia
Femur

Supplementary figure 2. Similar features between primary TBA-isolated SRC and prospectively isolated SRC Notch+ cells

(a) Representative FACS histogram and statistical frequency of human lymphoid and myeloid cells generated in secondary transplants (n=10), **p<0.01. **(b)** Overview of genes not differentially expressed in both SRC-LBA and SRC-TBA from global gene expression analysis. Dashed lines represent significant absolute fold changes ≥ 2. **(c)** Amplification product and quantitative expression of Fringe glycosyltransferase from RT-PCR reactions for each cell subset. (No RT indicates no reverse transcriptase) (n=5). **(d)** Representative multilineage analysis of the human graft in mice transplanted with Lin- CD34+Notch- cells and Lin- CD34+Notch+ cells is shown after gating based on human (CD45+) cells from engrafted mice for lymphoid (CD20 and CD19), myeloid (CD33 and CD15), and primitive (CD34 and CD38) cells (n=10).

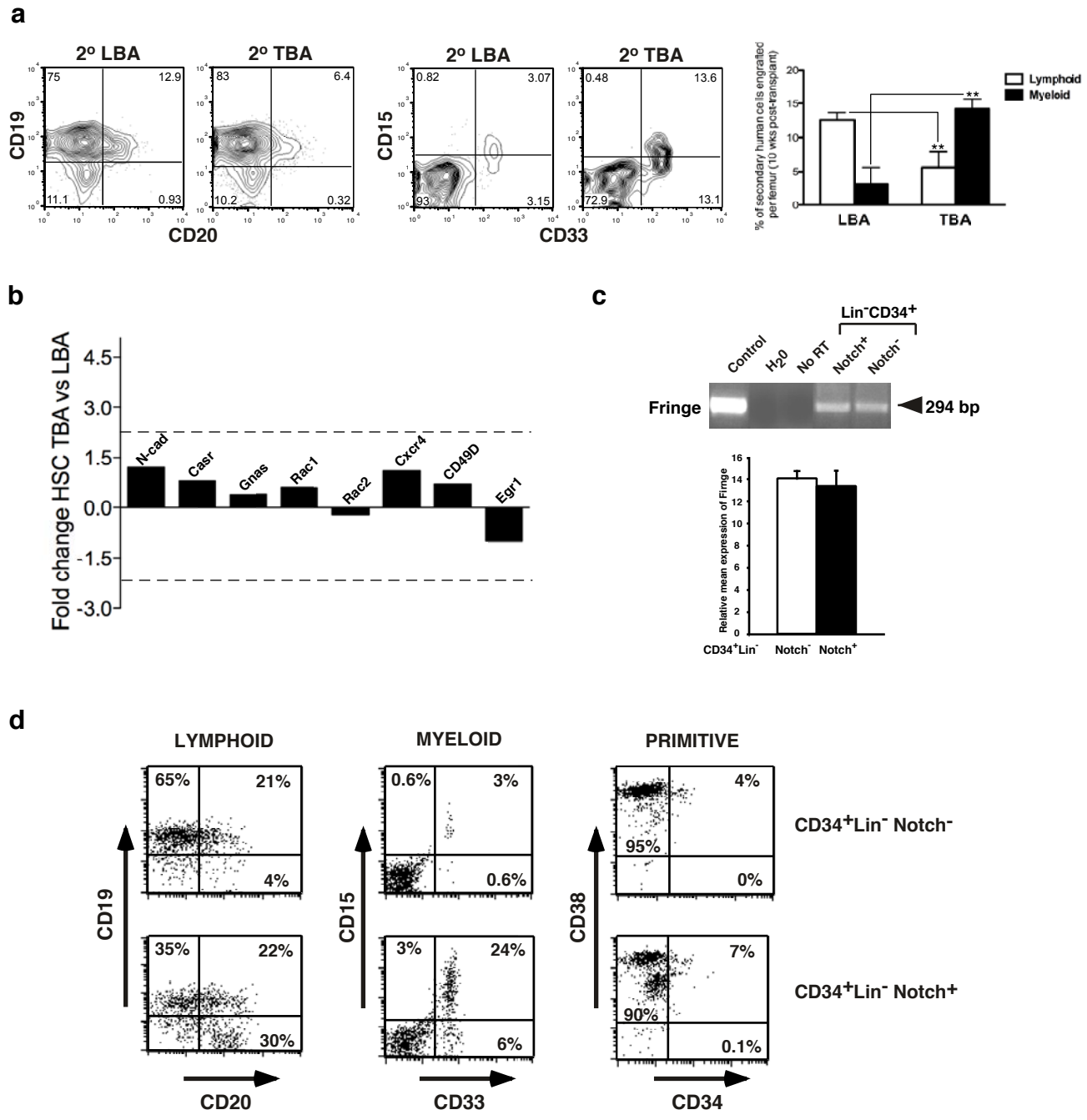

a
2º LBA
2º TBA
2º LBA
2º TBA
CD19
CD20
CD15
CD33
75
12.9
11.1
0.93
83
6.4
10.2
0.32
0.82
3.07
93
3.15
0.48
13.6
72.9
13.1
% of secondary human cells engrafted per femur (10 wks post-transplant)
Lymphoid
Myeloid
LBA
TBA
b
Fold change HSC TBA vs LBA
N-cad
Casr
Gnas
Rac1
Rac2
Cxcr4
CD49D
Egr1
c
Lin-CD34+
Control
H20
No RT
Notch+
Notch-
Fringe
294 bp
Relative mean expression of Fringe
CD34+Lin-
Notch-
Notch+
d
LYMPHOID
MYELOID
PRIMITIVE
CD19
CD20
CD15
CD33
CD38
CD34
65%
21%
4%
35%
22%
30%
0.6%
3%
0.6%
3%
24%
6%
4%
95%
0%
7%
90%
0.1%
CD34+Lin- Notch-
CD34+Lin- Notch+

Table A

Gene	Forward primer	Reverse primer
GAPDH	5'-TGCACCACCAACTGCTTAGC-3'	5'-GGCATGGACTGTGGTCATGAG-3'
Notch-1	5'-CAACGCCTACCTCTGCTTCT-3'	5'-ACATGCTCCCTGTGTAGCC-3'
Notch-2	5'-CGTCAGGGGTTAATTGTGAAAT-3'	5'-GGTGAGCAGACACAACTGTAGC-3'
Fringe	5'-CGTGGACGATGACAACTACG-3'	5'-ATGTAGCCGATGGTGCAGTC-3'

Table B

Gene	Forward primer	Reverse primer
Notch-1	5'-GATGCCAACATCCAGGACAACATGGG-3'	5'-GGCAGGCGGTCCATATGATCCGTGAT-3'
Notch-2	5'-ACATCATCACAGACTTGGTC -3'	5'-CATTATTGACAGCAGCTGCC-3'
Notch-3	5'-TGTCAGAGTAGCCCCTGTGTCAAC-3'	5'-CGTCAATGTTCACTTCGCAGTTC-3'
Notch-4	5'-TCGGGACTTCTGTTCAGCCAAC-3'	5'-GGGTCTCACACTCATCCACATCTTC-3'
Jagged-1	5'-GATCCTGTCCATGCAGAACG-3'	5'-GGATCTGATACTCAAAGTGG-3'
Jagged-2	5'-ACGGCTACTCGGGCAGGAAC-3'	5'-GCATGGCTTCCCTTCACACT-3'
Delta-1 (DLL1)	5'-TGCAGGAGTTCGTCAACAAG-3'	5'-TCCGTAGTAGTGTTCGTCAC-3'
Delta-3 (DLL3)	5'-TGGCCTGGCACCTTCTCTTT-3'	5'-TCTAGGCATCGGCATTCACC-3'
Delta-4 (DLL4)	5'-GCAAACCAGCACCCTCACAA-3'	5'-TTCTTGCATGGGGAGTGGTG-3'
Presenilin-1	5'-ACCCAGAGGAAAGGGGAGTA-3'	5'-TGGTGGTGTTCCAGTCTCCA-3'
Lunatic Fringe	5'-CGTGGACGATGACAACTACG-3'	5'-ATGTAGCCGATGGTGCAGTC-3'
Radical Fringe	5'-CCCGACCTCTCGGTGACA-3'	5'-GGTGGAAGCCTGTTCCCG-3'
HES-1	5'-AAGCTGGAGAAGGCGGACAT-3'	5'-GTGCCGCTGTTGCTGGTGTA-3'
β-Glucuronidase	5'-ACTATCGCCATCAACAACACACTCACC-3'	5'-GCTCTGAATAATGGGCTTCTG-3'
β-actin	5'-GCACAGAGCCTCGCCTTT-3'	5'-GGAATCCTTCTGACCCATGC-3'

Supplemental table 1. PCR primer sequences used for RT-PCR and qPCR

Chapter 4

Eradication of self-renewing human leukemia-initiating cells through niche replacement

Preamble

This chapter consists of an article prepared for submission to *Leukemia*. The authors are **Campbell CJ,** Levadoux-Martin M, Fiebig A, Foley R, Leber B and Bhatia M.

All experiments presented in the manuscript were designed by Dr. Mick Bhatia and myself. I performed all experiments, co-analyzed all of the data, assembled the figures, and wrote the manuscript. Dr. Marilyne Levadoux-Martin assisted with analyzing flow cytometry data. Aline Fiebig and Merry Wang provided technical assistance with *in vivo* experiments. Dr. Ronan Foley and Dr. Brian Leber both provided vital research reagents and contributed intellectual input. Dr. Mick Bhatia contributed significant intellectual input and support.

A great deal of clinical and experimental evidence suggests that the *in vivo* microenvironment has a role in human cancer (Lane et al., 2009). It has now been revealed that a large number human cancers display functional heterogeneity, where tumours are initiated and maintained by rare cells termed CSCs, due to their stem cell-like properties of self-renewal and robust proliferation (Dick, 2008). In the context of the emerging role of the *in vivo* microenvironment or niche in normal stem cell function, it has been suggested that CSCs are dependent upon the same *in vivo* niches as their normal tissue counterparts (Ho and Wagner, 2006; Lane et al., 2009). Furthermore, it has been

proposed that competition for niches, or displacement of normal stem cells from their supportive niches, is a significant factor in the disease process (Schuettpelz and Link, 2011). Given our findings in **Chapters 2 and 3** of this thesis regarding the role of the *in vivo* microenvironment in hHSC function, we sought to establish 1) whether normal hHSCs compete with LSCs for niche occupancy; 2) how this competition may play a role in the leukemogenic process; and 3) whether niche competition could be exploited for therapeutic targeting of self-renewing leukemic stem cells. Previous evidence in drosophila (Jin et al., 2008) and in the mouse (Bhattacharya et al., 2006; Wright et al., 2001) indicates that normal stem cells appear to compete for a limited number of niches to maintain tissue homeostasis, and clinical evidence from double CB transplants suggests that hHSCs appear to compete for engraftment in human patients. We therefore initially investigated whether highly purified hHSCs compete for niche occupancy *in vivo*, using the NS xenotransplant assay. Based upon the evidence described, we postulated that if hHSCs were competing for a limited number of niches, we would see a dose dependent engraftment effect when cotransplanting HSCs from two CB donors. Our *in vivo* findings revealed that such as dose-dependent competition for niches does occur *in vivo*, although HSCs from some donors appear to be dominant and have an engraftment advantage, revealing that some HSCs may possess properties that allow for an enhanced ability to occupy niches. We then went on to investigate whether purified normal hHSCs compete with primary LSCs isolated from human patients for niche occupancy *in vivo*. We found that in three independent patient-donor pairs, hHSCs dynamically compete with LSCs for niche occupancy *in vivo* as part of the leukemic initiation process. Through rigorous *in*

vivo serial transplantation studies, we observed that self-renewing LSCs could be eradicated in a dose-dependent manner using normal CB hHSCs. In remarkable similarity to clinical observations, we observed that the leukemic graft altered the myeloid/lymphoid differentiation axis in the CB graft; however, normal hematopoietic differentiation was restored in secondary recipients concomitant with the loss of self-renewing LSCs. Our work for the first time functionally demonstrates that primary LSCs compete with normal hHSCs for niche occupancy as part of the leukemia-initiation process, and further that this step can theoretically be targeted to eradiate LSCs and reduce the leukemic burden.

Eradication of self-renewing human leukemia-initiating cells through niche replacement

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Abstract

There has been paradigm shift in the field of cancer biology with the identification of extremely rare self-renewing cells that are capable of initiating human tumors known as cancer stem cells (CSCs). It has been postulated that CSCs such as those isolated from human acute myelogenous leukemia (AML) patients may be dependent upon a specific *in vivo* niche in order to initiate and maintain neoplasia, and therefore targeting the CSC niche could have unexploited therapeutic potential. We investigated whether AML leukemic stem cells (LSCs) isolated from primary human patient samples compete with normal human hematopoietic stem cells (HSCs) for occupancy of bone marrow (BM) niches *in vivo* as part of the neoplastic process. Co-transplantation of highly purified lineage-depleted (lin-) cord-blood (CB) cells with patient-derived leukemic blasts into NOD/SCID mice significantly reduced the ability of LSCs to initiate leukemic grafts in a dose-dependent manner in some samples tested. This decrease in engraftment occurred at the expense of increased normal HSC engraftment and led to a significant reduction or complete eradication of self-renewing LSCs as measured by serial transplantation. Furthermore, analogous to clinical findings, the leukemic graft altered hematopoietic differentiation within the HSC graft in primary recipients, but a normal differentiation pattern was restored in secondary recipients concomitant with the loss of LSC self-renewal. Our findings for the first time provide evidence that competition for niche occupancy between normal and transformed human stem cells *in vivo* is a critical step in the neoplastic process, and that this step can be targeted using normal adult stem cells. These findings have relevance both to the basic biology of human cancer and to

therapeutic strategies aimed at eradicating human CSC.

Introduction

Investigation into the etiology and pathogenesis of human cancer has been at the forefront of scientific research for over 25 years (Weinstein and Case, 2008). During this time, critical insight has been gained into the molecular biology underlying the process of neoplastic transformation (Hanahan and Weinberg, 2000; Luo et al., 2009), leading to the development of novel targeted therapies that have achieved success in treating several human malignancies such as chronic myelogenous leukemia (CML) and some types of breast cancer (Aggarwal, 2010). Despite these notable successes, to date many cancers continue to present a poor clinical prognosis (Jemal et al., ; Koch et al., 2010), and the mainstay of cancer treatment consisting of non-specific cytotoxic agents has changed little in the past quarter century (DeVita and Chu, 2008; Koch et al., 2010). The field of cancer biology continues to focus on unraveling the basic molecular mechanisms that govern the neoplastic process, and uncovering novel paradigms that may help to establish innovative approaches to cancer therapy.

The discovery that rare cells exist in some acute myelogenous leukemia (AML) patients that uniquely possess the ability initiate the disease represented a significant new concept in human cancer biology (Dick, 2008; Lapidot et al., 1994). Multiple studies have revealed that similar to normal hematopoiesis, human AML is organized in a cellular hierarchy, at the top of which are rare cells termed leukemic stem cells (LSCs) which initiate human AML and self-renew *in vivo* (Dick, 2008; Hope et al., 2004b). Subsequent studies have demonstrated the existence of similar cancer stem cells (CSCs) in many other types of human tumours (Ishizawa et al., 2010; Lapidot et al., 1994), revealing

functional heterogeneity in these neoplasms that mirrors the hierarchical organization of their normal counterpart tissues. While it is evident that the discovery of CSCs has intractably changed the fields of both human stem cell and cancer biology (Dick, 2008; Gupta et al., 2009), the clinical relevance of CSCs remains unknown. Clearly, a better understanding of how these cells function and contribute to the neoplastic process *in vivo* may hold unexploited therapeutic potential.

