

INTERACTIVE PROPERTIES OF LEUKEMIA STEM CELLS

WITH THEIR ENVIRONMENT

**INTERACTIVE PROPERTIES OF LEUKEMIA STEM CELLS
WITH THEIR ENVIRONMENT**

BY

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Descriptive Note

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Abstract

Rare cancer stem cells are thought to be responsible for relapsed disease in many malignancies due to their preferential ability to evade cytotoxic therapy. The hierarchical organization of cancer has been most thoroughly described in acute myeloid leukemia (AML), in which leukemia stem cells (LSCs) have been shown to both self-renew, and generate short-lived dysfunctional progeny. To date, most investigational efforts to eradicate these cells have focused on their intrinsic attributes. However, stem cell identities in the normal hematopoietic system are uniquely maintained through associations with the microenvironment. Similar context-dependent behaviour may also apply to malignant stem cells, although this has not been well explored in AML. Therefore, *I hypothesized that AML-LSCs are sensitive to the conditions of their external environment, and that these influences can be used to both understand chemotherapy resistance mechanisms, and to devise novel therapeutic strategies.*

We began by providing evidence that LSCs cannot be distinguished from healthy hematopoietic stem cells (HSCs) based on a signaling pathway that has been clinically shown to have therapeutic promise in AML. Instead, we showed that non-hematopoietic cells in the bone marrow (BM) are more sensitive to these signals, which may impact neighbouring LSCs in a non-cell-autonomous manner. We then investigated whether LSCs interact with abnormal “niches” in BM, using sophisticated xenograft modeling. Based on *in situ* tissue analysis and *in vivo*

functional assays, we found that LSCs directly compete with healthy HSCs for shared niche occupancy in BM, and equally require niche support to sustain their self-renewal. Furthermore, this dependence could be exploited by cytokine dissociation of LSC-niche interactions during transplantation therapy, to promote competitive replacement by healthy HSCs.

Finally, we investigated the *in vivo* cellular events that lead to leukemic relapse after chemotherapy, a process thought to occur due to niche-mediated protection of quiescent LSCs. Our findings however challenge the conventional view that LSCs are preferentially spared by cytotoxic therapy. While primitive leukemic cells are initially suppressed following chemotherapy, we show that a unique population assembles to regenerate the disease at a rate disproportionate to healthy recovery. Focusing on these cells, we have described novel molecular hallmarks of leukemic regeneration, and demonstrated that they can be targeted in combination with chemotherapy to durably suppress leukemic growth. Collectively, the data presented within this thesis offer a broader conceptualization of LSCs as interactive components of the BM environment. This has revealed novel therapeutic paths to ultimately enable LSC targeting on multiple fronts.

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Abbreviations

AML	Acute myeloid leukemia
ANOVA	Analysis of variance
APL	Acute promyelocytic leukemia
AraC	Cytarabine
BM	Bone marrow
CB	Cord blood
CEBPA	CCAAT/enhancer binding protein α
CFU	Colony forming unit
CFU-S	Colony forming unit - spleen
CML	Chronic myeloid leukemia
CR	Complete remission
CSC	Cancer stem cell
CXCL12	C-X-C ligand 12
CXCR4	C-X-C receptor 4
DHH	Desert hedgehog
DRD2	Dopamine receptor D2
FAB	French-American-British
FACS	Fluorescence activated cell sorting
FLT3	Fms-related tyrosine kinase 3
G-CSF	Granulocyte stimulating factor
G-MPB	G-CSF mobilized peripheral blood
GEO	Gene expression omnibus
GUSB	Beta glucuronidase
GSEA	Gene set enrichment analysis
Hh	Hedgehog
HLA-A2	Human leukocyte antigen A2
HPC	Hematopoietic progenitor cell
HSC	Hematopoietic stem cell
HSCT	Hematopoietic stem cell transplantation
HSPCs	Hematopoietic stem and progenitor cells
IHH	Indian hedgehog
IMDM	Iscove's modified Dulbecco's medium
IV	Intravenous
L-IC	Leukemia initiating cell
LBA	Long bone area
Lin ⁻	lineage-depleted
LRC	Leukemic regenerating cell
LSC	Leukemic stem cell
LTC-IC	Long-term culture initiating cell
MFI	Mean fluorescence intensity
MDS	Myelodysplastic syndrome

MNC	Mononuclear cell
MPB	Mobilized peripheral blood
MSC	Mesenchymal stem cell
MSigDB	Molecular signatures database
NH	Non-hematopoietic
NOD/SCID	Non-obese diabetic/Severe combined immunodeficiency
NOD	Non-obese diabetic
NPM1	Nucleophosmin 1
NSG	NOD/SCID/IL2R γ ^{null}
PB	Peripheral blood
PCA	Principal component analysis
PCR	Polymerase chain reaction
PTCH1	Patched 1
SCID	Severe combined immunodeficiency
SE	Standard error
SEM	Standard error of the mean
SHH	Sonic hedgehog
SMO	Smoothened
SL-IC	SCID leukemia initiating cell
SRC	SCID repopulating cell
TBA	Trabecular bone area
TDZ	Thioridazine
UCB	Umbilical cord blood
VLA-4	Very late antigen 4
WHO	World Health Organization

Chapter 1: Introduction

Preface

Portions of this Chapter have been previously published in an original article. Specifically, sections 1.3.1 and 1.3.2 have been adapted from a review originally published in *Current Opinion in Hematology*:

Boyd, A. L., & Bhatia, M. (2014). Bone marrow localization and functional properties of human hematopoietic stem cells. *Current Opinion in Hematology*, 21(4), 249–255. doi:10.1097/MOH.000000000000055 Copyright © Wolters Kluwer Health, Lippincott Williams & Wilkins.

Figure 1.1 was also originally presented in this publication, and was prepared by Claudia Hopkins.

1.1 Overview

Hematopoiesis presents an ideal model system to study tissue organization due to the ease of single cell purification and the availability of robust transplantation-based assays. Studies of the hematopoietic system have paved our understanding of somatic stem cell biology, and hematopoietic stem cell (HSC) transplantation remains the greatest clinical success in regenerative medicine (Gratwohl et al., 2015; Rouchi et al., 2015).

The assays and principles that have guided the HSC field have also been used to transform our perception of malignant hematopoiesis, particularly in acute myeloid leukemia (AML). We now understand that leukemic cells are heterogeneous, and possess a complex population structure that resembles the hierarchical organization of healthy hematopoietic tissue (Dick, 2008). Leukemia stem cells (LSCs) are hypothesized to provide a renewable source of dysfunctional cells, and are thought to be the basis of disease relapse (Jordan et al., 2006). However, these insights have yet to translate into changes in routine clinical practice, and therapy options for AML patients have remained relatively static over the last 30 years (Khan et al., 2012).

As the bone marrow (BM) microenvironment is recognized to strictly dictate HSC activity and cell fate decisions (Mendelson and Frenette, 2014), it is important to understand LSCs in this full tissue context as well. A careful characterization of the healthy BM environment has been a prominent research focus within the last ten

years, and this knowledge is now reaching maturity (Mendelson and Frenette, 2014). An extension of these concepts to leukemia biology is critical, and could identify novel avenues for therapeutic LSC targeting.

1.2 Healthy hematopoietic stem cells

HSCs are rare BM-resident cells that are responsible for sustainable blood cell production throughout life. This is achieved by balancing between states of self-renewal and differentiation according to changing demands for circulating immune cells and erythrocytes (Wilson et al., 2008). Specialized BM microenvironments or “niches” play a fundamental role in the coordination of HSC activity based on oscillating physiological conditions (Scadden, 2014). As a result, HSCs are considered to principally remain quiescent unless activated to enter the cell cycle by regulatory niche cues (Arai and Suda, 2007; Wilson et al., 2008). The direct cellular descendants of HSCs are transiently self-renewing progenitors that become progressively lineage-restricted and give rise to terminally differentiated hematopoietic cells. Accordingly, the hematopoietic system is structured as a functional hierarchy with HSCs situated at the apex (Doulatov et al., 2012).

Initial evidence of hierarchical organization was derived from observations that remarkable regeneration occurs following the intravenous transfer of hematopoietic tissue to animals that had received otherwise lethal doses of radiation (Ford et al., 1956; Till and McCulloch, 1961). Although it was originally believed that humoral factors were responsible for provoking this regeneration,

genetic studies indicated that hematopoietic reconstitution was in fact driven by the cellular fraction of donor material, despite the fact that the majority of hematopoietic cells are post-mitotic (Ford et al., 1956). In the past 60 years, the hematopoietic hierarchy and related BM microenvironments have been carefully characterized using a variety of *in vitro* and *in vivo* assays. Although they have been associated with characteristic cell surface phenotypes (Bhatia et al., 1997; Notta et al., 2011), HSCs cannot be recognized morphologically, and are only definitively defined based on their functional properties. Therefore, indirect methods are necessary in order to rigorously assess and quantify both HSCs and lineage-restricted progenitors, based on retrospective analysis of their functional capacity.

1.2.1 Original self-renewal assays

Researchers Till and McCulloch pioneered the first experimental assay that provided clear evidence for the existence of self-replenishing hematopoietic cell populations that could serve as reservoirs for continual blood production. They showed that intravenously transplanted mouse BM cells are capable of forming discrete macroscopic colonies in the spleens of syngeneic recipient mice pre-conditioned with total body irradiation (Till and McCulloch, 1961). Further examination showed that each colony contained as many as 10^6 cells, which were all direct genetic descendants of individual donor cells (Becker et al., 1963; Till and McCulloch, 1961). The donor cells capable of seeding these colonies (labeled spleen colony forming units, CFU-S) were determined to be rare BM cells, estimated on the order of 10

CFU-S per 10^5 nucleated cells (Till and McCulloch, 1961). Most importantly, dissociation of individual colonies revealed heterogeneous mixtures of maturing lineage-restricted precursors identifiable by histological examination (McCulloch, 1983), as well as infrequent cells able to seed new splenic colonies if transferred into a new recipient host (Siminovitch et al., 1963). This represented the first powerful demonstration that a single cell could give rise to both differentiated and self-renewing progeny, the two necessary and fundamental features of HSCs.

1.2.2 In vitro assays of self-renewal

Although the CFU-S model offered an unprecedented method to quantify cells with self-renewing potential, immune barriers between species precluded the use of this transplantation-based assay to study human hematopoiesis at the time. However, several years later, *in vitro* systems were developed to study colony forming activity of mouse hematopoietic cells through culture in semisolid agar or methyl cellulose preparations in which hematopoietic colonies could grow (Bradley and Metcalf, 1966; Ichikawa et al., 1966; Pluznik and Sachs, 1965). This *in vitro* colony forming unit (CFU) assay was rapidly adapted to study human hematopoietic cells as well (Pike and Robinson, 1970). Because of the viscosity of the semisolid media used, cultures could be plated to ensure distributed suspensions of single cells, and limited dispersal of their progeny. Therefore, each cellular aggregate that emerged following 10-14 days of incubation could be reasoned to represent a colony of descendant cells that were all derived from a single progenitor (Bradley and Metcalf,

1966), a conclusion reinforced by genetic colony analysis (Fauser and Messner, 1978). Like the CFU-S assay, individual colonies contained both mature myeloid cells, as well as rare cells that were capable of initiating new colonies upon physical collection and re-plating as single cells (Ash et al., 1981; Terstappen et al., 1991). However, despite the value offered by the CFU-S and *in vitro* CFU assays to understand the relationships between self-renewal and differentiation, both assays predominantly focused on myeloid differentiation capacity, and it was unclear whether myeloid and lymphoid lineages shared a common multipotent stem cell ancestor (Abramson et al., 1977; Löwenberg and De Zeeuw, 1979). Additionally, studies of mouse BM exposed to the cytoreductive agent 5-fluorouracil demonstrated that this treatment eliminates CFU-S cells yet preserves a precursor subset that can repopulate BM and give rise to CFU-S *in vivo* (Hodgson and Bradley, 1979). These observations suggested that a more primitive multipotent HSC may exist.

Further refinement of *in vitro* and *in vivo* assays to detect more primitive populations of HSCs required supportive conditions involving cellular elements of the BM microenvironment, highlighting the cell extrinsic basis of HSC maintenance. In order to extend the otherwise limited ability to propagate primitive hematopoietic cells *ex vivo*, long-term co-culture systems were developed in which mouse or human hematopoietic cells were cultured in direct physical contact with adherent non-hematopoietic cells from the BM (Dexter et al., 1973; Dexter et al.,

1980; Sutherland et al., 1990). This adherent support provided enhanced preservation of cells with CFU capacity, and long-term culture initiating cells (LTC-IC) were shown to represent upstream precursors relative to CFU cells (Andrews et al., 1990; Sutherland et al., 1990). However, LTC-IC cultures could not be extended beyond 8-10 weeks (Sutherland et al., 1990), ultimately failing to reflect the longevity of HSC populations *in vivo*.

1.2.3 *In vivo* assays of self-renewal

The most influential advance towards understanding human HSC biology was the development of immune-deficient mouse strains that would allow multi-lineage repopulation of the human hematopoietic system in a murine host, and provide long-term maintenance of CFU populations *in vivo* (Kamel-Reid and Dick, 1988; Lapidot et al., 1992; McCune et al., 1988). Early xenotransplantation studies with human hematopoietic cells were performed using recipient mice that were homozygous for the severe-combined immunodeficiency (SCID) mutation, preventing the development of cellular or humoral immune responses to human antigens due to impaired genetic recombination ability in lymphocytes (McCune et al., 1988). In order to improve the low levels of engraftment achieved using SCID mice, this strain was crossbred with non-obese diabetic (NOD) mice to generate the NOD/SCID strain (Shultz et al., 2005). In addition to the lymphocyte defects associated with the SCID strain, NOD/SCID mice additionally have reduced natural killer cell activity, and suppressed innate immune function (Shultz et al., 1995),

allowing for more robust human engraftment (Larochelle et al., 1996). This strain has remained popular for xenograft applications, and allowed the first quantitative measurement of multi-potent repopulating cells by transplanting incrementally limited hematopoietic cell numbers (Wang et al., 1997). These operationally defined SCID-repopulating cells (SRCs) were confirmed to have self-renewal capacity as demonstrated by serial transplantation into secondary recipient mice (Guenechea et al., 2001), and have been shown to be more primitive than both CFU and LTC-ICs based on retroviral marking studies (Larochelle et al., 1996).

Serial transplantation and limiting dilution quantification remain the gold standards to measure the most primitive human HSC populations *in vivo*. Since these initial foundational studies, further modification to increase the sensitivity of the assay has involved cell delivery via intra-femoral transplantation (Mazurier et al., 2003), as well as the development of increasingly permissive mouse strains. These include the addition of the IL2R γ^{null} mutation on the NOD/SCID background (NSG mice) (Shultz et al., 1995), as well as the genetic introduction of human-specific cytokines (Miller et al., 2012). In addition to these improvements, both retroviral marking techniques and cell purification technologies have set the basis for detailed phenotypic mapping of the human hematopoietic hierarchy (Doulatov et al., 2012).

1.2.4 Purification of hematopoietic stem and progenitor cells

The advent of flow cytometry and cell sorting techniques profoundly advanced the ability to characterize primitive hematopoietic populations based on prospective

isolation using cell surface antigen expression, followed by functional interrogation with *in vitro* and *in vivo* assays (Dick, 2008). Consistent with the immature cytomorphology of hematopoietic stem and progenitors cells (HSPCs), these techniques established that HSPC populations widely lack mature lineage-specific markers in both humans and mice (Andrews et al., 1990; Spangrude et al., 1988). Therefore, cell purification measures to isolate primitive hematopoietic cells routinely involve the physical removal of differentiated cell types using antibody cocktails directed towards a collection of mature cell markers, a process called lineage depletion (Lin⁻) (Spangrude et al., 1988). Beyond the absence of lineage marker expression, the cell surface antigens that define human and mouse HSCs are somewhat distinct. In the mouse system, the expression of stem cell antigen-1 and c-kit are most widely associated with HSPCs and the identification of additional antigens has led to HSC purification at nearly a single cell level (Benveniste et al., 2003; Kiel et al., 2005; Matsuzaki et al., 2004; Spangrude et al., 1988).

In the human system, CD34 was identified as a surface marker associated with morphologically primitive hematopoietic cells (also referred to as blasts) (Civin et al., 1984), and the first phenotype described to enrich for cells with functional BM repopulation ability was Lin⁻Thy1⁺CD34⁺ (Baum et al., 1992). Further quantitative analysis demonstrated that functional repopulating cells were enriched in the Lin⁻CD34⁺CD38⁻ fraction, while Lin⁻CD34⁺CD38⁺ cells only contained CFU progenitor capacity (Bhatia et al., 1997; Hao et al., 1995). Recently, a refined cell surface

phenotype was reported to purify human repopulating cells to a frequency of approximately 1 in 4 cells, based on multi-lineage reconstitution of NSG mice upon intra-femoral transplantation of single cells. These cells were distinguished by Lin⁻CD34⁺CD38⁻CD45RA⁻Thy1⁺CD49f⁺ surface marker expression and high rhodamine dye efflux activity, and were shown to self-renew by serial transplantation (Notta et al., 2011). However, it is important to note that although this may represent a nearly pure fraction of HSCs, analysis of this population may not always provide a comprehensive representation of the entire HSC pool, as repopulating cells also exist within other phenotypic fractions, although at lower frequencies (e.g. CD34⁺; Bhatia et al., 1998).

1.2.5 Signaling pathways critical to self-renewal

The processes of tissue pattern formation during embryogenesis and tissue repair in adulthood share common regulatory pathways that dictate stem cell fate decisions (Beachy et al., 2004). The Wnt, Hedgehog (Hh), and Notch signaling pathways represent morphogen signals that are critical to embryonic development, and have also been implicated in the regulation of HSC homeostasis (Campbell et al., 2008). The Hh pathway has recently received particular attention as an appealing target in several hematopoietic neoplasms (Lin et al., 2009), therefore it has become important to clarify its role and requirement for healthy adult hematopoiesis.

The Hh pathway is structured with multiple ligand-dependent feedback loops, which contribute to a sensitive level of regulatory control (**Figure 1.1**). The

pathway becomes activated when one of three Hh ligands (Sonic Hh, Desert Hh, or Indian Hh) bind to the transmembrane protein Patched 1, which interrupts its repression of Smoothed, another transmembrane protein. Smoothed activity results in the nuclear translocation of Gli transcription factors, of which target genes include pathway components Gli1 and Ptch1, as well as cell cycle and cell fate regulators cyclinD1, Bmp4, and Bcl2 (Duman-Scheel et al., 2002; Hooper and Scott, 2005).

While *in vitro* studies have indicated that Hh stimulation may be sufficient to directly promote the expansion of human HSCs (Bhardwaj et al., 2001), mouse models suggest that it may not be a necessary condition, as genetic inactivation of the pathway throughout the hematopoietic system produces no remarkable phenotype (Gao et al., 2009; Hofmann et al., 2009). However, mice with germline Hh mutations affecting all tissue lineages manifest distinct alterations in their ability to recover from hematopoietic injury resulting from chemotherapy treatment (Merchant et al., 2010; Trowbridge et al., 2006). This suggests that intact Hh signaling may instead be required within elements of the BM microenvironment that support hematopoietic regeneration. In fact, in some solid tissues, it is well established that epithelial cells secrete Ihh to the mesenchyme, which responds by releasing factors that stimulate epithelial cell proliferation (Fabian et al., 2012; Lamm et al., 2002; Shin et al., 2011). Although BM osteoblasts have been shown to be particularly responsive to Hh ligands (Kiuru et al., 2009), it remains to be

determined whether HSC function is influenced by paracrine Hh signaling within the niche, as is seen in solid tissues.

1.3 The HSC microenvironment

The mechanisms that either maintain self-renewing HSCs or direct their differentiation are largely cell extrinsic in nature, as well evidenced by the loss of functional HSC capacity that accompanies prolonged *in vitro* culture (Walasek et al., 2012; Williams, 1993). In order to explain the rapid exhaustion of primitive cell pools cultured *ex vivo* or through serial transplantation of CFU-S, Raymond Schofield first theorized that the cellular identities of HSCs can only be maintained in the context of their BM niches (Schofield, 1978). This concept of HSCs as “fixed tissue cells” has since gained increasing experimental support. Together, *in vivo* analyses employing genetic models, *in situ* high-resolution microscopy and sophisticated transplantation experiments have proven to be invaluable towards the identification of key specialized BM elements contributing to long-term HSC preservation (Joseph et al., 2013).

1.3.1 Micro-architecture of the HSC microenvironment

Other than vasculature and neural innervation that are common to all tissues, hematopoietic cells reside amongst specific mesenchymal cell lineages in the BM, which form important structural components of bone. The heterogeneous cell types that compose BM mesenchymal populations are often collectively referred to as “stromal cells” (Krebsbach et al., 1999), however the diverse identifies of these cells

and their relevance to HSC biology has been established with greater precision in recent years. Although the hierarchical structure of mesenchymal populations is not as well defined as in the hematopoietic system, mesenchymal stem cells (MSCs) have been functionally described, which possess both self-renewal and multi-lineage differentiation potential (Méndez-Ferrer et al., 2010). MSCs have been shown to give rise to adipocytes, chondrocytes, and osteoblasts, and can be passaged *in vivo* through serial transplantation (Méndez-Ferrer et al., 2010). In both humans and mice, MSCs are most commonly identified by their adherence to plastic in culture, as well as typical CD105⁺CD73⁺CD90⁺ cell surface phenotypes (Dominici et al., 2006).

Since the initial theoretical reasoning supporting the existence of HSC-specific niches in BM (Schofield, 1978), concentrated efforts have systematically evaluated the HSC-supportive capacities of BM-resident cell types on an individual lineage basis. Such studies, informed mostly by promoter-driven cell ablation techniques and static *in situ* microscopy, included the consideration of osteoblasts (Calvi et al., 2003; Zhang et al., 2003), endothelial cells (Kiel et al., 2005), osteoclasts (Lymperi et al., 2011), other BM-resident macrophages (Winkler et al., 2010), megakaryocytes (Olson et al., 2013), non-myelinating Schwann cells (Yamazaki et al., 2011), T-regulatory cells (Fujisaki et al., 2011), adipocytes (Naveiras et al., 2009), and MSCs (Mendez-Ferrer et al., 2010) (**Figure 1.2**). Of these cell types, osteoblasts and endothelial cells initially emerged as promising candidates that attracted the most attention as fundamental HSC regulators. In this context, endosteal and

vascular BM regions were often juxtaposed as mutually exclusive territories, largely based on the premise that the endosteum offers a hypoxic microenvironment, and therefore must be poorly perfused (Eliasson and Jonsson, 2010). Current understanding, however, favors a more integrated view between vasculature and the endosteum. Endothelial cells have been reported to situate in close association with osteoblasts in both the calvarium (Lo Celso et al., 2009) and the long bones (Ellis et al., 2011; Hooper et al., 2009; Lassailly et al., 2013; Nombela-Arrieta et al., 2013; Xie et al., 2009), with suggestions that osteoblast-rich trabeculated areas may even be more vascularized than central BM cavities (Lassailly et al., 2013; Xie et al., 2009). Both osteoblasts and endothelial cells have been suggested to directly contribute to the maintenance of HSC quiescence by supplying factors such as Stem Cell Factor and Angiopoietin (Arai et al., 2004; Ding and Morrison, 2013; Zheng et al., 2011),

More recently, important interactions between HSCs and functionally primitive MSCs have been described within the BM (Mendez-Ferrer et al., 2010). However, it remains to be resolved whether MSCs unilaterally influence HSCs, or whether these two cell types are reciprocally dependent, or are mutually regulated by other sources. Under homeostatic conditions, both HSCs (Guezguez et al., 2013; Kunisaki et al., 2013; Nombela-Arrieta et al., 2013) and MSCs (Kunisaki et al., 2013) have been consistently observed to localize peripherally near the bone surface rather than in central medullary regions. The interface between vasculature and the

endosteum represents a site of arteriolar sympathetic innervation (Kunisaki et al., 2013) and circulatory exchange between arteriolar and venous vessels (Nombela-Arrieta et al., 2013). Therefore, the positioning of HSCs and MSCs at these bone-proximal perivascular areas may in fact poise them to receive instructive signals from neural (Katayama et al., 2006) and endocrine (Nakada et al., 2014) sources.

Although the exact BM microdomains that house HSCs have yet to be discretely visualized, indirect evidence suggests that they are limited within BM and can become saturated when the supply of HSCs exceeds available niches (Czechowicz et al., 2007). This property has enabled the use of competitive transplantation models to measure relative BM repopulation abilities between different HSC populations with genetically identifiable markers (Harrison, 1980). Such transplantation systems have even shown that metastatic solid tumor cells can compete with HSCs to seed common BM niches (Shiozawa et al., 2011), but **competitive dynamics relative to malignant hematopoietic cells have not been well studied.**

1.3.2 Macro-architecture of the HSC microenvironment

In addition to detailed characterization of the cellular complement in the immediate vicinity of phenotypic HSCs, multiple groups have also observed heterogeneous localization of functionally defined HSCs across the gross anatomical topography of BM space (Ellis et al., 2011; Grassinger et al., 2010; Guezguez et al., 2013; Haylock and Nilsson, 2005). Using rigorous xenotransplantation assays, phenotypic and

functional analyses by our group have revealed that transplanted human HSCs non-randomly localize with preference to the trabecular-rich metaphyses of murine long bones. In fact, primitive human CD34⁺CD38⁻ cells isolated from trabecular bone ends contained higher frequencies of repopulating cells over those isolated from the central bone cavity, regardless of identical cell surface phenotypes (Guezguez et al., 2013b). Others have come to similar conclusions using congenic mouse systems, identifying enhanced reconstitutive capacities of HSCs based on their physical isolation from metaphyseal rather than diaphyseal bone sites (Grassinger et al., 2010; Haylock and Nilsson, 2005; Lord et al., 1975). Critically, this finding was reinforced by the examination of primitive hematopoietic populations according to trabeculation gradients within primary human trephine biopsies, confirming the direct clinical relevance of HSC spatial distribution within BM (Guezguez et al., 2013).

The selectively HSC-supportive potential of trabecular regions has been collectively attributed to the unique molecular composition of regional osteoblasts including elevated Jagged-1 expression (Guezguez et al., 2013), enriched bone remodeling activity in the long bone extremities (Lassailly et al., 2013), and the metaphyseal abundance of hyaluronic acid (Ellis et al., 2011), a cognate ligand for HSC-expressed CD44 (Avigdor et al., 2004)(**Figure 1.2**). Ultimately, these studies provide strong evidence that spatial BM localization can allow for the isolation of functionally superior HSC populations despite cell surface phenotypic equivalence

(Grassinger et al., 2010; Guezguez et al., 2013; Haylock and Nilsson, 2005), highlighting the instructive role of the microenvironment in defining and maintaining HSC cellular identities.

1.3.3 Trafficking and mobilization of HSCs

In early ontogenetic development of mammalian organisms, HSCs undergo successive colonization of different microenvironmental sites before settling in BM niches, where they reside throughout adult life (Mikkola and Orkin, 2006). The first “definitive HSCs” capable of repopulating the BM of irradiated adult recipients initially appear in the aorta-gonad-mesonephros region within the developing embryo, and then seed the fetal liver, where they expand dramatically prior to colonizing the BM at birth (Dzierzak and Speck, 2008; Medvinsky and Dzierzak, 1996). In addition to the migratory behaviour of HSCs during developmental ontogeny, HSCs are thought to traffic in and out of the BM throughout life, particularly in response to requirements for elevated hematopoietic cell production (Lemoli and D'Addio, 2008). Several adhesion molecules have been identified to play critical roles in regulating the homing and retention of HSCs in BM, including C-X-C chemokine receptor type 4 (CXCR4), very late antigen 4 (VLA-4), and CD44 (Méndez-Ferrer and Frenette, 2007). Of these, genetic ablation of CXCR4 produces the most profound hematopoietic phenotype, leading to embryonic lethality (Arroyo et al., 1999; Protin et al., 1999; Zou et al., 1998). This developmental defect is also mirrored by the genetic deletion of the CXCR4 ligand, C-X-C ligand 12

(CXCL12)(Nagasawa et al., 1996). In both CXCR4^{-/-} and CXCL12^{-/-} mice, embryonic HSC development proceeds normally to the fetal liver stage, however HSC colonization of BM is severely impaired (Nagasawa et al., 1996; Zou et al., 1998). In BM, CXCL12 is highly expressed on osteoblasts, vascular cells, and MSCs (Greenbaum et al., 2013) and its expression on these cells is regulated dynamically in response to injury and hematopoietic demand (Casanova-Acebes et al., 2013; Dominici et al., 2009; Mendez-Ferrer et al., 2008).

The CXCR4-CXCL12 axis plays a fundamental role in promoting HSC egress from the BM under conditions of stress. Part of the hematopoietic injury response involves the production of granulocyte colony stimulating factor (G-CSF) by endothelial and hematopoietic cells (Cook et al., 2013), which stimulates the release of proteolytic enzymes that cleave CXCL12 (Petit et al., 2002). As a result of disrupted niche anchorage, HSCs become mobilized into the periphery (Petit et al., 2002). G-CSF also suppresses CXCL12 mRNA and protein levels within osteoblastic populations, although this mechanism is incompletely understood (Katayama et al., 2006; Semerad et al., 2005). Because of the potency of G-CSF mobilization, this cytokine has been adopted in clinical settings to induce HSC egress from the BM to facilitate HSC collection from venous circulation for transplantation purposes (Méndez-Ferrer and Frenette, 2007). Other clinical mobilization strategies include small molecule antagonists of CXCR4, such as AMD3100, which can also synergize with G-CSF (Broxmeyer, 2005). While treatments targeted towards other adhesion

molecules have also been shown to provoke HSC mobilization (e.g. VLA-4 antibodies) (Craddock et al., 1997), the CXCR4-CXCL12 axis remains the most important molecular target of current clinical mobilization protocols.

1.4 Acute Myeloid Leukemia

AML is a blood disorder characterized by uncontrolled proliferation of non-functional myeloid blasts arrested at various stages of maturation (Döhner et al., 2010). AML cells are well suited to experimental manipulation using *in vivo* models, and leukemic subpopulations have been empirically demonstrated to be functionally heterogeneous (Bonnet and Dick, 1997; Hope et al., 2004; Lapidot et al., 1994). For these reasons, AML has become an appealing paradigm to better understand the biological significance of hierarchical organization within neoplastic cell populations (Dalerba et al., 2007; Felipe Rico et al., 2012). Since the original description of LSCs (Bonnet and Dick, 1997; Hope et al., 2004; Lapidot et al., 1994), evidence for similar concepts of functional diversity has been pursued in multiple solid tumors such as the breast and the brain (Al-Hajj et al., 2003; Singh et al., 2004). Therefore, novel conceptual insights derived from AML studies have the potential to extend beyond the scope of leukemia, and could be applied to other hierarchically organized cancers. It is critical to develop novel therapy strategies to clinically target LSCs and related cancer stem cell populations, as these unique and rare cells have been hypothesized to underlie therapy resistance and disease relapse in both leukemia patients and in solid tumors (Horton and Huntly, 2012; Jordan et al., 2006).

1.4.1 Clinical characteristics and pathophysiology

AML is the most common form of adult leukemia and has a higher incidence among older individuals, with newly diagnosed patients having a median age of 70 years old (Estey and Döhner, 2006). Patients with AML frequently present with symptoms related to underlying conditions of pancytopenia, including general fatigue, weakness, and bleeding (Estey and Döhner, 2006; Meyers et al., 2005). Untreated AML can ultimately result in serious infection as a consequence of the non-functional nature of leukemic blasts, as well as inhibited maturation of healthy HSCs (Estey and Döhner, 2006). These suppressive mechanisms of leukemic cells towards healthy HSCs are likely to extend beyond physical crowding alone, as the percentages of leukemic blasts do not reliably correlate with the severity of cytopenia (Estey and Döhner, 2006).

Diagnostic assessments of suspected AML cases routinely comprise complete blood counts and morphological examination of smears prepared from peripheral blood (PB) and BM aspirates (Döhner et al., 2010). Multicolor flow cytometric immunophenotyping of BM and PB cells are used to confirm the myeloid nature of the disease and to determine blast cell lineages (Döhner et al., 2010; Estey, 2011). BM cells are additionally subjected to structural cytogenetic analysis, and are often also analyzed by molecular cytogenetic techniques such as fluorescence in situ hybridization, and molecular genetics to detect fusion genes or somatic mutations by polymerase chain reaction (Döhner et al., 2010). Unless characteristic chromosomal

rearrangements are detected such as t(8;21) or inv(16), a BM or PB blast percentage of $\geq 20\%$ is required for a definitive diagnosis of AML (Döhner et al., 2010; Harris et al., 1999; Vardiman et al., 2002).

1.4.2 Etiological factors and risk stratification

There are few hereditary risk factors known to contribute to AML pathogenesis other than germline trisomy 21 abnormalities (Nickels et al., 2013; Xavier et al., 2009). Recognized environmental risk factors include histories of exposure to benzene, ionizing radiation, and chemotherapy (Estey and Döhner, 2006). As a result, intensive cytotoxic therapy for solid tumors and other hematopoietic disorders can cause predispositions towards developing AML as a secondary malignancy (Larson, 2007). Progression to AML is particularly common among patients with myelodysplastic syndromes (MDS), a group of clonal malignant diseases leading to ineffective cell maturation in one or more hematopoietic lineages (Corey et al., 2007; Shih and Levine, 2011). In addition to hallmark dysplastic hematopoiesis, a BM blast percentage threshold of less than 20% represents the main feature that distinguishes MDS from AML (Harris et al., 1999). Despite this distinction, transformation to overt AML eventually occurs in approximately 30% of MDS cases (Porwit and Saft, 2011).

Forms of AML that are secondary to antecedent neoplastic conditions are often more aggressive than *de novo* disease and reliably carry a poor prognosis (Godley and Larson, 2008; Larson, 2007). Other than previous history of malignant

disease, current patient sub-classification and risk stratification schemes in AML rely heavily on genetic characterization of leukemic cells (Harris et al., 1999; Vardiman et al., 2002). The historical French-American-British (FAB) system previously used to sub-stratify AML was originally founded upon cytomorphological and cytochemical analyses aimed to determine the cell lineages represented by leukemic blasts, as well as the extent of their maturation (Bennett, 1985; Bennett et al., 1976). However, more contemporary World Health Organization (WHO) standards consolidate cytomorphological, genetic, and disease history data for a more comprehensive classification of myeloid neoplasms according to features that will reliably predict disease progression (Harris et al., 1999; Vardiman et al., 2002; Vardiman et al., 2009).

Based on these WHO guidelines, the European LeukemiaNet organization has devised a prognostic scoring system to divide AML patients into three genetic risk tiers (Döhner et al., 2010). This defines several specific cytogenetic lesions as either reliably favorable (e.g. $t(8;21)(q22;q22)$) or unfavorable ($inv(3)(q21q26.2)$) prognostic indicators. Molecular genetics play an important role in appropriately categorizing patients with normal karyotypes (consisting of almost 50% of the AML patient population) (Döhner et al., 2010; Estey and Döhner, 2006). Mutations in recognized leukemia-associated genes such as CCAAT/enhancer binding protein α (CEBPA), nucleophosmin 1 (NPM1) and fms-related tyrosine kinase 3 (FLT3) determine whether normal karyotype patients fall into favorable or intermediate

prognostic categories (Döhner et al., 2010). Together, these distinctions directly inform therapeutic management decisions, particularly at the juncture of post-remission therapy to prevent relapse (Döhner et al., 2010).