It is now evident that the *in vivo* microenvironment, also known as the niche, plays a critical and indispensable role in normal stem cell function (Morrison and Spradling, 2008). Studies utilizing multiple tissue types in both vertebrate and invertebrate systems (Issigonis et al., 2009; Jin et al., 2008; Morrison and Spradling, 2008) have revealed that the stem cell fate decisions of self-renewal, survival and differentiation are a function of cell-extrinsic signals within the local *in vivo* microenvironment. The best studied mammalian stem cell niche is that of the mouse hematopoietic system, where hematopoietic stem cells (HSCs) reside in proximity to both the BM endothelium and the endosteal surface of the bone, and receive molecular signals from neighbouring cells that are essential for the maintenance of HSC self-renewal and hematopoietic regenerative capacities (Moore and Lemischka, 2006; Morrison and Spradling, 2008; Taichman, 2005). Independent of the experimental system used, numerous studies have demonstrated that niche occupancy is a requisite step for normal stem cell function *in vivo*.

In the context of the emerging concept of niche and normal stem cell function, it has been postulated that like their normal counterparts, CSCs may also display a

functional dependence on an *in vivo* niche, and that the niche may be involved in the neoplastic process (Lane et al., 2009; McGovern et al., 2009). Experimentally however, little is known as to how primary human CSCs may be dependant upon a niche for their function. One study using primary leukemic blasts transplanted into NOD/SCIDγc (NRG) mice suggested that leukemic cells with the CD34+CD38- phenotype home to and reside at the bone marrow endosteum similar to normal human HSCs (Ishikawa et al., 2007), where they evade chemotherapy-mediated apoptosis Another study using the NALM-6 acute lymphoblastic leukemia (ALL) cell line showed that ALL cells create aberrant niches that recruit and sequester normal CD34+ hematopoietic cells (Colmone et al., 2008). While these phenotypic progenitor cell studies suggested an association between niche occupancy and the leukemogenic process, the significance of these findings to human stem cell function remains unknown. Clinically, it has been observed that when cells from more than one umbilical cord blood (CB) donor are co-transplanted into a single recipient, one cord blood seems to dominate, indicating that there may be a competition for niche occupancy and engraftment among normal HSCs (Ballen et al., 2007; Haspel and Ballen, 2006b; Haspel et al., 2008; Tse et al., 2008b; Wagner, 2009). In addition, a characteristic finding in myeloid leukemia patients is the loss of normal HSCs within the bone marrow (BM) at the expense of transformed leukemic blast cells and LSCs (Lane et al., 2009; Liesner and Goldstone, 1997). Together, these observations suggest that transformed LSCs may compete with normal HSCs for niche occupancy as part of the leukemogenic process, a step that could represent a converging point in the process of neoplastic transformation and an ideal target for therapeutic intervention.

Accordingly, we investigated whether normal HSCs and transformed LSCs compete for BM niche occupancy *in vivo* using functional xenotransplant assays for human stem cell regenerative and self-renewal function. By identification of HLA-A2 disparate human CB donors and leukemic patients, we were able to track individual donors when co-transplanted into the NOD/SCID recipient mice. We observed that highly purified human HSCs from different CB donors exhibited evidence of competition for engraftment, although similar to clinical findings, this competition was limited by the dominance of one of the CB donors in the graft. Subsequent co-transplantation of HSCs with primary LSCs revealed a dose-dependent competition for engraftment, with an incremental reduction in leukemic engraftment occurring at the expense of increased normal hematopoietic engraftment in some patient samples tested. Serial transplantation of co-transplanted grafts into secondary recipients revealed an eradication of self-renewing LSCs, and an increase in the self-renewing normal HSC fraction. Similar to clinical observations, the leukemic graft caused altered hematopoietic differentiation in the normal hematopoietic graft, favouring expanded lymphopoiesis and suppressed myelopoiesis, an effect unique to AML-CB cotransplants that was not seen in CB-CB cotransplants. The normal myeloid-lymphoid balance in the HSC graft was restored in secondary recipients in parallel with the loss of self-renewing LSCs. Our findings show that like normal HSCs, transformed LSCs are functionally dependent upon an *in vivo* niche, and that they compete with normal HSCs for niche occupancy as part of the leukemic initiation and maintenance process.

Results

Self-renewing human hematopoietic stem cells compete for the bone marrow niche *in vivo*

A major limitation to the use of human CB in clinical transplants is the inability to obtain sufficient numbers of HSCs from a single CB donor to effectively reconstitute recipient hematopoiesis (Haspel and Ballen, 2006b). Consequently, a number of trials investigating double CB transplants have been implemented, where cells from two CB donors are infused into a single human transplant recipient (Ballen et al., 2007; Eldjerou et al., 2010a; Haspel and Ballen, 2006b; Haspel et al., 2008; Tse et al., 2008b; Wagner, 2009). A consistent finding of such trials is that one CB donor tends to dominate the recipient graft, giving rise to the majority of hematopoietic cells within the graft (Haspel and Ballen, 2006b). It has been proposed that this dominance may be due a T-cell mediated graft-versus-graft effect (Eldjerou et al., 2010a) or alternatively, may represent a competition among rare HSCs for occupancy of BM niches that support stem cell hematopoietic regenerative and self-renewal capacity (Haspel and Ballen, 2006b).

In order to directly assess whether normal human HSCs compete for BM niche occupancy to initiate and maintain hematopoiesis, we co-transplanted highly purified lineage-depleted (lin-) CB cells from independent HLA-A2 disparate human donors into recipient NOD/SCID mice (figure 1 and table 1). The NOD/SCID xenograft model represents an ideal system for studying stem cell competition *in vivo,* as human grafts within these mice do not give rise to mature functional lymphocytes (Manz and Santo, 2009; Shultz et al., 1995) and are completely devoid of the T-cell lineage (Manz and

Santo, 2009; Shultz et al., 1995), thereby excluding any potential graft-versus-graft effect. In this xenograft model, human HSCs are defined functionally as Scid Repopulating Cells or SRCs, which represent extremely rare cells that uniquely possess the ability to reconstitute human hematopoiesis in recipient mice (Larochelle et al., 1996). The purified lin- population of human CB is highly enriched for SRCs (Bhatia et al., 1997b), and devoid of all mature lineage-specific hematopoietic cells. Co-transplantation of 50 000 lin- CB cells from two CB donors (100 000 total cells) was not additive, and resulted in engraftment levels equivalent to 50 000 lin- cells transplanted from single-donor controls (figure 2A-B and table 1, two independent CB donor pairs, four independent CB donors). This effect was unique to the lin- SRC fraction, as co-transplantation of the lin+ progenitor fraction with the lin- SRC fraction did not affect SRC engraftment levels (data not shown). This indicated that rare SRCs from these donors were competing for short-term engraftment in the bone marrow niche. To investigate whether this effect was dose-dependent, we titrated a high dose (200 000) of lin- CB cells against a low dose (50 000) of lin-CB cells in each donor pair. In one of the donor pairs, we observed that one CB donor was able to out-compete the other CB donor for engraftment in a dose-dependent manner, with a trend toward decreased engraftment in the low-dose CB donor (figure 2A, donors 1 and 2). A similar trend was noted in the frequency of CD34+CD38- cells in the bone marrow of recipient mice (supplementary figure 1), suggesting that the observed effect on human engraftment was directly related to the size of the SRC compartment *in vivo*, and not simply an expansion of the human graft. However, the second donor (donor 2) was not able to out-compete the first donor in a dose-dependent manner (figure 2A,

donors 1 and 2). For the second donor pair (figure 2B, donors 3 and 4), competition for engraftment did not appear to be dose-dependent (figure 2B and supplemental figure 2, donors 3 and 4), despite having 4-fold the number of cells injected (table 1). This indicated a donor-specific dominance effect, similar to that widely reported in human patients receiving double CB transplants, which was again reflected in the CD34+CD38- compartment *in vivo* (supplemental figure 2) (Haspel and Ballen, 2006b). Interestingly, we also observed that co-transplantation of 2 CB donors tended to enhance total levels of human chimerism (figure 2A-B, left panels). Overall, these findings indicated that in some cases, rare human CB SRCs compete for the BM niche to establish short-term engraftment *in vivo*, although similar to clinical observations in human patients (Eldjerou et al., 2010a; Haspel and Ballen, 2006b; Tse et al., 2008b) the ability to compete for engraftment appears to be heterogeneous and donor-specific.

Transformed leukemic stem cells compete with normal hematopoietic stem cells for the bone marrow niche to initiate leukemogenesis *in vivo*

Human AML is an aggressive hematopoietic malignancy where an impaired differentiation program results in the accumulation of leukemic blast cells in the bone marrow and peripheral blood of affected patients (Liesner and Goldstone, 1997). These patients are also affected by the loss of normal HSCs in their bone marrow, a poorly understood process that has been described as a competitive "crowding out" of HSCs from their bone marrow niches by transformed LSCs as part of the neoplastic process (Lane et al., 2009). Similar to normal human HSCs, transformed LSCs are defined

functionally in the NOD/SCID xenograft model as Scid Leukemia Initiating Cells or SL-ICs (McGovern et al., 2009), which are rare self-renewing cells that have undergone neoplastic transformation in human patients and can initiate leukemic grafts *in vivo*. In order to assess whether transformed SL-ICs compete with normal human SRCs for the bone marrow niche to initiate and maintain leukemogenesis *in vivo*, we co-transplanted lin- CB cells with HLA-A2 disparate primary leukemic blasts isolated from the peripheral blood of AML patient donors (figure 3 and tables 2-3; 3 independent patient samples matched with 3 independent CB donors). In two of three patient samples tested, we observed an incremental and dose-dependent decrease in CD45+ leukemic blast cells as lin- CB was titrated at increasing doses (table 2 and figure 4A-B). This decrease in leukemic blasts occurred at the expense of increased levels of normal SRC-derived hematopoietic engraftment (figure 4A-B). In the third patient sample however, which was an FAB M2 classified relapsed leukemia, we did not observe decreased leukemic engraftment when transplanted with increasing doses of lin- CB in primary recipients (figure 4C), as the leukemic fraction dominated the human graft. These findings together indicated that transformed human SL-ICs dynamically compete with normal CB SRCs for the bone marrow niche to initiate leukemogenesis *in vivo*, and similar to normal SRCs, the ability to compete for and establish short-term engraftment is heterogeneous and patient-specific.

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Although conventional chemotherapy can often reduce leukemic blast counts and induce clinical remission (<5% leukemic blasts in the marrow), many AML patients are affected by aggressive and refractory relapsed disease, which has been attributed to the inability of current therapies to target LSCs (2010; Koch et al., 2010; Milas and Hittelman, 2009). If rare LSCs are dependent on BM niches that support stem cell leukemogenic and self-renewal function *in vivo*, then loss of niche occupancy through competition with normal HSCs should affect LSC self-renewal capacity. To assess whether our phenotypic observations in primary recipients translated into a functional effect on SL-IC self-renewal capacity, we performed secondary transplants of co-transplanted grafts. In all leukemic patient samples tested, we observed a dose-dependent eradication of self-renewing SL-ICs at the expense of self-renewing normal SRCs, indicating that human SL-ICs compete with SRCs for BM niches that support long-term self-renewal capacity and maintenance of leukemogenesis (figure 5A-C). In the second leukemic patient sample tested, we observed complete eradication of all self-renewing SL-ICs by co-transplantation of CB-derived SRCs, as demonstrated by the abolition of secondary leukemic engraftment (figure 5B). Furthermore, even though decreased engraftment levels were not evident in primary recipients, co-transplantation of CB SRCs with the third leukemic patient sample nonetheless resulted in a trend toward reduced LSC self-renewal capacity compared to controls, highlighting the importance of the functional self-renewal assay versus phenotypic analysis in human stem cell biology (figure 4C and

figure 5C). Similar to observations in primary recipients, the loss of self-renewing LSCs occurred at the expense of increased SRC self-renewal capacity and normal hematopoietic engraftment (figure 5). In two of the patient samples tested, the leukemic blast counts were reduced to <5% blasts in the bone marrow of secondary recipients, which is the current definition of clinical remission for AML (Campana and Pui, 1995) (figure 5A and B). Overall, these findings revealed that rare human SRCs isolated from highly purified lin-CB samples can compete with transformed human SL-ICs for the bone marrow niche *in vivo*, leading to the eradication of the self-renewing LSC fraction in primary human patient samples.

Niche competition leads to restoration of normal marrow hematopoiesis

A clinical hallmark of human myeloid leukemia is the accumulation of primitive malignant human blasts in affected patients, and the concomitant loss or suppression of normal myelopoiesis (Liesner and Goldstone, 1997). We observed a similar effect in the bone marrow of NOD/SCID mice co-transplanted with both normal SRCs and transformed SL-ICs (figure 6A, left panel). Analogous to clinical findings, the human grafts in bone marrow of primary recipient mice exhibited a suppression of SRC-derived myelopoiesis, with a shift toward an expanded primitive lymphoid graft (figure 6A, left panel). Accordingly, the SL-IC- derived leukemic graft dominated the myeloid lineage *in vivo* at the expense of SRC-derived myelopoiesis (figure 6A and B, right panels), consistent with clinical findings in AML, and myelodyplastic syndrome (MDS) were the myeloid fraction in the marrow is dominated by an dysplastic or neoplastic clone (Tiu et

al., 2007). This effect was exclusive to SRC/SL-IC co-transplanted grafts, as normal SRC co-transplanted grafts did not show altered contributions to myeloid or lymphoid differentiation (supplemental figure 3), demonstrating that the leukemic graft uniquely possessed the ability to alter lineage differentiation in the HSC-derived graft as part of the leukemogenic process. In addition, when SRCs and SL-ICs samples were co-transplanted into recipient mice, there was a significant reduction in the size of the primitive self-renewing CD34+ fraction in both the SRC and SL-IC derived grafts, strongly supporting our functional data indicating that there was a competition among rare cells in the stem cell compartment for the bone marrow niche. Again consistent with our functional *in vivo* findings, there was restoration in the balance of SRC-derived hematopoiesis toward the myeloid lineage in secondary recipients, concomitant with the loss of self-renewing SL-ICs (figure 5 and figure 6B). Together, these findings demonstrated that by targeting leukemic stem cell niche occupancy we were able to reduce the self-renewing LSC fraction and shift the balance back toward normal hematopoiesis *in vivo*.

Discussion

Most human cancers remain incurable to date (Jemal et al.), and consequently much research effort continues to focus on unraveling the basic molecular biology underlying neoplastic transformation, and also on discovering novel paradigms that may lead to innovative cancer therapies. It is now clear that stem cells are dependent upon signals within the *in vivo* niche for their function, and it has been suggested that CSCs may also be dependent upon a niche to support their function (Lane et al., 2009; McGovern et al.,

2009). A stem cell niche, as originally defined by Raymond Schofield (Schofield, 1978a), is a supportive *in vivo* microenvironment that functions to maintain and control stem cell fate decisions such as quiescence, survival, and self-renewal. As there are no defined surface markers for individual human HSCs (Dick et al., 1997), a true analysis of the *in vivo* niche can only be accomplished through functional studies of stem cell tissue regenerative and self-renewal capacities. Our functional *in vivo* data provide the first evidence that primary human CSCs are indeed dependent upon an *in vivo* niche for their function, and compete with normal human stem cells for niche occupancy as part of the neoplastic process. We show that by targeting the ability of primary LSCs to occupy the BM niche using highly purified normal human HSCs, it is possible to eradicate the self-renewing LSC fraction in some cases. These findings have implications on the basic biology of normal and transformed human stem cells, and also toward the development of new therapies that target CSC niche occupancy.

It was previously not known whether the competitive or dominance effect seen in clinical CB blood transplants was immune-mediated, or rather due to rare HSCs competing for niche occupancy in the BM (Eldjerou et al., 2010a; Haspel and Ballen, 2006b; Tse et al., 2008b). Our findings indicate that at least part of this process is due to the competition of HSCs for BM niches that support stem cell tissue regenerative and self-renewal function. While it is almost certain that there are also other factors that influence engraftment kinetics in human recipients where immune cell maturation occurs normally, our data for the first time show that some component of this process is due to HSC competition for the BM niche. Although it was clear that competition occurred, it

was also evident from our findings that HSCs from some CB donors seem to be dominant, or have an enhanced ability to compete for niches. Whether this is simply due to a higher SRC frequency in these donors, or other genetic or epigenetic elements that may enhance the ability of these cells to occupy niches remains to be addressed. Certainly, such inherent differences would be important factors that may predispose cells to neoplastic transformation, and warrant further investigation in future studies.

It has been postulated that the phenomenon of suppressed hematopoiesis in leukemic and dysplastic syndrome patients is the result of HSCs being displaced from their BM niche by transformed LSCs in a competitive process (Lane et al., 2009; McGovern et al., 2009). Given the clinical and experimental evidence that normal HSCs compete for niche occupancy, it follows that these findings might be extended to transformed LSCs as part of the neoplastic process. Our findings show that rare LSCs dynamically compete with normal HSCs for the BM niche to initiate leukemogenesis *in vivo*, and that by using high numbers of normal HSCs, it is possible to replace LSCs in the BM niche with normal HSCs and re-establish hematopoiesis. These data imply both that BM niche occupancy is a critical and requisite step for LCS self-renewal function, and that LSCs can compete for the same niches that support normal HSC function. They also support a model in which stem cell niche occupancy is a stochastic process that is dependent to some degree upon the actual numbers of normal or transformed stem cells that are available to potentially occupy a niche and initiate hematopoiesis or leukemogenesis (see figure 2 in Chapter 5). Whether the observed alteration of normal hematopoiesis was due to a loss of the appropriate HSC niche, or to a change in the

availability of hematopoietic cytokines in the microenvironment due to the leukemic graft is not known, and will require additional investigation such as gene expression analysis. Nonetheless, our data show that replacing LSCs with normal HSCs in the niche is sufficient to restore the balance toward normal hematopoiesis (figure 6).

Our findings using HSC and LSC co-transplantation also serve to reiterate that stem cell function cannot be assessed by short-term engraftment levels and phenotypic studies, but must be analyzed in the context of long-term self-renewal capacity using serial passage. Although one relapsed patient sample seemed refractory to increased HSC numbers in primary recipients (figure 4, donor / patient pair 3), it was apparent in secondary grafts that a dose-dependent competition did occur, where high numbers of normal HSCs resulted in a trend toward a reduced long-term self-renewing LSC fraction (figure 5). This observation may have direct clinical relevance, as the self-renewing fraction is currently thought to be one of the factors responsible for resistance to therapy and leukemic relapse (Milas and Hittelman, 2009). It is known that the niche interactions of mouse HSCs are dictated not only by parameters such as HSC numbers (Purton and Scadden, 2007; Reya et al., 2003; Zhang et al., 2003b), but also by complex molecular interactions that occur between HSCs and cells that make up the niche such as endothelial cells and osteoblasts (Purton and Scadden, 2007; Reya et al., 2003; Zhang et al., 2003b). It is therefore possible that during the transformation process, LSCs acquire an enhanced ability to occupy BM niches and out-compete their normal HSC counterparts, leading to loss of HSCs and normal hematopoiesis. There is also clinical and experimental evidence that LSCs may alter the BM niche to disrupt normal hematopoiesis and HSC function

(Colmone et al., 2008). In combination with our findings, the evidence to date supports a multifactoral role for the niche in leukemogenesis that involves both an increased propensity of LSCs to occupy niches, and a change in the structure of the BM niche to favour the leukemogenic process.

There are hundreds of different mutations that have been found in AML patients alone (Lane et al., 2009). Our work and other studies are now revealing that the transformation process in neoplasms like AML is not entirely cell-autonomous, but involves additional non-cell -autonomous factors in the microenvironment (Colmone et al., 2008; Ho and Wagner, 2006; Ishikawa et al., 2007; Lane et al., 2009). The ideal therapy for such cancers is one that could target a common step, or converging point, in the transformation process regardless of the upstream mutations that are involved. Our study supports a therapeutic approach where the ability of a CSC to occupy a niche that supports stem function could be blocked, potentially by using a normal tissue stem cell counterpart, thereby targeting the neoplasm at the stem cell level and re-establishing normal tissue growth. Further work examining the efficacy of such a strategy in established neoplasms *in vivo* may lead the way to clinical trials utilizing this approach.

Materials and methods

Mice

We used NOD/Prkdc^{scid} (NOD/SCID)[25] and ß2 microglobulin knockout (NOD/SCID/B2^{null}) [26] mice. Mice were bred and maintained in the human Stem Cell and Cancer Research Institute (SCC-RI) animal barrier facility at McMaster University. All

animal procedures received the approval of the animal ethics board at McMaster University.

Patient samples

Human umbilical cord blood (CB) mononuclear cells (MNC) were isolated as described previously[13].MNCs were harvested from the peripheral blood of adult AML patients (AML-PB) as described[13], using centrifugation on Ficoll-Paque. All patient samples were part of the McMaster SCC-RI stem cell bank, and were obtained with the approval of local human subject research ethics boards at McMaster University.

Purification of primitive human hematopoietic cells

Lin- cells were purified from whole MNC populations by negative selection using a custom antibody cocktail kit (StemCell Technologies). In cases where the CD34+CD38- fraction was utilized, purification was obtained by using a custom CD38 negative selection antibody cocktail kit (StemCell Technologies).

Flow cytometry

Flow cytometry was performed on a FACScalibur flow cytometer or FACSaria cell sorter (Becton Dickenson). Anti-human CD45, CD33, CD19, CD34, CD38 and HLA-A2 were obtained from Becton Dickenson. Viability was assessed using the stain 7AAD.

In vivo hematopoietic repopulation assays

Human and mouse lin- cells and human AML MNC were transplanted into sub lethally irradiated (325 – 350 cGy,^{137}Cs) NOD/SCID or NOD/SCID/B2^{null} recipient mice by intravenous tail vein injection and analyzed at 6 – 8 weeks post –transplant for human or mouse donor engraftment as described previously[12]. Quantities of cells used for IV transplants are indicated in the text. All CB donor samples used contained equal frequencies of CD34+CD38- cells. For secondary transplants, whole BM isolated from primary recipient mice and transplanted into secondary recipient mice.

Data and statistical analysis

Flow cytometry data were analyzed using the software Flowjo (Tree Star). Statistically analysis and plots were made using the software Prism. The significance of any differences between groups was assessed using the Student's t-test, and $p < 0.05$ was considered to be statistically significant.

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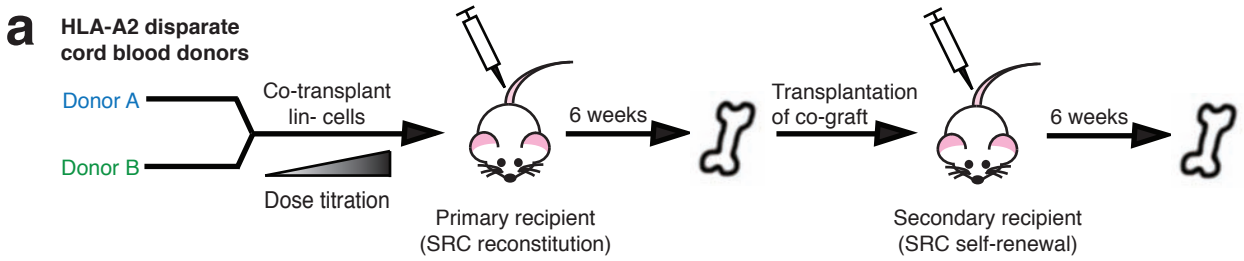
Figure 1. Experimental and flow cytometry gating strategies used to study *in vivo* niche competition between SRCs derived from different human cord blood donors

A) Experimental strategy used to study the effects of niche competition on human SRC regenerative and self-renewal capacities *in vivo*. Highly purified human lin- cells from two HLA-A2 disparate cord blood donors were co-transplanted into NOD/SCID recipient mice at equal doses, or were titrated against one another at increasing doses. At 6 weeks post-transplantation, contributions of each donor to multilineage human engraftment were analyzed. Grafts were then serially transplanted into secondary NOD/SCID recipients, and at 6 weeks the contribution of each donor to the human graft was analyzed to assess self-renewal capacity of SRCs derived from individual cord blood donors.

B) Flow cytometry strategy used to analyze the contributions of individual HLA-A2 disparate human cord blood donors to the human graft *in vivo*.


a
HLA-A2 disparate cord blood donors
Donor A
Donor B
Co-transplant lin- cells
Dose titration
6 weeks
Primary recipient (SRC reconstitution)
Transplantation of co-graft
6 weeks
Secondary recipient (SRC self-renewal)

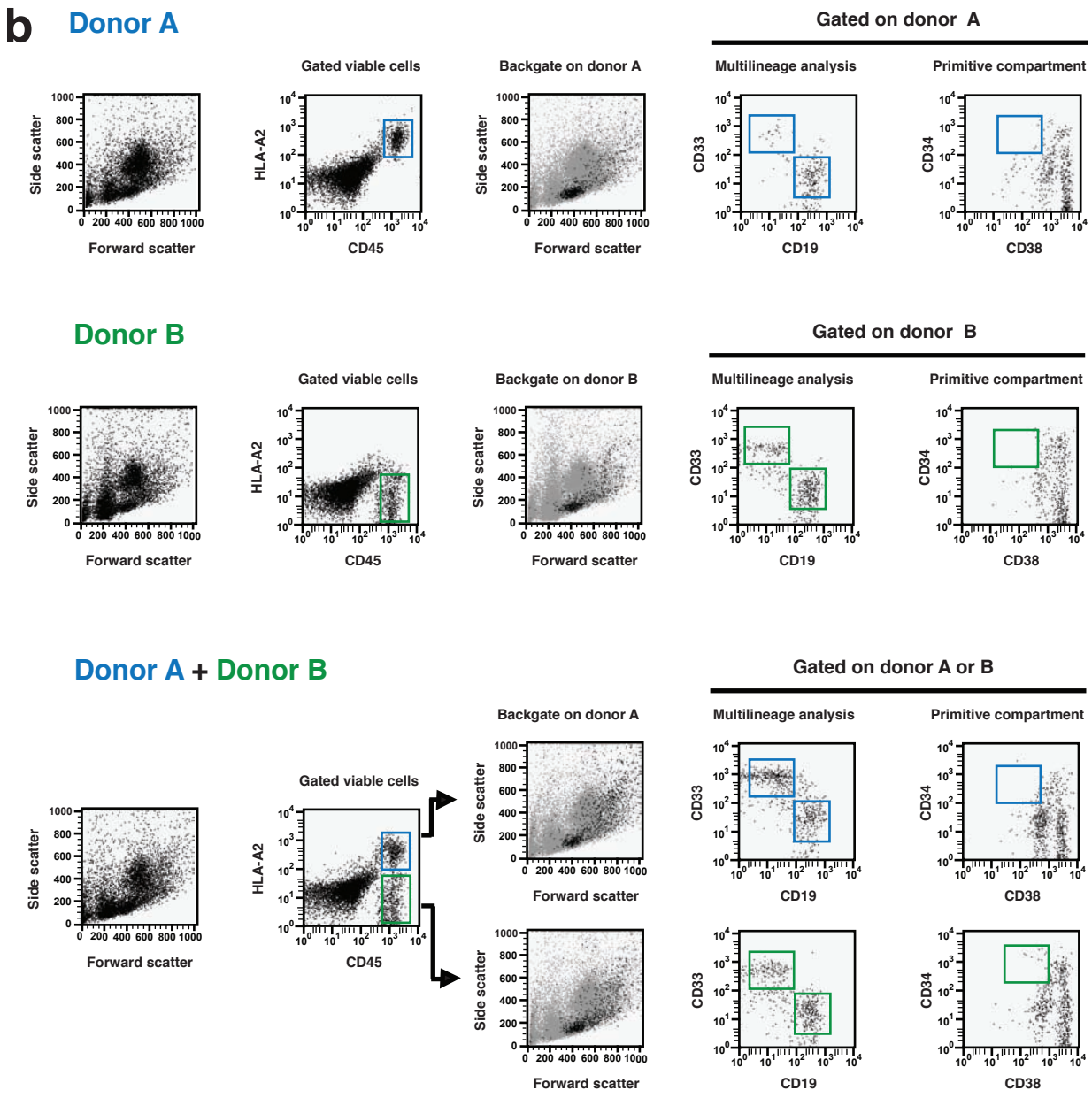

b
Donor A
Gated on donor A
Gated viable cells
Backgate on donor A
Multilineage analysis
Primitive compartment
Side scatter
Forward scatter
HLA-A2
CD45
CD33
CD19
CD34
CD38
Donor B
Gated on donor B
Backgate on donor B
Donor A + Donor B
Gated on donor A or B

Figure 2. Self-renewing human HSCs compete for the bone marrow niche *in vivo*

A-B) Average individual and relative contributions of each cord blood donor to the human graft in the bone marrow of NOD/SCID recipient mice for two independent donor pairs (4 independent donors). Mice were co-transplanted with SRCs from two HLA-A2 disparate cord blood donors, or with cells from each single donor alone and their bone marrow was analyzed for human CD45 and HLA-A2 expression 6 weeks later. Transplanted cohorts are labeled under each column on the x-axis. The colored bars represent the average individual donor engraftment (left panels) or relative contribution of each donor to the total human graft (right panels) in each respective cohort. The mean individual or total engraftment is significantly different relative to control group for donor 3* with $p < 0.05$. The mean individual or total donor engraftment is significantly different relative to control group for donors 1** and 2 or 4^{++} respectively with $p < 0.01$. n = 3-5 mice per group.

C = control; EQ = equal doses of both donors; H = high dose. The cell doses input into primary recipient mice for each cohort are outlined in **Table 1.**

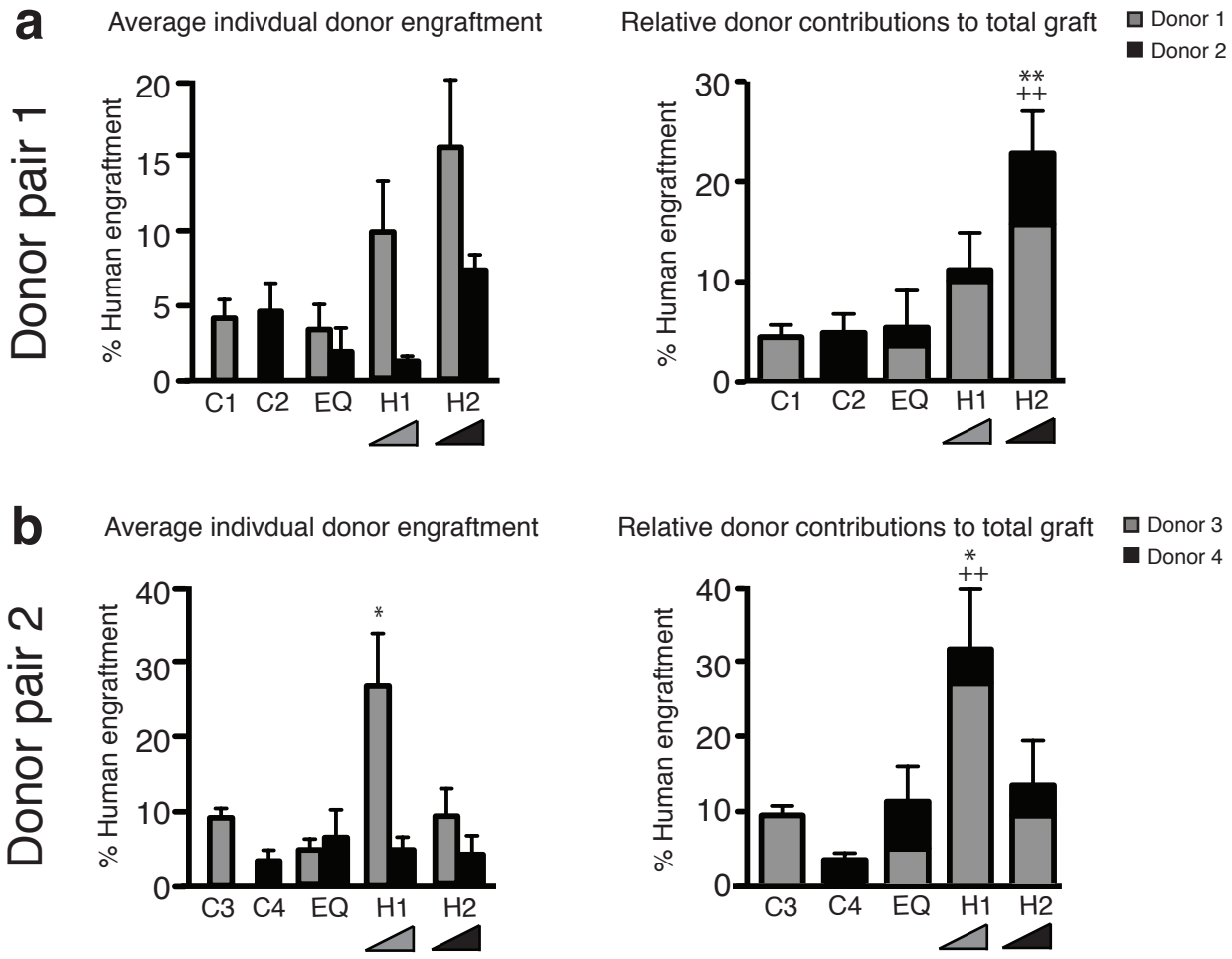
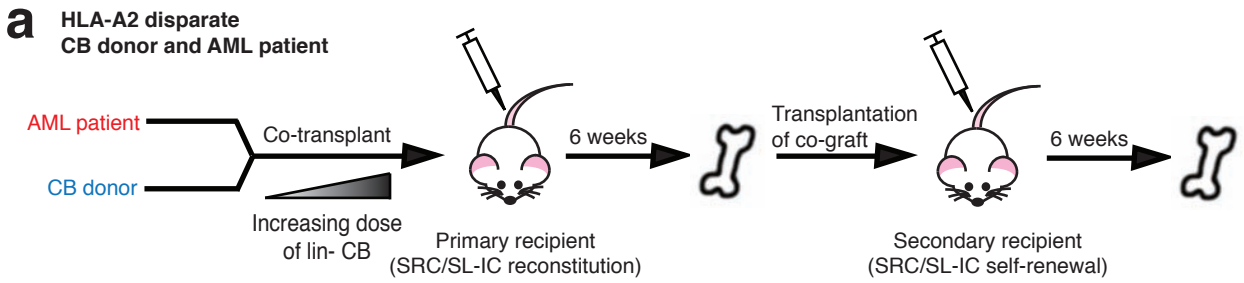