1.4.3 Current standard of care and therapy response assessment

Upon diagnosis, therapeutic intervention is divided into two phases when pursued with curative intent: induction therapy aimed to establish a state of remission, followed by consolidation therapy designed to prevent recurrence of disease if remission is successfully achieved (Estey and Döhner, 2006). Genetically defined acute promyelocytic leukemia (APL) represents a unique subtype of myeloid leukemia that is adequately managed by treatment with all-trans retinoic acid, which can be delivered in combination with other agents such as arsenic trioxide (Coombs et al., 2015). With this exception, the majority of AML cases are initially treated according to common standardized induction chemotherapy protocols, regardless of prognostic subtyping. Over the last 40 years, first line induction therapy for AML has involved the nucleoside analogue cytarabine (AraC), which is regularly paired with an anthracycline agent such as daunorubicin, typically delivered for first 3 of 7 days of AraC treatment (Estey and Döhner, 2006).

Following induction chemotherapy, complete remission (CR) criteria includes a reduction in leukemic blast percentages below 5% of BM nucleated cells, as well as restoration of absolute platelet and neutrophil counts to normal ranges (Cheson et al., 2003; Döhner et al., 2010). Generally, induction therapy successfully establishes

CR in 60-80% of younger patients below 60 years of age, and in 40-60% of older adults (Rowe, 2009). If CR is achieved, preferred strategies for consolidation therapy are informed by genetic risk stratification as well as patient age and physical condition (Estey and Döhner, 2006).

A common form of consolidation therapy consists of multiple courses of high intensity chemotherapy, of which AraC is a popular choice (Rowe, 2009; Stone, 2013). Patients classified in unfavorable cytogenetic risk groups are often offered HSC transplantation (HSCT) from allogeneic donors, in the form of adult BM, G-CSF mobilized peripheral blood, or umbilical cord blood (Hamilton and Copelan, 2012). Although HSCT represents the most curative approach in AML, the benefits are balanced by high morbidity risks, and this option becomes less practical for elderly patients who have greater difficulty tolerating the procedure (Champlin et al., 2001; Hamilton and Copelan, 2012; Rowe, 2009). The high intensities of the preparative conditioning regimens and complications such as graft-versus-host disease result in high rates of treatment-related mortality (Champlin et al., 2001; Rowe, 2009). Therefore, **continued efforts to reduce toxicity associated with HSCT are strongly needed to advance post-remission therapy measures in AML, for application across a wider patient population** (Champlin et al., 2001). Unfortunately, despite promising initial CR induction rates, only 10-30% of AML patients benefit from durable disease-free survival over 5 years, due to frequent

leukemic relapse and therapeutic toxicities that limit dose escalation (Bloomfield et al., 1997; Rowe, 2009; Rowe and Tallman, 2010).

1.4.4 The development of experimental AML models

Other than immortalized cell lines which have been suggested to deviate from their original biology over time (Gillet et al., 2011), primary AML cells exhibit poor growth *in vitro* (Clutterbuck et al., 1985; Sawyers et al., 1992). However, mononuclear cells from AML patients have been shown to form colonies in semi-solid culture assays developed to study the normal hematopoietic hierarchy (Moore and Metcalf, 1973). In these studies, the leukemic origin of clonal growth has been confirmed by karyotype analysis identifying patient-specific aberrations (Moore and Metcalf, 1973). Although AML cells often give rise to morphologically irregular colonies, these colonies have been shown to produce progeny with moderate properties of both self-renewal and differentiation (Griffin and Löwenberg, 1986), reminiscent of healthy hematopoietic progenitors. These functional conclusions were based on the observations of serial re-plating ability from individual colonies (Buick et al., 1979), as well as the detection of cells that had acquired progressively differentiated surface phenotypes and cytomorphology (Marie et al., 1981; Moore and Metcalf, 1973). While only rare cells have the capacity to form colonies in semisolid media, and fewer maintain serial re-plating capacity (Buick et al., 1979), these assays are considered to read out short-term progenitors with restricted regeneration potential (Larochelle et al., 1996). Therefore, *in vivo* repopulation studies are

necessary to comprehensively study long-term self-renewal capacity in AML populations (**Figure 1.3**).

Initial efforts to transplant primary AML cells into SCID mice were challenged by the inability to create orthotopic xenografts by intravenous transplantation (Sawyers et al., 1992). However, this limitation was overcome by supplementing SCID mice with human cytokine injections following transplantation (Lapidot et al., 1992), and by the eventual application of the more permissive NOD/SCID strain (Bonnet and Dick, 1997). Using these assays, SCID leukemia-initiating cells (SL-ICs) were operationally defined to be rare cells with extensive regenerative capacity (Bonnet and Dick, 1997; Lapidot et al., 1994). Importantly, like their healthy SRC counterparts (Bhatia et al., 1997), SL-ICs were restricted to the CD34⁺CD38⁻ phenotypic fraction, with little to no engraftment activity within CD34⁺CD38⁺ or CD34⁻ cells (Bonnet and Dick, 1997; Lapidot et al., 1994). Engrafted human cells lacked evidence of healthy hematopoiesis based on the exclusively myeloid composition of reconstituted cells (Bonnet and Dick, 1997), and the consistent identification of patient-specific chromosomal abnormalities (Lapidot et al., 1994). A closer examination of leukemic grafts showed that the phenotypic diversity characteristic of original patient cells had been faithfully recapitulated in the mice (Bonnet and Dick, 1997), indicating a capacity for limited maturation along myeloid lineages. In addition to this modest differentiation potential, xenografted AML cells

also maintained self-renewal capacity, through serial transplantation and CFU assessments (Bonnet and Dick, 1997; Lapidot et al., 1994).

The finding that CD34⁺CD38⁻ cells could engraft NOD/SCID mice and maintain CFU capacity, but that CD34⁺CD38⁺ populations were restricted to CFU formation was initially suggestive of a hierarchical organization in AML (Lapidot et al., 1994). This hypothesis was further reinforced by lentiviral marking studies, which established that like the healthy hematopoietic system (Guenechea et al., 2001), the clonal lineages that compose the SL-IC pool possess variable capacities for self-renewal (Hope et al., 2004). These seminal studies therefore provided the first experimental evidence to support the hypothesis of rare LSCs that perpetuate the production of short-lived leukemic blasts *in vivo* (**Figure 1.3**). Murine models of AML have additionally been generated by the retroviral transduction of mouse BM progenitors with characteristic AML fusion genes (e.g., MLL-AF9) (Krivtsov et al., 2006). These studies also supported the emergence of an LSC-driven model in which disease initiation capacity is limited to specific phenotypic subsets (Krivtsov et al., 2006). While such engineered models of oncogene-driven AML offer valuable insights into the causal contributions to disease by individual genetic lesions, xenograft models have become favoured for pre-clinical testing of investigational therapeutics, as they can capture a broad spectrum of realistic genetic mutations (Sausville and Burger, 2006).

1.4.5 Clinical implications of the cancer stem cell theory

In contrast to hierarchical models of disease, stochastic models of tumorigenesis would predict that it would not be possible to purify specific sub-fractions with reliable tumor-initiating capacity or lack thereof. Despite the strong experimental evidence for the hierarchical organization of AML and other solid tumors (Al-Hajj et al., 2003; Bonnet and Dick, 1997; Hope et al., 2004; Lapidot et al., 1994; Singh et al., 2004), some groups have challenged the cancer stem cell theory (Kelly et al., 2007; Quintana et al., 2008). Their work suggested that the inter-species boundaries presented by xenotransplantation cause an underestimation of cancer stem cell frequencies. Accordingly, they provided demonstrations that when immune barriers were minimized, tumor-initiating capacity could be detected in the majority of cells from human melanoma and transgenic murine hematopoietic malignancies (Kelly et al., 2007; Quintana et al., 2008). This distinction has important clinical ramifications, as it informs whether it is necessary to uniformly target the entire tumor bulk or whether the development of novel therapies should be tailored towards rare functional subsets that may be molecularly distinct.

Despite these reports that some tumors may not be hierarchically arranged (Kelly et al., 2007; Quintana et al., 2008), several lines of evidence reinforce the continued relevance of the cancer stem cell model in many malignancies including AML. First, recent *in situ* lineage tracing experiments suggest that rare clones drive the growth of several types of murine tumors, with variable proliferative potential

among clonal lineages (Driessens et al., 2012; Schepers et al., 2012). Second, not all syngeneic transplantation models suggest that tumor-initiating populations are rare. Syngeneic mouse transplants of mammary carcinoma or retinoblastoma cells have shown that tumorigenic populations can be prospectively partitioned into tumor initiating versus benign subpopulations (Kruger et al., 2006). Furthermore, over 50 years ago, tumor biopsies of volunteer human patients were dissociated and autologously transplanted into their own thighs or forearms at various cell doses (Southam and Brunshwig, 1961). These human studies showed that $>10^6$ tumor cells were required to seed autologous tumor growth with no immune rejection barriers, consistent with rare frequencies of tumor initiating cells (Southam and Brunshwig, 1961). Third, increasing the immune deficiency of xenograft recipients does not always indicate that cancer stem cells are abundant. While the transplantation of human melanoma cells into NSG mice was shown to increase engraftment capacity by several orders of magnitude relative to the less immune compromised NOD/SCID strain (Quintana et al., 2008), the use of NSG mice as recipients did not alter LSC estimates as dramatically in AML (Vargaftig et al., 2011). Despite the moderately enhanced sensitivity of SL-IC detection in NSG mice, there remain AML patient samples that fail to engraft even under these more permissive conditions (current reports suggest sample engraftment rates of approximately 40%) (Shlush et al., 2014), and estimated LSC frequencies remain rare (Agliano et al., 2008; Ishikawa et al., 2007; Sanchez et al., 2009; Sarry et al., 2011; Vargaftig et al., 2011).

Although LSC frequency estimates are consistently low, the use of increasingly permissive recipient mice has however suggested that LSC phenotypes are not always restricted to CD34⁺CD38⁻ fractions, and can be variable across patients (Eppert et al., 2011; Sarry et al., 2011; Taussig et al., 2010). However, CD34⁺ cells have been consistently found to possess higher LSC frequencies than their CD34⁻ counterparts, preserving the relative relationships originally described in NOD/SCID mice (Eppert et al., 2011; Ishikawa et al., 2007; Martelli et al., 2010; Sarry et al., 2011; Taussig et al., 2010). A recent report has directly addressed the heterogeneity of LSC surface phenotypes, and systematically identified LSC-containing fractions across a collection of AML patient samples (Eppert et al., 2011). Independent of cell surface phenotypes, LSC-containing subsets were found to have a unique gene signature relative to those that were devoid of LSC activity. Furthermore, an application of this signature to a large cohort of unfractionated AML samples revealed that poor survival outcomes were correlated with the expression of LSC-related genes, emphasizing the clinical relevance of LSC biology (Eppert et al., 2011). Additional studies have also supported a relationship between patient survival and leukemia initiating ability, using either NOD/SCID or NSG mice as recipients (Monaco et al., 2004; Pearce et al., 2006; Vargaftig et al., 2011). These findings reinforce the notion that LSCs are likely involved in therapy resistance mechanisms in AML, as evidence of high LSC frequencies reliably predicts poor patient outcomes following treatment, regardless of mouse strain used.

1.4.6 Theories of therapy failure in AML

Relapsed disease becomes much less responsive to subsequent therapeutic intervention (Forman and Rowe, 2013), and is the most frequent cause of mortality among patients diagnosed with AML (Estey, 2012). There are several prevailing theories to explain therapy resistance in AML, either involving selective fitness advantages thought to be cell intrinsic in nature, or relating to the preferential sparing of LSCs through their associations with the BM microenvironment.

To understand the genetic basis of disease recurrence, several studies have performed longitudinal genomic profiling of AML cells retrieved from individual patients at diagnosis and also at the point of subsequent relapse. Using single nucleotide polymorphism arrays and whole genome sequencing, many patients across three studies were found to have acquired novel mutations upon relapse (Ding et al., 2012; Krönke et al., 2013; Parkin et al., 2013). Although this provided evidence of genetically evolving disease, relapsed leukemic populations were however found to reliably reflect the characteristic background mutations originally detected at diagnosis (Ding et al., 2012; Krönke et al., 2013; Parkin et al., 2013). This finding is consistent with similar reports in acute lymphoid leukemia (Mullighan et al., 2008) and suggests that rather than creating novel leukemic clones, chemotherapeutic interventions primarily fail due to incomplete eradication of the founding lineage of clonal leukemic cells. This does not eliminate the possibility that chemotherapy may preferentially select for specific genetic subsets of leukemic

disease. However, these studies were unable to identify distinct genetic lesions that reliably associate with the dominantly emerging subclones across different relapsed AML patients (Ding et al., 2012; Krönke et al., 2013; Parkin et al., 2013). As in-depth exploratory studies of gene expression patterns between paired diagnosis and relapse samples have not yet been performed, common molecular themes of resistant disease are also not understood at the more global pathway level. As a result, **the molecular attributes that underlie selective chemoresistance remain poorly characterized in AML, presenting a challenge for the design of novel preventative measures against relapse.**

More recent perspectives on chemoresistance in AML incorporate an understanding of the hierarchical structure of the disease. LSCs are thought to selectively survive chemotherapy due to their cell cycle quiescence, which attenuates the effectiveness of agents that selectively target actively proliferating cells (Jordan et al., 2006). In fact, AraC has been reported to less potently induce apoptosis within phenotypically primitive CD34⁺CD38⁻ AML cells relative to the bulk leukemic population (Costello et al., 2000; Guzman et al., 2001; Ishikawa et al., 2007), consistent with the relative cell cycle quiescence that has been documented within this phenotypic subset (Guan et al., 2003; Ishikawa et al., 2007). The close association between LSCs and the BM microenvironment has been specifically hypothesized to maintain their dormant non-proliferative states. Accordingly, *in vitro* experiments have shown that co-culture with BM-derived mesenchymal cells

can reduce cell cycle activity in AML cell lines (Konopleva et al., 2002; Ramasamy et al., 2007), and can protect primary patient AML cells from chemotherapy-induced apoptosis (Garrido et al., 2001; Konopleva et al., 2002). Therefore, increasing efforts have been directed to better define the relationship between LSCs and the BM microenvironment, to understand potential cell extrinsic mechanisms of therapy failure in AML.

1.4.7 Therapeutic targeting of LSCs

The ability to prospectively purify LSC-enriched populations has led to efforts to seek distinctive molecular features between HSCs and LSCs, for the ultimate development of novel selective therapies to limit disease relapse. Gene expression profiling or antigen screening using phage display libraries have identified several surface proteins preferentially expressed in phenotypically primitive leukemic cells relative to matched healthy populations (Bakker et al., 2004; Hosen et al., 2007; Jordan et al., 2000; Majeti et al., 2009; Saito et al., 2010a). These LSC-specific surface proteins include CD123, CD96, T-cell Ig mucin 3, C-type lectin-like molecule-1, variant isoforms of CD44, and the tyrosine kinase HCK (Hosen et al., 2007; Jan et al., 2011; Jin et al., 2006; Jordan et al., 2000; Saito et al., 2010a; van Rhenen et al., 2007). Antibody or small molecule inhibitors have been developed against several of these antigens, and have shown promise against AML in preclinical xenograft models (Jin et al., 2006; Jin et al., 2009; Kikushige et al., 2010; Saito et al., 2010a). Although no LSC-targeted therapies have yet been adopted in routine clinical practice, several of

these agents are currently being tested in Phase 1 or Phase 2 clinical trials (Pollyea et al., 2014). In addition to the targets already described, Hh pathway antagonists have also shown encouraging efficacy results in Phase 1 clinical trials in AML, and have been proposed to suppress LSC self-renewal (Jamieson et al., 2011). **However, this mechanism remains unclear as sensitivity to Hh signaling has yet to be demonstrated in AML-LSCs using *in vitro* or *in vivo* experimental systems.** In several solid cancers, Hh inhibition has been shown to restrain carcinogenesis by acting through the tumor microenvironment (Tian et al., 2009; Yauch et al., 2008), a possibility that has not been examined in AML.

1.5 The leukemic BM niche

Historically, malignant transformation has been conceptually linked with a cellular state of self-sufficiency (Hanahan and Weinberg, 2000), implying a lack of dependence on a supportive microenvironment. However, sustained *ex vivo* cell culture of AML and maintenance of primitive phenotypes has proven prohibitively difficult in the absence of cell line immortalization (Clutterbuck et al., 1985; Mayani et al., 2009; Pabst et al., 2014; Sawyers et al., 1992), but can be improved by culture on BM stromal cell lines (Konopleva et al., 2002). Together, these findings suggest that clonal leukemic growth is dependent upon extrinsic cues. In fact, contemporary views of cancer now more broadly recognize the importance of tumor microenvironments across many types of malignancies (Hanahan and Weinberg, 2011), and this is most central to cancers thought to be hierarchically organized,

such as AML (Plaks et al., 2015). Therefore, focus has shifted towards characterizing LSC-niche interactions, and distinguishing potential overlap between LSC and HSC niches.

1.5.1 Spatial localization of LSCs

While some studies have reported endosteal BM localization of phenotypically primitive leukemic cells (Ishikawa et al., 2007; Ninomiya et al., 2006), consistent with localization properties of normal HSCs, others have argued that LSCs are likely to reside in aberrant niches (Zipori, 2014). LSC retrieval from anatomical sites such as the spleen has been proposed to indicate that their niches must differ from HSCs, as these locations rarely house stable HSC-resident populations (Somerville and Cleary, 2006; Zipori, 2014). However, HSCs can alter their activity and spatial distribution in response to environmental conditions, and may similarly relocate to unconventional sites during leukemic disease states. Indeed, genetic MLL-AF9 mouse models have documented increased mobilization of phenotypic HSCs into both peripheral circulation and splenic sites as a consequence of leukemic infiltration (Hanoun et al., 2014). Xenograft experiments employing human leukemia cell lines have similarly suggested that HSCs become physically relocated to aberrant BM niches in leukemic conditions, based on dysregulated BM gradients of Stem Cell Factor secretion (Colmone et al., 2008). Therefore, the detection of LSCs in irregular anatomic sites may reflect microenvironmental restructuring that is

likely to occur during leukemia pathogenesis, and does not necessarily imply niche-interactive properties that are distinct from HSCs.

1.5.2 Targeting LSC interactions with the niche

Like healthy HSCs, LSCs have been reported to physically interact with mesenchymal BM cells through adhesion proteins such as CXCR4, CD44, and VLA-4 (Jin et al., 2006; Matsunaga et al., 2003; Tavor, 2004). These axes therefore represent attractive therapeutic targets that have been explored in preclinical xenograft studies. For example, antibody-mediated targeting of hyaluronan receptor CD44 has been shown to compromise LSC homing abilities and can diminish the self-renewal of established leukemic disease (Jin et al., 2006). Furthermore, antagonism of both VLA-4 and CXCR4 has been used to distance LSCs from their protective niches, as a chemotherapy sensitization strategy (Nervi et al., 2009; Saito et al., 2010b). However, these chemosensitization approaches have been controversial, as they are likely to lack selectivity against leukemic cells, and could be accompanied by increased toxicity to the normal hematopoietic system (Murashige et al., 2003). Therefore, **it is critical to clearly resolve the relationships between HSC and LSC niche properties in order to inform strategic design of niche-targeted therapy in AML.**

1.6 Summary of Intent

The focus of my studies was to understand leukemia as a dysfunctional tissue rather than at the level of individual mutated cells. This begins with an appreciation of the functional diversity within leukemia populations, and the hierarchical organization that mirrors healthy tissue structure (Dick, 2008). In the past 20 years, extensive effort has been invested to phenotypically and molecularly characterize the rare LSC populations that resist therapy and drive relapse. However, it is not known how sensitively LSCs are influenced by their surroundings, or how distinct spatial and temporally changing conditions in BM may affect their behaviour. These details are required to interpret sources of conventional therapy failure, and to appropriately direct novel therapeutic approaches. Recent advances in the study of healthy hematopoiesis have carefully detailed the spatial and molecular properties of HSC niches, as well as dynamic changes that occur when homeostasis is disturbed. Equipped with these insights, I sought to apply similar principles to understand leukemia biology. Three central questions provided me with a solid starting basis to characterize the importance of LSC interactions with their environment:

- 1) Can elements of the BM niche serve as therapeutic targets in AML?
- 2) What are the characteristics of the *in vivo* niches that house LSCs?
- 3) How do leukemic populations respond to injury states?

Based on emerging evidence, **I hypothesized that AML-LSCs are sensitive to the conditions of their external environment, and that these influences can be used to both understand chemotherapy resistance mechanisms, and to devise novel therapeutic strategies.** To test this hypothesis, I defined three discrete objectives, all with the explicit goal of generating new perspectives towards therapeutic innovation for AML (**Figure 1.4**):

- 1) Evaluate non-hematopoietic BM cells as putative targets of therapeutics shown to have clinical promise in AML.
- 2) Determine whether LSCs compete directly with HSCs to occupy shared BM niches.
- 3) Characterize the cellular and molecular chronology of leukemic responses to cytoreductive therapy.

As an initial foundation to examine the potential for niche-directed targeting in AML, small molecule inhibitors of the Hh signaling pathway offered an appealing entry point. This family of Hh pathway antagonists has been recently proposed to be clinically effective against AML (Jamieson et al., 2011), however the cellular mechanism of these effects have not been described. Furthermore, the significance of Hh signaling in the normal hematopoietic system has been controversial. Adjusted views now suggest that cell intrinsic Hh signaling is dispensable for healthy hematopoiesis (Gao et al., 2009; Hofmann et al., 2009). This has made the Hh pathway appealing as a potential selective target for diseased hematopoietic cells. However, this observation does not exclude the possibility that intact Hh signaling

may be required within other cell lineages in the BM, to indirectly support either normal or malignant hematopoiesis. Indeed, in other tissues it is well understood that Hh signaling is important within mesenchymal niche cells in order to support tissue-specific cell types or tumor cells in a non-cell autonomous fashion (Fabian et al., 2012; Lamm et al., 2002; Shin et al., 2011; Tian et al., 2009; Yauch et al., 2008).

In order to first clarify whether small molecule Hh inhibitors may be selectively discriminating between healthy and leukemic hematopoietic cells, we performed molecular analyses of bulk mononuclear cells and purified primitive fractions, as well as progenitor assays to test the functional effects of pharmacological Hh inhibition. These assessments failed to provide evidence for selective cell intrinsic effects against AML. However, gene expression and protein analysis showed that non-hematopoietic cells from BM were highly enriched for Hh signaling machinery, and are therefore much more likely targets of small molecule Hh inhibitors. This work supported the hypothesis that individual cellular components of the BM niche could represent indirect conduits of LSC modulation. However, it did not address whether the niche structures that house LSCs are compositionally unique, which is an important determination for selective targeting efforts.

In order to directly examine whether LSCs occupy unique niches *in vivo*, we built upon strengths in the lab initially developed to characterize the healthy HSC microenvironment (Guezguez et al., 2013). High-resolution multi-parameter

imaging of AML-xenografted bones suggested remarkable overlap between LSC and HSC spatial distribution profiles. In order to test this on a functional level, we applied a unique co-transplantation model to capture both HSCs and LSCs of human origin within individual murine hosts. This indicated a direct competition between healthy and malignant stem cells for shared BM niche space. We then used xenograft preclinical modeling to demonstrate the proof-of-principle that this shared property of BM niches could be used to advantage HSC repopulation of LSC-occupied sites, by G-CSF cytokine displacement.

Recognizing that LSCs are remarkably sensitive to their spatial surroundings, I sought to understand how they adapt to changing conditions over time in response to cytoreductive BM injury. This is critical, as current therapeutic failure has been attributed to chemoresistant LSCs that are sheltered within BM niches. Although this logic is intuitive, it lacks empirical support, as the cellular events that occur between therapy and relapse have not been previously explored. We provide the first longitudinal survey of leukemic populations recovering from chemotherapy, both in human patients and in clinically-inspired xenograft models. We applied this controlled experimental system to demonstrate that leukemic recovery operates distinctly from the disciplined regeneration of healthy hematopoietic cells, as leukemic growth rates become accelerated in response to AraC but then do not appropriately subside. This did not appear to involve selective sparing of LSC populations, as primitive leukemic cells were dramatically suppressed at immediate

time points post-therapy. However, we identified a sensitive time frame in which unique regenerating populations emerge, and we found that molecular signatures associated with this process are shared across human patients and xenografted mice. These molecular markers of leukemic regeneration provide novel therapeutic targets for combined chemotherapy, which we have already demonstrated by proof-of-concept preclinical modeling.

Together, this thesis advances our knowledge of LSC positioning within microenvironments in the BM, and highlights surprising similarities with HSCs. It also presents a new understanding of leukemic recurrence following therapy. These findings offer concrete value to the field by clarifying important mechanisms of conventional therapy failure, and by identifying multiple promising avenues to combat AML through indirect LSC targeting.

Figures

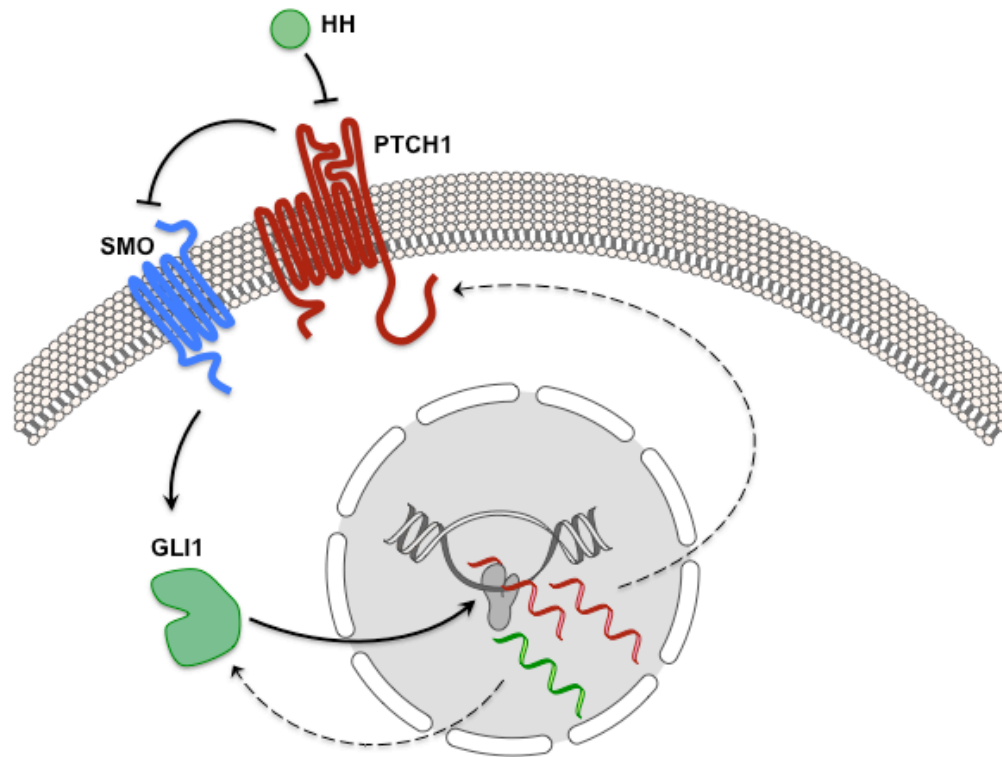


Figure 1.1. The hedgehog signaling pathway.

HH ligands bind to the transmembrane receptor Patched 1 (PTCH1), which causes a conformational change that prevents PTCH1 inhibition of Smoothed (SMO). As a result, SMO activates the nuclear translocation of GLI transcription factors, triggering transcription of downstream targets. GLI and PTCH1 are among these targets, forming feedback loops indicated by the dotted lines.

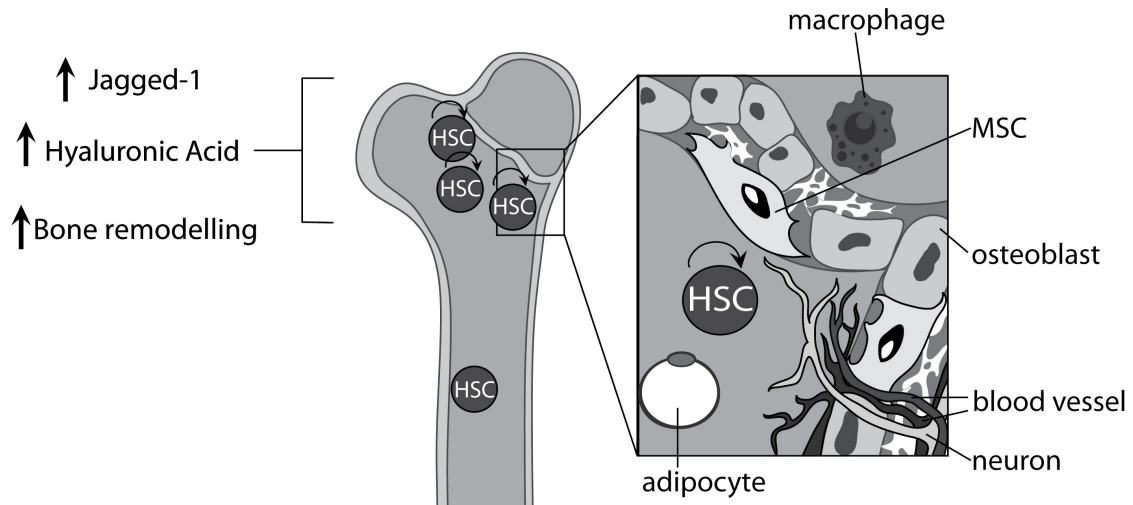


Figure 1.2. Macro-anatomical and micro-anatomical features of HSC BM niches..

Self-renewing HSCs are preferentially located at the extremities of the long bones, which offer unique BM microenvironments characterized by enriched numbers of Jagged-1⁺ osteoblasts, abundant hyaluronic acid, and high levels of bone remodeling activity. Other cell types that have additionally been described to associate with HSCs include macrophages, osteoblasts, blood vessels, neurons, adipocytes, and MSCs.

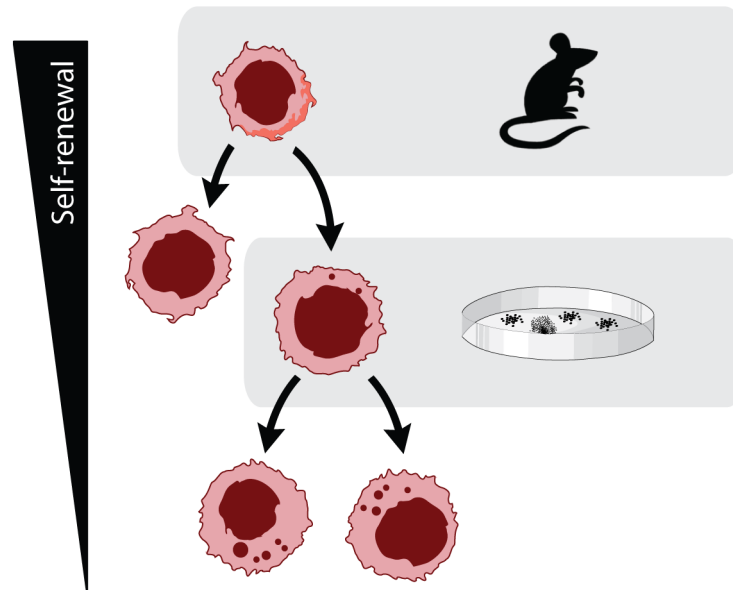


Figure 1.3. The leukemic stem cell hierarchy.

The most primitive LSCs with long-term self-renewal capacity are exclusively read out using *in vivo* repopulation assays. These assays involve transplantation into immune deficient mice. LSC progeny have variably restricted capacities for self-renewal, with short-term leukemic progenitors having sufficient clonogenic potential to form colonies when tested using *in vitro* CFU assays.

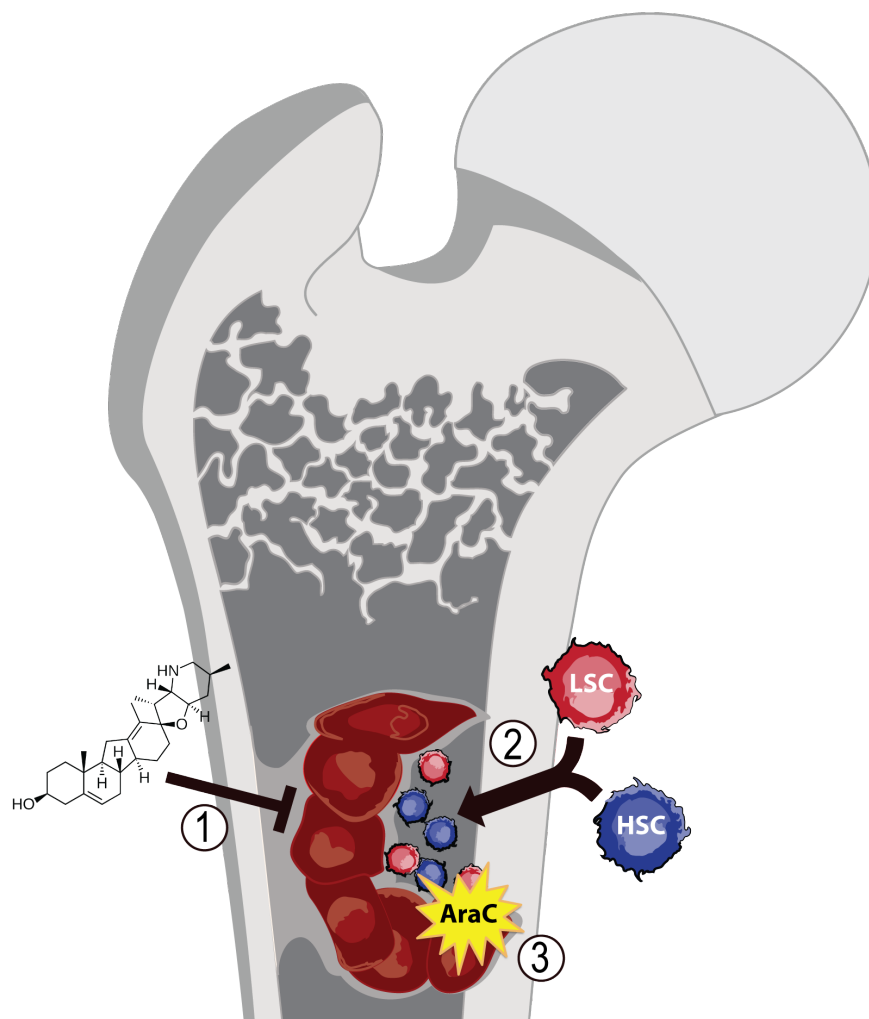


Figure 1.4. Summary of thesis objectives.

The overarching theme of this thesis is a common focus on the context-dependent behaviour of LSCs within the BM environment. This diagram illustrates my three specific objectives: 1. Evaluating the potential for indirect targeting of LSCs using Hh inhibitors 2. Examining overlap between HSC and LSC niches with competitive BM repopulation assays 3. Profiling leukemic regeneration in BM following conventional chemotherapy treatment.