a
Donor pair 1
Average indivdual donor engraftment
% Human engraftment
20
15
10
5
0
C1 C2 EQ H1 H2
Relative donor contributions to total graft
Donor 1
Donor 2
30
20
10
0
**
++
b
Donor pair 2
Average indivdual donor engraftment
40
30
20
10
0
C3 C4 EQ H1 H2
*
Relative donor contributions to total graft
Donor 3
Donor 4
*
++

Figure 3. Experimental and flow cytometry gating strategies used to study *in vivo* niche competition between cord blood derived SRCs and SL-ICs isolated from primary patient samples

A) Experimental strategy used to study the effects of niche competition on human SRC and SL-IC regenerative and self-renewal capacities *in vivo.* Highly purified human lin- cord blood cells were co-transplanted with HLA-A2 disparate leukemic blasts into NOD/SCID recipient mice. Lin- cells were titrated against leukemic blasts at increasing doses, and at 6 weeks contributions of each donor to multilineage human engraftment were analyzed. Grafts were then serially transplanted into secondary NOD/SCID recipients, and at 6 weeks the contribution of each donor to the human graft was analyzed to assess self-renewal capacity of both SRCs and SL-ICs.

B) Flow cytometry gating strategy used to analyze the contributions of HLA-A2 disparate SRCs and SL-ICs to the human graft *in vivo*.


a
HLA-A2 disparate
CB donor and AML patient
AML patient
CB donor
Co-transplant
Increasing dose
of lin- CB
6 weeks
Primary recipient
(SRC/SL-IC reconstitution)
Transplantation
of co-graft
6 weeks
Secondary recipient
(SRC/SL-IC self-renewal)

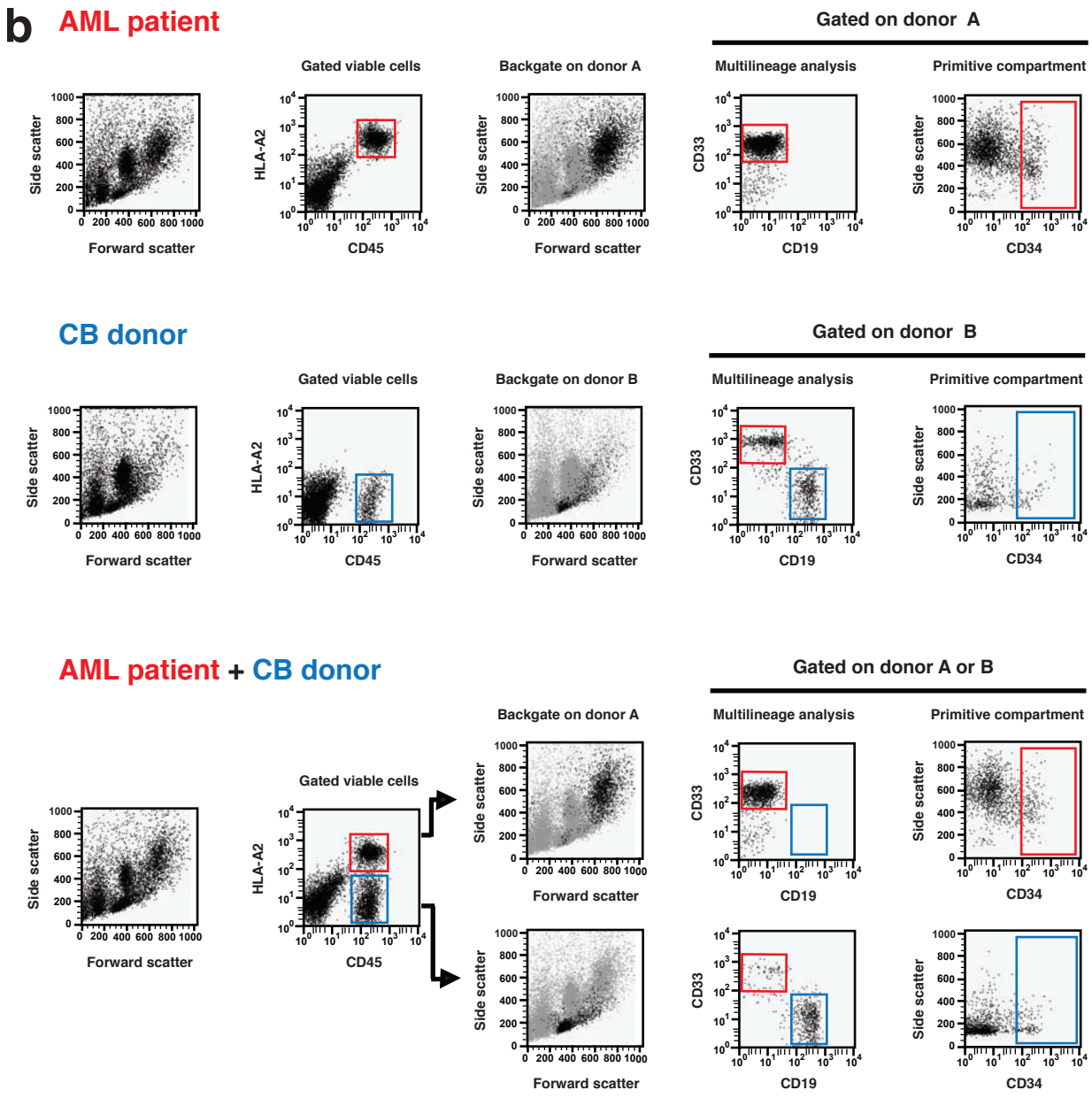

b
AML patient
Gated on donor A
Gated viable cells
Backgate on donor A
Multilineage analysis
Primitive compartment
Side scatter
Forward scatter
HLA-A2
CD45
CD33
CD19
CD34
CB donor
Gated on donor B
Backgate on donor B
AML patient + CB donor
Gated on donor A or B

Figure 4. Transformed leukemic stem cells compete with normal hematopoietic stem cells for the bone marrow niche to initiate leukemogenesis *in vivo*

A-C) Average individual total and relative contributions of SRCs and SL-ICs to the human graft in the bone marrow of NOD/SCID recipient mice for three independent donor pairs (6 independent cord blood donors and 3 independent AML patients). Mice were co-transplanted with HLA-A2 disparate SRCs and SL-ICs, or with cells from each single donor or patient alone and their bone marrow was analyzed for human CD45 and HLA-A2 expression 6 weeks later. Transplanted cohorts are labeled under each column on the x-axis. The colored bars represent the average individual donor engraftment (left panels) or relative contribution of each donor to the total human graft (right panels) in each respective cohort. In the right panels, each bar represents the total engraftment and relative engraftment of each donor in the bone marrow of a single recipient mouse. * indicates the mean individual engraftment is significantly different relative to control group for that donor with $p < 0.05$; ** indicates the mean individual donor engraftment is significantly different relative to control group for that donor with $p < 0.01$, and *** indicates the mean individual donor engraftment is significantly different relative to control group for that donor with $p < 0.001$. $n = 5 – 6$ mice per group.

CBL = low dose cord blood. CBH = high dose cord blood. The input into primary recipient mice for each cohort is outlined in **Table 2.**

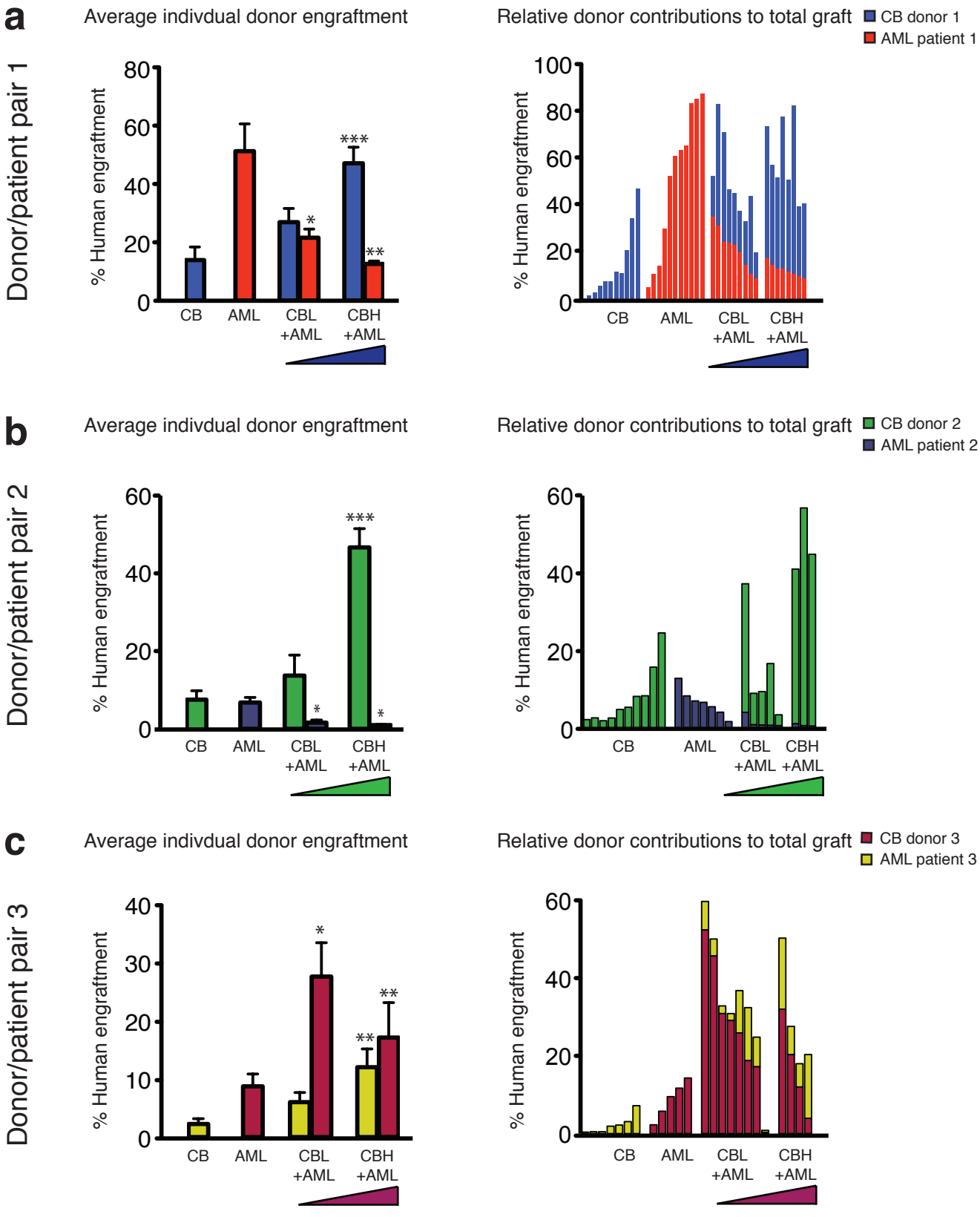

a
Average indivdual donor engraftment
Relative donor contributions to total graft
CB donor 1
AML patient 1
Donor/patient pair 1
% Human engraftment
CB
AML
CBL +AML
CBH +AML
b
Average indivdual donor engraftment
Relative donor contributions to total graft
CB donor 2
AML patient 2
Donor/patient pair 2
% Human engraftment
c
Average indivdual donor engraftment
Relative donor contributions to total graft
CB donor 3
AML patient 3
Donor/patient pair 3
% Human engraftment

Figure 5. Eradication of self-renewing human leukemia-initiating cells through niche replacement

A-C) Average individual total and relative contributions of SRCs and SL-ICs to the secondary human graft in the bone marrow of NOD/SCID recipient mice for three independent donor pairs (6 independent cord blood donors and 3 independent AML patients). Grafts from mice co-transplanted with HLA-A2 disparate SRCs and SL-ICs, or with cells from each single donor alone were serially transplanted into secondary NOD/SCID recipient mice and their bone marrow was analyzed for human CD45 and HLA-A2 expression 6 weeks later. The colored bars represent the average individual donor engraftment (left panels) or relative contribution of each donor to the total human graft (right panels) in each respective cohort. In the right panels, each bar represents the total engraftment and relative engraftment of each donor in the bone marrow of a single recipient mouse. * indicates the mean individual engraftment is significantly different relative to control group for that donor with $p < 0.05$; ** indicates the mean individual donor engraftment is significantly different relative to control group for that donor with $p < 0.01$. n = 4 -5 mice per group. The input into the primary recipients whose marrow was serially transplanted in each cohort is labeled under each column on the x-axis and is outlined in **Table 2.**

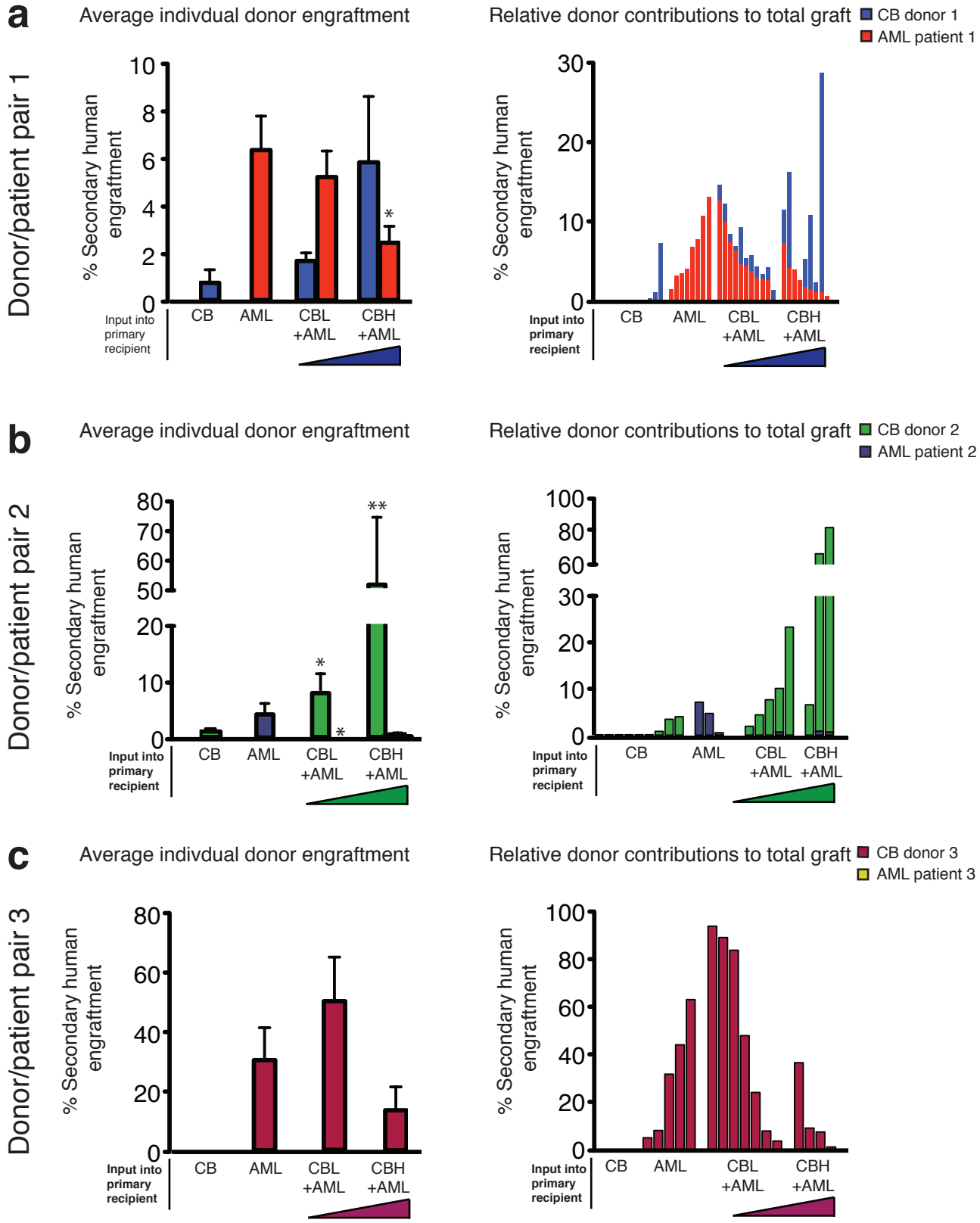
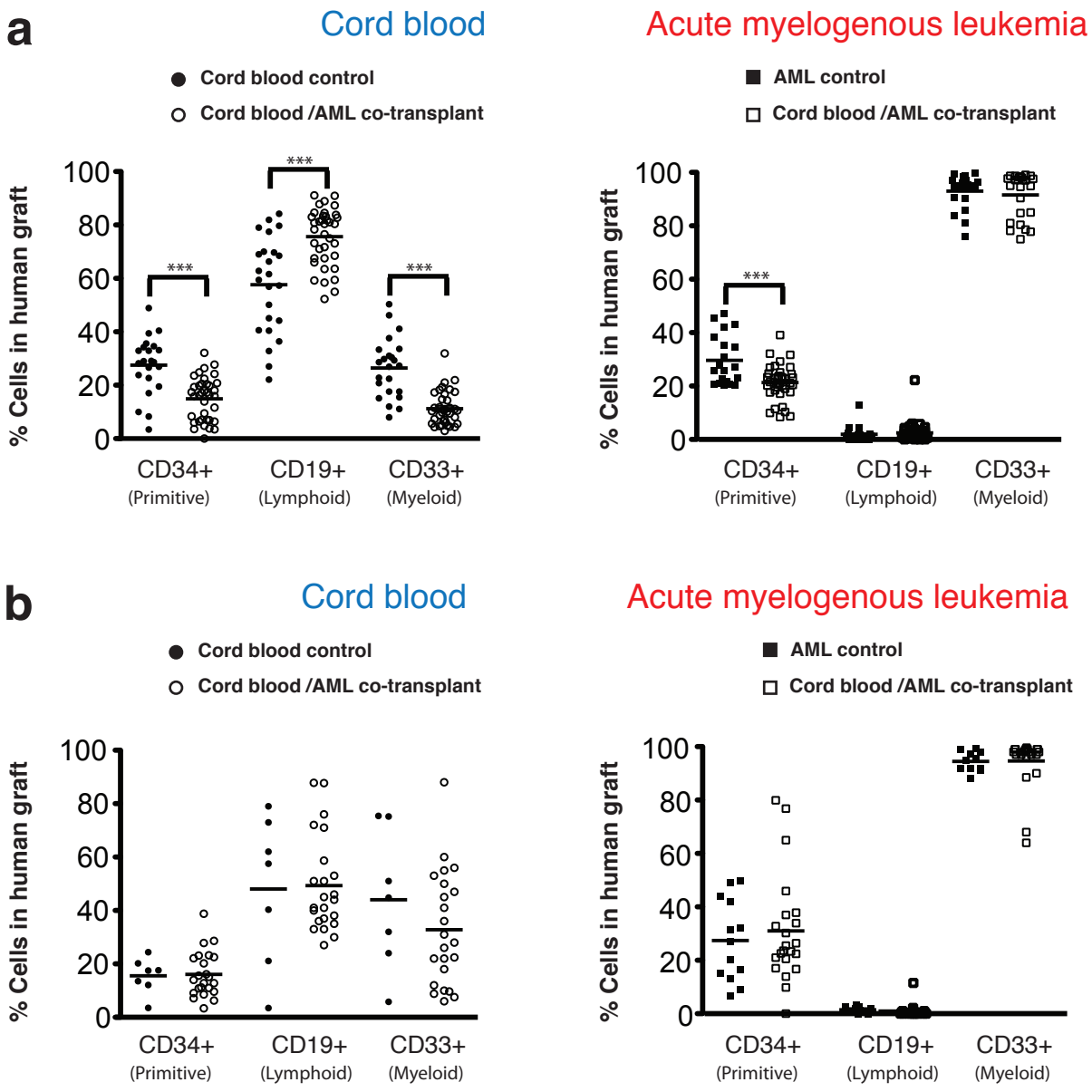

a
Average indivdual donor engraftment
Relative donor contributions to total graft
CB donor 1
AML patient 1
Donor/patient pair 1
% Secondary human engraftment
Input into primary recipient
CB
AML
CBL +AML
CBH +AML
b
Average indivdual donor engraftment
Relative donor contributions to total graft
CB donor 2
AML patient 2
Donor/patient pair 2
c
Average indivdual donor engraftment
Relative donor contributions to total graft
CB donor 3
AML patient 3
Donor/patient pair 3

Figure 6. Restoration of normal hematopoiesis through niche competition

A) Average individual contributions of SRCs and SL-ICs to the primitive (CD34+), lymphoid (CD19+) and myeloid (CD33+) lineages of the human graft in the bone marrow of primary NOD/SCID recipient mice. Mice were co-transplanted HLA-A2 disparate SRCs and SL-ICs, or with cells from each single donor alone and their bone marrow was analyzed for human CD34, CD19 and CD33 expression within the human (CD45+) graft. Each cord blood donor or AML patient sample was analyzed alone (control) or in a co-graft as indicated in the legends. Each dot represents the cord blood donor or AML patient sample derived multilineage engraftment in the bone marrow of a single transplanted mouse. * indicates the mean individual engraftment is significantly different relative to control group for that donor with $p < 0.05$; ** indicates the mean individual donor engraftment is significantly different relative to control group for that donor with $p < 0.01$, and *** indicates the mean individual donor engraftment is significantly different relative to control group for that donor with $p < 0.001$.

B) Average individual contributions of SRCs and SL-ICs to the primitive (CD34+), lymphoid (CD19+) and myeloid (CD33+) lineages of the human graft in the bone marrow of secondary NOD/SCID recipient mice. Grafts from primary recipient mice co-transplanted with HLA-A2 disparate SRCs and SL-ICs, or with cells from each single donor alone were serially transplanted into secondary NOD/SCID recipient mice and their bone marrow was analyzed for human CD34, CD19 and CD33 expression within the

human (CD45+) graft. Self-renewal in each cord blood donor or AML patient sample was analyzed alone (control) or in a co-graft as indicated in the legends. Each dot represents the cord blood donor or AML patient sample derived multilineage engraftment in the bone marrow of a single transplanted secondary recipient mouse.


a
Cord blood
Acute myelogenous leukemia
Cord blood control
Cord blood /AML co-transplant
AML control
Cord blood /AML co-transplant
% Cells in human graft
100
80
60
40
20
0
***
***
***
***
CD34+
(Primitive)
CD19+
(Lymphoid)
CD33+
(Myeloid)
b
Cord blood
Acute myelogenous leukemia
Cord blood control
Cord blood /AML co-transplant
AML control
Cord blood /AML co-transplant
% Cells in human graft
100
80
60
40
20
0
CD34+
(Primitive)
CD19+
(Lymphoid)
CD33+
(Myeloid)

	Donor A control	Control B control	Equal dose A and B	High dose donor A	High dose donor B
Donor A cell dose	**50 000**	**-**	**50 000**	**50 000**	**200 000**
Donor B cell dose	**-**	**50 000**	**50 000**	**200 000**	**50 000**

Table 1. Input cell doses used in cord blood co-transplantation experiments. Doses represent numbers of lin-cells used from each donor.

	Cord blood donor control	AML patient control	Low dose cord blood	High dose cord blood
Cord blood cell dose	**50 000**	**-**	**50 000**	**5×10^6**
Leukemic cell dose	**-**	**5×10^6**	**5×10^6**	**200 000**

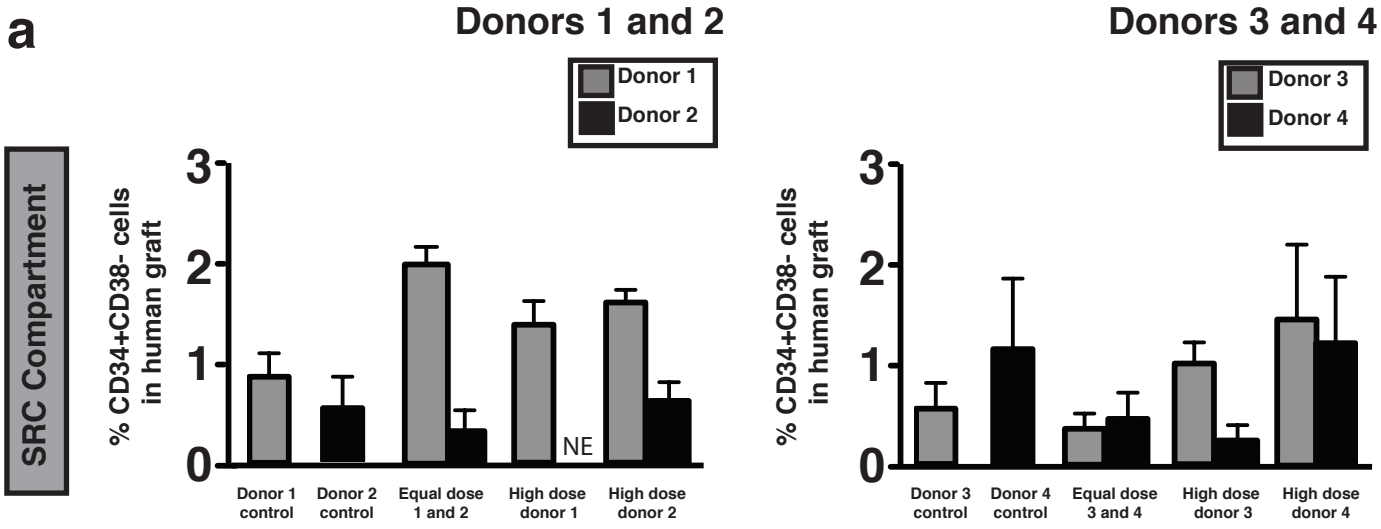
Table 2. Input cell doses used in cord blood / AML co-transplantation experiments.Doses represent numbers of lin-cells or AML peripheral blood cells used from each donor.

	Sample type	Cytogenetics	Mutations	AML subtype
Patient 1	Diagnosis	Normal	FLT3-ITD +	M4
Patient 2	Diagnosis/ antecedent MDS	Monosomy 7	-	M2
Patient 3	Relapse	Normal	-	M4

Table 3. Cytogenetics, molecular profiling and FAB classification of AML patient samples used.

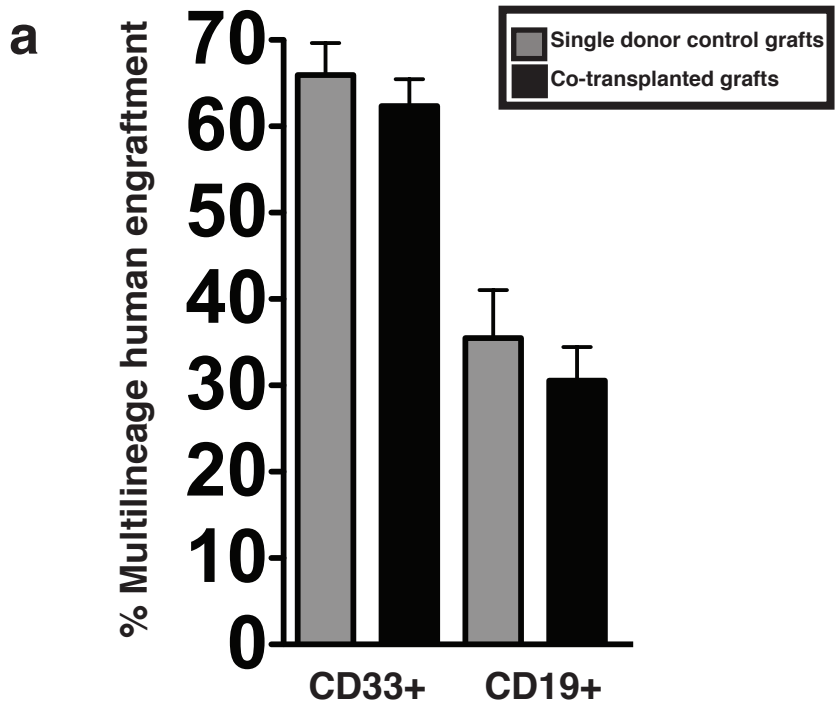
Supplementary figure 1. Competition and single cord blood dominance are reflected in the primitive lin-CD34+CD38- phenotype *in vivo*

Average individual contributions of each cord blood donor to the lin-CD34+CD38- compartment of the human graft in the bone marrow of NOD/SCID recipient mice for two independent donor pairs (4 independent donors). Mice were co-transplanted with SRCs from two HLA-A2 disparate cord blood donors, or with cells from each single donor alone. Transplanted cohorts are labeled under each column on the x-axis. The input into primary recipient mice for each cohort is outlined in **Table 1.** The colored bars represent the average individual donor engraftment in each respective cohort. n = 3-4 mice per group.


a
SRC Compartment
Donors 1 and 2
Donor 1
Donor 2
% CD34+CD38- cells in human graft
3
2
1
0
NE
Donor 1 control
Donor 2 control
Equal dose 1 and 2
High dose donor 1
High dose donor 2
Donors 3 and 4
Donor 3
Donor 4
% CD34+CD38- cells in human graft
3
2
1
0
Donor 3 control
Donor 4 control
Equal dose 3 and 4
High dose donor 3
High dose donor 4

Supplementary figure 2. Co-transplantation of cord blood SRCs does not affect lineage differentiation *in vivo*

Average individual contributions of each cord blood donor to the myeloid (CD33+) and lymphoid (CD19+) compartment of the human graft in the bone marrow of NOD/SCID recipient mice for two independent donor pairs (4 independent donors). Mice were co-transplanted with SRCs from two HLA-A2 disparate cord blood donors, or with cells from each single donor alone and their bone marrow was analyzed for human CD19, CD33 and HLA-A2 expression within the human graft (CD45+). The colored bars represent the average donor engraftment in controls or when co-transplanted with a second donor. $n > 20$ mice per group.


a
% Multilineage human engraftment
70
60
50
40
30
20
10
0
Single donor control grafts
Co-transplanted grafts
CD33+
CD19+

Chapter 5

Discussion

Experimental manipulation of human stem cells for therapeutic applications is often cited as the goal of stem cell research. Furthermore, with advent of the CSC theory, the field also commonly cites understanding the biology of CSCs as requisite step to eradicating malignancies at the stem cell level. It is now clear, after decades of research, that 1) hSSC do not retain their stem cell properties when manipulated *in vitro*; 2) phenotypically identical cell populations enriched for hSSCs demonstrate significant functional heterogeneity; and 3) *ex vivo* modification of hSSC fate decisions to expand stem cell numbers, direct differentiation, and enhance regenerative function to a clinically relevant extent has not yet been realized for any type of human stem cell. Together, these findings suggest that when stem cells are removed from their unique, highly complex and dynamic *in vivo* setting and placed in a comparatively simplistic *in vitro* setting, some essential regulatory elements that control their unique stem cell properties are lost. Experimental credence to this theory can be found in multiple vertebrate and invertebrate model systems that have demonstrated the existence of and defined the unique *in vivo* microenvironment, known as the stem cell niche, that functions to control stem cell fate decisions. Logically, the concept of the niche should extend to human stem cell function as well, but apart from the *in vitro* hESC system (Bendall et al., 2007), studies to date have not addressed the structure and function of the *in vivo* human stem cell niche in either the normal or transformed setting. Characterizing the human stem cell niche will

almost certainly be essential towards moving forward in the field of human stem cell biology and realizing the long-touted therapeutic potential of stem cells.