Chapter 2: Non-hematopoietic cells represent a more rational target of *in vivo* hedgehog signaling affecting normal or acute myeloid leukemic progenitors

Preface

This Chapter is an original published article. It is presented in its published form.

This research was originally published in *Experimental Hematology*:

Boyd, A. L., Salci, K. R., Shapovalova, Z., McIntyre, B. A. S., & Bhatia, M. (2013). Nonhematopoietic cells represent a more rational target of *in vivo* hedgehog signaling affecting normal or acute myeloid leukemia progenitors. *Experimental Hematology*. doi:10.1016/j.exphem.2013.05.287. Copyright © Elsevier.

I designed the study and wrote the paper together with Dr. Mick Bhatia. I performed all cell sorting and qPCR experiments, as well as functional *in vitro* progenitor assays. Kyle Salci isolated and characterized BM mesenchymal cells, Dr. Zoya Shapovalova performed gene expression analysis, and Dr. Brendan McIntyre helped with immunofluorescence staining.

When I began my studies, the hedgehog (Hh) signaling pathway was gaining attention as an attractive target of leukemic stem cell (LSC) self-renewal in chronic myeloid leukemia (CML). Based on these studies in CML (Dierks et al., 2008; Zhao et al., 2009), small molecule Hh inhibitors were advancing to clinical trials for a range hematopoietic malignancies including acute myeloid leukemia (AML), in which

preliminary reports of efficacy were promising (Jamieson et al., 2011; Merchant et al., 2010). However, unlike in CML, molecular evidence for direct sensitivity to Hh pathway inhibition had not been described in AML-LSCs.

At the same time, two reports of engineered mouse models had recently shown that genetic inactivation of Hh signaling throughout the hematopoietic lineage had no negative consequences for healthy hematopoietic stem and progenitor cell (HSPC) function (Gao et al., 2009; Hofmann et al., 2009). This supported a notion that Hh inhibition of LSCs could safely spare healthy hematopoiesis. However, these findings contrasted a previous publication from our lab, which suggested that a germline haploinsufficiency of the Patched 1 receptor had a profound effect on HSC responses to cytoreductive injury (Trowbridge et al., 2006). Therefore, I hypothesized that the disruption of Hh signaling in additional cell lineages within the bone marrow (BM) microenvironment could have contributed to the more profound hematopoietic phenotypes observed here relative to other studies. This would be consistent with a well described stromal role for Hh signaling during regeneration states in other tissues (Fabian et al., 2012; Lamm et al., 2002; Shin et al., 2011). As Hh-activated stromal cells have also been proposed to support tumor cell growth in solid cancers (Tian et al., 2009; Yauch et al., 2008), an understanding of Hh signaling within the BM niche could also be relevant to AML biology.

Therefore, I pursued an investigation of the potential relationships between Hh signaling, AML, and the BM microenvironment, which all represent strong areas of expertise in the lab. We first used gene expression analysis and functional progenitor assays to rule out a basis for molecular selectivity between healthy HSPC populations versus primitive AML cells. We then performed a molecular examination of the Hh signaling pathway in BM mesenchymal cells, ultimately providing evidence that Hh modulation could be impacting healthy and malignant hematopoietic cells in a non-cell-autonomous manner.

Non-hematopoietic cells represent a more rational target of *in vivo* hedgehog signaling affecting normal or acute myeloid leukemic progenitors

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Abstract

Recent work has shown that leukemic stem cell self-renewal in chronic myeloid leukemia (CML) is dependent on cell-intrinsic hedgehog (Hh) signaling, and early clinical trials suggest targeting this pathway is also therapeutic in acute myeloid leukemia (AML) patients. Here, we aimed to better understand Hh signaling in normal hematopoiesis and AML by molecularly and functionally analyzing over 200 primary human AML patient samples compared to non-leukemic controls. Gene expression analysis indicated that Hh pathway transcripts were similarly regulated in AML and non-leukemic controls, whether samples were purified based on primitive phenotypes or not. Consistent with these results, pharmacological inhibition of SMO did not preferentially reduce *in vitro* colony formation of AML versus normal progenitors. Using a unique analytic approach, mRNA expression of membrane receptor Smoothed (SMO) was found to be unexpectedly rare within all hematopoietic samples analyzed, indicative of heterogeneity at the level of Hh signaling machinery. In contrast, abundant SMO expression could be readily detected in the non-hematopoietic fraction of human and murine bone marrow (BM) cells. Our predictions of increased SMO⁺ cell frequencies within non-hematopoietic BM fractions were further supported by single-cell protein analyses. Although we did not find support for cell-autonomous sensitivity of AML cells to Hh pathway inhibition, we alternatively suggest that non-hematopoietic BM cells may represent

an indirect target through which primitive normal and leukemic cells can be modulated. These findings suggest current approaches applying Hh inhibition should be carefully re-evaluated to take into account BM niche cell regulation that may be selectively Hh responsive.

Background

AML is a hematopoietic malignancy composed of a cellular hierarchy that structurally resembles the normal hematopoietic system, with cells at different hierarchical levels having variable self-renewal properties and phenotypes [1-3]. While the majority of leukemic blasts possess limited ability to propagate leukemic disease, leukemia-initiating capacity has been shown to be restricted to rare cell populations known as leukemic stem cells (LSCs; [1,4,5]). Signaling pathways known to regulate developmental or cell fate processes are of particular interest in this disease, as they play important roles in the transitions of normal hematopoietic stem cells (HSCs) between quiescent and active states under conditions of injury, and could thus be exploited by neoplastic stem cells to fuel their expansion. The Hedgehog, Wnt, and Notch pathways represent strong candidate targets to eradicate AML-LSCs, as they convergently regulate HSC proliferation and cell fate decisions, and have also been identified as putative modulators of LSC behaviour [6,7]. Of these pathways, hedgehog (Hh) signaling is gaining considerable attention in the clinic as a therapeutic target for myeloid malignancies [8-11], despite the fact that its role in normal and malignant human hematopoiesis remains poorly defined and controversial [12-17].

Three different mammalian Hh ligands have been identified; Sonic Hedgehog (Shh), Indian Hedgehog (Ihh) and Desert Hedgehog (Dhh). In the presence of Hh ligands, the transmembrane receptor Patched (Ptch) releases its inhibition of

another transmembrane protein called Smoothed (Smo), allowing Smo to assume an active conformation. In this activated state, Smo transduces signals that cause the nuclear translocation of the Gli family of transcription factors (Gli1, Gli2, or Gli3), with ultimate influences on cell cycling and fate decision processes in many mammalian tissues [18]. Following initial reports of Shh's modulatory role in adult blood stem cell fate choices [19], transgenic mouse studies have since shown that conditional deletion of Smo from hematopoietic cells has no negative consequences for the normal adult blood system under homeostatic conditions [12,13]. However, this morphogenic pathway may be of more importance upon departures from homeostasis; following injury the hematopoietic regenerative response is augmented in mice with activating mutations in the pathway [16], and suppressed by the knockout of positive downstream effectors [15]. In other organs, Hh signal transduction ability is not restricted to tissue-specific stem cells, but also becomes active in stromal cell types following local injury [20-22], a paracrine process that has furthermore been implicated in solid tissue tumorigenesis [23-25]. These latter observations suggest potential non-cell-autonomous roles for Hh in certain tissues.

Recent evidence, however, suggests that the self-renewal capacity of CML-LSCs is dependent on Hh signaling in a cell-autonomous manner, characterized by increased mRNA expression of *GLI1* and *PTCH* in primary CML cells compared to healthy human control samples, as well as demonstrated sensitivity to Hh pathway blockade *in vitro* using cyclopamine, a SMO antagonist [17,26]. While early clinical trial results indicate promising effects of single-agent Hh inhibition in AML patients

in addition to CML patients [10], a mechanistic understanding of Hh signaling in AML and in normal HSCs remains elusive. Elevated Hh pathway gene expression in CD34⁺ cells of autologous stem cell transplant patients has been found to precede development of myelodysplastic syndrome and AML [27], but causal relationships to disease have not been identified. Studies of established AML revealed the presence of SHH- and GLI1-expressing cells in bone marrow (BM) smear preparations, in contrast to lymphoblastic leukemia samples [28], and *IHH*, *SMO*, *PTCH* and *GLI1* transcripts have also been detected in certain AML cell lines [29] and patient samples [8]. However, the expression levels of Hh pathway components have yet to be quantitatively evaluated or analyzed in functionally relevant populations of primary patient samples, relative to normal phenotypically-matched counterparts (hematopoietic stem and progenitor cells; HSPCs).

Accordingly, in the present study we have purified phenotypically primitive subsets of both human AML and healthy blood samples to clarify whether there is molecular evidence that the Hh pathway could represent a selective target in AML-LSCs. This was accompanied by clonal assays to characterize any functional divergence between cell-autonomous pathway dependence of AML versus normal blood progenitors. Based on our molecular and functional findings that this pathway does not represent a direct selective target for primitive AML cells, we examined the possibility of paracrine Hh signaling within BM cells isolated from mouse strains known to support human hematopoiesis, and we detected enriched expression of genes involved in the transduction of Hh signals within the non-hematopoietic BM

fraction. This was further validated in human BM-derived cells at both the transcript and protein levels. This finding could ultimately be of significance for understanding both normal injury responses in the healthy hematopoietic system, as well as interactions between AML-LSCs and the BM microenvironment.

Materials and Methods

Primary human samples

Primary blasts from AML patients were obtained from peripheral blood (PB), BM, or therapeutic leukapheresis. Table 2.1 outlines patient characteristics and how each sample was used experimentally. Healthy hematopoietic samples were isolated from early gestational cord blood, full term umbilical cord blood (UCB), or G-CSF mobilized peripheral blood samples (G-MPB). Informed consent was obtained from all sample donors in accordance with Research Ethics Board-approved protocols at McMaster University and the London Health Sciences Centre (Research Ethics Board #08-330 and #08-042). Additionally, healthy human BM samples were purchased from Lonza (Cedarlane, Burlington, ON Canada). Mononuclear cells (MNC) were recovered by density gradient centrifugation (Ficoll-Paque Premium; GE Healthcare, Cooksville, ON, Canada), and remaining red blood cells were lysed using ammonium chloride solution (StemCell Technologies, Vancouver, BC, Canada). Lineage depletion was performed using a commercially available kit (StemCell Technologies, Vancouver, BC, Canada), according to manufacturer's instructions.

Isolation of non-hematopoietic human BM cells

MNCs from healthy human donor BM were seeded at a density of 3×10^7 cells per T150 tissue culture flask in non-hematopoietic BM (NH-BM) media (MEM alpha supplemented with 20% fetal bovine serum (FBS) and 2 mM L-glutamine) and incubated at a humidity of 37°C with 5% CO₂. After five days, an equal volume of fresh media was added. A complete media change was performed after three to five additional days of culture. When the cell culture achieved 80% confluence, adherent cells were dissociated using TrypLE, washed, depleted of contaminating CD45⁺ hematopoietic cells using EasySep Human Whole Blood CD45 depletion kit (StemCell Technologies, Vancouver, BC, Canada), and re-plated in NH-BM media at a concentration of 7×10^3 cells per cm². Using flow cytometry, adherent non-hematopoietic cells were verified to express CD73 and CD105, and lack pan-hematopoietic marker CD45 (Supplementary Figure 2.1). All non-hematopoietic BM cells were observed to have fibroblast-like spindle morphology, without any adipocyte-like characteristics.

Mouse BM samples

All experimental procedures were approved by the Animal Council of McMaster University (Animal Use Protocol #13-02-05). Tibias, femurs, and iliac crests were dissected from 3-4 month-old immunodeficient (NOD SCID, NSG) and wildtype (C57BL/6) mice, and whole BM was recovered by crushing in Iscove's Modified Dulbecco's Medium (IMDM) supplemented with 3% FBS. Residual bone fragments were then incubated for one hour at 37°C in IMDM with 10% FBS and 3 mg/mL

collagenase in order to enzymatically dissociate additional non-hematopoietic BM cells. Recovered collagenase-treated cells were then pooled with mechanically dissociated cells, and red blood cells were lysed using ammonium chloride (StemCell Technologies, Vancouver, BC, Canada).

Fluorescence-activated cell sorting and flow cytometric analysis

Human hematopoietic samples (AML-PB, AML-BM, UCB, and G-MPB) were purified based on CD34 and CD38 expression using APC-conjugated anti-CD34 (clone 581), and PE-conjugated anti-CD38 (clone HB7; BD, Mississauga, ON, Canada). Both CD34⁺CD38⁻ and CD34⁺CD38⁺ fractions were collected for RNA extraction. Whole BM murine samples were sorted using PE-conjugated anti-Ter119 and APC-conjugated anti-CD45 (Clone 20-F11; BD, Mississauga, ON, Canada). The CD45⁻Ter119⁻ non-hematopoietic fractions were collected for RNA extraction, as well as the CD45⁺ or Ter119⁺ hematopoietic cells.

In order to evaluate the effects of target cell frequency on expression levels of an example gene of interest (CD3e), T cells were purified from human hematopoietic samples using PE-conjugated anti-CD3 (clone UCHT1; Beckman Coulter, Mississauga, ON, Canada). These CD3⁺ T cells were then combined with CD3⁻ myeloid cells at varying ratios (0.1%, 1%, 10%, 50%, and 100%) for RNA extraction.

The cell surface phenotype of non-hematopoietic human BM cells was assessed using Brilliant violet-conjugated anti-CD73 (Clone AD2), PE-conjugated anti-CD105 (Clone 266), and APC-conjugated anti-CD45 (Clone 2D1; BD, Mississauga, ON, Canada). For all experiments, cells were incubated with antibodies for 30 minutes at 4°C were then washed prior to flow cytometry, and 7-Aminoactinomycin D was used to exclude non-viable cells. A FACSaria II (BD, Mississauga, ON, Canada) was used for all cell purification experiments, and an LSRII Flow Cytometer (BD, Mississauga, ON, Canada) was used for experiments that did not require purification.

Quantitative real-time polymerase chain reaction

Total RNA was extracted from cell populations using a Total RNA Purification Kit (Norgen Biotek Corp, Thorold, ON, Canada), and this was followed by first-strand cDNA synthesis with qScript cDNA SuperMix (Quanta Biosciences, Gaithersburg, MD, USA), following manufacturer's instructions. Synthesized cDNA was added to Platinum SYBR Green qPCR SuperMix (Life Technologies, Burlington, ON, Canada) for quantitative real-time PCR, which was run on a Stratagene Mx3000P thermocycler (Agilent Technologies, Mississauga, ON, Canada). All real-time PCR primer sequences were designed to either span or flank introns, and all primer pairs have been confirmed to have an amplification efficiency of at least 87% at the temperatures used (refer to Table 2.2 for primer sequences and melting temperatures). Human primer sets were further validated by separating PCR

products on a 2% agarose gel, followed by gel extraction, and sequencing of PCR products, which confirmed the specificity of all human Hh pathway primers tested (Supplementary Figure 2.2). Relative gene expression was calculated using the $\Delta\Delta C_t$ method. *GAPDH* and *β -actin* were used as housekeeping genes for human and mouse experiments, respectively.

Methylcellulose colony forming unit assay (CFU)

AML MNCs and cord blood Lin⁻ cells were plated at 50 000 cells/mL and 200-1000 cells/mL, respectively, in semisolid methylcellulose media supplemented with cytokines (Methocult GF H4434; Stem Cell Technologies, Vancouver, BC, Canada) following established protocols [3]. Cyclopamine (Toronto Research Chemicals Inc., Toronto, ON, Canada) was dissolved in methylcellulose at concentrations ranging from 2 to 50 μ M, while control wells received 0.1% and 0.5% DMSO. Cells were incubated at 37°C for 14 days, and then scored for the presence of colonies using standard morphological criteria. Final colony counts were normalized to appropriate DMSO percentage controls (0.1% DMSO for 2 and 10 μ M cyclopamine concentrations, and 0.5% DMSO for 50 μ M cyclopamine).

Analysis of Affymetrix Gene Arrays

Eleven different gene expression series were downloaded from the Gene Expression Omnibus repository (GEO; www.ncbi.nlm.nih.gov/geo), representing human hematopoietic cells from AML patients and non-leukemic individuals, as well as non-hematopoietic cells from human BM (Table 2.3). Hematopoeitic collections included

both unpurified MNCs or subpopulations isolated based on stem, progenitor, or stem/progenitor cell surface phenotypes. This strategy of hematopoietic dataset selection was designed to determine whether the purity of functionally primitive cells influences the gene expression signature for the Hh pathway. The selection of non-hematopoietic collections were deliberately chosen from seven independent published sources to ensure that the technical variability within experimental conditions (e.g. non-hematopoietic BM cells) would be equal to that between experimental conditions (e.g. hematopoietic versus non-hematopoietic cells). Several non-hematopoietic BM collections contained samples subjected to various experimental treatment conditions. In these cases, experimentally treated samples were omitted from analyses and only non-treated controls were used (as outlined in Table 2.3).

The type of microarray platform was consistent across all eleven gene expression series (HG-U133 Plus 2.0). Agilent GeneSpring GX-11 software (Santa Clara, CA USA) was used for data normalization and analysis. Data were log₂ transformed following RMA normalization. A principal component analysis (PCA) was performed on the entire collection of eleven datasets after filtering genes based on a curated Hh pathway gene list (PID_HEDGEHOG_2PATHWAY) from the Pathway Interaction Database (M211), with the addition of genes *GLI1*, *GLI3*, and *SUFU*. Additionally, the gene expression intensities for selected Hh pathway genes (*HHIP*, *SMO*, and *PTCH*) were compared between non-diseased BM MNCs and non-hematopoietic BM samples.

Immunofluorescence

Human cord blood MNCs and plastic adherent non-hematopoietic BM cells were fixed in 4% paraformaldehyde for 20 minutes at 4°C. Cells were permeabilized for 5 minutes in 0.01% Triton X-100. Blocking was performed using 5% FBS/PBS. Rabbit polyclonal anti-SMO primary antibody (H-300; Santa Cruz Biotechnology, Dallas, Texas, USA) was diluted 1:250 and incubated overnight at 4°C in blocking solution. Samples were washed three times for 5 minutes in PBS-T (PBS + 0.01% Tween-20) and incubated in secondary Alexa Fluor 647 goat anti-rabbit antibody (Life Technologies, Burlington, ON, Canada) at 1:500 for 1 hour at room temperature. Controls used to set background thresholds were incubated with secondary antibody only. Samples were then washed three times in PBS-T, counterstained with DAPI and visualized using an Olympus inverted spinning disk confocal microscope (Richmond Hill, ON, Canada). Images were captured using Metamorph software (Molecular Devices, Sunnyvale, CA, USA) and multi-channel overlays were made using the open source ImageJ software suite.

Statistical analysis

Data are represented as mean \pm SEM. Unpaired two-tailed Student's t-tests were used for statistical comparisons of gene expression data, with the exception of the purified hematopoietic versus non-hematopoietic cell gene expression, which was analyzed with paired two-tailed Student's t-tests. CFU assays were analyzed using One-way ANOVAs, followed by Newman-Keuls multiple comparison tests. Prism

(version 5.0a, GraphPad, San Diego, CA, USA) software was used for all statistical analyses, and the criterion for statistical significance was $p < .05$.

Results

Gene expression analyses suggest that cell-autonomous dependence on Hh signaling is unlikely within primitive AML cells.

Hh pathway transcripts were profiled in a quantitative manner, in highly purified AML patient samples enriched for LSCs, and compared to purified populations of normal hematopoietic cells enriched for HSCs and progenitors. We chose to evaluate both CD34⁺CD38⁻ and CD34⁺CD38⁺ purified populations (Figure 2.1A), as these immunophenotypes respectively represent stem and progenitor fractions of normal HSCs, and LSCs have been reliably found to reside in highest frequencies within these subsets [1,4,5,30].

The Hh pathway gene expression profile was almost identical between AML samples and normal G-MPB, except for a down-regulation of *SMO* within AML samples in both the CD34⁺CD38⁻ and CD34⁺CD38⁺ fractions (Figure 2.1B). This was consistent across AML samples despite variable stages of disease (progression from MDS, new diagnosis, therapy-resistant, and relapse), and different tissue sources (PB or BM; Table 2.1). To confirm our findings of reduced *SMO* expression in AML cells relative to normal CD34⁺ cells, we repeated cDNA synthesis from G-MPB donors and 4 of the AML patients originally assessed. We further expanded our sample size and diversity by purifying cells from 4 additional healthy hematopoietic samples (2 G-

MPB and 2 UCB) and 2 additional AML samples, totaling 6 AML patients in our overall analysis. In this analysis, we compared *SMO* expression to that of beta glucuronidase (*GUSB*), a uniformly expressed housekeeping gene with a low abundance of transcripts on a per-cell basis, with the fidelity to detect single cell transcript contributions [19,31]. *GUSB* is often used diagnostically to molecularly monitor the frequency of BCR-ABL-containing cells in chronic myeloid leukemia blood samples [32,33]. As shown in Figure 2.1C, *SMO* expression was consistently higher in normal hematopoietic controls relative to AML, regardless of stage of HSPC ontogeny (both adult and fetal sources of normal blood were used in this analysis). As expected of a housekeeping gene, *GUSB* expression did not differ between normal and leukemic samples, but was found to be more highly expressed than *SMO* in both AML and healthy controls (Figure 2.1C, 2.1D, and Supplementary Figure 2.3). That *SMO* expression is less than that of a gene that is uniformly expressed at low levels suggests that the expression of *SMO* transcripts is likely heterogenous, and is restricted to a sub-fraction of the already relatively purified populations assessed. This in-depth analysis is unlike previous reports in CML and suggests that only rare subfractions of AML-LSCs and normal HSCs utilize Hh signaling.

AML progenitors lack cell-autonomous sensitivity to SMO inhibition in vitro.

We next sought to evaluate the functional response of pharmacological Hh pathway inhibition in normal and leukemic cells at the clonal level. Cyclopamine, a *SMO* antagonist, was dissolved in semisolid methylcellulose media at doses ranging from

2 to 50 μM and compared to appropriate volume ratios of vehicle control (DMSO). Neither normal nor leukemic colony formation was influenced by SMO inhibition, except at the 50 μM concentration, which suppressed the formation of leukocyte colonies in cord blood but had minimal effects on AML colony formation (Figure 2.2). Since cyclopamine suppressed normal progenitors to a greater extent than AML after being evaluated beyond concentrations expected to generate off-target effects [17], this suggests that Hh pathway blockade is not a direct selective target of primary AML cells. This lack of cell-autonomous dependence of AML cells on Hh signaling is consistent with our gene expression data of reduced *SMO* mRNA and unchanged levels of downstream transcripts in phenotypically primitive cells. However, paracrine Hh signaling has been detected in several solid tumors [24,25] and cannot be ruled out by our molecular or functional analyses.

Hh signal-transduction machinery is more abundantly expressed in non-hematopoietic BM cell fractions.

Given the rarity of human AML-LSCs and HSC-enriched cells that express cell surface Hh pathway components, we evaluated the possibility that non-hematopoietic BM cells may be able to support paracrine Hh signaling. We performed Hh pathway expression profiling of non-hematopoietic (CD45⁻Ter119⁻) compared to hematopoietic (CD45⁺ or Ter119⁺) cells isolated from the BM of mice used for the functional detection of both AML-LSCs and normal HSCs [1,4,5] (Figure 2.3A). Relative Hh gene expression patterns between these BM fractions of

immunodeficient mice were similar to those found in wildtype mice, despite differences in hematopoietic cell composition between strains [34] (Supplementary Figure 2.4). In comparison to hematopoietic cells, CD45⁻Ter119⁻ BM cells had almost 13-fold higher expression of *Smo*, and 38-fold higher expression of *Ptch* (Figure 2.3B). Notably, both receptors were detected in higher levels than *Gusb* in non-hematopoietic BM cells, whereas *Smo* and *Ptch* were less abundant than *Gusb* in the hematopoietic fraction (as was seen in the primitive human samples). This suggests that Smo⁺ cells may be more frequent in the non-hematopoietic BM fraction than within developing blood cell populations.

In order to confirm the significance of the *GUSB* threshold as a predictive measure of gene expression heterogeneity, we evaluated mRNA levels in hematopoietic populations with known frequencies of cells expressing an example gene of interest. CD3 protein-expressing human T cells were combined at various ratios with hematopoietic cells lacking CD3 protein expression (Figure 2.3C). *CD3e* mRNA levels were then measured in these mixtures by qPCR and compared to those of *GUSB*. *CD3e* expression was only detected below that of *GUSB* once the CD3⁺ cells represented 10% or less of the assayed population, supporting our conclusion that *Ptch* and *Smo*-expressing cells are more frequent in non-hematopoietic versus hematopoietic mouse BM populations. As the non-hematopoietic fraction of BM represents a substantially heterogeneous group of different cell types, it is surprising that *Smo* and *Ptch* were detected above the *Gusb* threshold, considering that both remained well below *Gusb* levels even in the more purified primitive

human hematopoietic populations. It is most likely that the underrepresentation of *Smo* transcripts in hematopoietic BM fractions reflects a combination of reduced numbers of transcripts per cell as well as reduced numbers of transcript-expressing cells (as interpreted by relative *Gusb* expression).

In order to extend our observations of Hh receptor enrichment within non-hematopoietic cells in murine BM, we accessed publicly available human mRNA expression profiles from the GEO database. A principal component analysis was performed on Hh pathway gene expression from eleven merged datasets, including hematopoietic samples from AML patients and non-leukemic individuals, as well as non-hematopoietic BM-derived cells (Figure 2.4A). Both bulk MNCs and purified primitive populations were represented within hematopoietic datasets. Despite the diversity represented among seven different non-hematopoietic BM datasets evaluated, the Hh profiles of non-hematopoietic fractions consistently clustered together, distinctly from all hematopoietic-derived cells. Furthermore, there were no appreciable differences in Hh signaling between AML samples and non-diseased hematopoietic cells. This was true regardless of whether samples were enriched for stem/progenitor phenotypes or not, supporting our earlier observations. We then more specifically assessed the relative expression levels of genes critical to the Hh signal transduction process in hematopoietic MNCs compared to non-hematopoietic fractions of human BM. Consistent with murine gene expression data, human non-hematopoietic BM cells expressed higher transcript levels of cell surface receptors

SMO and *PTCH*, and also *HHIP*, a protein involved in cell surface binding of Hh ligands (Figure 2.4B).

Finally, we validated the *SMO* transcript expression patterns that were observed in both mouse and human BM by assessing *SMO* at the protein level in human samples. Non-hematopoietic cells were isolated from human BM by plastic adherence, and were confirmed to lack any contaminating blood cells (Supplementary Figure 2.1). Protein immunofluorescence indicated that these non-hematopoietic cells expressed appreciable levels of *SMO*, and that this expression was consistent across the population. In contrast, normal human MNCs expressed *SMO* protein in a more heterogeneous fashion (Figure 2.4C), as had been predicted by gene expression levels relative to *Gusb*. This higher abundance of *SMO* expression in non-hematopoietic BM fractions was consistent across all transcript and protein level evaluations of both human and mouse tissue, as measured across three independently collected datasets.

Collectively, our results indicate that cells expressing Hh signal transduction machinery may even be more numerous in the BM microenvironment niche cells compared to committed or uncommitted blood populations. This provides unexpected evidence of possible paracrine Hh signaling within the marrow that could better explain the potential role of Hh modulators shown to be efficacious in AML and CML patients [10]. Accordingly, *SMO*⁺ cells in the BM niche could support LSCs in a manner similar to that in which myofibroblast-secreted factors have been shown to maintain cancer stem cell features in colorectal cancer [35].

Discussion

We have molecularly and functionally evaluated Hh signaling using over 200 different primary AML samples and we conclude that cell-intrinsic addiction to the Hh pathway is unlikely, particularly within primitive human AML cells. This is as evidenced by reduced signal-transduction machinery compared to normal controls, without compensatory overexpression of downstream GLI factors by alternate mechanisms. Importantly, we found that molecular Hh pathway signatures do not discriminate AML samples from normal hematopoietic cells whether or not they are subjected to stringent purification based on primitive cell surface phenotypes. The lack of Hh pathway dependence was further substantiated by similar responses to pharmacological pathway blockade between normal HSPCs and AML throughout a range of inhibitor concentrations. However, we did find evidence of Hh signal-transduction capacity in non-hematopoietic BM cells of both mouse and human samples, with Hh receptor-expressing cells appearing surprisingly abundant in this heterogeneous fraction, especially in comparison to their low abundance among purified primitive hematopoietic cells.

Our data contrast those of Kobune et al. [29], who were able to demonstrate chemosensitizing properties of cyclopamine in AML cell lines and a small number of patient samples. One source of variability between studies is that the analysis by Kobune et al. [29] was exclusively based on unpurified samples, and our results do not preclude the possibility that the more differentiated AML blasts may be functionally sensitive to SMO inhibition. We chose to instead focus on populations

responsible for leukemic relapse and prolonged maintenance of disease, due to the greater clinical significance. This approach demonstrated that the molecular determinants of AML-LSC self-renewal differ from those of CML-LSCs, which do appear to be cell-autonomously dependent on Hh signaling [17,26]. However, our data do support a potential role for paracrine Hh signaling between the BM microenvironment and HSCs, complementing findings generated using conditional knockout mice in which the ablation of *Ptch1* in osteoblastic lineages had more dramatic effects on hematopoiesis than *Ptch1* deletion in hematopoietic lineages [36]. Suppression of Hh signals in the BM microenvironment could also be relevant to AML-LSC behaviour, and such a mechanism could explain the clinical effectiveness of Hh inhibitors in the treatment of AML [10] despite the absence of LSC cell-intrinsic targets of these inhibitors.

While the efficacy of direct therapeutic targeting of AML-LSCs through the Hh pathway is unlikely, our findings suggest that paracrine signaling may exist between non-hematopoietic BM cells and HPSC/LSC populations, which could be of therapeutic significance. Since most AML patients experience anemia and cytopenia as part of the clinical course of the disease [37], this should stimulate hematopoietic injury-recognition and supportive response mechanisms within the BM microenvironment, which likely involves Hh signaling. This proposed model is well supported by stromal-parenchymal interactions in other normal and malignant tissues [20-25,35], and complements recent evidence that cells in tumor microenvironments can actively maintain the phenotypic properties of cancer stem

cells [35]. In order to properly understand whether Hh signaling represents a target to therapeutically disrupt LSC-microenvironment interactions, it will first be necessary to more carefully dissociate the importance of Hh signaling within HSCs versus non-hematopoietic BM cells under normal and stressful conditions. *In vivo* experimentation will ultimately be critical to confirm our prediction that any therapeutic utility of the Hh signaling pathway in AML will be through paracrine, rather than autocrine, signaling mechanisms.

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References

1. Eppert K, Takenaka K, Lechman ER, Waldron L, Nilsson B, van Galen P, Metzeler KH, Poepl A, Ling V, Beyene J, Canty AJ, Danska JS, Bohlander SK, Buske C, Minden MD, Golub TR, Jurisica I, Ebert BL, Dick JE (2011) Stem cell gene expression programs influence clinical outcome in human leukemia. *Nature Medicine* 17:1086
2. Hope KJ, Jin L, Dick JE (2004) Acute myeloid leukemia originates from a hierarchy of leukemic stem cell classes that differ in self-renewal capacity. *Nature Immunology* 5:738
3. Sachlos E, Risueno RM, Laronde S, Shapovalova Z, Lee JH, Russell J, Malig M, McNicol JD, Fiebig-Comyn A, Graham M, Levadoux-Martin M, Lee JB, Giacomelli AO, Hassell JA, Fischer-Russell D, Trus MR, Foley R, Leber B, Xenocostas A, Brown ED, Collins TJ, Bhatia M (2012) Identification of drugs including a dopamine receptor antagonist that selectively target cancer stem cells. *Cell* 149:1284
4. Bonnet D, Dick JE (1997) Human acute myeloid leukemia is organized as a hierarchy that originates from a primitive hematopoietic cell. *Nature Medicine* 3:730
5. Lapidot T, Sirard C, Vormoor J, Murdoch B, Hoang T, Caceres-Cortes J, Minden M, Paterson B, Caligiuri MA, Dick JE (1994) A cell initiating human acute myeloid leukaemia after transplantation into SCID mice. *Nature* 367:645
6. Campbell C, Risueño RM, Salati S, Guezguez B, Bhatia M (2008) Signal control of hematopoietic stem cell fate: Wnt, Notch, and Hedgehog as the usual suspects. *Current Opinion in Hematology* 15:319
7. Sengupta A, Banerjee D, Chandra S, Banerji SK, Ghosh R, Roy R, Banerjee S (2007) Deregulation and cross talk among Sonic hedgehog, Wnt, Hox and Notch signaling in chronic myeloid leukemia progression. *Leukemia* 21:949
8. Fiedler W, Vohwinkel G, Krauter J, Vettorazzi E, Loges S, Fuhrmann L, Seyfert P, Bokemeyer C, Wellbrock J (2011) Expression of Gli2, a Downstream Mediator of the Activated Hedgehog Pathway, Represents a Negative Prognostic Marker in AML. *ASH Annual Meeting Abstracts* 118:3535
9. Irvine DA, Copland M (2012) Targeting hedgehog in hematological malignancy. *Blood* 119:2196
10. Jamieson C, Cortes JE, Oehler V, Baccarani M, Kantarjian HM, Papayannidis C, Rice KN, Zhang X, Shaik N, Courtney R, Levin WJ, Martinelli G (2011) Phase 1 Dose-

Escalation Study of PF-04449913, An Oral Hedgehog (Hh) Inhibitor, in Patients with Select Hematologic Malignancies. ASH Annual Meeting Abstracts 118:424

11. Lin TL, Matsui W (2012) Hedgehog pathway as a drug target: Smoothened inhibitors in development. *OncoTargets and therapy* 5:47

12. Gao J, Graves S, Koch U, Liu S, Jankovic V, Buonamici S, El Andaloussi A, Nimer SD, Kee BL, Taichman R, Radtke F, Aifantis I (2009) Hedgehog Signaling Is Dispensable for Adult Hematopoietic Stem Cell Function. *Cell Stem Cell* 4:548

13. Hofmann I, Stover EH, Cullen DE, Mao J, Morgan KJ, Lee BH, Kharas MG, Miller PG, Cornejo MG, Okabe R, Armstrong SA, Ghilardi N, Gould S, de Sauvage FJ, McMahon AP, Gilliland DG (2009) Hedgehog Signaling Is Dispensable for Adult Murine Hematopoietic Stem Cell Function and Hematopoiesis. *Cell Stem Cell* 4:559

14. Mar BG, Amakye D, Aifantis I, Buonamici S (2011) The controversial role of the Hedgehog pathway in normal and malignant hematopoiesis. *Leukemia* 25:1665

15. Merchant A, Joseph G, Wang Q, Brennan S, Matsui W (2010) Gli1 regulates the proliferation and differentiation of HSCs and myeloid progenitors. *Blood* 115:2391

16. Trowbridge JJ, Scott MP, Bhatia M (2006) Hedgehog modulates cell cycle regulators in stem cells to control hematopoietic regeneration. *Proceedings of the National Academy of Sciences* 103:14134

17. Zhao C, Chen A, Jamieson CH, Fereshteh M, Abrahamsson A, Blum J, Kwon HY, Kim J, Chute JP, Rizzieri D, Munchhof M, VanArsdale T, Beachy PA, Reya T (2009) Hedgehog signalling is essential for maintenance of cancer stem cells in myeloid leukaemia. *Nature* 458:776

18. Hooper JE, Scott MP (2005) Communicating with Hedgehogs. *Nature Reviews Molecular Cell Biology* 6:306

19. Bhardwaj G, Murdoch B, Wu D, Baker DP, Williams KP, Chadwick K, Ling LE, Karanu FN, Bhatia M (2001) Sonic hedgehog induces the proliferation of primitive human hematopoietic cells via BMP regulation. *Nature Immunology* 2:172

20. Fabian SL, Penchev RR, Jacques BS, Rao AN, Sipilä P, West KA, McMahon AP, Humphreys BD (2012) Hedgehog-Gli Pathway Activation during Kidney Fibrosis. *The American Journal of Pathology* 180:1441

21. Omenetti A, Diehl AM (2008) The Adventures of Sonic Hedgehog in Development and Repair. II. Sonic hedgehog and liver development, inflammation, and cancer. *AJP: Gastrointestinal and Liver Physiology* 294:G595
22. Shin K, Lee J, Guo N, Kim J, Lim A, Qu L, Mysorekar IU, Beachy PA (2011) Hedgehog/Wnt feedback supports regenerative proliferation of epithelial stem cells in bladder. *Nature* 472:110
23. O'Toole SA, Swarbrick A, Sutherland RL (2009) The Hedgehog signalling pathway as a therapeutic target in early breast cancer development. *Expert Opinion on Therapeutic Targets* 13:1095
24. Tian H, Callahan CA, DuPree KJ, Darbonne WC, Ahn CP, Scales SJ, de Sauvage FJ (2009) Hedgehog signaling is restricted to the stromal compartment during pancreatic carcinogenesis. *Proceedings of the National Academy of Sciences of the United States of America* 106:4254
25. Yauch RL, Gould SE, Scales SJ, Tang T, Tian H, Ahn CP, Marshall D, Fu L, Januario T, Kallop D, Nannini-Pepe M, Kotkow K, Marsters JC, Rubin LL, de Sauvage FJ (2008) A paracrine requirement for hedgehog signalling in cancer. *Nature* 455:406
26. Dierks C, Beigi R, Guo G-R, Zirlik K, Stegert MR, Manley P, Trussell C, Schmitt-Graeff A, Landwerlin K, Veelken H, Warmuth M (2008) Expansion of Bcr-Abl-Positive Leukemic Stem Cells Is Dependent on Hedgehog Pathway Activation. *Cancer Cell* 14:238
27. Li L, Li M, Sun C, Francisco L, Chakraborty S, Sabado M, McDonald T, Gyorffy J, Chang K, Wang S, Fan W, Li J, Zhao LP, Radich J, Forman S, Bhatia S, Bhatia R (2011) Altered Hematopoietic Cell Gene Expression Precedes Development of Therapy-Related Myelodysplasia/Acute Myeloid Leukemia and Identifies Patients at Risk. *Cancer Cell* 20:591
28. Bai L-Y, Chiu C-F, Lin C-W, Hsu N-Y, Lin C-L, Lo W-J, Kao M-C (2008) Differential expression of Sonic hedgehog and Gli1 in hematological malignancies. *Leukemia* 22:226
29. Kobune M, Takimoto R, Murase K, Iyama S, Sato T, Kikuchi S, Kawano Y, Miyanishi K, Sato Y, Niitsu Y, Kato J (2009) Drug resistance is dramatically restored by hedgehog inhibitors in CD34⁺ leukemic cells. *Cancer Science* 100:948
30. Ishikawa F, Yoshida S, Saito Y, Hijikata A, Kitamura H, Tanaka S, Nakamura R, Tanaka T, Tomiyama H, Saito N, Fukata M, Miyamoto T, Lyons B, Ohshima K, Uchida N, Taniguchi S, Ohara O, Akashi K, Harada M, Shultz LD (2007) Chemotherapy-

resistant human AML stem cells home to and engraft within the bone-marrow endosteal region. *Nature Biotechnology* 25:1315

31. Oshima A, Kyle JW, Miller RD, Hoffmann JW, Powell PP, Grubb JH, Sly WS, Tropak M, Guise KS, Gravel RA (1987) Cloning, sequencing, and expression of cDNA for human beta-glucuronidase. *Proc Natl Acad Sci U S A* 84:685

32. Lee JW, Chen Q, Knowles DM, Cesarman E, Wang YL (2006) β -Glucuronidase Is an Optimal Normalization Control Gene for Molecular Monitoring of Chronic Myelogenous Leukemia. *The Journal of Molecular Diagnostics* 8:385

33. Wang YL, Lee JW, Cesarman E, Jin DK, Csernus B (2006) Molecular monitoring of chronic myelogenous leukemia: identification of the most suitable internal control gene for real-time quantification of BCR-ABL transcripts. *The Journal of molecular diagnostics : JMD* 8:231

34. Shultz LD, Schweitzer PA, Christianson SW, Gott B, Schweitzer IB, Tennent B, McKenna S, Mobraaten L, Rajan TV, Greiner DL, et al. (1995) Multiple defects in innate and adaptive immunologic function in NOD/LtSz-scid mice. *J Immunol* 154:180

35. Vermeulen L, De Sousa E Melo F, van der Heijden M, Cameron K, de Jong JH, Borovski T, Tuynman JB, Todaro M, Merz C, Rodermond H, Sprick MR, Kemper K, Richel DJ, Stassi G, Medema JP (2010) Wnt activity defines colon cancer stem cells and is regulated by the microenvironment. *Nature Cell Biology* 12:468

36. Siggins SL, Nguyen NY, McCormack MP, Vasudevan S, Villani R, Jane SM, Wainwright BJ, Curtis DJ (2009) The Hedgehog receptor Patched1 regulates myeloid and lymphoid progenitors by distinct cell-extrinsic mechanisms. *Blood* 114:995

37. Liesner RJ, Goldstone AH (1997) ABC of clinical haematology. The acute leukaemias. *Bmj* 314:733

38. Cheung AMS, Chow HCH, Liang R, Leung AYH (2009) A comparative study of bone marrow and peripheral blood CD34⁺myeloblasts in acute myeloid leukaemia. *British Journal of Haematology* 144:484

39. Majeti R, Becker MW, Tian Q, Lee T-LM, Yan X, Liu R, Chiang J-H, Hood L, Clarke MF, Weissman IL (2009) Dysregulated gene expression networks in human acute myelogenous leukemia stem cells. *Proceedings of the National Academy of Sciences* 106:3396

40. Gentles AJ, Plevritis SK, Majeti R, Alizadeh AA (2010) Association of a leukemic stem cell gene expression signature with clinical outcomes in acute myeloid leukemia. *JAMA* 304:2706
41. Mills KI, Kohlmann A, Williams PM, Wieczorek L, Liu W-M, Li R, Wei W, Bowen DT, Loeffler H, Hernandez JM, Hofmann W-K, Haferlach T (2009) Microarray-based classifiers and prognosis models identify subgroups with distinct clinical outcomes and high risk of AML transformation of myelodysplastic syndrome. *Blood* 114:1063
42. Sacchetti B, Funari A, Michienzi S, Di Cesare S, Piersanti S, Saggio I, Tagliafico E, Ferrari S, Robey P, Riminucci M, Bianco P (2007) Self-Renewing Osteoprogenitors in Bone Marrow Sinusoids Can Organize a Hematopoietic Microenvironment. *Cell* 1:324
43. Kubo H, Shimizu M, Taya Y, Kawamoto T, Michida M, Kaneko E, Igarashi A, Nishimura M, Segoshi K, Shimazu Y, Tsuji K, Aoba T, Kato Y (2009) Identification of mesenchymal stem cell (MSC)-transcription factors by microarray and knockdown analyses, and signature molecule-marked MSC in bone marrow by immunohistochemistry. *Genes Cells* 14:407
44. Grundberg E, Brändström H, Lam KCL, Gurd S, Ge B, Harmsen E, Kindmark A, Ljunggren O, Mallmin H, Nilsson O, Pastinen T (2008) Systematic assessment of the human osteoblast transcriptome in resting and induced primary cells. *Physiol. Genomics*. 2008 33:301
45. Mrugala D, Dossat N, Ringe J, Delorme B, Coffy A, Bony C, Charbord P, Haupl T, Daures JP, Noel D, Jorgensen C (2009) Gene expression profile of multipotent mesenchymal stromal cells: Identification of pathways common to TGFbeta3/BMP2-induced chondrogenesis. *Cloning Stem Cells*. 2009 11:61
46. Wagner W, Bork S, Horn P, Krunic D, Walenda T, Diehlmann A, Benes V, Blake J, Huber F-X, Eckstin V, Boukamp P, Ho AD (2009) Aging and Replicative Senescence Have Related Effects on Human Stem and Progenitor Cells. *PLoS ONE* 4:e5846
47. Rivron NC, Raiss CC, Liu J, Nandakumar A, Sticht C, Gretz N, Truckenmuller R, Rouwkema J, van Blitterswijk CA (2012) Sonic Hedgehog-activated engineered blood vessels enhance bone tissue formation. *Proceedings of the National Academy of Sciences* 109:4413
48. Guilloton F, Caron G, Ménard C, Pangault C, Amé-Thomas P, Dulong J, De Vos J, Rossille D, Henry C, Lamy T, Fouquet O, Fest T, Tarte K (2012) Mesenchymal stromal cells orchestrate follicular lymphoma cell niche through the CCL2-dependent recruitment and polarization of monocytes. *Blood* 119:2556

Figures and Tables

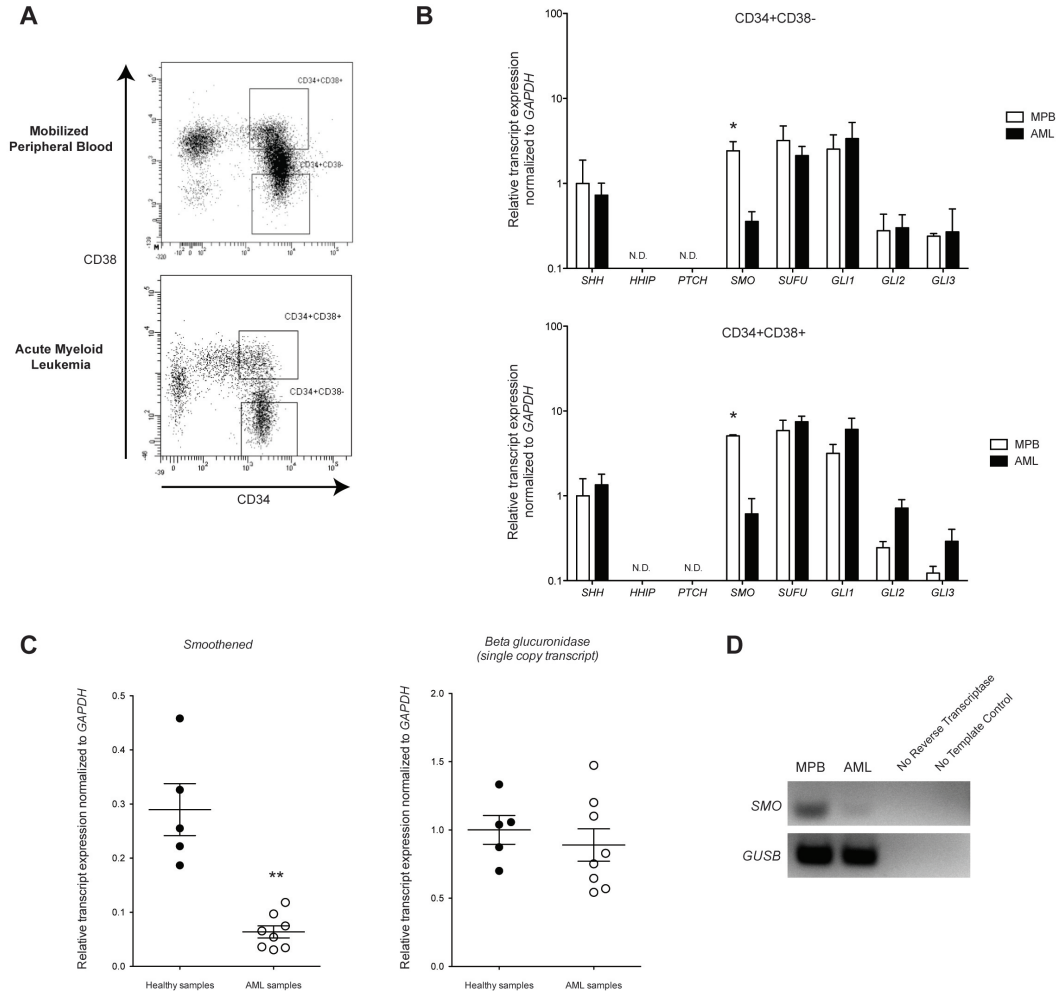


Figure 2.1. Hh signaling is not increased at the mRNA level in primitive AML cells. (A) Gating strategy used to isolate CD34⁺CD38⁻ and CD34⁺CD38⁺ populations from normal and leukemic hematopoietic samples. **(B)** Relative expression levels of Hh pathway transcripts in CD34⁺CD38⁻ and CD34⁺CD38⁺ of G-MPB and AML. $n = 2$ G-MPB samples, 6 AML patient samples. Expression levels were normalized to *GAPDH*. N.D.: not detected; * $p < .05$ relative to phenotypically matched G-MPB samples. **(C)** Relative expression levels of *SMO* and *GUSB* mRNA in CD34⁺ cells isolated from AML patients, as well as normal controls (UCB or G-MPB). $n = 5$ healthy samples, 8 samples from 6 AML patients. The scales of both graphs were adjusted such that the average *GUSB* expression in normal controls is equivalent to 1. ** $p < .005$. **(D)** Agarose gel electrophoresis of *SMO* and *GUSB* products in CD34⁺ G-MPB and AML samples following 30 cycles of PCR, including no reverse transcriptase and no cDNA controls.

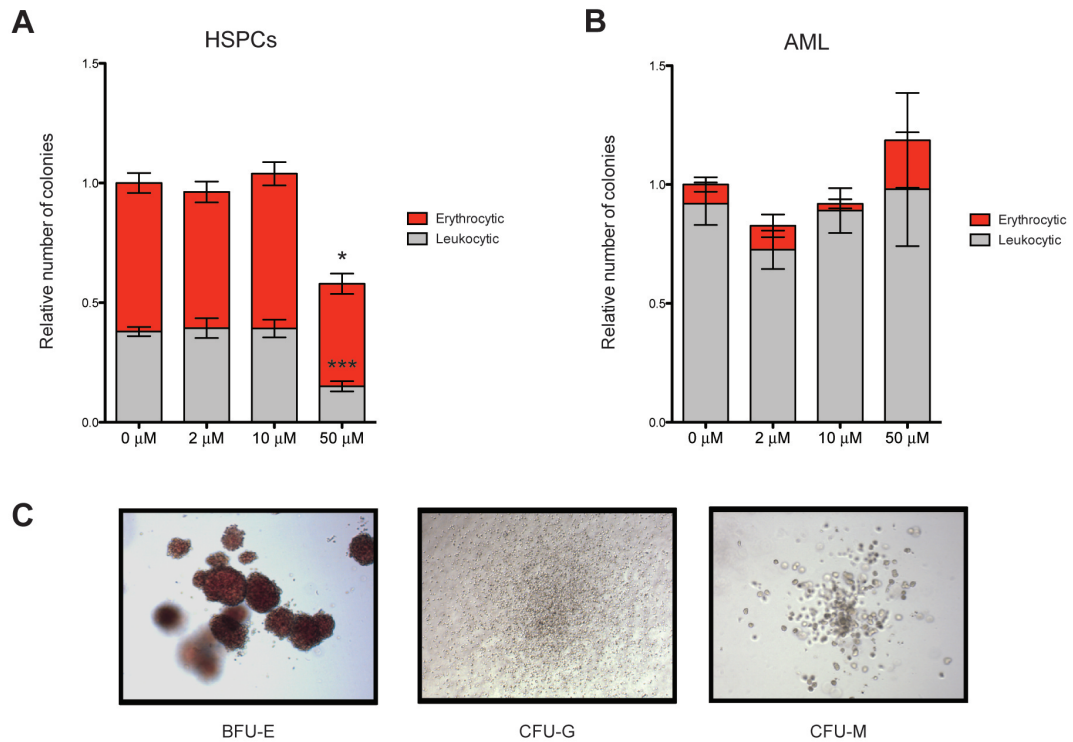


Figure 2.2. AML progenitors are not selectively sensitive to pharmacological inhibition of Hh signaling *in vitro*. Relative erythrocytic and leukocytic colony forming ability of cord blood HSPCs (A) and AML (B) samples in the presence of 2-50 μM cyclopamine. $n=5$ cord blood samples, 6 AML samples. *** $p<.0001$ relative to DMSO control leukocyte colony number, * $p<.05$ relative to DMSO control erythrocyte colony number. (C) Representative images of erythrocytic and leukocytic (granulocyte and monocyte) colonies.

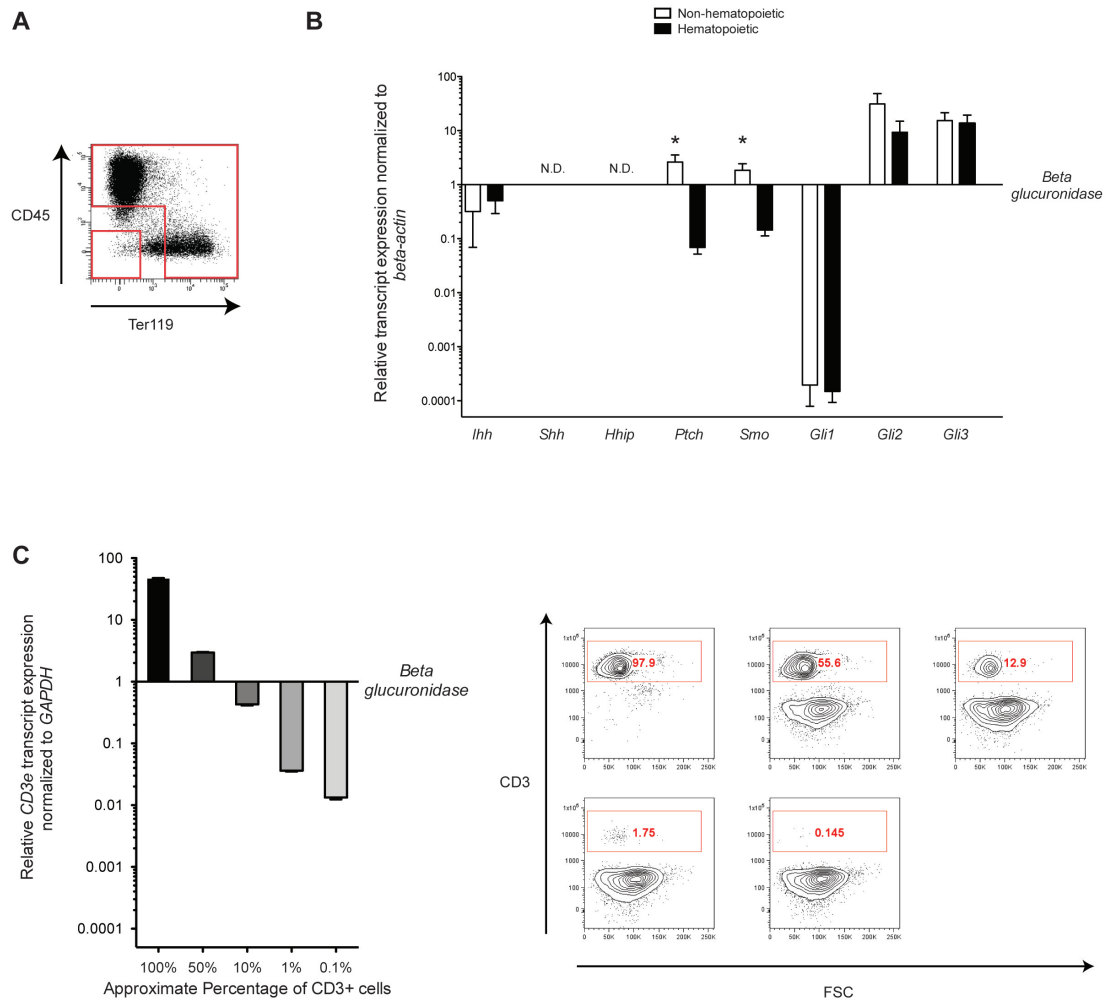


Figure 2.3. Hh pathway receptors are enriched in non-hematopoietic fractions of mouse BM. (A) Gating strategy to isolate hematopoietic (CD45⁺ or Ter119⁺) and non-hematopoietic (CD45⁻Ter119⁻) cells from mechanically and enzymatically dissociated BM. (B) Relative expression levels of Hh pathway transcripts between hematopoietic (CD45⁺ or Ter119⁺) and non-hematopoietic (CD45⁻Ter119⁻) mouse BM cells ($n=5-9$). Expression levels were normalized to *Actb*, and scaled to reflect transcript levels of *Gusb*. N.D.: not detected; * $p<.05$. (C) CD3 protein-expressing T cells were diluted at varying concentrations among non-expressing myeloid cells to evaluate the sensitivity of relative *Gusb* expression to detect changes in the cell frequency of an example population (CD3⁺ T cells). *CD3e* mRNA expression was found to drop below that of *GUSB* once the frequency of the CD3⁺ target population fell below 10%. Expression levels were normalized to *GAPDH*. Flow cytometry plots indicate the precise frequencies of CD3⁺ cells contained within each cell mixture.

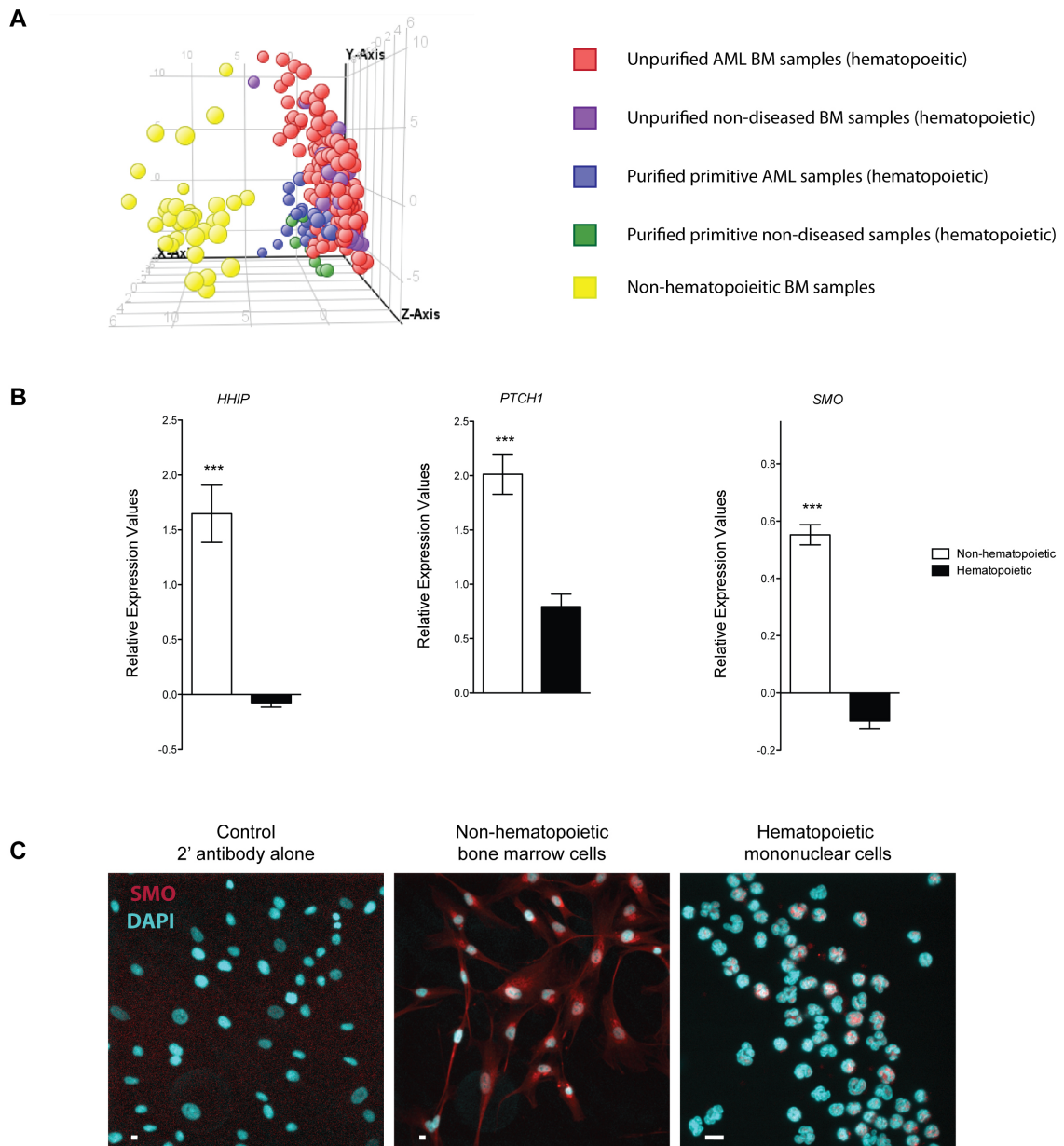
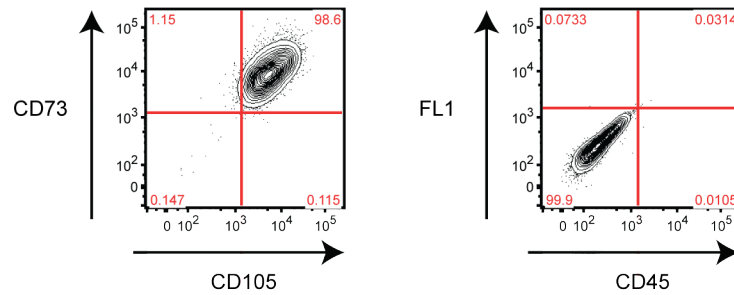
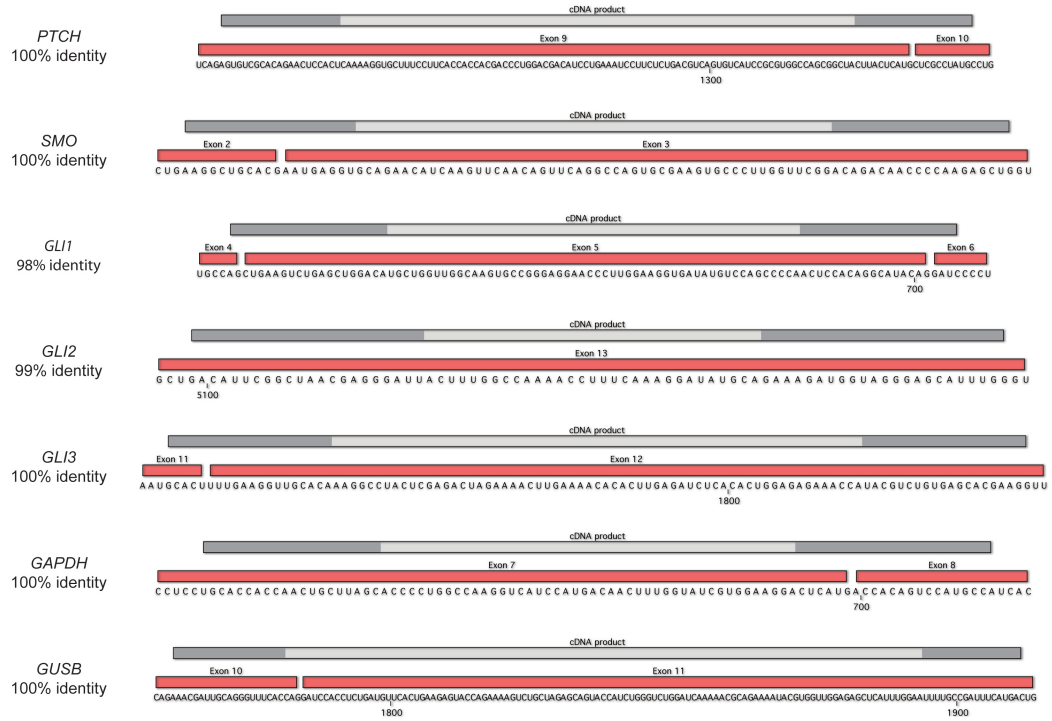


Figure 2.4. Hh pathway receptors are enriched in non-hematopoietic fractions of human BM. (A) The three principal components of Hh pathway gene expression are graphically represented. Substantial differences in Hh signaling are evident in non-hematopoietic human BM cells ($n=40$), as compared to hematopoietic samples from leukemic ($n=229$) or non-leukemic ($n=76$) sources. Leukemic and non-leukemic hematopoietic cells clustered together regardless of whether functionally primitive fractions were isolated or not. (B) Relative to non-leukemic hematopoietic BM cells ($n=69$), non-hematopoietic BM cells ($n=40$) have enriched transcript expression for genes typical of Hh-responding cells (*SMO*, *PTCH*, and *HHIP*). (C) *SMO*

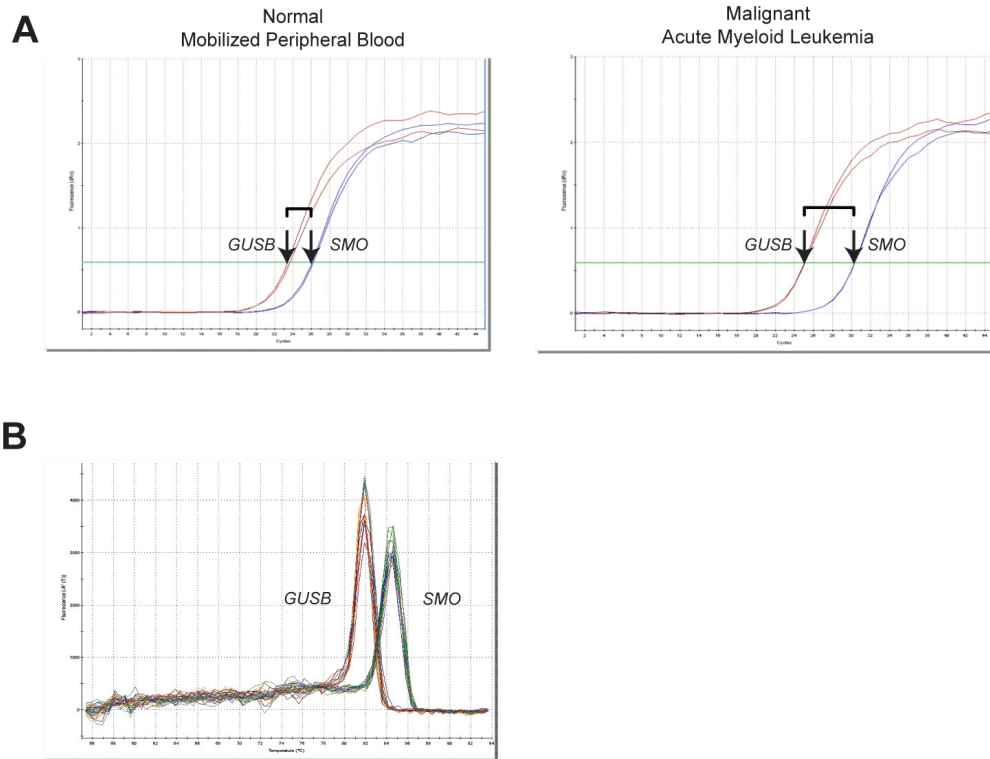
protein expression is consistently detected in non-hematopoietic human BM cells, while expression is heterogeneous in mononuclear blood cells. Scale bar, 10 μm .



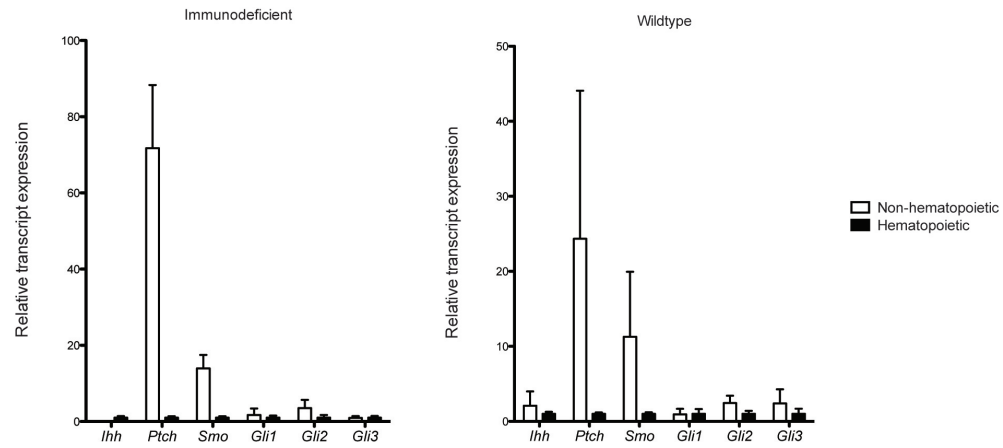
Supplementary Figure 2.1. Adherent cells derived from human BM samples are non-hematopoietic. By flow cytometry, adherent BM cells were confirmed to express characteristic BM mesenchymal markers CD105 and CD73 while lacking pan-hematopoietic marker CD45.



Supplementary Figure 2.2. Sequenced qPCR products have been validated to specifically align to human Hh pathway transcript sequences. Grey bars represent sequenced gel-extracted qPCR products, with primers being indicated by shaded segments. Human mRNA GenBank sequences are shown in light red, with labeled exon numbers. Sequence identity was determined using the BLASTn program.



Supplementary Figure 2.3. *SMO*-expressing cells are rare within normal and leukemic $CD34^+$ cells. (A) Example *SMO* and *GUSB* amplification plots from representative G-MPB and AML samples. (B) Dissociation curves showing specific *SMO* and *GUSB* amplification products from normal and leukemic hematopoietic sources



Supplementary Figure 2.4. Mouse strain does not influence the cell fraction-specific BM gene expression profiles for the Hh pathway. Hh pathway gene expression in hematopoietic (CD45⁺ or Ter119⁺) and non-hematopoietic (CD45⁻ Ter119⁻) BM cell fractions is shown separately for immunodeficient mice and wildtype (C57BL/6) mice. All gene expression values are normalized such that hematopoietic gene expression is equal to 1. This represents the same data shown in Figure 3B, represented differently to show the consistency of Hh gene expression across strains.

Table 2.1. Clinical details of AML patients. AML samples were obtained from either BM, PB, or therapeutic leukapheresis throughout a variety of disease progression stages. Figure numbers indicate the experimental use of each sample.

Patient #	Disease status	Tissue source	Gender	Figures
1	progressed from myelofibrosis	BM	Female	2.1B, 2.1C
2	new diagnosis	PB	Male	2.1B
3	therapy resistant	BM	Female	2.1B, 2.1C
4	relapse	PB	Female	2.1B, 2.1C
5	unknown	PB	Female	2.1B, 2.1C
6	new diagnosis	BM	Male	2.1B
7	unknown	BM	Male	2.1C
8	unknown	BM	Male	2.1C
9	follow-up	BM	Male	2.2
10	new diagnosis	PB	Female	2.2
11	new diagnosis	BM	Male	2.2
12	new diagnosis	PB	Male	2.2
13	progressed from MDS	Leukapheresis	Male	2.2
14	relapse	BM	Male	2.2

Table 2.2. Primer sequences and melting temperatures for Hh pathway primers. Human and mouse primers listed were used by applying the following cycling parameters: 50°C for 2 minutes, 95°C for 10 minutes, followed by 45 cycles of 95°C for 15 seconds and T_m for 60 seconds. Dissociation curves were run following amplification.