Until now, most studies investigating novel molecular regulators of human stem cell fate decisions either extrapolated from studies in non-human systems, or from *in vitro* studies that did not specifically address the role of these regulators *in vivo*. In some cases, there was little correlation between *in vitro* findings and *in vivo* function (Bhardwaj et al., 2001; Dahlberg et al., 2011; Delaney et al., 2010), and a number of regulators claimed to be essential for stem cell function *in vitro* have shown to be dispensable *in vivo* (Hofmann et al., 2009; Maillard et al., 2008; Orkin and Zon, 2008). This thesis addressed these discrepancies via an *in vivo* approach to pathways regulating human stem cell fate decisions that demonstrated the functional dependence of hSSCs on a specific molecular pathway in the *in vivo* setting, and specifically contrasted this with the function of this pathway *in vitro*. Subsequently, this thesis went on to characterize and define the nature of the human stem cell niche *in vivo*, in order to progress the *in vivo* human stem cell niche from a hypothetical concept to an experimentally defined entity with specific anatomic, cellular and molecular characteristics. Once the molecular and microenvironmental parameters of the *in vivo* hSSC niche were explored, it followed that the role of the niche in CSC function and human transformation should be investigated. This thesis showed that at least in some cases, CSCs are dependent upon the same *in vivo* niches as their normal tissue counterparts, and that this niche dependence can be exploited for therapeutic applications. Overall, the work presented here allows for several key

conclusions to be made regarding the role of the niche in regulating normal and transformed human stem cell fate decisions.

5.1 Molecular regulators of human stem cell fate decisions and the *in vivo* microenvironment

Studies addressing the molecular regulation of hSSC fate decisions to date have been limited by the lack of appropriate *in vivo* model systems and reconstitution assays (Dick et al., 1997; Pastrana et al., 2011; van Os et al., 2004). Such difficulties have led to great deal of research generated from model systems such as the mouse, as well as *in vitro* interrogation of hSSC regulatory pathways. In some cases, regulatory pathways deemed essential for stem cell function in the mouse did not translate into human systems (Czechowicz et al., 2007), and likewise pathways deemed essential for hSSC function *in vitro* have been found to be redundant in other model systems *in vivo* (Hofmann et al., 2009; Maillard et al., 2008; Orkin and Zon, 2008). The apparent inability to maintain and manipulate hSSC outside of their natural *in vivo* setting is in itself an important indicator of critical role that this *in vivo* setting has in regulating their function, and in the context of the aforementioned difficulties, necessitated a clear delineation that the regulatory pathways which control hSSC fate decisions function uniquely in an *in vivo* setting. In other words, if hSSC are indeed dependent upon non-cell-autonomous parameters in the niche which regulate their cell fate decisions, then 1) it is essential that molecular pathways which regulate hSSC function be identified and studied in an *in vivo* context;

and 2) these pathways may function uniquely within an *in vivo* setting when compared to an *in vitro* setting.

The Bcl-2 family member MCL-1 was an ideal target to be interrogated as a starting point for such an approach. As discussed above, the Bcl-2 regulatory pathway can be modulated through both cell-autonomous parameters such as DNA damage (Youle and Strasser, 2008), and non-cell autonomous signals such as morphogens and cytokines (Youle and Strasser, 2008). MCL-1 is a traditional pro-survival Bcl-2 family member (Adams and Cory, 2007), with a well-established role of a regulator of critical cell fate decisions such as apoptosis, transformation and differentiation in human cell lines (Yang et al., 1996), and appears to have a unique function beyond survival in primitive cell populations and in embryonic development (Opferman et al., 2005b). Therefore, through a combined *in vivo* gene knockdown approach we sought to establish 1) whether MCL-1 was a regulator of hHSC function within the *in vivo* microenvironment; and 2) whether the role of MCL-1 in hHSC was unique to this *in vivo* setting, thereby demonstrating that novel molecular pathways regulating human stem cell fate decisions could be identified in and shown to function uniquely in the context of non-cell-autonomous parameters within the *in vivo* microenvironment. Our findings revealed MCL-1 as an essential, indispensable regulator of human hHSC self-renewal *in vivo*, and showed that a functional dependence on MCL-1 *in vivo* hierarchically delineated true hHSCs from primitive HPCs **(Chapter 2)**. Identification of this unique and previously unappreciated role for MCL-1 as a regulator of human stem cell self-renewal was entirely dependent upon the *in vivo* setting, as previous *in vitro* studies in the mouse system (Opferman et al.,

2005b) (Opferman et al., 2003) as well as our *in vitro* data clearly indicated that MCL-1 functions uniquely and distinctly *in vivo* as opposed to *in vitro.* It is not surprising that MCL-1 exhibited a unique and novel role as a regulator of human stem cell self-renewal *in vivo,* as a previous study in the mouse system showed that knockout of MCL-1 in mouse blastocyst cells *in vivo* led to developmental arrest at the blastocyst stage as opposed to increased apoptosis (Rinkenberger et al., 2000). Again, this was in contrast to the *in vitro* phenotype of MCL-1 knockout in mHPCs, which rapidly led to massive apoptosis in these cells (Opferman et al., 2005b) (Opferman et al., 2003). In our study, knockdown of MCL-1 hHPCs *in vitro* revealed a trend towards decreased survival, although this was not significant (**Chapter 2)**. Only when hHSCs were challenged to regenerate hematopoiesis *in vivo* was the unique role of MCL-1 in regulating stem cell self-renewal apparent **(Chapter 2).** Further work investigating the mechanistic links between non-cell-autonomous signaling such as cytokines and morphogens within the *in vivo* microenvironment and the Bcl-2 family axis will help to fully delineate its role in human stem cell function *in vivo*. Insight into this role can be found in recent studies demonstrating regulation of MCL-1 by GSK-3, providing a potential relationship between MCL-1 function and a pathway implicated in stem cell self-renewal in the niche (Maurer et al., 2006a; Rattis et al., 2004; Trowbridge et al., 2006c). At the moment, the work presented in this thesis has revealed that it is essential to study molecular regulation of human stem cell fate decisions within the *in vivo* microenvironment, and further has provided a proof-in-principle that human stem cell fate decisions are controlled in a non-cell-autonomous manner within this unique *in vivo* setting.

5.2 Constructing the human stem cell niche *in vivo*

Prior to the work presented in this thesis, the *in vivo* human stem cell niche was a hypothesis. There have been no studies either delineating the unique regulation of hSSC fate decisions in the *in vivo* microenvironment **(Chapter 2)**, or characterizing the cellular, molecular and anatomical parameters that constitute a true human stem cell niche *in vivo*. Our current knowledge of the human stem cell microenvironment is derived from *in vitro* studies of hESCs, which clearly reveal an essential role for a supportive microenvironment in the regulation of hESC fate decisions (Bendall et al., 2007). We therefore sought not only to establish that molecular pathways regulating hSSC fate decisions functioned distinctly *in vivo* **(Chapter 2)**, but further to begin characterizing the parameters that define a human stem cell niche *in vivo* **(Chapter 3).** Examination of previous studies characterizing stem cell niches in drosophila and murine model systems elicits several salient features of stem cell niches that appear to be evolutionarily conserved. Firstly, there appear to be multiple, physically distinct types of niches, comprised of different cell types in both systems (Kiel et al., 2005; Lo Celso et al., 2009; Xie and Spradling, 2000). In drosophila, unique roles for cap, hub and escort cells in stem cell function have been described (Xie and Spradling, 2000). In mice, several types of niches have been described, including the EN (Calvi et al., 2003; Zhang et al., 2003), the VN (Kiel et al., 2005) and niches that include both endosteal and vascular constituents (Kiel et al., 2005; Lo Celso et al., 2009; Xie et al., 2009), although the functional implications of these distinct niches is not understood (Kiel and Morrison, 2008). Secondly, the developmental morphogen signaling pathways such as WNT and Notch

have been implicated in regulating stem cell function within the niche in both of these systems (Carlesso and Cardoso, 2010; Morrison and Spradling, 2008; Weber and Calvi, 2010). Together these model systems provided a tangible starting point for investigation of the *in vivo* hSSC niche, taking the hypothesis that these features would be continue to be conserved in the human stem cell niche.

5.2.1 The *in vivo* microenvironment and human stem cell fate decisions

Imaging studies of engrafted hHPCs in the NS microenvironment revealed a distribution of primitive hHPCs to the bone marrow endosteum (EN), the vascular endothelium (VN) as well as the interstitium between these areas (interniche, IN) **(Chapter 3).** This observation for the first time provided evidence of the *in vivo* distribution of primitive hHPCs, and revealed remarkable pattern of conservation from the murine system. We additionally found that this pattern of micro-distribution between individual cell types was set within a larger framework of anatomical location. Our imaging studies revealed that the anatomical location of hHPCs within the BM had a profound influence on their distribution pattern at the cellular level, as well as on their molecular and gene expression patterns. hHPCs preferentially distributed to the trabecular region of the bone (TBA) as opposed to the cortical long bone region (LBA) **(Chapter 3)**, and were in close proximity with osteoblasts expressing the Notch ligand Jagged-1, whereas in the LBA, hHPCs interacted with non-Jagged-1 expressing osteoblasts. Recent imaging studies in the mouse hematopoieitic system have also reported that that mHPCs home to the endosteal surface of the TBA, again revealing a high level of conservation between the mouse and

human systems (Lo Celso et al., 2009; Xie et al., 2009). These analogous findings in the mouse and human systems suggest that anatomical location constitutes an additional and previously unappreciated layer of complexity to the *in vivo* stem cell niche, where localization within the TBA versus the LBA can influence both distribution of hHPCs between cell types into individual micro-niches (EN versus IN versus VN), as well as the molecular signaling which regulates hHPC function within the niche (Jagged-1 expression in osteoblasts).

As discussed throughout this thesis, stem cells can only be measured by their function, which in the case of HSCs is to generate long-term hematopoiesis *in vivo*. A niche, by definition, regulates stem cell fate decisions *in vivo* (Schofield, 1978). Using the NS assay, we demonstrated that there was significant enrichment of SRCs within the TBA, and that SRCs with enhanced *in vivo* regenerative capacity could be prospectively isolated based upon a Notch ligand binding propensity **(Chapter 3).** Consistent with these functional observations, we further found that Notch pathway target genes, as well as other genes previously implicated in stem cell quiescence and self-renewal, demonstrated increased expression in the TBA **(Chapter 3).** Together, these findings define and characterize specific anatomic, functional, and molecular parameters that regulate human stem cell fate decisions *in vivo*. Therefore, these characteristics describe a true human stem cell niche as a functional entity, and move the human stem cell niche from a theoretical concept to an experimentally defined system. The strong degree of agreement between these and previous findings in the mouse system suggest that additional paradigms derived from the mouse will have high relevance to the human stem cell niche.

Furthermore, investigating how altering the composition of the microenvironment may influence the expression of both genes involved in non-cell-autonomous signaling in the niche such as cytokines and morphogens, and of pathways regulating stem cell fates *in vivo* such as Notch, WNT and the Bcl-2 family will help to unravel the dynamics of signaling in normal homeostasis and under conditions of stress or disease.

5.2.2 Reevaluating the concept of niche

In the mouse system it has been hypothesized that distinct regions such as the endosteum and vascular endothelium may represent unique types of niches (Calvi et al., 2003; Kiel et al., 2005; Sugiyama et al., 2006; Zhang et al., 2003), although functionally this theory has yet to be validated. There has been significant debate as to whether these micro-domains represent unique niches, or rather represent a number of functionally redundant niches that serve to support and maintain stem cell function (Kiel and Morrison, 2008). Of note, while there have been many elegant genetic and imaging studies investigating the niche in the mouse hematopoietic system, the link between bone marrow location and *in vivo* regenerative function has yet to be investigated in the mouse. Until the capability to purify HSCs at the single cell level from micro-domains such as EN or VN exists, the question of whether there is any functional difference between, or relevance to, these microdomains will remain unresolved. However, the work in this thesis has revealed that in the human system, the concept of EN versus VN may not have the functional importance previously suggested. We have demonstrated that the *in vivo* human stem cell niche is largely a function of anatomical location versus specific cell type, and therefore

this may be a more relevant framework within which the human niche can be modeled. From this perspective, the actual location of a cell type within a tissue, versus its specific identity, is a critical determining factor in its ability to function as a niche constituent. In this context, a stem cell niche would be viewed as both a general region within a tissue that regulates stem cell function, as well as the supportive cell types *per se* which reside in those locations. This notion is evident in a number of mathematical approaches that have been used to examine stem cell behaviour at the population level (Mangel and Bonsall, 2008; TILL et al., 1964; Vogel et al., 1969). In these models, the concept of stemness is viewed as a function of a cell population in a tissue, versus a set of pre-determined epigenetic characteristics of individual cells (Lander, 2009). In this case, a number of "potential" stem cells could acquire stem cell properties based upon signaling and feedback control within their infinitely complex *in vivo* microenvironment, which encompasses both cellular and anatomical parameters (Lander, 2009). Furthermore, the BM is comprised of a large number of other cell types in addition to osteoblasts and endothelial cells, such as fibroblasts, MSCs, and adipocytes (Clarke, 2008). Again in the mouse system, studies have begun to reveal roles for these other cell types comprising the BM microenvironment in controlling HSC fate decisions. Overall, together with the findings presented in this thesis, it appears that the niche is a heterogeneous and multidimensional entity, whose complexity lies in both individual cell types as well as the broader regional characteristics of the tissue itself.