Gene	Forward sequence	Reverse sequence	T _m
<i>Human primers</i>			
<i>GUSB</i>	5' AAA CGA TTG CAG GGT TTC AC 3'	5' CAG TCA TGA AAT CGG CAA AA 3'	55
<i>SHH</i>	5' TGA CCC CTT TAG CCT ACA AGC A 3'	5' CCT TCA TAC CTT CCG CTG GC 3'	60
<i>HHIP</i>	5' AGG CCA TAT TCC AGG TTT CC 3'	5' GAA AGC ACA ACC CAC CAT CT 3'	60
<i>PTCH</i>	5' AGT GTC GCA CAG AAC TCC ACT 3'	5' GCA TAG GCG AGC ATG AGT AAG 3'	55
<i>SMO</i>	5' AAG GCT GCA CGA ATG AGG T 3'	5' CAG CTC TTG GGG TTG TCT GT 3'	55
<i>SUFU</i>	5' CCT CCA GAT CGT TGG TGT CT 3'	5' TCC GCA TGT CAG TTA TCA GC 3'	55
<i>GLI1</i>	5' AGC TGA AGT CTG AGC TGG ACA 3'	5' GAT CCT GTA TGC CTG TGG AGT 3'	60
<i>GLI2</i>	5' GAC ATT CGG CTA AGG AGG GAT T 3'	5' CCA AAT GCT CCC TAC CAT CTT TC 3'	55
<i>GLI3</i>	5' GCA CTT TTG AAG GTT GCA CA 3'	5' CCT TCG TGC TCA CAG ACG TA 3'	60
<i>CD3e</i>	5' ATG AAA CAA AGA TGC AGT CG 3'	5' GTC TGT GTA ATA CCA CCC ATT 3'	55
<i>GAPDH</i>	5' TGC ACC ACC AAC TGC TTA GC 3'	5' GGC ATG GAC TGT GGT CAT GAG 3'	55-60
<i>Mouse primers</i>			
<i>Ihh</i>	5' GCT TCG ACT GGG TGT ATT ACG 3'	5' GCT CGC GGT CCA GGA AAA T 3'	52
<i>Shh</i>	5' TCA CAA GAA ACT CCG AAC GAT TT 3'	5' GAT GTC CAC TGC TCG ACC C 3'	52
		5' GCA GTT GTT TGA AGA AGA CCT GG 3'	
<i>Hhip</i>	5' CTG GAT GGG GAC CTA GCA CTT 3'		55
<i>Ptch</i>	5' TCC GAG TGG CTG TAA TTG AGA 3'	5' CTG AAT CAA CAT CTG GGA GGT G 3'	55
<i>Smo</i>	5' GAG CGT AGC TTC CGG GAC TA 3'	5' CTG GGC CGA TTC TTG ATC TCA 3'	55
<i>Gli1</i>	5' TCG ACC TGC AAA CCG TAA TCC 3'	5' TCC TAA AGA AGG GCT CAT GGT A 3'	52
<i>Gli2</i>	5' CAG TGG AAT GAG GTG AGT TCT G 3'	5' GCT CTG CAC GGA TTG TGG ATT 3'	55
<i>Gli3</i>	5' TGC CCA TCA GCT ACT CAG TG 3'	5' TTG TTG CAG AGT GAG GTT GC 3'	55
<i>Actb</i>	5' AGC CTT CCT TCT TGG GTA TG 3'	5' GGT CTT TAC GGA TGT CAA CG 3'	52-55

Table 2.3. Human gene expression datasets retrieved from GEO

GEO Accession number	Reference	Number of replicates	Sample description
<i>Purified primitive AML samples (hematopoietic)</i>			
GSE12326	Cheung et al., 2009 [38]	10	Leukemic stem/progenitor phenotype: CD34 ⁺ cells isolated from AML patient PB or BM samples (a total of 5 individual AML patients)
GSE17054	Majeti et al., 2009 [39]	9	Leukemic stem cell phenotype: Lin ⁻ CD34 ⁺ CD38 ⁻ CD90 ⁻ cells isolated from AML patient PB or BM samples
GSE24006	Gentles et al., 2010 [40]	8	Leukemic progenitor cell phenotype: Lin ⁻ CD34 ⁺ CD38 ⁺ leukemic progenitor cells isolated from AML patient leukapheresis or peripheral blood samples
<i>Purified non-diseased samples (hematopoietic)</i>			
GSE17054	Majeti et al., 2009 [39]	4	Normal hematopoietic stem cell phenotype: Lin ⁻ CD34 ⁺ CD38 ⁻ CD90 ⁺ cells isolated from normal human BM
GSE24006	Gentles et al., 2010 [40]	3	Normal hematopoietic stem cell phenotype: Lin ⁻ CD34 ⁺ CD38 ⁻ CD90 ⁺ CD45RA ⁻ cells isolated from normal human cord blood
<i>Unpurified AML BM samples (hematopoietic)</i>			
GSE15061	Mills et al., 2009 [41]	202	AML BM mononuclear cells
<i>Unpurified non-diseased BM samples (hematopoietic)</i>			
GSE15061	Mills et al., 2009 [41]	69	Non-leukemic BM mononuclear cells

<i>Non-hematopoietic BM samples</i>			
GSE6460	Sacchetti et al., 2007 [42]	4	Non-hematopoietic CD146-expressing subendothelial cells isolated from human BM (untreated controls only)
GSE9451	Kubo et al., 2009 [43]	3	Human iliac mesenchymal stem cells purchased from BioWhittaker Inc
GSE10311	Grundberg et al., 2008 [44]	9	Non-hematopoietic plastic adherent cells isolated from human BM (untreated controls only)
GSE10315	Mrugala et al., 2009 [45]	6	Non-hematopoietic plastic adherent cells isolated from human BM (unstimulated controls only)
GSE12274	Wagner et al., 2009 [46]	3	Non-hematopoietic plastic adherent cells isolated from human BM
GSE22293	Rivron et al., 2012 [47]	3	Co-culture of non-hematopoietic plated adherent cells isolated from human BM (92%) with human umbilical vein endothelial cells (8%; untreated controls only)
GSE35331	Guilloton et al., 2012 [48]	12	Non-hematopoietic plastic adherent cells isolated from human BM (healthy donors only)

Chapter 3: Niche displacement of human leukemic stem cells uniquely allows their competitive replacement with healthy HSPCs

Preface

This Chapter is an original published article. It is presented in its published form.

This research was originally published in *The Journal of Experimental Medicine*:

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I designed the study with Dr. Mick Bhatia, and with considerable participation of Dr. Clinton Campbell. I performed competitive xenotransplantation experiments and G-CSF administration studies. I also performed *in situ* spatial analysis of xenografted mouse bone marrow. Dr. Clinton Campbell screened patient and donor samples for HLA-A2 expression and performed initial competitive transplantation experiments. Aline Fiebig-Comyn, Jelena Ulemek, and Claudia Hopkins also assisted with animal experiments. Claudia Hopkins, Jennifer Russell, and Dr. Tony Collins helped with *in situ* bone marrow imaging. Drs. Ronan Foley, Brian Leber, and Anargyros Xenocostas

provided human leukemia samples and intellectual input. I wrote the paper with Dr. Mick Bhatia.

As I had participated in our lab's study showing that healthy hematopoietic stem cells (HSCs) localize to discrete trabecular regions in bone marrow (BM) (Guezguez et al., 2013), an extension of these findings to acute myeloid leukemia (AML) was a logical progression. This also complemented the themes of AML and the BM microenvironment that began with **Chapter 2**.

The *in vivo* microenvironments that house LSCs have been poorly defined, and it was not clear whether features of LSC niches would closely resemble those of HSCs. We therefore began by applying the same techniques that we used to characterize HSC niche attributes (Guezguez et al., 2013). We then pursued competitive transplantation xenograft studies, to more definitively evaluate any functional overlap between HSC and LSC niches. This provided a new framework to understand interactions between HSCs and LSCs in the BM niche, and provides insights to potentially improve therapeutic transplantation strategies in AML.

Niche displacement of human leukemic stem cells uniquely allows their competitive replacement with healthy HSPCs

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Running head: Competitive replacement of LSC niches

Abstract

Allogeneic hematopoietic stem cell transplantation (HSCT) is currently the leading strategy to manage acute myeloid leukemia (AML). However, treatment-related morbidity limits the patient generalizability of HSCT use, and the survival of leukemic stem cells (LSCs) within protective areas of the bone marrow (BM) continues to lead to high relapse rates. Despite growing appreciation for the significance of the LSC microenvironment, it has remained unresolved whether LSCs preferentially situate within normal hematopoietic stem cell (HSC) niches or whether their niche requirements are more promiscuous. Here we provide functional evidence that the spatial localization of phenotypically primitive human AML cells is restricted to niche elements shared with their normal counterparts, and that their intrinsic ability to initiate and retain occupancy of these niches can be rivaled by healthy hematopoietic stem and progenitor cells (HSPCs). When challenged in competitive BM repopulation assays, primary human leukemia initiating cells (L-ICs) can be consistently outperformed by HSPCs for BM niche occupancy in a cell-dose dependent manner that ultimately compromises long-term L-IC renewal and subsequent leukemia-initiating capacity. The effectiveness of this approach could be demonstrated using cytokine-induced mobilization of established leukemia from the BM that facilitated the replacement of BM niches with transplanted HSPCs. These findings identify a functional vulnerability of primitive leukemia cells, and suggest that clinical development of these novel transplantation

techniques should focus on the dissociation of L-IC-niche interactions to improve competitive replacement with healthy HSPCs during HSCT towards increased survival of patients.

Introduction

AML is a hematological neoplasm with a hierarchical cellular structure that is reminiscent of the normal hematopoietic system (Bonnet and Dick, 1997; Hope et al., 2004; Lapidot et al., 1994). LSCs, which sit at the top of this hierarchy, are particularly resistant to conventional therapeutic measures, contributing to minimum residual disease and ultimately causing patient relapse (Guzman et al., 2002). More recent insights suggest that the BM microenvironment plays a fundamental role in sheltering LSCs (Konopleva et al., 2002) and specifying their self-renewal properties (Kode et al., 2014; Raaijmakers et al., 2010; Schepers et al., 2013). Therefore, niche-targeted consolidation treatment strategies represent a promising mechanism to effectively compromise LSC self-renewal and eliminate minimum residual disease in AML. In order to inform novel therapeutic efforts towards this goal, it is necessary to develop a thorough understanding of LSC niche characteristics, in relation to those of HSCs.

We have previously characterized geographical and molecular features that functionally define the HSC niche *in vivo* (Guezguez et al., 2013), and in this study we extend these observations by reporting that LSC-enriched populations share an equivalent spatial and functional distribution in BM. Critically, we show that HSPCs can rival L-ICs to populate vacant sites within the BM, which has been described to contain a limited number of saturable niches (Colvin et al., 2004; Czechowicz et al., 2007). We further demonstrate that in the context of established leukemic disease, it

is necessary to dissociate leukemia-niche interactions prior to HSCT, in order to achieve competitive healthy reconstitution at the expense of LSC self-renewal.

Results and Discussion

Spatial overlap exists between normal and leukemic stem cell-enriched populations in the BM

We have recently described anatomical boundaries within the BM that discretely define the functional localization of healthy HSCs (Guezguez et al., 2013). Relative to diaphyseal long bone areas (LBA), the cellular composition of trabecular bone (TBA) provides a unique molecular microenvironment that preferentially accommodates self-renewing HSCs. Applying the same analytical techniques, we comparatively interrogated whether phenotypically immature leukemic cells share this non-uniform distribution in BM, using xenografted immunodeficient mice established as a reliable surrogate model. Following transplantation with primary cells from AML patients or normal human donors, xenografted femurs were dissected along axial planes that delineate the borders between TBA and LBA regions (Fig. 3.1 A). Flow cytometric measurement of primitive CD45⁺CD34⁺ human hematopoietic cells indicated that like their normal counterparts, immature leukemic cells were markedly more predominant in the cancellous TBA (Fig. 3.1, B and C). Longitudinal sectioning of frozen decalcified femurs further allowed more precise comparison of microanatomical distribution patterns of normal and leukemic hematopoiesis *in situ*. Using a high-resolution fluorescence-based imaging platform (Guezguez et al.,

2013), human-specific CD45⁺CD34⁺ cells could be sensitively and accurately detected, paralleling our flow cytometry analysis (Fig. S3.1, A and B). Enrichment of CD34⁺ leukemic cells was evident along the surface area of the endosteum (Fig. 3.1, D and E), a geographical arrangement that has been previously described for both human and murine HSCs (Guezguez et al., 2013; Nombela-Arrieta et al., 2013). A customized quantitative localization analysis based on endosteal proximity (Fig. S3.1 C) showed that the spatial frequency distribution of CD34⁺ AML cells is indistinguishable from that of normal HSPC donors (Fig. 3.1, E and F). This assessment extends observations of general associations of primitive AML cells with paratrabecular features (Ishikawa et al., 2007; Ninomiya et al., 2006; Fig. 3.1, B and C), and more specifically predicts that the regional distribution of normal and leukemic self-renewal niches are physically superimposed.

Healthy HSPCs can out-compete L-ICs to populate common functional BM sites

Although spatial profiling of phenotypically primitive cells provides compelling evidence that HSPCs and L-ICs share common BM niches, ultimately self-renewing cells are stringently defined based on their *in vivo* performance, requiring rigorous competitive transplantation assays to comprehensively address their predicted functional co-localization. To this end, human leukocyte antigen A2 (HLA-A2) served as a faithful antigen which allowed us to combine and track primary cells from genetically distinct human individuals within mixed chimeric xenografts (Guzman et al., 2002; Fig. S3.2). We initially performed competitive transplantation experiments

between pairs of healthy cord blood (CB) donors, to first establish whether this co-transplantation xenograft model would accurately allow for the unbiased examination of BM niche repopulation dynamics (Fig. 3.2 A). Cell dose titration of co-injected donor cells revealed that relative initial CD34⁺ cell content was strongly predictive of BM graft dominance (Fig. 3.2, B and C), reflecting clinical observations made in the context of human transplantation with mixed CB cells from two combined donors (Ballen et al., 2007). Furthermore, earlier transplantation provided a competitive repopulation advantage if donor cells were instead transplanted in a successive fashion (data not shown), again recapitulating clinical reports (Avery et al., 2011; Ballen et al., 2007). In no instance did we observe evidence of immune recognition between donor cells (Yahata et al., 2004), as assured by the use of lineage-depleted (Lin⁻) CB samples lacking CD2⁺ T cells (Fig. 3.2 B), and the application of the NOD SCID recipient background, which precludes terminal maturation of immune competent lymphocytes (Shultz et al., 2005).

Using this clinically relevant system, we then co-transplanted constant cell numbers of AML with escalating doses of CB Lin⁻ cells to determine whether normal and leukemic repopulating cells can compete with each other to seed common BM niches (Fig. 3.2 D). All AML samples chosen for competitive repopulation experiments generated exclusively myeloid leukemic grafts (Fig. S3.3 A), and co-transplanted CB-HSPCs consistently gave rise to multi-lineage hematopoiesis (Fig. S3.3 B). Leukemic cell doses were chosen which would generate considerable levels of disease burden, to ensure that our model would accurately represent clinical

situations of poor prognosis that would require HSCT therapy. Similar to our observations in paired CB donor-transplantation experiments, the proportions of AML patient-derived cells within the human grafts were consistently reduced by CB competition in a cell dose-dependent manner (Fig. 3.2, E and F). Remarkably, this translated into suppression of the absolute leukemic burden at higher CB Lin⁻ cell doses, unless the overall human reconstitution was low (AML Patient #1; Fig. 3.2, G and H). In this particular case of low human reconstitution, the presence of any competing CB cells was sufficient to compromise leukemic engraftment regardless of CB cell dose used (Fig. 3.2 I), and this observation was not unlike absolute engraftment profiles observed in double CB co-transplant experiments (Fig. 3.2 J). Overall, this demonstrates that normal and leukemic repopulating cells can compete dynamically to populate vacant BM niches and that L-ICs can be outcompeted by CB-HSPCs in a cell dose-dependent manner as long as niche availability is limiting. Our finding that L-ICs do not appear to have a superior affinity for niche repopulation was striking considering that primitive AML blasts have been reported to express elevated levels of antigens involved in adhesion and chemoattraction (De Waele et al., 1999; Jin et al., 2006).

When human cells from healthy/leukemic chimeras were serially transplanted into secondary recipients, we found that leukemic repopulation was equal to or less than that in primary recipient mice, while the self-renewal of AML-alone controls was uncompromised or elevated (Fig. 3.2, K and L). Therefore, the competitive pressure provided by co-injected HSPCs was apparently able to jeopardize L-IC

fitness in a durable fashion. This would be an unlikely finding if L-ICs had the capacity to resist HSPC competition by relocation to alternative BM niches. When our model was instead adjusted to simulate the clinical context of pre-established leukemic disease followed by irradiation-conditioned CB Lin⁻ transplantation, leukemic engraftment was able to recur in secondary recipients, indicating less effective replacement of L-ICs (Fig. 3.2 M). This provides a model of leukemic relapse and complements our CB co-transplant studies, suggesting that equal competition for BM niches is compromised if leukemic engraftment is pre-established.

Mobilizing agents can displace leukemic cells from BM niches

We next reasoned that better capitalization of such unanticipated HSPC competitive properties would offer the most promising direction to improve therapeutic targeting of residual AML-LSCs during HSCT. Murine studies have suggested that small molecule or cytokine-induced mobilization of indigenous HSCs can effect dramatic niche exchange and HSC redistribution throughout BM (Verovskaya et al., 2014) and can also act as a preparative treatment to allow allogeneic HSC engraftment (Chen et al., 2006). We therefore sought to apply the same approach in a xenograft setting, to evaluate its therapeutic value to promote competition of transplanted HSPCs versus niche-occupant L-ICs. Initially, we assessed whether engrafted HSPCs and L-ICs would respond to mobilization treatment in similar ways based on their mutual dependence on CXCR4-CXCL12 for anchorage and retention in the BM (Peled et al., 1999; Petit et al., 2002; Tavor et al., 2004). Starting with CB-

engrafted mice, we applied a mobilization regimen using two CXCR4-CXCL12 antagonists (granulocyte colony-stimulating factor; G-CSF and AMD3100), adapted from Petit et al. (2002) and Broxmeyer et al. (2005; Fig. 3.3 A). Consistent with previous reports (Nervi et al., 2009), we found that CXCR4 antagonism effectively mobilized xenografted human cells from the BM into the periphery within one hour of treatment (Fig. 3.3 B). Although human hematopoietic cells were present in splenic isolates from both mobilized and control-treated mice, transplantation of these cells into secondary recipients revealed functional repopulating activity only among human cells that had been recovered from the spleens of mobilized mice (Fig. 3.3 C). This rigorous assessment of primitive functional characteristics is evidence that mobilization treatment successfully caused physical displacement of human long-term repopulating cells out of the BM, causing their redistribution into peripheral hematopoietic sites. We next applied the same treatment strategy to xenografts of primary human AML to establish whether leukemic cells could equally be displaced from the mouse BM (Fig. 3.3 D). Consistent with the niche similarities shared by normal and leukemic cells, mobilization treatment was also able to effect movement of human leukemic cells from the BM into the peripheral blood and spleens of diseased chimeric mice (Fig. 3.3 E). Recovered AML cells expressed appropriate tissue-specific and treatment-specific levels of CXCR4 (with the highest expression observed within the BM of mobilized mice; Petit et al., 2002; Fig. S3.4). Together, this suggests that the functional integrity of the CXCR4-CXCL12 axis is intact and targetable in primitive AML cells.

This evidence that BM-resident leukemic cells can successfully be physically dislodged from their inhabited niches offers promise that leukemic mobilization could represent a mechanism to promote better competitive repopulation by HSPCs during therapeutic transplantation. Due to the inability of serial transplantation to discriminate between LSCs localized within specialized niches versus those transiting through non-niche BM space during mobilization, we explored the effects of mobilization on niche accessibility by challenging injected CB-HSPCs to home to the marrow after leukemic graft displacement. In our initial leukemia mobilization experiments, we found that within one day post-treatment, the spatial configuration of the leukemic grafts had begun returning to a normal non-mobilized state, and that AML cells had completely re-assumed their original tissue distribution by one week post-mobilization (data not shown). We therefore reasoned that rapid infusion of competitive CB-HSPCs would be necessary after leukemic mobilization. One hour following mobilization-treatment of AML-engrafted mice, lipophilic dye-labeled CB Lin⁻ cells were injected intravenously (IV) and the dissemination of these fluorescently labeled cells was assessed one day later (Fig. 3.3 F). Although a significant proportion of dye-labeled cells were localized to the spleen under both conditions at this time point, there was a modest reduction of these cells in pre-mobilized mice (data not shown). This was reflected by a two-fold increase in CB Lin⁻ cells homed to the BM (Fig. 3.3 G), suggesting that leukemic displacement led to increased niche availability. The observation of enhanced HSPC homing ability as a consequence of leukemic niche displacement supports our earlier interpretation

that primitive normal and leukemic cells do share and compete for common microanatomical niches, and reinforces the prediction that leukemic mobilization can enhance competitive L-IC replacement by transplanted HSPCs.

Leukemic mobilization facilitates competitive reconstitution and leukemia elimination by transplanted HSPCs

Next, we evaluated the long-term effectiveness of pre-HSCT mobilization in the context of established leukemic disease by injecting large numbers of healthy CB-HSPCs after leukemic mobilization, in order to saturate the newly vacant BM niches (Fig. 3.4 A). Importantly, the injected CB Lin⁻ cell doses were chosen such that the numbers of infused CD34⁺ cells/kg body weight were representative of realistic cellular doses achievable in clinical human transplantation from adult HSPC sources (Korbling et al., 1995; Scheid et al., 1999; Table 3.1). Across three different patients, mobilization pre-conditioning consistently ameliorated the ability of injected HSPCs to competitively reconstitute the leukemic xenograft microenvironment relative to non-mobilized controls (Fig. 3.4 B). In each case, this was accompanied by a statistically significant reduction in overall leukemic burden, as measured by overall leukemic cell frequencies in BM (Fig. 3.4 C) or total leukemia cell numbers per mouse (data not shown). Established healthy human grafts could be similarly suppressed by mobilization followed by transplantation with either normal or leukemic human cells (Fig. 3.4, D-G). This supports the notion that the temporal sequence of events is a fundamental determinant of competitive repopulation, and

suggests that this is independent of the normal or leukemic nature of competing stem or progenitor cells. Critically, the superior therapeutic effects of mobilization pre-conditioning persisted after each individual chimeric graft was serially transplanted into a single secondary recipient mouse (Fig. 3.4, H and I; Table 3.2). In several cases, mobilization pre-treatment involved complete elimination of L-IC repopulative capacity, with robust CB engraftment serving as a powerful positive internal control. This suggests that leukemic BM mobilization represents a promising HSCT preconditioning strategy, which can improve long-term management of leukemic disease.

Finally, in order to put our findings into a broader context for clinical application, we considered the predictive value of pre-treatment disease levels towards therapeutic outcomes of mobilization-HSCT. The robust therapeutic responses described for AML Patients #4 and #5 were associated with marginal frequencies of peripherally circulating leukemic blasts in primary mice at the time of treatment (Fig. 3.5 A), which provides an accurate surrogate representation of BM infiltration (Fig. 3.5 B). In contrast, mice treated under conditions of abundant circulating blasts (Patient #6; Fig. 3.5 A) ultimately manifested aggressively disseminated disease with extramedullary leukemic burdens that exceeded the cellular capacity of the BM space (Fig 3.5 C), despite mobilization-HSCT intervention (Fig. 3.5 D). This extreme example reinforces the principal suitability of mobilization-HSCT as a consolidation measure after cytoreduction (Rowe, 2009), and parallels clinical reports that poor disease-free survival is predicted by residual

BM blast levels $\geq 30\%$ at the time of conventional HSCT (Kebriaei et al., 2005; Sierra et al., 2000). Of fundamental significance, the therapeutic resistance of AML Patient #6 xenografts was accompanied by the notable absence of healthy CB self-renewal upon serial transplantation, despite successful healthy repopulation in primary recipients (Fig. 3.5 D). Therefore, transient repopulation by healthy hematopoietic progenitors is not sufficient to reverse leukemic progression, which instead requires durable niche population and engraftment by functionally-defined HSCs (Fig. 3.4 I). These critical functional readouts provide evidence that HSCs rather than HPCs are most likely the direct LSC competitors, complementing recent insights implying functional similarities between HSC and LSC niches based on BM conditions that exacerbate murine AML (Krause et al., 2013) in a manner similar to niche-dependent HSC expansion (Calvi et al., 2003).

On the basis of *in situ* localization analysis and careful functional repopulation assays performed *in vivo*, we propose a model in which HSPCs and LSCs share and compete for common protective niches within the BM. Because the functional property of self-renewal is cell-extrinsically maintained (Guezguez et al., 2013), our finding of common regulatory niches between normal and leukemic stem cells presents a considerable barrier towards selective pharmacological targeting of LSC versus HSPC self-renewal. We instead suggest that novel approaches to cell-based therapy offer a more promising consolidation strategy to challenge LSC survival within the BM. To our knowledge, this represents the first demonstration that similar functional properties shared by LSCs and HSCs can be used as a therapeutic

advantage in a preclinical transplantation model. Our study identifies a currently unexploited ability of HSPCs to rival L-ICs for BM niche territory, achievable by mobilization of resident L-ICs prior to healthy HSPC transplantation. This repositions transplanted HSPCs as powerful therapeutic effectors that can actively participate in LSC elimination, in contrast to their traditional reconstitutive role that is secondary to aggressive anti-leukemic therapy. The value in this novel mechanistic strategy is that LSC elimination can be accomplished without cytotoxic myeloablation, enhancing the safety and therapeutic index of HSCT through the repurposing of agents already known to be well-tolerated for patient administration (Bradley et al., 2012).

This potential for improved reduced-intensity conditioning could allow larger patient populations to be considered as HSCT candidates, and complements other toxicity-limiting strategies such as techniques that selectively deliver radiation targeted specifically to the BM, sparing other organs (Wong et al., 2006). The clinical application of leukemia mobilization for the purpose of HSCT is currently unprecedented, unlike the strategy of G-CSF-mediated chemotoxic sensitization (Saito et al., 2010), which has been subject to numerous patient trials over the past decade that have led to somewhat conflicting interpretations regarding efficacy (Buchner et al., 2004; Estey et al., 1999; Lowenberg et al., 2003). Based on the anticipated safety and the promising pre-clinical observations reported here, the testing and optimization of mobilization pre-conditioning as a new HSCT approach for AML patients is currently planned, including the evaluation of adult BM as a

compatible HSPC source due to the greater CD34⁺ cell numbers that can be procured (Korbling et al., 1995; Scheid et al., 1999; Wagner et al., 2002). Additionally, although a single round of mobilization-primed transplantation provided an effective technique of disease suppression under conditions of considerable leukemic burden (AML Patients #3 and 5), further refinement of this procedure could involve repeated cycles of mobilization and HSPC infusion (Bhattacharya et al., 2009; Colvin et al., 2004) to capitalize on multiple windows of opportunity to competitively eliminate L-ICs. Ultimately, the use of mobilization pre-treatment either alone or in conjunction with low-intensity HSCT regimens offers a novel strategy towards the development of more tolerable AML transplantation therapy with superior eradication of residual disease.

Materials and Methods

Primary human samples. Primary blasts were obtained from peripheral blood (PB) apheresis or BM aspirates of AML patients (Table S3.1), and healthy hematopoietic cells were isolated from CB samples. Informed consent was obtained from all sample donors in accordance with Research Ethics Board-approved protocols at McMaster University and the London Health Sciences Centre (Research Ethics Board #08-330 and #08-042). Mononuclear cells were recovered by density gradient centrifugation (Ficoll-Paque Premium; GE Healthcare, Cooksville, Canada), and remaining red blood cells were lysed using ammonium chloride solution (StemCell Technologies, Vancouver, Canada). Lineage depletion of CB samples was performed using a

commercially available kit (StemCell Technologies, Vancouver, Canada), according to manufacturer's instructions.

Xenotransplant assays. NOD/Prkdcscid and NOD/SCID/B2Mnull mice were used as xenotransplantation recipients. Mice were bred in a barrier facility and all experimental protocols were approved by the Animal Care Council of McMaster University (Animal Use Protocol #13-02-05). Immunodeficient mice aged 6-10 weeks old were sublethally irradiated (350 rads, 137Cs) 24 hours prior to initial transplantation in order to induce and establish either healthy or leukemic human hematopoiesis (Bonnet and Dick, 1997; Lapidot et al., 1994). Pairs of human samples were selected based on disparate HLA-A2 expression, and were transplanted IV either separately or mixed together, according to established protocols (Guezguez et al., 2013; Sachlos et al., 2012). In some cases, mice received a second HLA-A2-mismatched human transplant after a period of three weeks, as outlined in the text. Between three to ten weeks after original transplantation, mice were killed and cells from the BM and spleen were recovered by mechanical dissociation. Following red blood cell lysis, species-specific CD45 antibodies were used to determine levels of human chimerism by flow cytometry. HLA-A2 targeted antibodies were then used to distinguish individual donor contributions to human grafts, and multi-lineage analysis involved antibodies directed towards CD33, CD19, CD34, and CXCR4 (BD Biosciences, Mississauga, Canada). An LSRII flow cytometer (BD Biosciences,

Mississauga, Canada) was used for data acquisition, and all flow cytometry analysis was performed using FlowJo Software (version 9.3.2 Tree Star Inc., Ashland, USA).

In order to assess self-renewal and evaluate the long-term persistence of L-ICs, normalized numbers of BM cells from individual engrafted mice were transplanted IV into single secondary recipients. In one experiment, cells recovered from the spleens of primary mice were transplanted intrafemorally into secondary recipients, by normalizing cell input based on organ volume. Engraftment of all secondary recipients was assessed six to eight weeks following transplantation using flow cytometry. The threshold used for human engraftment was 0.1% human CD45⁺ chimerism within bone marrow (Notta et al., 2010).

Quantitative immunofluorescent microscopy. Whole femurs extracted from mice xenografted with healthy or leukemic hematopoietic cells were fixed overnight in 4% paraformaldehyde at 4°C, followed by overnight decalcification in formic acid (Immunocal, Decal, Tallman, USA). Femur specimens were subsequently snap frozen in OCT compound (Sakura, Tokyo, Japan) and sectioned at a 5- μ m thickness with a cryostat microtome, using the CryoJane tape-transfer system (Leica, Wetzlar, Germany). After blocking with 20% donkey serum (Jackson ImmunoResearch, West Grove, USA) and mouse Fc receptor blocking (BD Biosciences, Mississauga, Canada), slides were incubated with anti-human CD45 rat monoclonal antibody ab30446 and anti-human CD34 rabbit monoclonal antibody ab81289 (both 1:50 dilution; Abcam, Cambridge, USA). Donkey-raised secondary antibodies conjugated to Alexa488 or

Alexa-647 fluorophores were used for the detection of immunopositive cells (Life Technologies, Burlington, Canada). Fluorescent montage images of immunostained bone sections were acquired at 20x using an Operetta high content imaging system (Perkin Elmer, Waltham, USA), and assembled with Columbus analysis software (Perkin Elmer, Waltham, USA). Quantitative proximity analysis relative to endosteal bone regions was performed as previously described (Guezguez et al., 2013), using customized scripts in Acapella (Perkin Elmer, Waltham, USA) and MATLAB (MathWorks, Natick, USA) software. Briefly, DAPI signal intensity was used to identify individual cell nuclei, and the fluorescence intensities of the remaining channels were then quantified for each nuclear and perinuclear region. Negative staining controls were used to set positive signal thresholds. Endosteal cells were defined as CD45⁻CD34⁻ cells in areas with low surrounding nuclear density and high perinuclear autofluorescence in the DAPI channel. X-Y spatial nuclei coordinates were then used to calculate the distance of each human CD45⁺CD34⁺ cell to the nearest endosteal-defined cell to generate spatial distribution histograms.

In vivo mobilization treatment of xenografts. Following the establishment of human grafts, mice were injected subcutaneously with mobilization agents adapted according to published treatment schedules (Broxmeyer, 2005; Petit et al., 2002). This consisted of three consecutive days of subcutaneous G-CSF injections at 300 µg/kg, followed by a single SQ injection of AMD3100 (Sigma-Aldrich, Oakville, Canada; 5 mg/kg) on the fourth day. Vehicle control animals were injected with

equivalent volumes of saline. One to two hours after the final injection, mice were either transplanted IV with cord blood Lin⁻ cells (or saline), or were killed for analysis of human cell tissue distribution.

Hematology. Peripheral blood was collected from the mandibular vein of mice one hour following treatment with mobilization agents or with vehicle. WBC counts in peripheral circulation were evaluated using a Nexcelom Cellometer (Lawrence, USA) following acridine orange staining of diluted whole blood samples. Total WBC numbers were then expressed per unit volume of blood.

Homing assay. CB Lin⁻ cells were incubated with Vybrant DiO cell labeling solution (Life Technologies, Burlington, Canada), following manufacturer's instructions. 1.5×10^5 labeled Lin⁻ cells per mouse were transplanted IV into either mobilized or control-treated animals that had been engrafted with AML three weeks prior. Twenty-four hours following transplantation, the relative homing of labeled CB Lin⁻ cells was assessed in dissociated spleen and bone marrow cells by flow cytometry.

Statistical Analysis. Data are represented as mean \pm standard error of the mean (SEM). Unpaired two-tailed Student's t-tests, one-way ANOVAs, or linear regressions were used for statistical comparisons, with the exception of the localization-based frequency distributions of normal versus leukemic CD45⁺CD34⁺ cells, which were statistically compared using Chi square analysis. Statistical analyses were performed using Prism (version 5.0a, GraphPad, San Diego, USA) or MATLAB (MathWorks, Natick, USA) software, and the criterion for statistical significance was $P < 0.05$.

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References

- Avery, S., W. Shi, M. Lubin, A.M. Gonzales, G. Heller, H. Castro-Malaspina, S. Giralt, N.A. Kernan, A. Scaradavou, and J.N. Barker. 2011. Influence of infused cell dose and HLA match on engraftment after double-unit cord blood allografts. *Blood* 117:3277-3285.
- Ballen, K.K., T.R. Spitzer, B.Y. Yeap, S. McAfee, B.R. Dey, E. Attar, R. Haspel, G. Kao, D. Liney, E. Alyea, S. Lee, C. Cutler, V. Ho, R. Soiffer, and J.H. Antin. 2007. Double unrelated reduced-intensity umbilical cord blood transplantation in adults. *Biology of Blood and Marrow Transplantation* 13:82-89.
- Bhattacharya, D., A. Czechowicz, A.G.L. Ooi, D.J. Rossi, D. Bryder, and I.L. Weissman. 2009. Niche recycling through division-independent egress of hematopoietic stem cells. *Journal of Experimental Medicine* 206:2837-2850.
- Boggs, D.R. 1984. The total marrow mass of the mouse: a simplified method of measurement. *Am J Hematol* 16:277-286.
- Bonnet, D., and J.E. Dick. 1997. Human acute myeloid leukemia is organized as a hierarchy that originates from a primitive hematopoietic cell. *Nature Medicine* 3:730-737.
- Bradley, A.M., A.M. Deal, L.W. Buie, and H. van Deventer. 2012. Neutropenia-associated outcomes in adults with acute myeloid leukemia receiving cytarabine consolidation chemotherapy with or without granulocyte colony-stimulating factor. *Pharmacotherapy* 32:1070-1077.
- Broxmeyer, H.E. 2005. Rapid mobilization of murine and human hematopoietic stem and progenitor cells with AMD3100, a CXCR4 antagonist. *Journal of Experimental Medicine* 201:1307-1318.
- Buchner, T., W.E. Berdel, and W. Hiddemann. 2004. Priming with granulocyte colony-stimulating factor--relation to high-dose cytarabine in acute myeloid leukemia. *N Engl J Med* 350:2215-2216.
- Calvi, L.M., G.B. Adams, K.W. Weibrecht, J.M. Weber, D.P. Olson, M.C. Knight, R.P. Martin, E. Schipani, P. Divieti, F.R. Bringhurst, L.A. Milner, H.M. Kronenberg, and D.T. Scadden. 2003. Osteoblastic cells regulate the haematopoietic stem cell niche. *Nature* 425:841-846.

Chen, J., A. Larochelle, S. Fricker, G. Bridger, C.E. Dunbar, and J.L. Abkowitz. 2006. Mobilization as a preparative regimen for hematopoietic stem cell transplantation. *Blood* 107:3764-3771.

Colvin, G.A., J.F. Lambert, M. Abedi, C.C. Hsieh, J.E. Carlson, F.M. Stewart, and P.J. Quesenberry. 2004. Murine marrow cellularity and the concept of stem cell competition: geographic and quantitative determinants in stem cell biology. *Leukemia* 18:575-583.

Czechowicz, A., D. Kraft, I.L. Weissman, and D. Bhattacharya. 2007. Efficient transplantation via antibody-based clearance of hematopoietic stem cell niches. *Science* 318:1296-1299.

De Waele, M., W. Renmans, K. Jochmans, R. Schots, P. Lacor, F. Trullemans, J. Otten, N. Balduck, K. Vander Gucht, B. Van Camp, and I. Van Riet. 1999. Different expression of adhesion molecules on CD34⁺ cells in AML and B-lineage ALL and their normal bone marrow counterparts. *European Journal of Haematology* 63:192-201.

Estey, E.H., P.F. Thall, S. Pierce, J. Cortes, M. Beran, H. Kantarjian, M.J. Keating, M. Andreeff, and E. Freireich. 1999. Randomized phase II study of fludarabine + cytosine arabinoside + idarubicin +/- all-trans retinoic acid +/- granulocyte colony-stimulating factor in poor prognosis newly diagnosed acute myeloid leukemia and myelodysplastic syndrome. *Blood* 93:2478-2484.

Guezguez, B., C.J. Campbell, A.L. Boyd, F. Karanu, F.L. Casado, C. Di Cresce, T.J. Collins, Z. Shapovalova, A. Xenocostas, and M. Bhatia. 2013. Regional localization within the bone marrow influences the functional capacity of human HSCs. *Cell Stem Cell* 13:175-189.

Guzman, M.L., C.F. Swiderski, D.S. Howard, B.A. Grimes, R.M. Rossi, S.J. Szilvassy, and C.T. Jordan. 2002. Preferential induction of apoptosis for primary human leukemic stem cells. *Proc Natl Acad Sci U S A* 99:16220-16225.

Hope, K.J., L. Jin, and J.E. Dick. 2004. Acute myeloid leukemia originates from a hierarchy of leukemic stem cell classes that differ in self-renewal capacity. *Nat Immunol* 5:738-743.

Ishikawa, F., S. Yoshida, Y. Saito, A. Hijikata, H. Kitamura, S. Tanaka, R. Nakamura, T. Tanaka, H. Tomiyama, N. Saito, M. Fukata, T. Miyamoto, B. Lyons, K. Ohshima, N. Uchida, S. Taniguchi, O. Ohara, K. Akashi, M. Harada, and L.D. Shultz. 2007. Chemotherapy-resistant human AML stem cells home to and engraft within the bone-marrow endosteal region. *Nature Biotechnology* 25:1315-1321.

Jin, L., K.J. Hope, Q. Zhai, F. Smadja-Joffe, and J.E. Dick. 2006. Targeting of CD44 eradicates human acute myeloid leukemic stem cells. *Nature Medicine* 12:1167-1174.

Kebriaei, P., J. Kline, W. Stock, K. Kasza, M.M. Le Beau, R.A. Larson, and K. van Besien. 2005. Impact of disease burden at time of allogeneic stem cell transplantation in adults with acute myeloid leukemia and myelodysplastic syndromes. *Bone Marrow Transplant* 35:965-970.

Kode, A., J.S. Manavalan, I. Mosialou, G. Bhagat, C.V. Rathinam, N. Luo, H. Khiabani, A. Lee, V.V. Murty, R. Friedman, A. Brum, D. Park, N. Galili, S. Mukherjee, J. Teruya-Feldstein, A. Raza, R. Rabadan, E. Berman, and S. Kousteni. 2014. Leukaemogenesis induced by an activating beta-catenin mutation in osteoblasts. *Nature* 506: 240-244.

Konopleva, M., S. Konoplev, W. Hu, A.Y. Zaritskey, B.V. Afanasiev, and M. Andreeff. 2002. Stromal cells prevent apoptosis of AML cells by up-regulation of anti-apoptotic proteins. *Leukemia* 16:1713-1724.

Korbling, M., Y.O. Huh, A. Durett, N. Mirza, P. Miller, H. Engel, P. Anderlini, K. van Besien, M. Andreeff, D. Przepiorka, A.B. Deisseroth, and R.E. Champlin. 1995. Allogeneic blood stem cell transplantation: peripheralization and yield of donor-derived primitive hematopoietic progenitor cells (CD34+ Thy-1dim) and lymphoid subsets, and possible predictors of engraftment and graft-versus-host disease. *Blood* 86:2842-2848.

Krause, D.S., K. Fulzele, A. Catic, C.C. Sun, D. Dombkowski, M.P. Hurley, S. Lezeau, E. Attar, J.Y. Wu, H.Y. Lin, P. Divieti-Pajevic, R.P. Hasserjian, E. Schipani, R.A. Van Etten, and D.T. Scadden. 2013. Differential regulation of myeloid leukemias by the bone marrow microenvironment. *Nat Med* 19:1513-1517.

Lapidot, T., C. Sirard, J. Vormoor, B. Murdoch, T. Hoang, J. Caceres-Cortes, M. Minden, B. Paterson, M.A. Caligiuri, and J.E. Dick. 1994. A cell initiating human acute myeloid leukaemia after transplantation into SCID mice. *Nature* 367:645-648.

Lowenberg, B., W. van Putten, M. Theobald, J. Gmur, L. Verdonck, P. Sonneveld, M. Fey, H. Schouten, G. de Greef, A. Ferrant, T. Kovacovics, A. Gratwohl, S. Daenen, P. Huijgens, and M. Boogaerts. 2003. Effect of priming with granulocyte colony-stimulating factor on the outcome of chemotherapy for acute myeloid leukemia. *N Engl J Med* 349:743-752.

Nervi, B., P. Ramirez, M.P. Rettig, G.L. Uy, M.S. Holt, J.K. Ritchey, J.L. Prior, D. Piwnicka-Worms, G. Bridger, T.J. Ley, and J.F. DiPersio. 2009. Chemosensitization of acute

myeloid leukemia (AML) following mobilization by the CXCR4 antagonist AMD3100. *Blood* 113:6206-6214.

Ninomiya, M., A. Abe, A. Katsumi, J. Xu, M. Ito, F. Arai, T. Suda, H. Kiyoi, T. Kinoshita, and T. Naoe. 2006. Homing, proliferation and survival sites of human leukemia cells in vivo in immunodeficient mice. *Leukemia* 21:136-142.

Nombela-Arrieta, C., G. Pivarnik, B. Winkel, K.J. Canty, B. Harley, J.E. Mahoney, S.Y. Park, J. Lu, A. Protopopov, and L.E. Silberstein. 2013. Quantitative imaging of haematopoietic stem and progenitor cell localization and hypoxic status in the bone marrow microenvironment. *Nat Cell Biol* 15:533-543.

Notta, F., S. Doulatov, and J.E. Dick. 2010. Engraftment of human hematopoietic stem cells is more efficient in female NOD/SCID/IL-2Rgc-null recipients. *Blood* 115:3704-3707.

Peled, A., I. Petit, O. Kollet, M. Magid, T. Ponomaryov, T. Byk, A. Nagler, H. Ben-Hur, A. Many, L. Shultz, O. Lider, R. Alon, D. Zipori, and T. Lapidot. 1999. Dependence of Human Stem Cell Engraftment and Repopulation of NOD/SCID Mice on CXCR4. *Science* 283:845-848.

Petit, I., M. Szyper-Kravitz, A. Nagler, M. Lahav, A. Peled, L. Habler, T. Ponomaryov, R.S. Taichman, F. Arenzana-Seisdedos, N. Fujii, J. Sandbank, D. Zipori, and T. Lapidot. 2002. G-CSF induces stem cell mobilization by decreasing bone marrow SDF-1 and up-regulating CXCR4. *Nature Immunology* 3:687-694.

Raaijmakers, M.H., S. Mukherjee, S. Guo, S. Zhang, T. Kobayashi, J.A. Schoonmaker, B.L. Ebert, F. Al-Shahrour, R.P. Hasserjian, E.O. Scadden, Z. Aung, M. Matza, M. Merckenschlager, C. Lin, J.M. Rommens, and D.T. Scadden. 2010. Bone progenitor dysfunction induces myelodysplasia and secondary leukaemia. *Nature* 464:852-857.

Rowe, J.M. 2009. Optimal induction and post-remission therapy for AML in first remission. *The Education Program of the American Society of Hematology*. 396-405.

Sachlos, E., R.M. Risueno, S. Laronde, Z. Shapovalova, J.-H. Lee, J. Russell, M. Malig, J.D. McNicol, A. Fiebig-Comyn, M. Graham, M. Levadoux-Martin, J.B. Lee, A.O. Giacomelli, J.A. Hassell, D. Fischer-Russell, M.R. Trus, R. Foley, B. Leber, A. Xenocostas, E.D. Brown, T.J. Collins, and M. Bhatia. 2012. Identification of Drugs Including a Dopamine Receptor Antagonist that Selectively Target Cancer Stem Cells. *Cell* 149:1284-1297.

Saito, Y., N. Uchida, S. Tanaka, N. Suzuki, M. Tomizawa-Murasawa, A. Sone, Y. Najima, S. Takagi, Y. Aoki, A. Wake, S. Taniguchi, L.D. Shultz, and F. Ishikawa. 2010. Induction

of cell cycle entry eliminates human leukemia stem cells in a mouse model of AML. *Nature Biotechnology* 28:275-280.

Scheid, C., A. Draube, M. Reiser, A. Schulz, J. Chemnitz, S. Nelles, M. Fuchs, S. Winter, P.D. Wickramanayake, V. Diehl, and D. Schöningh. 1999. Using at least 5×10^6 /kg CD34+ cells for autologous stem cell transplantation significantly reduces febrile complications and use of antibiotics after transplantation. *Bone Marrow Transplantation* 23:1177-1181.

Schepers, K., E.M. Pietras, D. Reynaud, J. Flach, M. Binnewies, T. Garg, A.J. Wagers, E.C. Hsiao, and E. Passegue. 2013. Myeloproliferative neoplasia remodels the endosteal bone marrow niche into a self-reinforcing leukemic niche. *Cell Stem Cell* 13:285-299.

Shultz, L.D., B.L. Lyons, L.M. Burzenski, B. Gott, X. Chen, S. Chaleff, M. Kotb, S.D. Gillies, M. King, J. Mangada, D.L. Greiner, and R. Handgretinger. 2005. Human lymphoid and myeloid cell development in NOD/LtSz-scid IL2R gamma null mice engrafted with mobilized human hemopoietic stem cells. *J Immunol* 174:6477-6489.

Sierra, J., B. Storer, J.A. Hansen, P.J. Martin, E.W. Petersdorf, A. Woolfrey, D. Matthews, J.E. Sanders, R. Storb, F.R. Appelbaum, and C. Anasetti. 2000. Unrelated donor marrow transplantation for acute myeloid leukemia: an update of the Seattle experience. *Bone Marrow Transplant* 26:397-404.

Tavor, S., I. Petit, S. Porozov, A. Avigdor, A. Dar, L. Leider-Trejo, N. Shemtov, V. Deutsch, E. Naparstek, A. Nagler, and T. Lapidot. 2004. CXCR4 Regulates Migration and Development of Human Acute Myelogenous Leukemia Stem Cells in Transplanted NOD/SCID Mice. *Cancer Research* 64:2817-2824.

Verovskaya, E., M.J. Broekhuis, E. Zwart, E. Weersing, M. Ritsema, L.J. Bosman, T. van Poele, G. de Haan, and L.V. Bystrykh. 2014. Asymmetry in skeletal distribution of mouse hematopoietic stem cell clones and their equilibration by mobilizing cytokines. *J Exp Med* 211:487-497.

Wagner, J.E., J.N. Barker, T.E. DeFor, K.S. Baker, B.R. Blazar, C. Eide, A. Goldman, J. Kersey, W. Krivit, M.L. MacMillan, P.J. Orchard, C. Peters, D.J. Weisdorf, N.K. Ramsay, and S.M. Davies. 2002. Transplantation of unrelated donor umbilical cord blood in 102 patients with malignant and nonmalignant diseases: influence of CD34 cell dose and HLA disparity on treatment-related mortality and survival. *Blood* 100:1611-1618.

Wong, J.Y., A. Liu, T. Schultheiss, L. Popplewell, A. Stein, J. Rosenthal, M. Essensten, S. Forman, and G. Somlo. 2006. Targeted total marrow irradiation using three-dimensional image-guided tomographic intensity-modulated radiation therapy: an

alternative to standard total body irradiation. *Biol Blood Marrow Transplant* 12:306-315.

Yahata, T., K. Ando, H. Miyatake, T. Uno, T. Sato, M. Ito, S. Kato, and T. Hotta. 2004. Competitive repopulation assay of two gene-marked cord blood units in NOD/SCID/gammac(null) mice. *Mol Ther* 10:882-891.

Figures and Tables

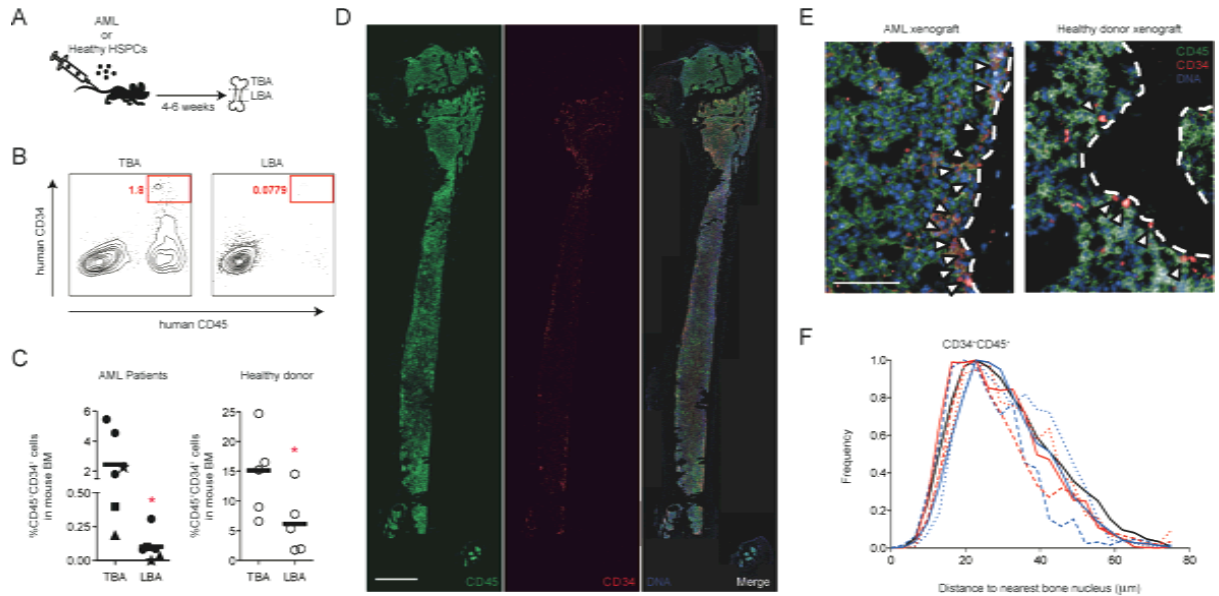


Figure 3.1. Healthy and leukemic human CD34⁺ cells spatially overlap within the BM cavity. (A) Experimental design to study the location of healthy or leukemic human hematopoietic cells within the TBA and LBA of xenografted mouse femurs. (B) Representative flow cytometry plots showing human CD45⁺CD34⁺ AML cells within TBA and LBA regions. (C) Scatter plots showing the TBA- and LBA-specific frequencies of human CD45⁺CD34⁺ cells derived from AML patients or normal human donors. Each data point represents an individual mouse, and each symbol represents an independent primary human sample. * $P < 0.05$, paired Student's t test. (D) Representative whole-bone section images of AML-engrafted mouse femurs immunostained with human-specific CD45 and CD34. Scale bar, 1 mm. (E) Representative high magnification images of AML or healthy donor-engrafted mouse femurs immunostained with human-specific CD45 and CD34. Dotted lines indicate the endosteal boundary. Scale bar, 50 μm . (F) Proximity distribution of healthy or leukemic human CD45⁺CD34⁺ cells relative to the nearest bone nucleus. Black line indicates the distribution of cord blood CD45⁺CD34⁺ cells (averaged across 4 replicates), and each colored line represents an independent AML-engrafted femur. Each color represents a different primary human AML sample. All P values ≥ 0.20 when comparing AML CD45⁺CD34⁺ versus CB CD45⁺CD34⁺ distributions (Chi square).

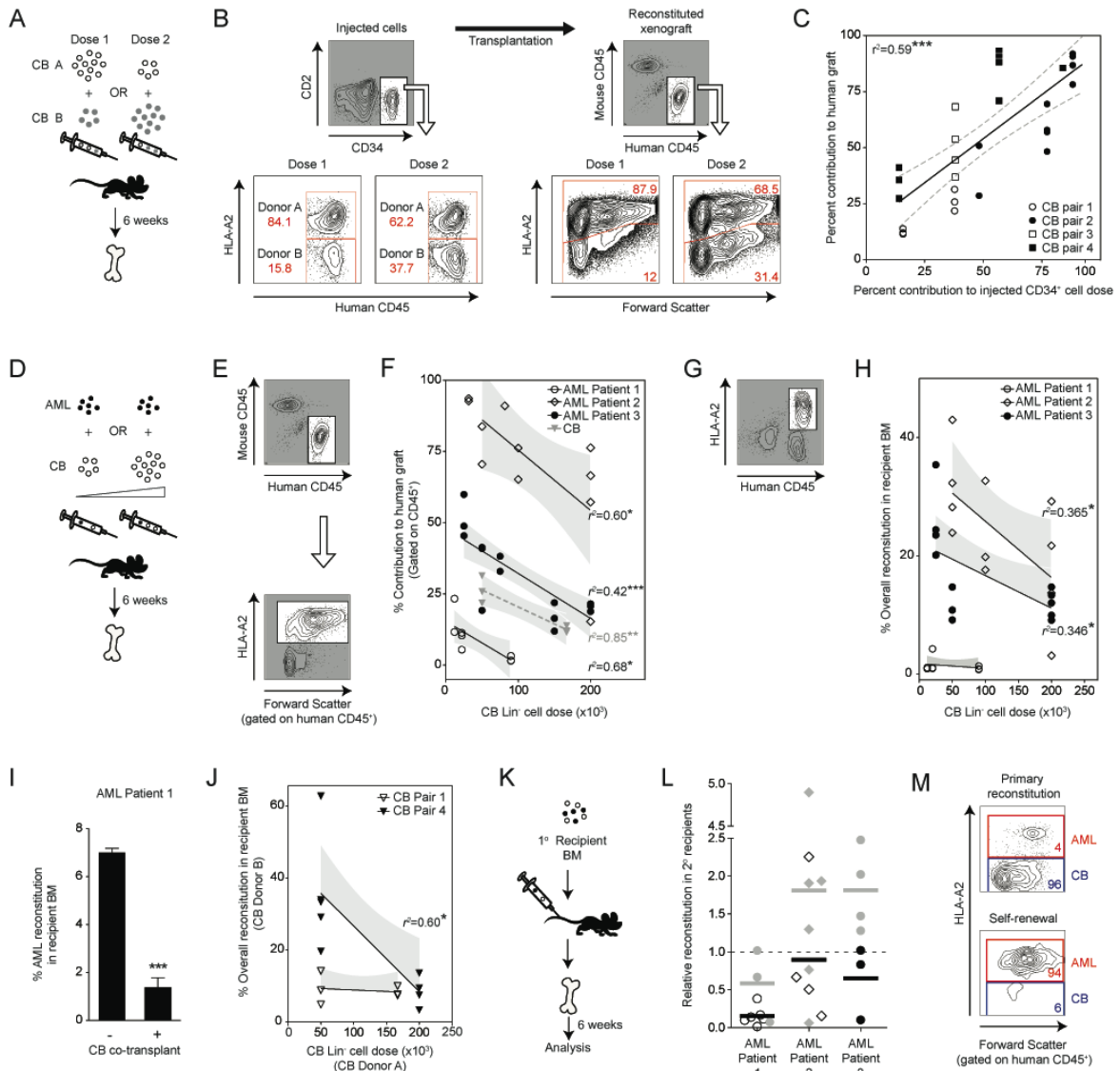


Figure 3.2. Co-transplantation of healthy HSPCs with AML MNCs can competitively reduce leukemic progression. (A) Experimental design to study competitive niche repopulation between HSPCs enriched from pairs of healthy human CB donors. (B) Gating strategy and representative flow cytometry plots showing relative donor cell proportions within human populations before and after co-injection into xenograft recipients. Upper left plot shows that CD2⁺ T cells were absent from all injected Lin⁻ populations, precluding any immune reactions within the recipient mice. (C) Scatter plot of flow cytometry data showing that in mouse BM grafts, the relative donor CD45⁺ cell frequencies closely recapitulated their original proportions within injected CD34⁺ populations, consistently across 4 independent experiments using different CB donor pairs. Dotted lines represent 95% confidence

intervals. Each data point represents an individual transplanted mouse. *** $P < 0.0001$, linear regression. **(D)** Experimental design to study the effects of BM niche competition between healthy and leukemic human repopulating cells. AML cell numbers were kept constant with increasing dose titrations of CB Lin⁻ cells. **(E)** Example gating strategy to assess the relative reconstitution of primary AML samples in the presence of competing CB-HSPCs, expressed as a proportion of the total human graft. **(F)** Flow cytometry data showing that HSPC competition reduces the leukemic contribution to human grafts in a cell dose-dependent manner for all AML samples tested (three independent experiments). Co-transplantation of CB Pair #1 is also shown for comparison. Shaded areas represent 95% confidence intervals. Each data point represents an individual mouse. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, linear regression. **(G)** Example gating strategy to assess the absolute BM reconstitution levels of AML samples in the presence of CB-HSPC competition. **(H)** Flow cytometry data showing that absolute leukemic reconstitution is reduced by HSPC competition in a cell dose-dependent manner. Shaded areas represent 95% confidence intervals. Each data point represents an individual mouse. * $P < 0.05$, linear regression. **(I)** Flow cytometry data showing that relative to control mice transplanted with AML cells alone, the total leukemic reconstitution of AML Patient #1 is reduced in the presence of competing HSPCs at all competing cell doses tested (single transplant $n=2$, co-transplant $n=8$). *** $P < 0.0005$, unpaired Student's t -test. **(J)** Flow cytometry data showing that absolute CB reconstitution levels are also influenced dose-dependently by competing CB donor cells only if human chimerism levels are high. Each data point represents an individual mouse. * $P < 0.05$, linear regression. **(K)** Experimental design to study the effects of BM niche competition on the self-renewal ability of AML L-ICs by serial transplantation. **(L)** Flow cytometry data showing that in the presence of CB competition, leukemic chimerism following serial transplantation is either lower than or equal to leukemic reconstitution in primary recipients. Leukemic engraftment in secondary recipients was normalized to that of primary recipients (dotted line). Relative engraftment of control AML cells transplanted in the absence of cord blood is shown in gray. Each data point represents an individual mouse. **(M)** Representative flow cytometry plots gated on human CD45 show robust normal human reconstitution subsequent to therapeutic irradiation-conditioned CB Lin⁻ transplantation of AML-engrafted mice (top), followed by AML recurrence after serial transplantation (bottom). Plots are representative of 6 mice that received irradiation-conditioned HSPC transplantation three weeks post disease initiation, all of which had persistent leukemia after treatment.

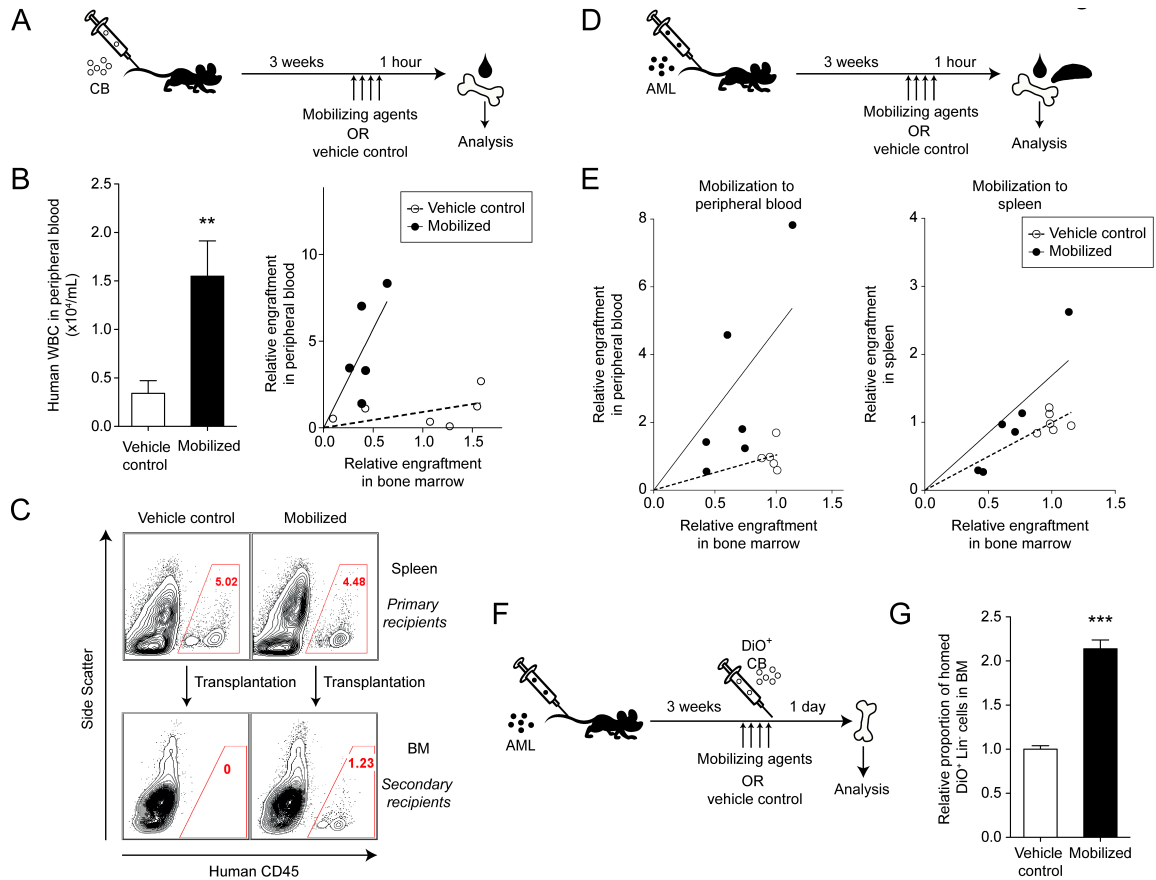


Figure 3.3. Mobilization treatment displaces normal and leukemic human repopulating cells from the xenograft BM niche, facilitating competitive homing by subsequent HSPC transplants. (A) Experimental design to investigate the effects of mobilization treatment on established human CB grafts. (B) Human white blood cell (WBC) distribution one hour following final treatment of CB xenografts with mobilization agents or vehicle (saline $n=6$, mobilization $n=5$). ** $P < 0.01$, unpaired Student's t -test (bar graph). Each data point represents an individual mouse and slopes were significantly different (scatter plot; linear regression $P < 0.05$). (C) Representative flow cytometry plots show that human CD45⁺ cells can be detected in the spleens of xenografted mice one hour following treatment with either vehicle or mobilization agents (top row). However, only splenic populations from mobilized mice possess repopulating activity, as measured by intrafemoral transplantation into secondary recipients (bottom row). Plots are representative of serial transplantation performed from spleens of 18 xenografted primary mice. (D) Experimental design to investigate the effects of mobilization agents on established human AML grafts. (E) Tissue distribution of human leukemic CD45⁺ cells one hour following final treatment with mobilization agents or vehicle. (F) Experimental design to investigate the effects of mobilization agents on established human AML grafts. (G) Relative proportion of homed DIO⁺ Lin⁻ cells in BM. *** $P < 0.001$, unpaired Student's t -test (bar graph).

Each data point represents an individual mouse. Slopes were significantly different, linear regression $P < 0.05$ (peripheral blood) and $P < 0.0005$ (spleen). **(F)** Experimental design to test whether mobilization of established human leukemic grafts can facilitate competitive niche replacement by subsequent HSPC transplants (CB Lin⁻ cells). **(G)** Relative frequency of DiO-labeled CB Lin⁻ cells homed to the BM of AML-engrafted mice following mobilization or vehicle treatment ($n=3$ each). *** $P < 0.005$, unpaired Student's *t*-test.

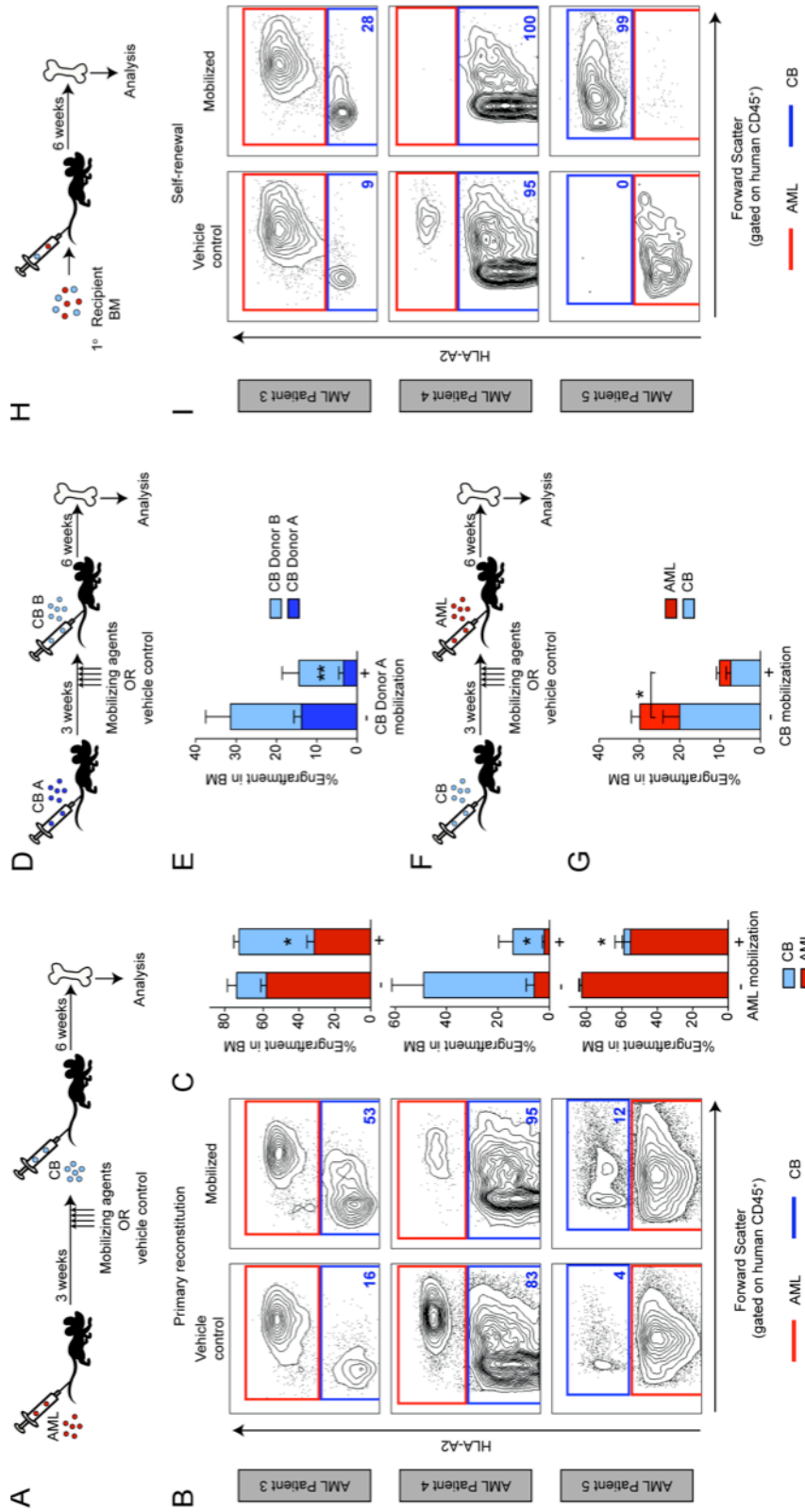


Figure 3.4. Leukemia mobilization facilitates competitive reconstitution by subsequent HSPC transplants at the expense of leukemic self-renewal. (A) Experimental design to test whether mobilization treatment of established AML grafts can facilitate competitive reconstitution by subsequent HSPC transplants (CB Lin⁻ cells). (B) Representative flow cytometry plots gated on human CD45⁺ populations. Gate frequencies indicate percent CB composition of human grafts (blue gates). (C) Summary of absolute human CB and leukemic engraftment levels showing that the total AML engraftment of three individual patients is reduced following mobilization-primed HSCT ($n=4-5$ per group for each patient). Engraftment values are gated within all live events in BM. * $P<0.05$, unpaired Student's t -test. (D) Experimental design to test whether mobilization treatment of established CB grafts can facilitate competitive reconstitution by subsequent healthy HSPC transplants. (E) Summary of absolute donor-specific engraftment levels showing that the chimerism levels of initial healthy donor grafts are reduced following mobilization-primed HSPC transplantation ($n=4$ per group). Engraftment values are gated within all live events in BM. ** $P<0.01$, unpaired Student's t -test. (F) Experimental design to test whether mobilization treatment of established CB grafts can facilitate competitive reconstitution by subsequent AML transplantation. (G) Summary of absolute human CB and leukemic engraftment levels showing that the chimerism levels of initial healthy donor grafts are reduced following mobilization-primed AML transplantation ($n=4$ per group). Engraftment values are gated within all live events in BM. * $P<0.05$, unpaired Student's t -test. (H) Experimental design to assess the self-renewal potential of primary human AML grafts that were exposed to competitive repopulation by healthy HSPCs either with or without pre-mobilization of leukemic cells. (I) Representative flow cytometry plots gated on human CD45 showing preferential self-renewal of HSPCs at the expense of AML L-ICs following mobilization-conditioned HSC transplantation of leukemic mice. Gate frequencies indicate percent CB composition of human grafts (blue gates).