5.2.3 The niche and *ex vivo* manipulation of human somatic stem cells

The most important limitations to the *ex vivo* manipulation of hSSCs to date have been 1) the rapid loss of stem cell properties and apoptosis when these cells are removed from their *in vivo* setting; and 2) the significant functional heterogeneity found among phenotypically homogeneous cell populations enriched for stem cell function. The findings presented in this thesis help to address both of these issues. Our combined *in vivo* imaging and functional data show that the cell fate decisions of hHSCs are directly influenced by their anatomical location *in vivo* and the unique molecular interactions that occur in this setting **(Chapter 3)**. Moreover, phenotypically identical cell populations enriched for hHSCs isolated from different *in vivo* locations (TBA versus LBA) demonstrated unique functional properties and gene expression patterns **(Chapter 3).** In other words, functional heterogeneity in these phenotypically identical cell populations was related to *in vivo* niche propensity. These findings imply that the niche must necessarily have a central role in future efforts to purify and manipulate hSSCs, and efforts to modify hSSC fate decisions will only be realized through understanding and better characterizing their niche. Functional heterogeneity may therefore be better understood by viewing a human stem cell as a fixed tissue cell (Schofield, 1978), which only functions as a stem cell when residing in an appropriate niche within the tissue supports its function (Schofield, 1978). Depending on the molecular characteristics of its microenvironment, a stem cell will exhibit unique cell fate decisions, or in other words, demonstrate functional heterogeneity. This notion is further supported by findings in mouse HSC biology, where it has been shown that 1) HSCs constantly recirculate in and

out of niches *in vivo* (Wright et al., 2001); and 2) regardless of the maximum dose of HSCs that are injected into an irradiated recipient, steady state hematopoiesis is always achieved (Jordan and Lemischka, 1990; Smith et al., 1991). In the context of the findings in this thesis, this would suggest that stem cell maintenance of tissue homeostasis is a function of niche availability, as well as the molecular and gene expression properties of the niche components *per se* in the context of a defined anatomical location *in vivo*. Together, these non-cell-autonomous parameters would combine with cell-autonomous gene expression parameters to define the functional heterogeneity inherent cell populations enriched for hSSC.

5.2.4 A working model of the human hematopoietic stem cell niche

The findings in Chapters 1 and 2 of this thesis allow for the construction of a preliminary model of the hHSC niche *in vivo* (figure 1). In this model, hHSCs reside in both the TBA and LBA of the bone, but are enriched in the TBA. Within both of these anatomic microenvironments, hHSCs are found in micro-domains or "micro-niches" in close proximity to the bone marrow endosteum (EN), the vascular endothelium (VN) and within the interstitium (IN) (figure 1). hHSCs are more frequently found close to the EN and IN within the TBA, and uniquely interact with Jagged-1 expressing osteoblasts in the EN of TBA via Notch receptors (figure 1). Here, gene expression programs controlled through the Notch pathway and other yet-to-be identified pathways function to regulate hHSC fate decisions such as self-renewal, survival, differentiation and quiescence in the niche. Differentially expressed genes in TBA versus LBA identified in **Chapter 3**

provide evidence of these gene expression programs, such as the cell cycle regulators PTEN and cdc42. Regulators of hHSC fate decisions that function uniquely *in vivo*, such as MCL-1, are also candidates for regulation within the niche. Other pathways identified as playing a critical role in HSC homing to and retention in the niche, such as CXCR4/SDF-1 and integrin/cadherin interactions, remain to be fully characterized as to the precise nature of their role in the human HSC niche in the context of both cell type (endothelial versus vascular) and anatomical location (TBA versus LBA) through additional *in vivo* studies. As discussed above, in the model proposed here, the niche is highly complex and dynamic microenvironment, consisting of multiple layers that encompass anatomical location, cell types, and molecular and gene expression parameters. This would support a model where heterogeneity in hSSC populations is better understood as niche heterogeneity, versus intrinsic epigenetic cellular heterogeneity. Although this work provides early model of an *in vivo* human stem cell niche, the possibility of a pathway being necessary versus sufficient to support stem cell function remains. As in the mouse system, it may be found that ablation of cell types or disruption of molecular pathways implicated as being niche constituents may turn out to be functionally redundant, as appears to be the case for N-cadherin, Hh and WNT signaling in the mouse system (Hofmann et al., 2009; Kiel et al., 2009; Maillard et al., 2008; Orkin and Zon, 2008). However, unlike studies in the mouse system, we have provided a proof-in-principle for the specific signals found to be important for hHSC fate decisions in the niche. Namely, we have used the Notch signaling axis to prospectively isolate hHSCs with enhanced regenerative function, thereby demonstrating the functional

relevance of this signaling pathway in the niche to hHSC fate decisions. Such rigorous *in vivo* functional approaches in the future will help to clarify functionally redundant versus essential pathways in the stem cell niche, regardless of the model system used.

5.3 The niche in cancer

Evidence that the *in vivo* microenvironment has functional role in the transformation process has existed for many years in the field of cancer biology. Early hematology studies revealed that BM stromal cells isolated from leukemic patients demonstrated altered morphology and physiology compared to those of healthy patients (Castro-Malaspina et al., 1982; Nagao et al., 1983), suggesting that the tumour cells may alter the physiology of their microenvironment. More recent studies have shown that human breast cancer cells recruit hMSCs to sites of metastasis *in vivo*, and alter their physiology to support tumour growth and enhance metastatic potential (Karnoub et al., 2007), and that human prostate cancer cells may disrupt the HSC niche *in vivo* (Shiozawa et al., 2011a). Years of clinical and pathological observations in leukemias, myelodysplastic syndromes, and myeloproliferative disorders have shown that in these hematological malignancies 1) the dysplastic, neoplastic or hyperplastic cells "fill up" the BM microenvironment, which is where HSCs normally reside; 2) HSCs and hematopoiesis concomitantly disappear from the BM, sometimes reappearing at extramedullary sites such the liver and spleen; and 3) when hematological malignancies such as AML enter clinical remission, HSCs and hematopoiesis reappear in the BM. Other recent work has shown that an AML cell line creates an aberrant microenvironment *in vivo* which recruits

and functionally inhibits hHPCs in the BM (Colmone et al., 2008; Sipkins et al., 2005). Elegant imaging studies in *drosophila* have demonstrated that dysplastic stem cells compete with normal GSCs for niche occupancy *in vivo*, eventually leading to tumour formation (Jin et al., 2008). These findings together strongly support the hypothesis that microenvironment plays a role in the transformation process, and that this process may involve a competitive displacement or sequestration of normal stems cells from their physiologic niche by dysplastic or neoplastic cells. The evidence presented in this thesis has functionally and phenotypically described the hHSC niche *in vivo* **(Chapters 2 and 3)**, and therefore we asked whether the concept of stem cell niche could be extended to the process of transformation.

5.3.1 Niche competition and human stem cells *in vivo*

Evidence to date provides two central hypotheses as to the role of the microenvironment and CSC function. Firstly, that the tumour microenvironment altars normal stem cell function, facilitating tumour growth and CSC function. Secondly, that CSCs compete with and displace normal stem cells from their niche as part of the transformation process. Clinical evidence derived from double CB transplants where cells from two CB donors were infused into single recipients showed graft dominance of one CB donor over another (Eldjerou et al., 2010a; Haspel and Ballen, 2006; Majhail et al., 2006). These studies also revealed that this effect was in some cases dose-dependent, with increased chimerism seen from the donor with the larger number of infused cells (Eldjerou et al., 2010a; Haspel and Ballen, 2006; Majhail et al., 2006). These observations support the

conclusion hHSCs compete for niche occupancy as part of the engraftment process. Further evidence from the mouse system has shown that HSCs constantly circulate in and out of niches *in vivo* (Wright et al., 2001), and are therefore essentially always competing for niches during normal hematopoiesis. We postulated that if competition for niches between HSCs occurs *in vivo,* co-transplantation of highly purified CB HSCs *in vivo* should reveal donor chimerism that corresponds to input HSC dose. We observed that engraftment from two CB donors was never additive, and that some donors were able to compete for engraftment in a dose-dependent manner **(Chapter 4),** providing the first direct functional evidence that hHSCs compete for niches *in vivo*. We also found that some CB donors seemed to dominate the graft, and could not be out-competed by high doses of cells from another donor **(Chapter 4).** One could interpret this as having some hHSCs with an enhanced ability to interact with or occupy a niche, or alternatively that hHSCs from some donors have a more robust proliferation capacity. The process of *in vivo* engraftment and hematopoiesis is clearly highly complex, but this thesis has shown that at least some component is a result of competition for niche space in the marrow, thereby experimentally confirming years of clinical observations.

Cancer has been described as a stepwise acquisition of characteristics that lead a normal healthy cell to a transformed state (Hanahan and Weinberg, 2011), resulting from genetic and epigenetic changes rendering molecular pathways that control cell fate decisions aberrantly functional (Hanahan and Weinberg, 2011). In this view, a cancer cell utilizes existing cell-autonomous programs to its advantage, to support properties of neoplasia such as evasion of apoptosis, increased proliferation and metastasis (Hanahan

and Weinberg, 2011). Extending this idea to stem cells, if stem cells are dependent upon a niche in their normal physiologic state, then niche occupancy would be a non-cell-autonomous parameter that a cancer stem cell could potentially utilize in the transformation process. In the context of our observations in normal stem cells **(Chapters 2, 3 and 4)**, we asked 1) similar to normal hHSCs, do CSCs display a functional dependence on a niche? 2) Do CSCs compete for niche occupancy with normal hHSCs as part of the transformation process? 3) Could this step be targeted as a potential therapy for human cancers? Our findings indicate that when hHSCs and LCSs are both given an opportunity to initiate a graft *in vivo*, graft initiation is both competitive and dose-dependent **(Chapter 4).** When high numbers of purified hHSCs are present in the marrow, self-renewing leukemic stem cells are permanently lost, and the balance of normal hematopoiesis is re-established. This work shows that CSCs are dependent upon a niche for their function, and that they compete for these niches with normal stem cells as part of the tumour-initiation process. This observation extended over three independent CB-AML donor patient pairs, suggesting that niche occupancy may be a converging point and potential therapeutic target in the leukemia. Acute leukamias such as AML often lead to bone marrow myelosuppression (or bone marrow failure), resulting in significant co-morbidities such as infection and thrombocytopenia (Chang et al., 1976; HERSH et al., 1965; Najman et al., 1991; Valent and Schiffer, 2011). In agreement with these observations, we found that the leukemic graft suppressed myeloid differentiation of the CB graft *in vivo*, an effect that was lost in serial transplantation concomitantly with the eradication of LSCs in secondary recipients **(Chapter 4).** These findings therefore

support a model where normal hHSCs are competitively displaced from their niche by LSCs, leading to altered hematopoietic differentiation and loss of stem cell function, and replacement of the normal marrow with a blast-filled leukemic marrow. This would not only be highly consistent with clinical findings, but also suggests that by blocking the ability of a LSC to occupy a niche, the leukemogenic process can be disrupted and potentially reversed. Whether this model of niche competition extends to other types of CSCs, such as breast (Chaffer and Weinberg, 2010), colon (O'Brien et al., 2007) and neural (Lathia et al., 2011) remains to be established, but if so could be seen as a critical underlying principle in the transformation process. Additional gene expression analyses will be highly informative as to genetic alterations that may occur in the niche which lead to altered differentiation within the normal stem cell compartment in a leukemic setting.

Although there is an evident role for the niche in human cancer, competition for niche occupancy is almost certainly only one aspect of this role, and was the only aspect addressed in this thesis. Other studies have demonstrated that the tumour microenvironment additionally displays altered physiology which may affect stem cell function (Colmone et al., 2008; Shiozawa et al., 2011a; Sipkins et al., 2005), and further work will be needed to better understand the complexities of both competition and niche physiology in the transformed state. Furthermore, investigation and characterization of the molecular signals and pathways that may give a CSC a competitive advantage over a normal stem cell to occupy and survive within a niche will need to be investigated. The CXCR4/SDF-1 axis has been implicated in the adhesion of LSCs in the BM niche (Tavor et al., 2004), and investigation of additional pathways that confer a self-renewal and

survival advantage on hHSCs *in vivo* such as the Bcl-2 pathway would be an ideal starting points for investigation into the molecular basis for augmented niche propensity in disease states.

5.3.2 A conceptual model for the niche and cancer stem cell fate decisions

Based upon the findings in this thesis and other findings to date (Colmone et al., 2008; Ishikawa et al., 2007; Shiozawa et al., 2011a; Sipkins et al., 2005), we propose a preliminary model for the niche and cancer stem cell function (figure 2). In this model, a LSC occupies a vacant niche, which supports its cell fate decisions, and renders it protected from chemotherapy. The LSC can divide asymmetrically, both self-renewing and giving rise to non-self-renewing blast cells which fill the marrow (Lapidot et al., 1994). At the same time, hHSCs either are displaced from their physiologic niches, or are sequestered in aberrant niches that exist within the tumour microenvironment, causing them to die or to transiently loose their stem cell function (Hu et al., 2009), resulting in the eventual loss of normal hematopoiesis. Cancer relapse is a poorly defined and understood process, but generally encompasses the return of chemotherapy-resistant tumour cells following a period of clinically undetectable disease (clinical remission or minimal residual disease). The cancer stem cell hypothesis has been proposed as a possible explanation for relapse, where CSCs reside in a niche and a maintained in a pro-survival and quiescent state, thereby resisting cytotoxic therapies which target cycling cells pre-disposed to apoptosis (Dick, 2008). This thesis supports this hypothesis, providing direct evidence that CSCs are dependent upon a niche for their *in vivo* function,

supporting a potential mechanism for chemotherapy resistance and relapse in cancer. In this model, leukemic relapse is either the result of the activation of a quiescent LSC residing in a niche, or the landing of a potential LSC in a vacant niche, possibly following the acquisition of additional transforming mutations. The inability to find an appropriate niche would lead to the loss of the self-renewing LSC fraction. Recent studies in human ALL support such a model, as they have revealed that 1) in many cases the relapse clone is unique and has only limited genetic similarity to the predominate clone at diagnosis (Anderson et al., 2010; Notta et al., 2011b) and 2) the relapse clone acquires additional mutations that contribute to its aggressive and refractory nature (Anderson et al., 2010; Notta et al., 2011b).

The cell of origin in the CSC theory is a highly controversial topic (Chaffer and Weinberg, 2010; Majeti and Weissman, 2011), and is not in the scope of this thesis. Whether the LSC is a transformed normal hHSC or a progenitor that acquires stem cell properties, and whether the transforming event occurs in the niche, remains to be addressed in future studies. Additionally, further delineating the role of signaling pathways shown to function in the niche to support normal hHSC function and retention, such as WNT, Notch **(Chapter 3)** and CXCR4/SDF-1, and the Bcl-2 family pathway will be essential towards fully understanding the role of the niche in transformation and in leukemic relapse, and developing potential niche-targeted therapies.

5.4 Concluding remarks: the niche and the future of stem cell research

Human stem cells are thought to hold tremendous therapeutic potential. This potential will only be fully realized through an understanding of how fate decisions such as survival, quiescence, differentiation and self-renewal are regulated in these cells. This thesis has 1) shown that molecular pathways that regulate human stem cell fate decisions are uniquely regulated by their *in vivo* microenvironment; 2) characterized for the first time *in vivo* anatomic, cellular and molecular parameters that define a human stem cell niche; 3) shown that the niche is a determinant of functional heterogeneity in the human stem cell compartment; and 4) provided functional evidence that competition for niches is part of the transformation process. Together, these findings have advanced the field of human stem cell biology by providing novel insight into the role of the *in vivo* human stem cell microenvironment in both normal physiologic and malignant settings. This work supports the hypothesis of Raymond Schofield, that stem cells function as fixed-tissue cells, and therefore can only be fully defined and understood in the context of their *in vivo* microenvironment. These findings have implications both in cancer research and regenerative medicine. The field of regenerative medicine has historically focused on *ex vivo* manipulation of human stem cells for therapeutic applications. From the viewpoint of a stem cell being a functional extension of a fixed tissue, the only way to understand and manipulate stem cell fate decisions will be through understanding and manipulating their niche. This may be attained, for example through studying and culturing stem cells as whole-tissue explants that include intact three-dimensional anatomical components of their microenvironment, such as live human bone and marrow tissue in the case of

hHSCs. Such niche-based approaches will likely be essential toward moving landmark discoveries such as induced pluripotency (iPS) from experimental modalities to feasible clinical therapies applicable to human patients. Furthermore, as demonstrated here **(Chapter 3),** understanding the molecular interactions between a stem cell and the niche can be exploited to allow for prospective isolation of human stem cells for transplantation purposes. The work in this thesis has provided only a small, preliminary glimpse of the full potential of such approaches.

Unraveling the molecular biology of human cancer ranks as one of the greatest scientific breakthroughs in recent history. Despite this extraordinary work, most human cancers continue to present a poor clinical prognosis, and with some notable exceptions such as CML (O'Brien et al., 2003), and breast cancer (Baselga, 2001), continue to be exclusively treated with non-specific cytotoxic chemotherapies. The work presented in this thesis as well as that of others (Colmone et al., 2008; Ishikawa et al., 2007; Shiozawa et al., 2011a; Sipkins et al., 2005) now indicates that the niche has a central role in the transformation process, and that niche occupancy may be a converging point that can be targeted therapeutically. In this context, pharmacological disruption of the interaction between a LSC and the niche may allow for replacement of an established leukemia with normal hematopoietic tissue at the stem cell level, thereby reversing the disease. An ideal target for such an approach would be the CXCR4/SDF-1 axis, which is required for homing and retention of LSCs in the BM (Tavor et al., 2004) and can be antagonized with AMD3100, a drug in clinical use for hHSC mobiliza tion from the BM niche (Broxmeyer et al., 2005). By causing the egress of a LSC from its niche, the work presented here

suggests that it may be possible to replace it with a HSC and restore normal hematopoiesis **(Chapter 4).** Our work has also revealed the MCL-1 axis as a potential target for CSC survival within the *in vivo* microenvironment, particularly in the context of studies revealing the up-regulation of MCL-1 at the time of leukemic relapse (Kaufmann et al., 1998). Such approaches in leukemia and other malignancies may hold promise for relapsed patients that are resistant to chemotherapy. Future work focusing on unraveling the role the niche in transformation and the molecular interactions and pathways involved will almost certainly revolutionize our understanding of and approach to treating human cancers.

In summary, the way in which stem cells are viewed is evolving. Rather than functioning as independent entities whose fate decisions are controlled by cell-autonomous programs, stem cells appear to function as fixed-tissue entities whose cell fate decisions are regulated by non-cell-autonomous parameters in their microenvironment, or niche. The niche appears to be a complex spatial environment that is a function of anatomical location, cellular identity, and molecular programs. We sit at the beginning of a new and exciting era in human stem cell and cancer biology, where the concepts of stem cells, cancer, and the niche are interdependent and contiguous.

Figure 1. A working model of the human hematopoietic stem cell niche *in vivo*

A representative section of a femur illustrating anatomical, cellular and molecular heterogeneity hHSC niche in vivo. hHSCs reside either in the LBA (LBA-SRC) or TBA (TBA-SRC), forming unique subsets of hHSCs with distinct self-renewal, regenerative and differentiation capacities, which correlates unique gene expression profiles. Within each anatomical region, hHSCs interact with osteoblasts, vascular endothelial cells, or are interspersed between these discrete locations. Further heterogeneity in the TBA-SRC subset arise from the Notch/Notch-ligand axis, where a subset of TBA-SRC interact with Jagged-1 expressing osteoblasts, and are enhances for self-renewal and regenerative function.

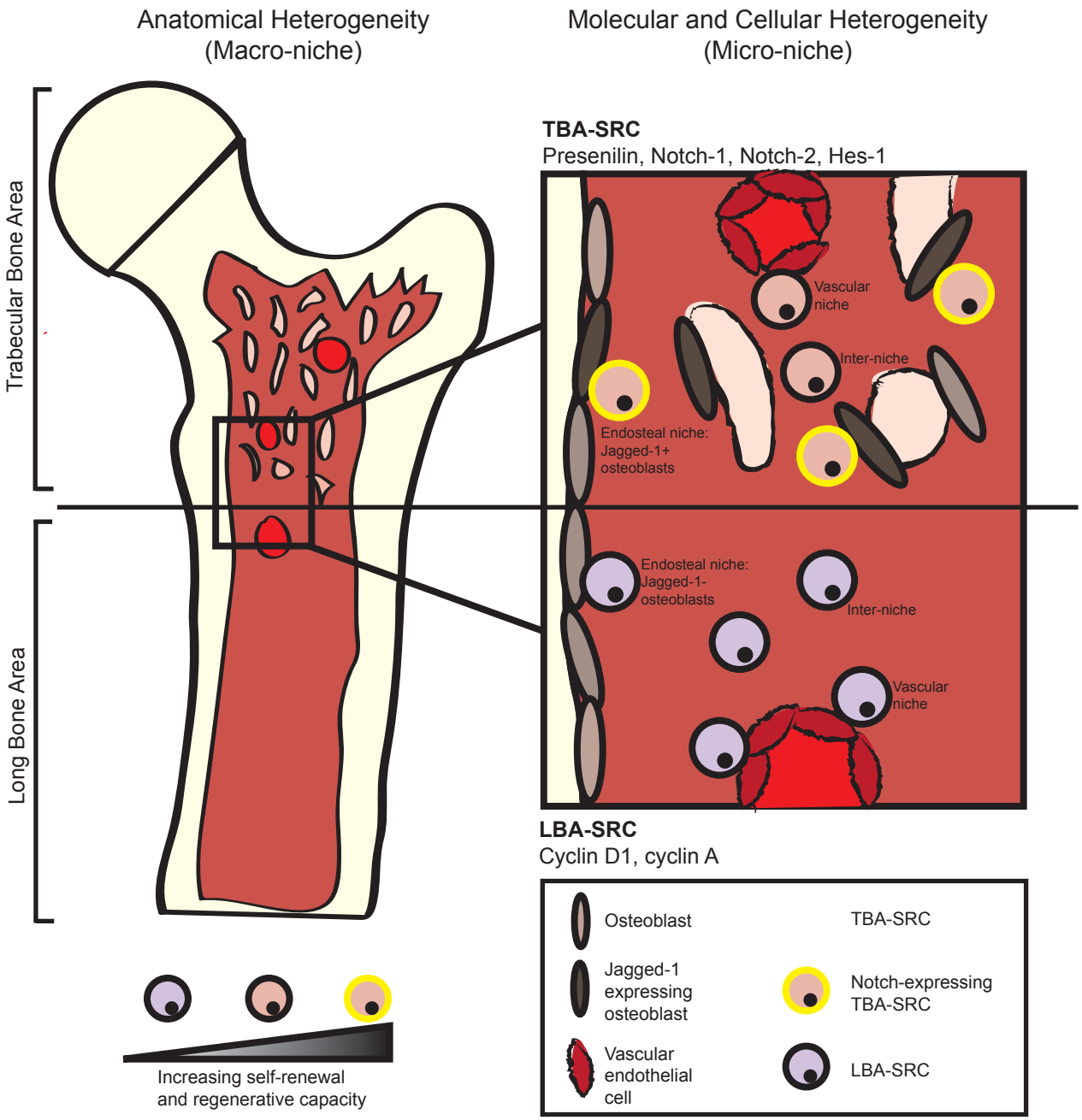

Anatomical Heterogeneity
(Macro-niche)
Molecular and Cellular Heterogeneity
(Micro-niche)
Trabecular Bone Area
Long Bone Area
TBA-SRC
Presenilin, Notch-1, Notch-2, Hes-1
Vascular niche
Inter-niche
Endosteal niche: Jagged-1+ osteoblasts
Endosteal niche: Jagged-1- osteoblasts
Inter-niche
Vascular niche
LBA-SRC
Cyclin D1, cyclin A
Osteoblast
TBA-SRC
Jagged-1 expressing osteoblast
Notch-expressing TBA-SRC
Vascular endothelial cell
LBA-SRC
Increasing self-renewal and regenerative capacity

Figure 2. Modeling the stem cell niche in cancer

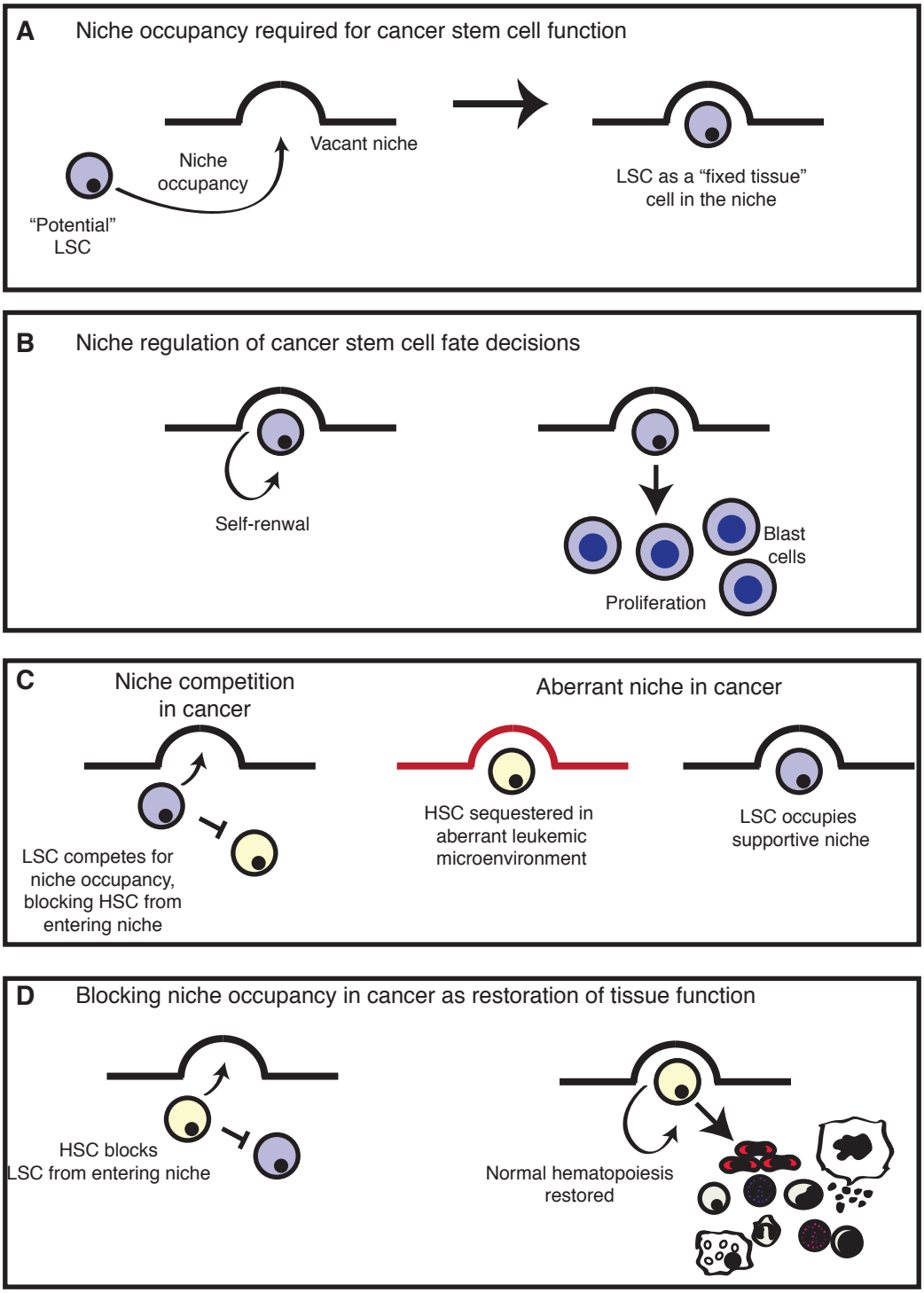
The work presented in this thesis and elsewhere (see text) allows for a preliminary model of the role of the stem cell niche in human cancer.

A. Similar to normal stem cells, cancer stem cells (CSCs) are do not functionally exist in isolation, rather, they exist as functional stem cells only when residing in a specific microenvironment or niche.

B. The niche functions to regulate CSC fate decisions, and maintains long-term self-renewal and proliferative function in these cells.

C. Evidence suggests that the niche has more than one role in human malignancy. Firstly, CSCs such as leukemic stem cells (LSCs) may compete with normal human hematopoietic cells (hHSCs) for niche occupancy, leading to loss of normal hematopoiesis. Secondly, an aberrant niche may exist within the leukemic environment, which sequesters and functionally inhibits normal hHSCs.

D. Replacing a LSC with a normal hHSC in the niche results in disruption of the leukemic process, and restoration of normal hematopoiesis.


A
Niche occupancy required for cancer stem cell function
Vacant niche
Niche occupancy
"Potential" LSC
LSC as a "fixed tissue" cell in the niche
B
Niche regulation of cancer stem cell fate decisions
Self-renwal
Blast cells
Proliferation
C
Niche competition in cancer
LSC competes for niche occupancy, blocking HSC from entering niche
Aberrant niche in cancer
HSC sequestered in aberrant leukemic microenvironment
LSC occupies supportive niche
D
Blocking niche occupancy in cancer as restoration of tissue function
HSC blocks LSC from entering niche
Normal hematopoiesis restored

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Appendix II

List of scientific publications and abstracts

Published, refereed papers (in chronological order)

Risueno RM, **Campbell CJ,** Dingwall S, Levadoux-Martin M, Leber B, Xenocostas A, Bhatia M. Identification of T-lymphocytic leukemia-initiating stem cells residing in a small subset of patients with acute myeloid leukemic disease. ***Blood*** (2011) 117(26): 112-120.

Campbell CJ, Lee JB, Levadoux-Martin M, Wynder T, Xenocostas A, Leber B, Bhatia M. The human stem cell hierarchy is defined by a functional dependence on Mcl-1 for self-renewal capacity. ***Blood*** (2010) 116(9): 1433-1442.

Campbell C, Risueno RM, Salati S, Guezguez B, Bhatia M. Signal control of hematopoietic stem cell fate: Wnt, Notch, and Hedgehog as the usual suspects. ***Current Opinion in Hematology*** (2008**)** 15(4):319-325

Manuscripts under review or in preparation

Guezguez B*, **Campbell CJ***, Karanu F, Smith MJ, Levadoux-Martin M, Bhatia M. *In vivo* bone marrow niche propensity reveals functional heterogeneity of the human hematopoietic stem cell compartment. Under review at ***Nature Immunology***.
*Equal author contributions.

Campbell CJ, Boyd A, Levadoux-Martin M, Fiebig A, Foley R, Leber B, Bhatia M. Eradication of human leukemia-initiating cells through niche replacement. Manuscript in preparation for submission to ***Leukemia.***

Scientific abstracts

Risueno, Ruth M, **Campbell, Clinton JV,** Levadoux Martin, Marilyne, Xenocostas, Anargyros, Leber, Brian, Bhatia, Mickie. Identification of T-Lymphocytic Leukemia-Initiating Stem Cells Residing in Patients with Acute Myeloid Leukemic Disease. International Society for Stem Cell Research (ISSCR) 9th Annual Meeting, Toronto, 2011.

Guezguez, Borhane*, **Campbell, Clinton JV*,** Karanu, Francis, Schnerch, Angelique, Smith, Mary-Jo, Levadoux-Martin, Marilyne, Bhatia, Mickie. *In Vivo* Bone Marrow

Niche Propensity Reveals Functional Heterogeneity of the Human Hematopoietic Stem Cell Compartment. International Society for Stem Cell Research (ISSCR) 9th Annual Meeting, Toronto, 2011.
*Equal author contributions.

Clint Campbell, Jung-Bok Lee, Marc Bosse, Marilyne Levadoux-Martin, Tracy Wynder and Mick Bhatia. MCL-1 is an essential regulator of human normal and malignant stem cell function. Canadian Stem Cell Network Annual General Meeting (AGM) Vancouver, 2008.

Aisha Shamas-Din, Scott Binder, **Clinton Campbell,** Brian Leber, David W. Andrews and Cecile Fradin . Exploring the membrane binding properties of the pro-apoptotic protein BID. Canadian Chemistry Conference and Exhibition, Biological and Medicinal Chemistry Division Edmonton, 2008.

Aisha Shamas-Din, **Clinton Campbell,** Brian Leber, David W. Andrews and Cecile Fradin . Exploring the membrane binding properties of the pro-apoptotic protein BID. Canadian Chemical Biophysics Symposium Toronto, 2008.