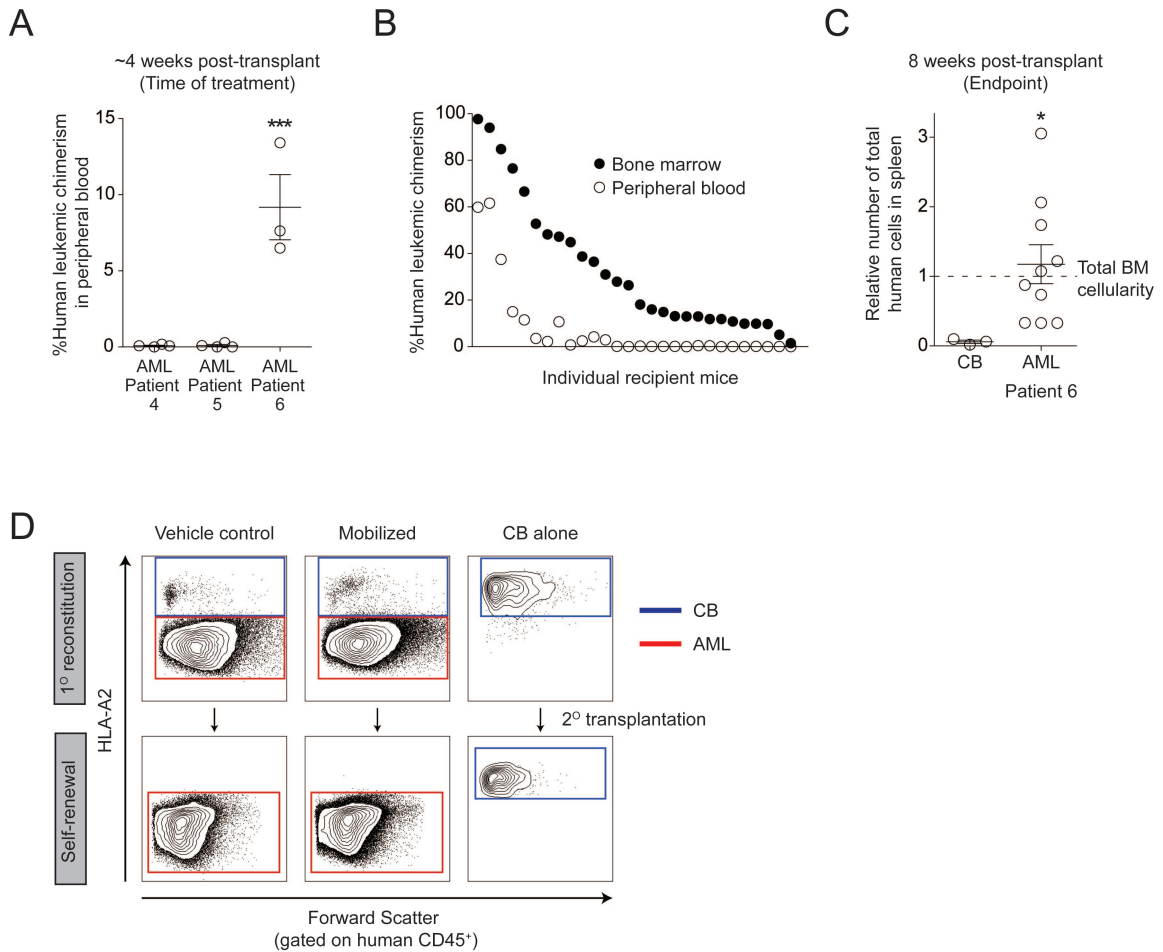


Figure 3.5. The leukemic burden at the time of mobilization-HSCT treatment influences therapeutic responses.

(A) Circulating leukemic cell frequencies detected in peripheral blood at 3-4 weeks post-disease initiation (when mobilization treatment and HSPC transplantation was performed). Each data point represents an individual mouse. *** $P < 0.0005$, one-way ANOVA. (B) Matched peripheral blood and BM chimerism levels plotted from individual AML-engrafted mice analyzed at various time points post-transplantation. Circulating blast levels increase dramatically once the leukemic infiltration of BM exceeds 50% chimerism. (C) The detection of substantial peripheral blood blast levels at 4 weeks post-transplantation (shown in A) is related to an extremely aggressive course of disease culminating in $96 \pm 1\%$ leukemic chimerism in BM with extramedullary leukemic dissemination and dramatic splenomegaly by 8 weeks post-disease initiation. The number of cells recovered from the spleens of mice transplanted with cells from AML Patient #6 approximated or even surpassed the cellularity from the entire BM (calculated according to Boggs, 1984). As a reference, the total abundance of human cells recovered from whole spleens is provided for CB-engrafted mice. * $P < 0.05$,

unpaired Student's *t*-test. **(D)** Representative flow cytometry plots gated on human CD45 showing detectable healthy (HLA-A2⁺) reconstitution in primary mice engrafted with AML Patient #6 cells and subsequently transplanted with healthy HSPCs. However, upon serial transplantation, HLA-A2⁺ CB cells failed to self-renew, suggesting inadequate niche colonization. Cell-intrinsic CB-HSPC deficits are ruled out by HLA-A2⁺ engraftment following serial transplantation of CB-alone controls. Plots are representative of 3-5 mice per group.

Table 3.1. CD34⁺ cell doses injected in mobilization-conditioned transplantation experiments, expressed per kg of recipient body weight. All doses are below reported clinically used values of 25.9×10^6 CD34⁺ cells/kg from adult HSPC sources (Scheid et al., 1999) and 40.1×10^6 CD34⁺ cells/kg (Korbling et al., 1995)

Sample	CD34⁺ cells x10⁶ /kg body weight
AML Patient 3	19.4 ± 0.4
AML Patient 4	10.9 ± 0.2
AML Patient 5	15 ± 1

Table 3.2. Self-renewal analysis of human AML-CB co-transplants with or without mobilization treatment. For each of three AML samples, it is indicated how many secondary transplant recipients were observed to have AML L-IC self-renewal, HSPC self-renewal, or both.

Engraftment Outcomes	AML Patient 3		AML Patient 4		AML Patient 5	
	Control	Mobilized	Control	Mobilized	Control	Mobilized
AML only	0/4	0/3	0/3	0/5	1/1	2/5
AML + cord blood	4/4	3/3	2/3	1/5	0/1	2/5
Cord blood only	0/4	0/3	1/3	4/5	0/1	1/5
Total mice	7		8		6	

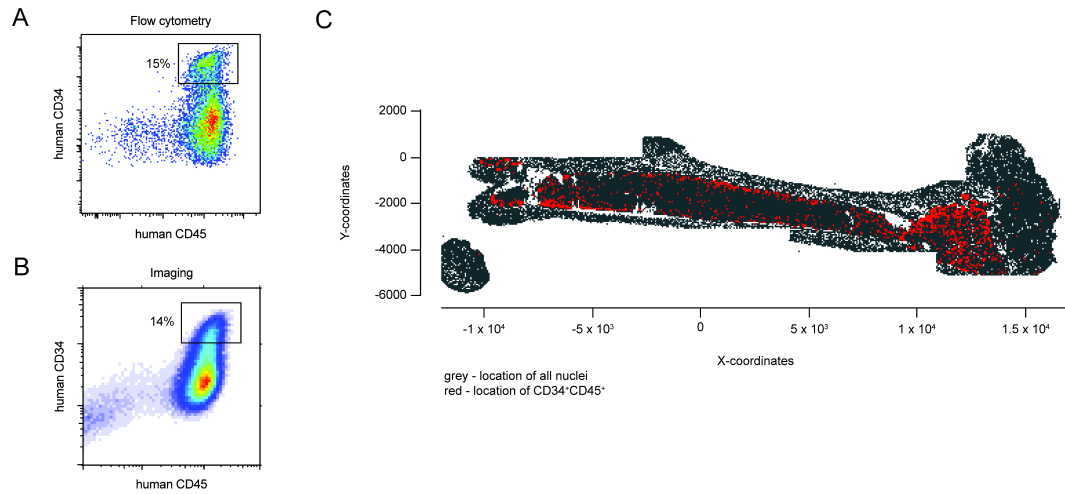


Figure S3.1. Gating strategies to quantify primitive leukemic cells in xenografted mouse femurs. Representative gating strategies showing similar frequencies of human CD45⁺CD34⁺ cells detected from different bones of the same mouse, as measured by flow cytometry (A), or whole-bone section imaging (B). (C) Representative total distribution of individually detected CD45⁺CD34⁺ cells within an AML-engrafted mouse femur.

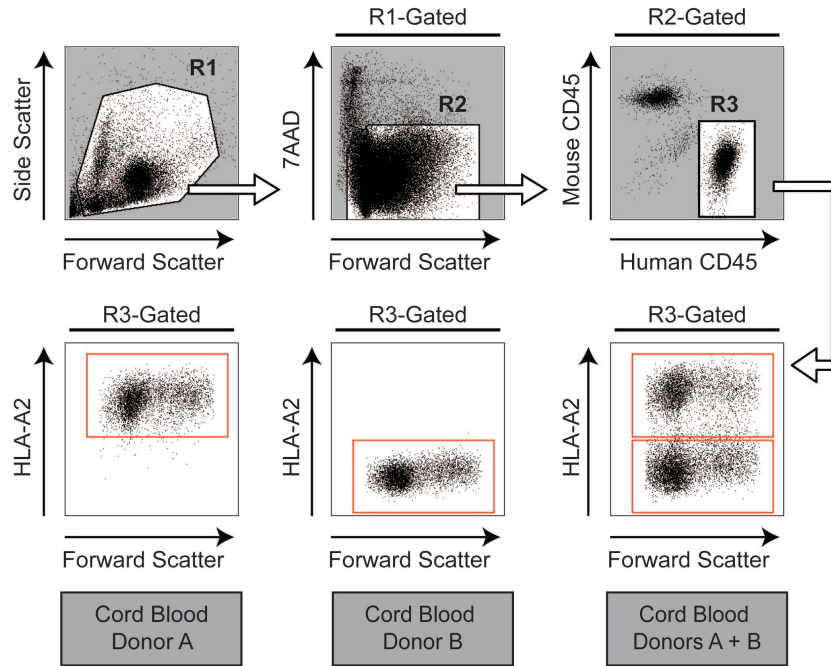


Figure S3.2. HLA-A2 mismatching provides a means to track hematopoietic cells from individual human donors transplanted into immunodeficient mice. Representative flow cytometry strategy to analyze the use of HLA-A2 disparity as a means of monitoring relative BM graft contributions of primary cells from pairs of different human individuals.

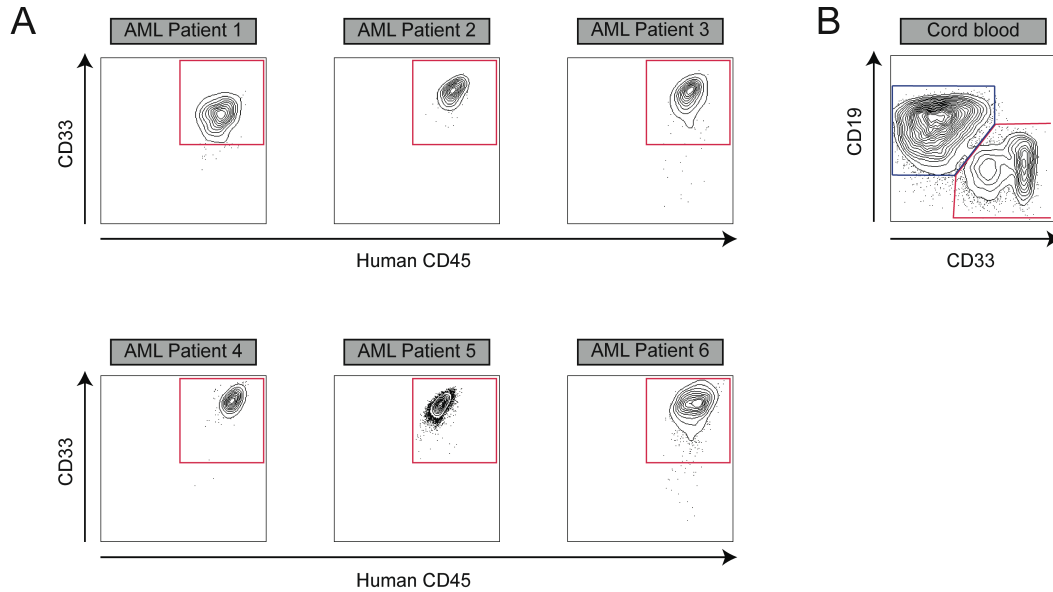


Figure S3.3. Multi-lineage gating strategy for co-transplanted human AML and CB samples. (A) Representative multi-lineage flow cytometry gating strategy for each primary AML patient sample evaluated in HSPC co-transplantation experiments show exclusive myeloid leukemic engraftment after patient-specific HLA-A2 gating. (B) Representative gating strategy shows that multi-lineage hematopoiesis is evident from healthy CB-HSPCs co-transplanted with AML, as determined by donor-specific HLA-A2 gating. Both myeloid (red gate) and lymphoid (blue gate) subpopulations were consistently detected.

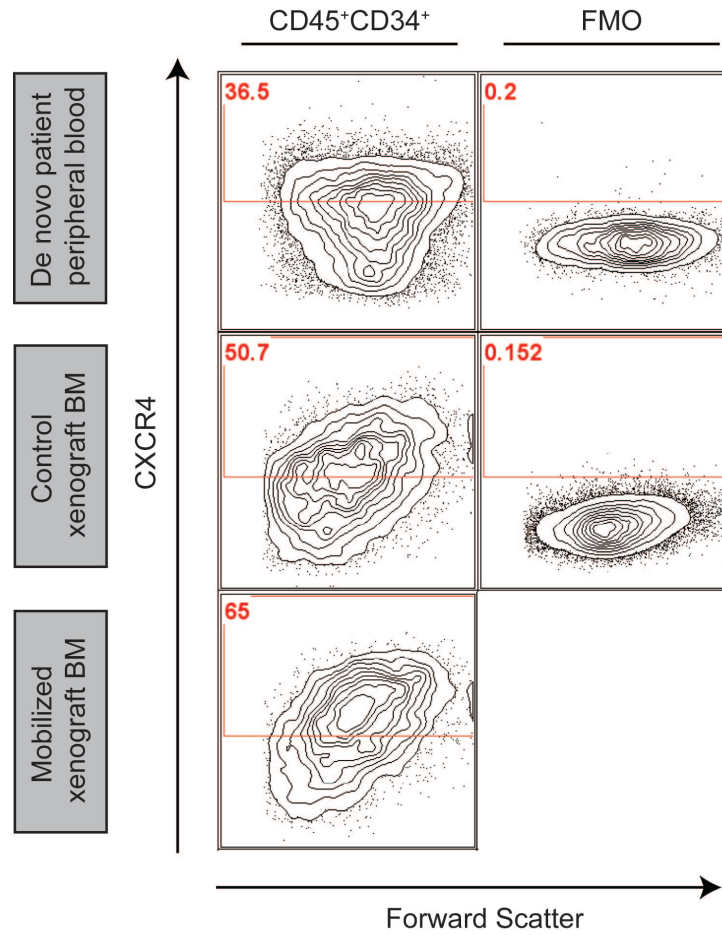


Figure S3.4. Gating strategy to evaluate the influence of AML graft mobilization on CXCR4 expression. Representative flow cytometry gating strategy showing that CXCR4 levels are appropriately increased on AML CD34⁺ cells one hour post-mobilization (Petit et al., 2002). Fluorescence minus one (FMO) controls are included to demonstrate gating strategy.

Table S3.1. Clinical details of AML samples used.

Sample	Tissue Source	Disease Stage	Patient Gender	Patient Age
AML 1	PB	Progressed from MDS	Female	83
AML 2	PB	Relapse (M4)	Female	51
AML 3	PB	New diagnosis (M4)	Male	56
AML 4	BM	Relapse (M5b)	Male	71
AML 5	PB	New diagnosis	Female	89
AML 6	Leukapheresis	Progressed from MDS	Male	48

Chapter 4: Cellular and Molecular Targeting of Recurrence in Acute Myeloid Leukemia

Preface

This chapter is prepared as an unpublished manuscript. The authors are: Allison L. Boyd*, Lili Aslostovar*, Aline Fiebig-Comyn, Zoya Shapovalova, Mohammed Almakadi, Jenn Reid, Fanny L. Casado, Kyle R. Salci, Anargyros Xenocostas, Ronan Foley, Brian Leber, and Mickie Bhatia

*Equal contribution

I designed the study and wrote the manuscript together with Dr. Mick Bhatia and Lili Aslostovar. Experiments were performed in equal collaboration with Lili Aslostovar, however Lili played a primary role in designing and executing all experiments related to DRD2 and thioridazine treatment, while I played a primary role in designing and interpreting the kinetic characterization of AraC chemotherapy responses. Aline Fiebig-Comyn and Jennifer Reid assisted with animal experiments. Dr. Zoya Shapovalova performed bioinformatic analysis. Dr. Mohammed Almakadi retrieved and analyzed clinical history data for treated AML patients. Fanny Casado performed thioridazine plasma analysis. Kyle Salci helped with cell purification experiments. Drs. Ronan Foley, Brian Leber, and Anargyros Xenocostas provided human leukemia samples and intellectual input.

Based on the observation that leukemia stem cells (LSCs) and healthy hematopoietic stem cells (HSCs) reside in common bone marrow niches (**Chapter 3**), I became interested to understand how they would respectively react to perturbations in the environment. Relative responses to standard AML chemotherapy were particularly relevant, as the mechanisms of LSC therapy resistance have not been well documented. Using kinetic analysis of AraC-treated xenografts, we modeled distinct regenerative responses between healthy and leukemic hematopoiesis. To understand the unique features of leukemic regeneration, we then performed longitudinal analysis of leukemic cells following chemotherapy treatment within individual patients, and also within xenografted mice. This represents the first comprehensive assessment of functional and molecular changes in leukemic populations as a response to standard of care chemotherapy, providing insights to direct novel combination therapy approaches.

Cellular and Molecular Targeting of Recurrence in Acute Myeloid Leukemia

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Allison L. Boyd and Lili Aslostovar contributed equally to this article.

Abstract*Background*

Despite decades of utilizing cytarabine-based chemotherapy regimens, recurrence of disease remains the outstanding clinical challenge in acute myeloid leukemia (AML). Unfortunately, the basis of relapse remains poorly characterized, thereby preventing effective therapeutic targeting.

Methods

Through longitudinal patient assessments combined with orthotopic xenograft modeling, we have characterized the cellular and molecular basis of therapy failure in AML that leads to recurrent disease. We performed gene expression analyses on 76 AML samples derived from 60 different AML patients, in addition to *in vitro* and *in vivo* assays of leukemic cell survival and self-renewal from 11 patients.

Results

Although transcriptional profiling captures patient diversity at diagnosis, the transcriptomes of AML patients at relapse converge to a common profile, hallmarked by a primitive stem cell state that is independent of tissue type. However, selective chemoresistance among these primitive leukemic subsets does not adequately explain the cellular dynamics of recurrence, as the proportions of these primitive cells equally declines following cytoreductive therapy. We have identified by molecular and functional analyses, the point of leukemic regeneration that supports recurrence. This is marked by the emergence of Leukemic

Regenerating Cells (LRCs) that arise as a consequence of cytoreductive chemotherapy. Preclinical modeling provided proof-of-principle for preventing AML recurrence by combination chemotherapy where unique LRC properties are targeted.

Conclusions

Leukemic relapse in AML patients is fueled by the emergence of LRCs, which can be targeted through combination therapies towards more effective and durable elimination of AML regrowth.

Background

Disease-free survival rates remain poor among acute myeloid leukemia (AML) patients. Following initial chemotherapy treatment, 20-50% of patients fail to achieve sufficient cytoreduction, thereby representing a refractory form of the disease¹. In addition, the majority of patients who do achieve complete remission (CR) relapse within several years². These two major paths of therapeutic failure ultimately lead to disease re-growth and overt leukemic recurrence. Prognosis is characterized at initial presentation based on specific cytogenetic and molecular genetic features, patient age, and immunophenotypic and morphological analyses¹. For patients that relapse, the duration of first CR may be an important predictive variable¹. However, risk stratification is poorly defined at recurrence^{3,4}, leaving patients with a uniformly poor prognosis and few therapeutic options^{1,5,6}. Therefore, it is necessary to better understand the mechanisms that drive therapy failure in order to rationally design more effective measures to prevent recurrence of AML.

A prominent hypothesis to explain the prevalent failure of cytoreductive chemotherapy in AML is the selective resistance of leukemia stem cells (LSCs)⁷ thought to be dormant and chemoprotected. Although functional LSCs are typically enriched in CD34⁺ immunophenotypic fractions, their cell surface phenotype is not consistent⁸⁻¹⁰. Accordingly, recent efforts have recently assigned a molecular signature of LSCs as a more reliable definition⁹, but this has yet to be characterized sufficiently for use in development of directed therapies. Furthermore, the series of

cellular and molecular events that occur between therapy and recurrence have not been thoroughly scrutinized, mainly due to the inability to phenotypically, functionally or genetically interrogate minimal residual leukemic cell populations that exist below the limit of detection during states of CR in patients¹¹. However, modeling therapeutic response using orthotopic human-mouse xenografts offers an enhanced ability to find and purify residual disease below the standard morphological detection limit of approximately 5% blasts in the bone marrow (BM), as human leukemia can be easily discriminated from murine hosts by immunophenotype to a high level of sensitivity and can be subjected to repeated sampling over several time points¹². Accordingly, xenograft models offer an experimental means to approximate human leukemic biology to uncover processes of recurrence in response to cytoreductive therapy that has not been possible in AML patients.

We have complemented direct cellular and molecular assessments of therapeutic response in leukemia patients with a novel xenograft modeling to understand the basis of AML recurrence. As the cytoreductive agent cytarabine (AraC) represents the single common element across different chemotherapy regimens used in the treatment of AML patients and is the only cytoreductive drug that is regularly administered as a monotherapy¹, we focused our studies of leukemic recurrence after treatment with AraC. We used human patients, as well as patient-derived xenografts to perform integrative cellular and molecular

transcriptional profiling¹³ to characterize the response to chemotherapy. With these insights, we established the origin of disease recurrence and successfully tested a new approach that uniquely targets AML patients at the point of leukemic regeneration.

Methods

Primary human AML samples

Disease recurrence is significantly less prevalent in acute promyelocytic leukemia (APL) and AML with favorable cytogenetic and molecular profiles, ranging from relapse rates of 15-25%, compared to upward of 50% of intermediate and high-risk groups¹⁴. Therefore, we have focused our examination on intermediate- and high-risk cytogenetic groups that represent the most urgent unmet clinical need. Primary AML specimens were obtained from peripheral blood apheresis or BM aspirates of AML patients. Clinical details of AML patient samples are outlined in Table 4.1 of the Supplementary Appendix. Healthy hematopoietic cells were isolated from BM and mobilized peripheral blood (MPB) of healthy donors or from umbilical cord blood. Samples were processed as described in the Supplementary Appendix. All samples were obtained from consenting donors in accordance with approved protocols by the Research Ethics Board at McMaster University and the London Health Sciences Centre, University of Western Ontario (Research Ethics Board #08-330 and #08-042).

Myeloid progenitor assay

The clonogenic capacity of hematopoietic progenitors was evaluated by colony-forming unit (CFU) assays. Briefly, AML mononuclear cells (MNCs; 5000-50000 cells) were seeded in semisolid methylcellulose media (Methocult GF #H4434; Stemcell Technologies) according to established protocols¹⁵. CFU assays of xenografted leukemic cells were performed following human cell purification as described in the Supplementary Appendix.

Murine recipients and xenograft assays

NOD/Prkdcscid (NOD/SCID) mice were used as xenograft recipients for AML recurrence modeling, and xenografts were initiated using previously described protocols¹⁶ (further outlined in the Supplementary Appendix). All experimental procedures were approved by the Animal Council of McMaster University (Animal Use Protocol #13-02-05). In combination regimens with AraC and thioridazine (TDZ), AraC (50 mg/ kg/ day) was administered daily between days 7-12 within the 21-day TDZ (22.5 mg/ kg/ day) regimen.

Gene expression profiling

RNA was extracted and hybridized to Affymetrix Gene Chip Human Gene 2.0 ST arrays (London Regional Genomics Centre, ON, Canada; further details provided in the Supplementary Appendix). Output data was normalized using the Robust Multichip Averaging algorithm with Partek Genomics Suite 6.6 software (Partek Inc). Gene expression data from 11 paired diagnosis-relapse samples¹⁷ (GSE66525), and diagnostic AML patient samples with predominantly normal karyotypes (GSE425,

Groups I and II¹⁸) were obtained from publically accessible data sets. Further analysis details are provided in the Supplementary Appendix.

Statistical analysis

Data are represented as mean \pm standard error (SE). Statistical comparisons were analyzed using unpaired two-tailed Student's t tests, analysis of variance tests (ANOVAs), linear regressions or Fisher's exact test. Prism software (version 5.0a; GraphPad) and ELDA¹⁹ were used for statistical analysis, and the criterion for statistical significance was $P < 0.05$.

Results

Molecular presentation of recurrent AML

Over a decade has passed since the transcriptional landscape was defined in cases of AML at diagnosis^{18,20}, however it remains critical to determine how recurrence of AML disease is positioned within this framework following cytoreductive chemotherapy. In order to define a molecular portrait of recurrence in AML, we performed gene expression analyses on matched diagnosis-recurrence samples from 15 individual AML patients with predominantly normal karyotypes (Table 4.1 in the Supplementary Appendix; Patients 1-15). Global transcriptional profiling revealed two consistent patterns between diagnosis versus subsequent recurrence of disease. While the recurrence-specific profiles of many patients closely resembled their initial diagnostic presentation, an equal proportion of cases reflected substantial molecular transitions that commonly converged among these patients (Fig. 4.1A).

This indicated a conserved biology among AML patients during the process of disease recurrence that is independent of initial patient heterogeneity.

A comprehensive analysis of all paired 30 samples confirmed the functional significance of these recurrence-driven changes resulting in transcriptional uniformity among AML patients. Unsupervised hierarchical clustering resolved the complete set of AML patient profiles into two major subgroups (Fig. 4.1B). One group's profile was anchored by mature healthy hematopoietic cells, indicating that cellular differentiation represents a defining axis that allows sample bifurcation. This observation was reminiscent of transcriptional subclasses previously described for newly diagnosed AML patients, which were also reported to reflect features of lineage commitment versus undifferentiated states¹⁸. Based on this similarity, we amalgamated the two datasets, which confirmed exceptional overlap between subgroup classifications (Fig. 4.1C), suggesting that these transcriptional boundaries represent robust and comprehensive molecular states of AML. Applying this classification scheme, we discovered that patients who initially presented with transcriptionally primitive disease at diagnosis usually remained in this state upon recurrence (Fig. 4.1B and Fig. 4.1D). However, patients with mature transcriptional features at diagnosis uniformly acquired primitive programs at recurrence (Fig. 4.1B and Fig. 4.1D), thus collapsing initial heterogeneity of patients seen at diagnosis.

The relationship between AML recurrence and primitive gene expression programs complements previously established associations between solid tumor

aggressiveness and transcriptional hallmarks of stemness. These studies have indicated that such stemness properties are not restricted to the tissue of tumor origin, but share overlapping features found in embryonic stem cells²¹. In the context of AML, we found that the gene expression of multiple pluripotent stem cell lines closely corresponds with the primitive AML subclass that is characteristic of recurrence. Thus, at recurrence, the transcriptional profiles of human AML patients become unified by primitive features that are not tissue- or lineage-specific. The specific transitions from mature to primitive molecular signatures from diagnosis to recurrence shown here would not have been predicted from genomic evolution studies of acquired mutations^{22,23}, demonstrating the value of global transcriptional-level analysis of the process of recurrence in AML.

Modeling AML recurrence in vivo

Convergence towards primitive transcriptional states at recurrence suggests that cytoreductive therapy may exert unifying influences across leukemias from different patients despite their heterogeneous origins. To better define the extent to which AraC influences the nature of leukemic cells that re-grow, we developed novel xenograft modeling of AML recurrence to parallel the clinical analysis of human patient responses. AML-xenografting techniques have traditionally served as invaluable tools to functionally describe leukemic subpopulations^{8,10}, however they have yet to be utilized to investigate the mechanisms leading to clinical failure after standard-of-care therapy that remains the major problem in AML. Xenografts

bearing patient leukemic cells were treated with optimized regimens of high-dose AraC²⁴ to emulate *in vivo* cytoreductive chemotherapy responses (Fig. 4.1 in the Supplementary Appendix). Using serial BM monitoring of individual recipient mice as a relevant response assessment, we were able to recreate the disease kinetics of both refractory and relapse states after therapy failure as seen in patients, with remarkable consistency among individual xenografts (Fig. 4.2A and 4.2B). Our CR criteria included restoration of healthy white blood cell counts (Fig. 4.2A in the Supplementary Appendix), as well as undetectable levels of leukemic blasts in BM aspirates¹ (Fig. 4.2B). This schema provides a potential powerful biological model of minimal residual disease, as leukemic cells temporarily escaped below the limits of detection in BM despite the extreme sensitivity of human versus murine cell discrimination in our system (eg. detection of cells as rare as 0.01%¹² versus approximately 5% based on standard morphological thresholds in patients¹; Fig. 4.2B).

To determine the extent to which *in vivo* modeling of chemotherapy response resembles the molecular processes of subsequent recurrence seen in AML patients, we purified human leukemic cells for transcriptional profiling at the point of overt leukemic reappearance after cytoreductive treatment of xenografts (Fig. 4.2C). These profiles were integrated into the framework of human AML gene expression for classification according to the mature versus primitive transcriptional states we defined (Fig. 4.1). Similar to our finding with clinical samples from relapsed patients,

there was little change in transcriptional profiles at recurrence modeled in xenografts if patient cells were in a primitive state prior to treatment (Fig. 4.2C; Fig. 4.2B in the Supplementary Appendix). In contrast, cells from another AML patient initially manifested a mature transcriptional program at diagnosis, however these cells could be provoked to transition to a primitive transcriptional state as a direct consequence of AraC-induced disease reemergence, which also recapitulated our direct analysis in patients (Fig. 4.2C and Fig. 4.2B in the Supplementary Appendix). Not only does this demonstrate that xenograft modeling reliably reflects human recurrent AML disease, but it also provides direct evidence that chemotherapy actively influences the molecular attributes of AML relapse.

The ability to accurately recreate AML patient therapy response provided us with an unprecedented opportunity to perform controlled comparisons between normal and leukemic regeneration not possible clinically. Longitudinal study of AraC-treated xenograft recipients indicated that leukemic patterns of unrestrained re-growth are notably disproportionate to the disciplined regeneration of the normal hematopoietic system. While healthy human grafts also recovered promptly from cytoreductive challenge after AraC treatment, they ultimately respected the boundary of their original levels of growth, even following a prolonged observation period (Fig. 4.2D). In contrast, AML cells rapidly exceeded the thresholds of their initial pre-treatment disease burden as consistently demonstrated across xenografts derived from each of three patients with distinct genetic profiles (Fig. 4.2D). This

indicates that despite the initial disease suppression noted after AraC therapy, AML cells respond in a unique manner that confers potent regeneration characteristics such that it exceeds normal hematopoietic recovery, thereby forming an integral part of overall disease recurrence.

Phenotypic and functional chronology of leukemic regeneration

To further characterize the unrestrained leukemic growth properties that uniquely define AML regeneration after cytoreductive therapy, we examined this compensatory response under different conditions of AraC treatment. In xenograft replicates of the same patient sample, re-emergence of aggressive leukemic disease surprisingly occurred in a uniform pattern among different chemotherapy-treated groups regardless of the timing of AraC intervention or the attainment of CR (Fig. 4.3A). In addition to the synchrony of leukemic re-emergence across AraC-treated groups, we also observed that the leukemic burden within all AraC-exposed recipients ultimately approached that of saline-treated controls. This was also mediated by a consistent three-fold acceleration in leukemic growth rates in response to AraC treatment (Fig. 4.3A), and reproducibly observed in other AMLs (Fig 4.3A in the Supplementary Appendix). This increased growth during relapse would not be evident in AML patients, as the obligation to treat precludes baseline kinetic characterization of therapy-naïve disease. However, the acquisition of exaggerated growth properties provides empirical data that is consistent with the clinical observations that relapsed disease becomes more aggressive and difficult to

manage in AML patients⁶. Our results indicate that leukemic growth can actively compensate for cytoreductive insult, and that the determinism and magnitude of this response is consistent across AML patients.

In order to refine therapeutic approaches to durably suppress leukemic regeneration, it is critical to understand the mechanistic basis that drives dysregulated leukemic growth in response to standard cytoreductive therapy. The perception that chemotherapy selectively preserves self-renewing LSCs⁷ offers a promising hypothesis to explain both the acquisition of primitive transcriptional profiles and rapid leukemic re-growth properties that characterize recurrent AML. The premise that LSCs are selectively chemoresistant would predict a relative enrichment of phenotypically and functionally primitive cells at the point of chemotherapy withdrawal. We formally tested this possibility by examining the expression of CD34 on BM cells in a clinical case of unsuccessful leukemia clearance, measured at the earliest evaluable opportunity following cytoreductive therapy²⁵. Surprisingly, not only did this assessment fail to show that CD34⁺ leukemic cells were preferentially spared, but also indicated instead that CD34⁺ frequencies were markedly reduced by cytoreductive therapy (Fig. 4.3B). This observation was reproduced under controlled conditions of human AML-xenografts (Fig. 4.3B), in which the dynamics of CD34 expression could be precisely measured over time in the absence of further intervention. Despite the initial marked reduction of CD34⁺ populations following cytoreductive AraC treatment, CD34-expressing cells

gradually became restored to levels equal to or above those of matched saline-treated controls (Fig. 4.3B in the Supplementary Appendix). This eventual predominance of phenotypically primitive cells is consistent with the development of the molecular features of primitiveness at recurrence (Fig. 4.1B-E). Furthermore, the kinetics of CD34 expression suggest that these features are gradually re-acquired during the regeneration process, as opposed to selection of primitive cells immediately after cytoreduction therapy.

Based on our phenotypic observations that do not support the postulation of primitive cell selection after chemotherapy, we initiated a rigorous functional assessment of AML cell subsets over time. We quantified colony-forming cells from two clinical cases of AML patient induction failure, which were captured at distinct time intervals after cytoreductive treatment. An early post-therapy assessment indicated that colony-forming potential was massively reduced by cytoreductive treatment. In contrast, subsequent time periods revealed that leukemic progenitor content in fact surpassed original pre-treatment levels (Fig. 4.3C). These clinical observations were recapitulated in AML-xenograft recurrence modeling, which provided a more seamless analysis of residual leukemic composition over the course of AraC response. Among xenografts derived from multiple AML patients, purified human populations replicated the trajectory of clonogenic depression that was similarly followed by acute recovery, the pattern noted in AML patients. Using the higher resolution provided by our AML-xenograft modeling, we observed that this

recovery phase culminated in a proportional expansion of the progenitor pool that exceeded untreated levels, prior to any evidence of bulk leukemia re-growth (Fig. 4.3D; Fig. 4.3C in the Supplementary Appendix). With the knowledge of these well-characterized timelines, we expanded our analysis at this unique time point that is defined by the lowest threshold of leukemic cells remaining in BM. From xenografts established from 5 out of 6 patients, we reproducibly detected the presence of highly clonogenic AML cells at this precise time point that precedes the accelerated expansion of leukemic disease regeneration (Fig. 4.3E). This sequence of events has been previously unappreciated due to the rare frequencies of recovering sufficient leukemic cells following cytoreduction, and the practical challenges associated with their accurate detection and isolation from humans²⁶. These results indicate that functional reconstruction of the primitive leukemic hierarchy precedes overt disease recurrence, thereby providing a unique window to examine potential therapeutic targets to combat residual disease in AML at this sensitive time of re-growth.

Targeting the onset of leukemic regeneration

The observation that cytoreductive chemotherapy transiently suppresses clonogenic proliferative leukemia cells suggests that a possible window of therapeutic opportunity may exist to prevent this initiation and onset of leukemic regeneration. We therefore sought to capture the molecular signature of these rare cells that form the origins of disease re-emergence. Leukemic populations were physically isolated following AraC-based cytoreduction from clinical samples or xenograft surrogates,

at this point of disease regeneration. Relative to matched therapy-naïve controls, cytoreductive therapy produced consistent transcriptional shifts across individual patients (Fig. 4.4A). This level of concordance was impressive, as the diversity of patients profiled included both de novo AML and progressive cases secondary to MDS, with diverse cytogenetic features ranging from normal karyotype to very poor prognostic abnormalities such as *inv(3)(q21q26.2)* rearrangements. Furthermore, the transcriptional consequences of AraC-based therapy were closely aligned between human patient and xenograft assessments.

We began a closer examination of regenerating leukemic populations by testing for molecular evidence of LSC gene expression programs, which have been suggested to correlate with the risk of recurrence⁹. Contrary to expectations that cytoreductive therapy would enrich for LSC-specific signatures after debulking the more rapidly proliferating subsets⁷, LSC-related genes were not represented in these rare leukemic populations (Fig. 4.4B). We then examined the transcriptional alterations noted with DNMT3A expression, as this gene has gained recent attention as an important driver of leukemogenesis both at initial presentation and at recurrence^{23,27}. Cytoreduction had no influence on DNMT3A gene expression in leukemic cells ($P=0.86$; data not shown). Unfortunately, as gene signatures relevant to DNMT3A-driven leukemic pathogenesis have not been detected in AML^{28,29}, no additional insights could be offered to determine the suggested impact of DNMT3A in AML regeneration processes. This inability to explain the functional properties of

leukemic recovery based on expected gene signatures suggests the emergence of a unique and unexplored transcriptional state that is fundamental to this stage of leukemic regeneration.

We next applied an unbiased exploratory approach to molecularly define Leukemic Regenerating Cells (LRCs) that arise as a consequence of cytoreductive therapy and that ultimately fuel recurrent AML disease. Across patients, the expression of 251 protein-coding genes was specifically elevated within LRC populations following AraC-based cytoreduction. To identify a clinically useful link between the transcriptional profiles and the functional process of recurrence, we further filtered these genes to identify known druggable targets using the Drug-Gene Interaction database³⁰. This refinement yielded 19 therapeutically targetable gene products (Fig. 4.4C). These genes were found exclusively in the regenerating transitional LRC state, as their unique expression levels were not evident in the bulk AML disease at diagnosis or at relapse, further highlighting the specific nature of the hierarchical process that drives recurrence (Fig. 4.4D). Among these 19 genes, DRD2 was identified and represents a cell surface receptor our group has recently characterized in human AML, that can be targeted using the drug thioridazine³¹ (TDZ) to suppress colony forming activity of AML. We therefore selected this drug for further proof-of-principle testing of novel combination therapy coupled with AraC cytoreduction.

Across three different AML patients, flow cytometry analysis of human AML-xenografts confirmed protein-level elevation of DRD2 within human leukemic cells at the point of LRC expansion after AraC treatment (Fig. 4.4E). In contrast, *in vivo* TDZ delivery induced opposing effects on DRD2 expression (Fig. 4.4E), using doses that were modeled to approximate clinically relevant circulating plasma levels³² (Fig. 4.4 in the Supplemental Appendix). We designed the AraC + TDZ combination therapy such that TDZ extended over the period of LRC emergence (Fig. 4.4F). Application of this combination regimen to AML-xenografts revealed that although similar levels of AraC-cytoreduction could be achieved between monotherapy and combination approaches, TDZ co-administration effectively prevented the accelerated re-growth (Fig. 4.4F) following AraC. Quantitative kinetic modeling confirmed that TDZ treatment significantly restrained leukemic growth rates when used incorporated with AraC (Fig. 4.4G).

Extensive further experimentation validated that the therapeutic benefits of TDZ were achieved through successful targeting of AraC-induced LRCs (Fig. 4.4H). At the peak of LRC outgrowth resulting from AraC treatment, residual leukemia cells were collected for the careful functional dissection of combined targeting with TDZ. As the property of disease initiation cannot be conclusively identified based on surface phenotypes alone⁹, serial xenotransplantation remains the only method to stringently quantify the cellular units responsible for *in vivo* regeneration. Using this assay, no residual leukemic re-initiation capacity was detectable after therapeutic

incorporation of TDZ, despite robust regeneration potential across AraC-monotherapy controls (Fig. 4.4H). In replicate experiments from two additional AML patients, re-growth of AML was suppressed so deeply that insufficient leukemic cells could be recovered for serial transplantation. In this case we used residual AML cells for colony forming assays that require fewer cells. In contrast to AraC-treated controls, no evidence of LRC re-growth was detectable from either patient sample if AraC therapy had been supplemented with TDZ (Fig. 4.4H). A final case demonstrated complete leukemia clearance by AraC + TDZ (Fig. 4.4H) where no AML cells were detectable, indicating that combination therapy was able to completely abolish leukemia in sharp contrast to AraC monotherapy. Using this predictive model of recurrence, our results indicate that co-treatment with TDZ decisively ensured that AML was consistently eradicated *in vivo* (Fig. 4.4H). This ultimately demonstrates the potential for systematic molecular targeting for improved efficacy to eliminate AML recurrence.

Discussion

In contrast to initial presentation in which patients are risk-stratified into multiple subgroups¹⁸, we have documented unifying shifts towards primitive molecular states at the time of AML relapse. We offer a model to conceptualize AML recurrence that includes therapeutic vulnerabilities and new opportunities during patient management (Fig. 4.5). Kinetic modeling revealed that transcriptional signatures were accompanied by accelerated leukemic growth in response to cytoreductive

therapy, and these regeneration characteristics were dissociable from healthy hematopoietic recovery. A thorough examination of this process provided a unique molecular account of leukemic regeneration after cytoreductive therapy, which was not predicted by LSC signatures⁹, or by restricting transcriptional analysis to the later overt stage of disease reappearance. Importantly, we have demonstrated the immediate clinical value of these novel molecular insights through proof-of-principle targeting of newly defined LRC properties using a pre-clinical model of AML recurrence. These results introduce the approach that leukemic recurrence can be prevented by rational combination therapies that can be selectively applied to inhibit this unique period of regeneration onset defined by LRCs during a discrete therapeutic window of vulnerability (Fig. 4.5). In conditions of overtly persistent leukemia, it was unexpected to find that CD34⁺ and colony-forming cells were not preferentially spared in the initial periods that followed AraC cytoreduction. Instead, subsequent restoration of this primitive compartment was first necessary to propel leukemic disease recovery, reinforcing the importance of understanding the hierarchical organization of this disease at the onset of recurrence post therapy. These findings complement emerging insights on chemotherapy-driven repopulation in solid tumors^{33,34}, highlighting that this represents a broad element of cancer therapy resistance that requires further attention. We propose a movement towards APL-like models of molecularly directed therapy³⁵ in AML that are adapted to target the specific transcriptional hallmarks of leukemic re-growth. The optimal duration of LRC-targeted therapy will require further investigation in

clinical trials, but could be informed by novel advances in minimum residual disease monitoring for AML³⁶. The potential for predictive measures of therapy response should also be pursued based on LRC gene set testing during remission states, to examine possible relationships with disease-free survival.

Our results suggest that we reshape our perspective of AML recurrence. As human patients also receive anthracyclines in their cytoreductive regimens, the striking conformity of these therapy response measures in xenograft surrogates versus AML patients suggests that the dynamics we have described are not limited to a single cytoreductive agent. We have provided an unprecedented demonstration of the clinical fidelity of AML xenografts, on the basis of transcriptional, phenotypic, and functional parameters throughout the therapeutic response to the current standard-of-care AraC therapy. This realistically accurate model forms a solid foundation upon which to test further candidate molecules for comparative efficacy and prioritization toward clinical testing. A Phase I safety study has been designed on the basis of these findings, and the exploration of LRC gene signatures presents a promising platform for additional therapeutic innovation in AML that we and others are currently pursuing.

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References

1. Döhner H, Estey EH, Amadori S, et al. Diagnosis and management of acute myeloid leukemia in adults: recommendations from an international expert panel, on behalf of the European LeukemiaNet. *Blood* 2010;115:453-74.
2. Shipley JL, Butera JN. Acute myelogenous leukemia. *Exp Hematol* 2009;37:649-58.
3. Breems DA, van Putten WLJ, Huijgens PC, et al. Prognostic index for adult patients with acute myeloid leukemia in first relapse. *J Clin Oncol* 2005;23:1969-78.
4. Szer J. The prevalent predicament of relapsed acute myeloid leukemia. *The Education Program of the American Society of Hematology* 2012; 43-8.
5. Ravandi F, Cortes J, Faderl S, et al. Characteristics and outcome of patients with acute myeloid leukemia refractory to 1 cycle of high-dose cytarabine-based induction chemotherapy. *Blood* 2010;116:5818-23.
6. Forman SJ, Rowe JM. The myth of the second remission of acute leukemia in the adult. *Blood* 2013;121:1077-82.
7. Jordan CT, Guzman ML, Noble M. Cancer stem cells. *N Engl J Med* 2006;355:1253-61.
8. Bonnet D, Dick JE. Human acute myeloid leukemia is organized as a hierarchy that originates from a primitive hematopoietic cell. *Nature Medicine* 1997;3:730-7.
9. Eppert K, Takenaka K, Lechman ER, et al. Stem cell gene expression programs influence clinical outcome in human leukemia. *Nature Medicine* 2011;17:1086-93.
10. Lapidot T, Sirard C, Vormoor J, et al. A cell initiating human acute myeloid leukaemia after transplantation into SCID mice. *Nature* 1994;367:645-8.
11. Ofran Y, Rowe JM. Introducing minimal residual disease in acute myeloid leukemia. *Current opinion in hematology* 2015;22:139-45.
12. van Galen P, Kreso A, Mbong N, et al. The unfolded protein response governs integrity of the haematopoietic stem-cell pool during stress. *Nature* 2014;510:268-72.
13. Gerstung M, Pellagatti A, Malcovati L, et al. Combining gene mutation with gene expression data improves outcome prediction in myelodysplastic syndromes. *Nat Comms* 2015;6:5901.
14. Stone RM. Acute myeloid leukemia in first remission: to choose transplantation or not? *J Clin Oncol* 2013;31:1262-6.

15. Boyd AL, Salci KR, Shapovalova Z, McIntyre BAS, Bhatia M. Nonhematopoietic cells represent a more rational target of in vivo hedgehog signaling affecting normal or acute myeloid leukemia progenitors. *Experimental Hematology* 2013;41:858-869.
16. Boyd AL, Campbell CJV, Hopkins CI, et al. Niche displacement of human leukemic stem cells uniquely allows their competitive replacement with healthy HSPCs. *Journal of Experimental Medicine* 2014;211:1925-35.
17. Hackl H, Steinleitner K, Lind K, et al. A gene expression profile associated with relapse of cytogenetically normal acute myeloid leukemia is enriched for leukemia stem cell genes. *Leuk Lymphoma* 2014:1-3.
18. Bullinger L, Döhner K, Bair E, et al. Use of gene-expression profiling to identify prognostic subclasses in adult acute myeloid leukemia. *N Engl J Med* 2004;350:1605-16.
19. Hu Y, Smyth GK. ELDA: extreme limiting dilution analysis for comparing depleted and enriched populations in stem cell and other assays. *Journal of immunological methods* 2009;347:70-8.
20. Valk PJM, Verhaak RGW, Beijen MA, et al. Prognostically useful gene-expression profiles in acute myeloid leukemia. *N Engl J Med* 2004;350:1617-28.
21. Ben-Porath I, Thomson MW, Carey VJ, et al. An embryonic stem cell-like gene expression signature in poorly differentiated aggressive human tumors. *Nat Genet* 2008;40:499-507.
22. Ding L, Ley TJ, Larson DE, et al. Clonal evolution in relapsed acute myeloid leukaemia revealed by whole-genome sequencing. *Nature* 2012;481:506-10.
23. Kronke J, Bullinger L, Teleanu V, et al. Clonal evolution in relapsed NPM1-mutated acute myeloid leukemia. *Blood* 2013;122:100-8.
24. Wunderlich M, Mizukawa B, Chou F-S, et al. AML cells are differentially sensitive to chemotherapy treatment in a human xenograft model. *Blood* 2013;121:e90-7.
25. Ravandi F. Primary refractory acute myeloid leukaemia - in search of better definitions and therapies. *British Journal of Haematology* 2011;155:413-9.
26. Zeijlemaker W, Gratama JW, Schuurhuis GJ. Tumor heterogeneity makes AML a “moving target” for detection of residual disease. *Cytometry* 2014;86:3-14.
27. Shlush LI, Zandi S, Mitchell A, et al. Identification of pre-leukaemic haematopoietic stem cells in acute leukaemia. *Nature* 2014;506:328-33.

28. Marcucci G, Metzeler KH, Schwind S, et al. Age-related prognostic impact of different types of DNMT3A mutations in adults with primary cytogenetically normal acute myeloid leukemia. *J Clin Oncol* 2012;30:742-50.
29. Ribeiro AF, Pratcorona M, Erpelinck-Verschueren C, et al. Mutant DNMT3A: a marker of poor prognosis in acute myeloid leukemia. *Blood* 2012;119:5824-31.
30. Griffith M, Griffith OL, Coffman AC, et al. DGIdb: mining the druggable genome. *Nat Meth* 2013;10:1209-10.
31. Sachlos E, Risueno RM, Laronde S, et al. Identification of drugs including a dopamine receptor antagonist that selectively target cancer stem cells. *Cell* 2012;149:1284-97.
32. Nagel D, Spranger S, Vincendeau M, et al. Pharmacologic inhibition of MALT1 protease by phenothiazines as a therapeutic approach for the treatment of aggressive ABC-DLBCL. *Cancer Cell* 2012;22:825-37.
33. Kim JJ, Tannock IF. Repopulation of cancer cells during therapy: an important cause of treatment failure. *Nat Rev Cancer* 2005;5:516-25.
34. Kurtova AV, Xiao J, Mo Q, et al. Blocking PGE2-induced tumour repopulation abrogates bladder cancer chemoresistance. *Nature* 2014;517:209-13.
35. Tallman MS, Altman JK. How I treat acute promyelocytic leukemia. *Blood* 2009;114:5126-35.
36. Klco JM, Miller CA, Griffith M, et al. Association Between Mutation Clearance After Induction Therapy and Outcomes in Acute Myeloid Leukemia. *JAMA* 2015;314:811-22.
37. Baumann B, Krell D, Dobrowolny H, Bielau H. Mechanisms of action in the prevention of recurrent mood disorders. *Pharmacopsychiatry* 2004;37 Suppl 2:S157-64.
38. Verlinden SF, van Es HH, van Bekkum DW. Serial bone marrow sampling for long-term follow up of human hematopoiesis in NOD/SCID mice. *Exp Hematol* 1998;26:627-30.
39. Vo T-T, Ryan J, Carrasco R, et al. Relative Mitochondrial Priming of Myeloblasts and Normal HSCs Determines Chemotherapeutic Success in AML. *Cell* 2012;151:344-55.

Figures and Tables

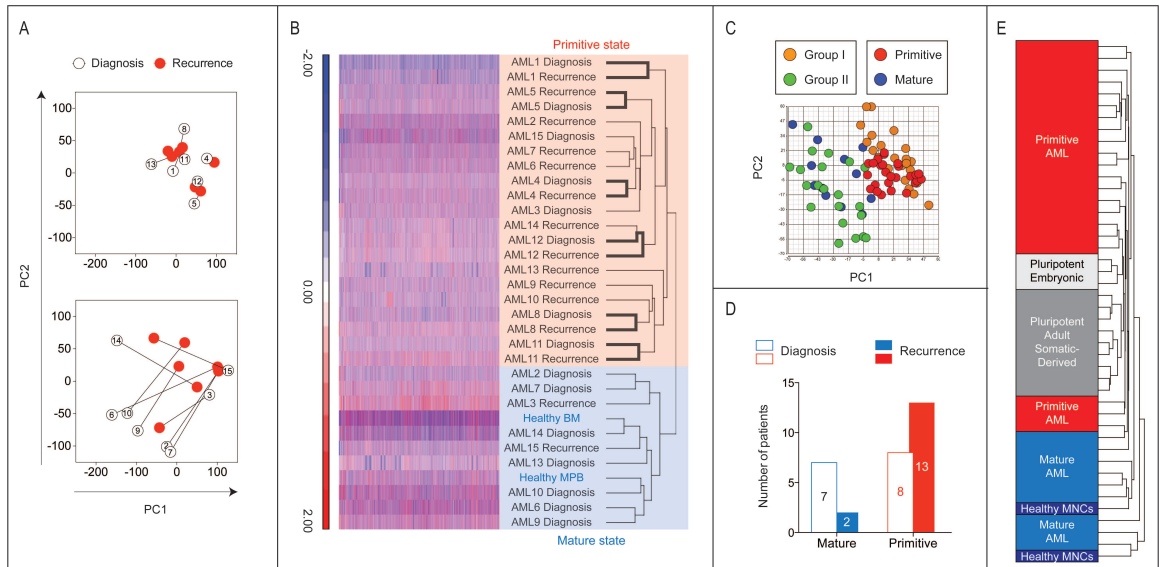


Figure 4.1. Transcriptional profiles of AML at diagnosis and recurrence

Panel A shows a principal component analysis (PCA) correlation biplot representing global gene expression for paired diagnosis and recurrence samples from 15 AML patients. For 7 patients, gene expression signatures were undistinguishable between diagnosis and recurrence (top). However for the remaining 8 patients, gene expression signatures were variable between the two sampling points, converging upon similar gene space across patients at recurrence (bottom). Both plots share the same components, which respectively explain 29.1% (PC1) and 10.3% (PC2) of the gene expression variation across the whole dataset of 15 patients. The numbers within datapoints indicate patient IDs. Panel B shows two major molecular subgroups resolved by unsupervised global hierarchical clustering analysis of all 30 paired diagnosis-recurrence AML samples together with two mononuclear cell (MNC) preparations from healthy donors (BM and MPB). The positioning of healthy MNCs within the second cluster suggests this may represent a state of mature hematopoietic cells. Bolded brackets highlight the transcriptionally stable patient samples presented in Panel A. Panel C shows a PCA correlation biplot of the paired diagnosis-recurrence AML patient samples presented in Panels A-B, merged with diagnostic AML patient samples reported in Bullinger et al.¹⁸ (N= 44 samples with predominantly normal karyotypes within GSE425; based on 6905 common genes across the different technology platforms). These two components explain 15% (PC1) and 9.42% (PC2) of the gene expression variation within the merged datasets. Data point colors represent gene expression states as defined in Panel B, or group membership defined in Bullinger et al.¹⁸, which were described to have primitive (Group I) or mature (Group II) characteristics. Panel D shows a summary outlining

that at diagnosis, there is an equal distribution of patients with mature versus primitive transcriptional signatures, whereas at recurrence the majority of patient samples reflect primitive signatures. Panel E shows an unsupervised hierarchical clustering analysis of the AML samples and healthy MNCs presented in Panels A-B, in addition to non-hematopoietic human pluripotent stem cells including both embryonic (N=3) and adult somatic cell-derived induced pluripotent stem cells (N=9). AML group membership matches that shown in Panel B.

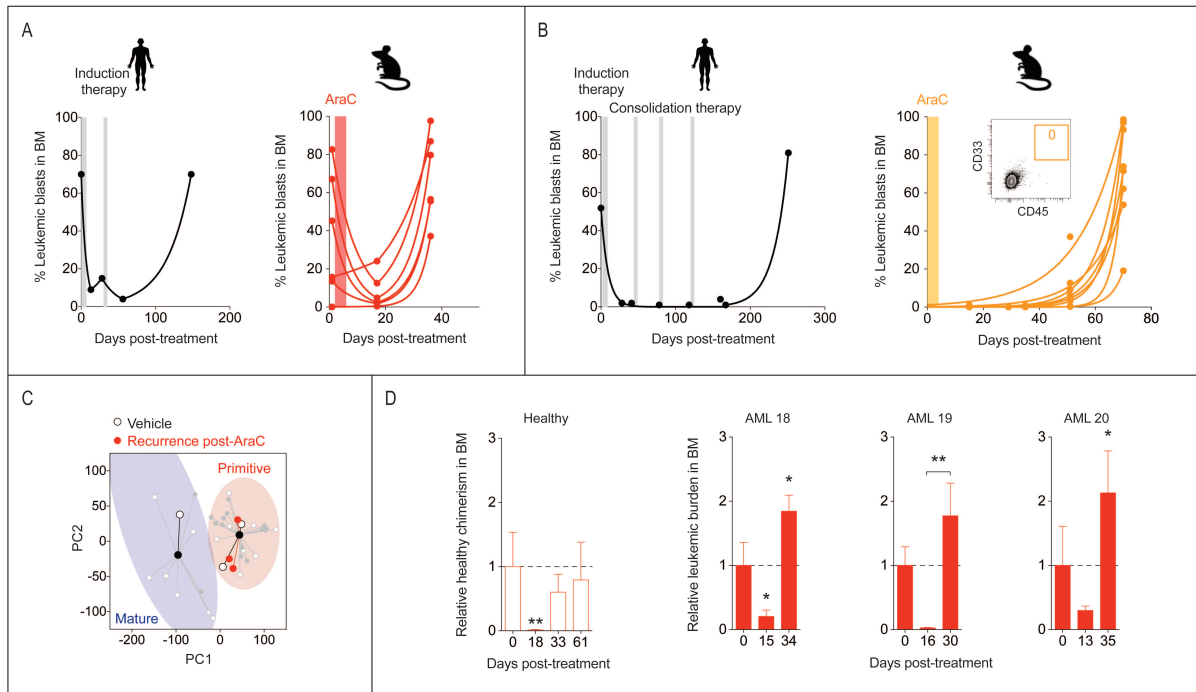


Figure 4.2. Modeling leukemic recurrence *in vivo*

Panel A shows clinical disease history representing a case of refractory AML (left; Patient 16) as determined by BM leukemic blast percentages according to clinical aspirate cytopathology reports. Gray bars indicate the timing and duration of AraC-based cytoreductive chemotherapy. Similar responses were recreated in xenografted mice following AraC therapy (right; Patient 18). Data represent flow cytometry measurements of human leukemic chimerism from serial BM aspirates. The red bar indicates the timing and duration of AraC administration (subcutaneous delivery of 50 mg/kg/day). Each curve represents an individual recipient mouse. Panel B shows a case of relapsed disease following a period of CR (left; Patient 17) as per documented clinical aspirate cytopathology reports. Gray bars indicate the timing and duration of AraC-based cytoreductive chemotherapy (including consolidation therapy delivered after achievement of CR). Similar remission-relapse scenarios were also captured in AML-xenografted recipients (right; Patient 18). The orange bar indicates the timing and duration of AraC administration (subcutaneous delivery of 50 mg/kg/day). Each curve represents an individual mouse. The inset shows a representative flow cytometry plot of a follow-up BM aspirate performed at Day 29 post-treatment, confirming a state of remission. Panel C shows a PCA correlation biplot representing xenografted AML cells from three different AML patients (Patients 18-20), either at the point of recurrence following *in vivo* AraC treatment, or from matched saline-treated controls. As seen in human analyses, AraC-treated leukemic cells consistently reflect a primitive molecular state while

saline controls are distributed between primitive and mature subspaces. Shaded ellipses identify the gene space defined by primitive and mature molecular signatures. Components are identical to those presented in Fig. 4.1A. Human AML samples from Fig. 4.1 are shown in gray as a reference (diagnostic samples are represented by open circles and recurrent samples are represented by closed circles). Panel D shows longitudinal flow cytometry analyses of healthy or leukemic human chimerism in xenograft BM following AraC treatment, normalized to pre-treatment levels. $N=5$ (healthy) or 6 (AML) per human sample at each time point. Chimerism was either assessed by BM aspirate sampling or by terminal BM analysis of replicate mouse cohorts. Data are expressed as mean \pm SE. * $P<0.05$, ** $P<0.01$ by one-way ANOVA.

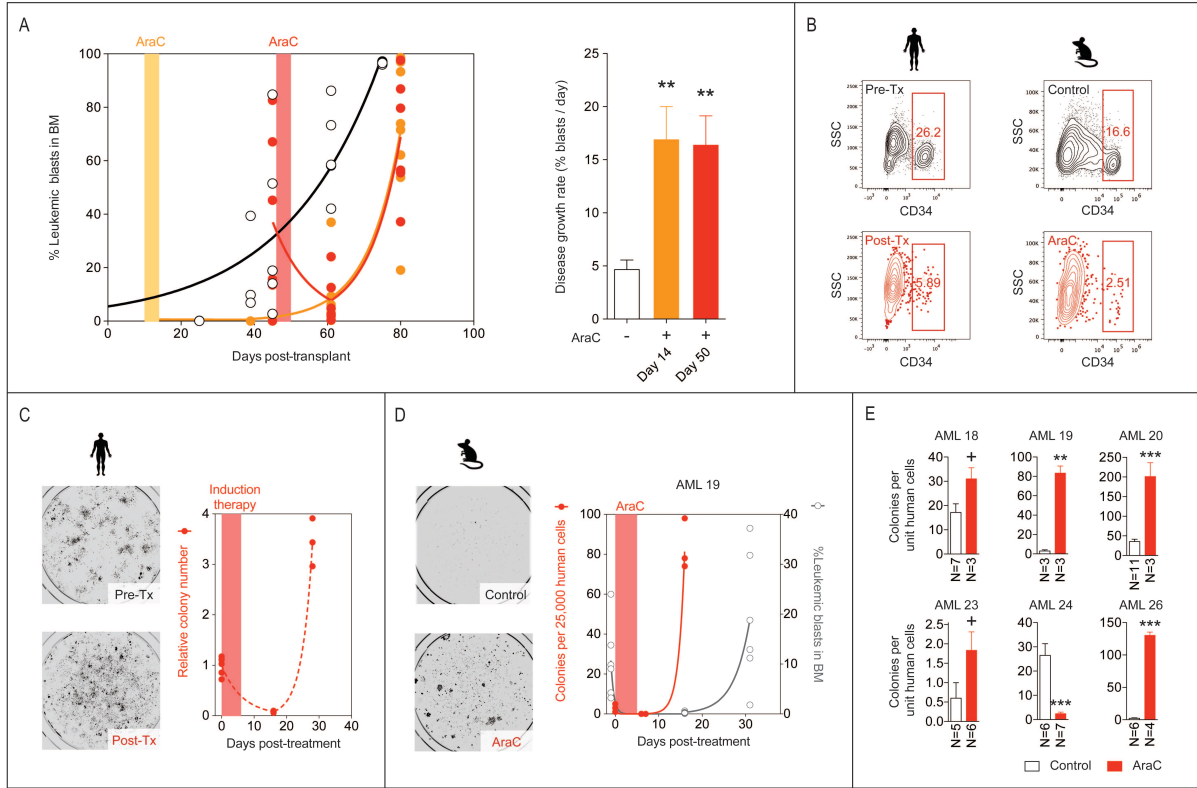


Figure 4.3. Characterizing residual leukemic cells after cytoreductive therapy

The left side of Panel A shows superimposed growth curves from AraC-treated xenografts presented in Fig. 4.2A-B, adjusted to reflect differences in the timing of AraC treatment between two independent experimental groups (as indicated by orange and red bars). Data points represent individual mice over time, and curves represent group averages. The graph on the right shows mean (\pm SE) leukemic growth rates calculated for individual mice, as derived from the rate constant “k” of the fitted exponential growth model $Y=Y_0(k^*X)$. $**P<0.01$ relative to saline control by one-way ANOVA. Panel B shows representative flow cytometry plots of CD34 expression. On the left, BM mononuclear cells were assessed from Patient 21 at diagnosis (“Pre-Tx”), and on Day 14 after the initiation of standard 3+7 induction therapy (“Post-Tx”; cytarabine 100 mg/m² IV by continuous infusion daily for 7 days, and daunorubicin 60mg/m² IV daily for 3 days). The repeat BM aspirate at Day 14 post-induction was indicated by peripheral monocytosis, and confirmed refractory disease. On the right, flow cytometry plots display BM cells that were recovered from Patient 18-xenografts, either two days after AraC treatment or from saline-treated controls. All events are gated on live human CD45⁺CD33⁺ populations. Panel C shows fluctuating changes in the colony-forming capacity of AML over time

after induction chemotherapy of human patients. On the left, representative whole-well colony forming unit (CFU) images from Patient 22 show elevated CFU potential at Day 28 following standard 3+7 induction therapy (“Post-Tx”), relative to the diagnostic sample (“Pre-Tx”). A peripheral blood film confirmed persistent leukemia at this time. On the right, data represent replicate CFU wells plated from Patient 21 (Day 14 post-induction) and Patient 22 (Day 28 post-induction), normalized to pre-treatment CFU levels. The red bar indicates the timing and duration of AraC administration. Panel D shows fluctuating changes in the colony-forming capacity of AML over time after AraC treatment of xenografts. On the left, representative whole-well CFU images from Patient 26-xenografts show elevated CFU potential at Day 16 post-AraC. On the right, data represent replicate CFU wells from BM pooled across multiple mice (Patient 19). Separate cohorts of mice were sacrificed at each time point shown. Gray circles and curves represent the recovery kinetics of the bulk leukemic population. The red bar indicates the timing and duration of AraC administration. Panel E shows average CFU counts from xenografts of six AML patients either 14-16 days following AraC treatment, or in the absence of AraC (saline controls). Data represent multiple CFU wells seeded from pooled BM across multiple mice (Patients 19, 20, 23, and 26) or individual CFU wells plated from multiple independent mice (Patients 18 and 24). All data are expressed as mean \pm SE.

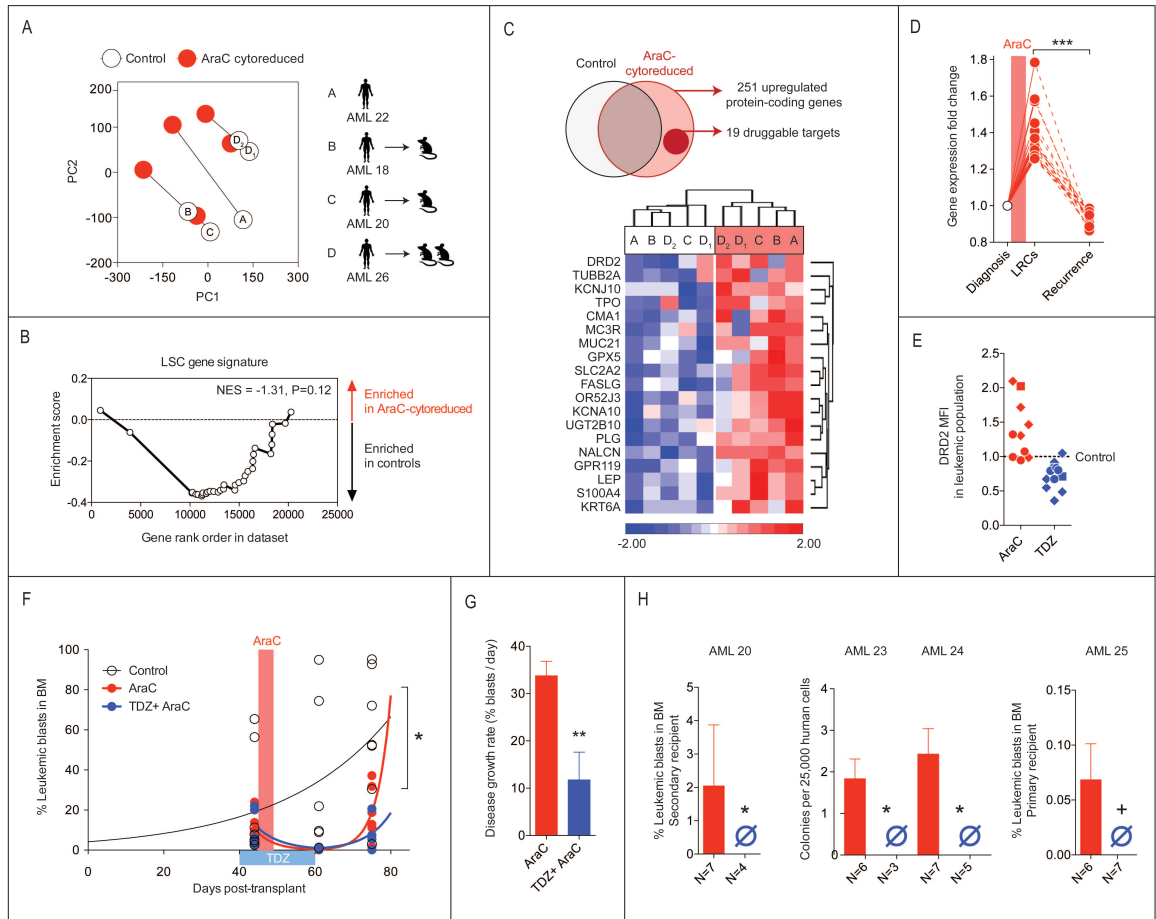


Figure 4.4. Novel targets for combination therapy

Panel A shows a PCA correlation biplot representing the two principal components that explain 23.3% (PC1) and 17.5% (PC2) of the variation in gene expression among purified human AML cells retrieved at the point of enhanced colony-forming capacity after cytoreductive therapy, versus matched untreated controls. Data points include a sample at Day 28 post-cytoreductive therapy and a diagnostic pre-treatment control from AML Patient 22, and xenografted AML samples at Day 14 post-AraC treatment or saline-treated controls. Panel B shows a gene set enrichment plot of samples presented in Panel A, indicating that gene signatures characteristic of LSCs⁹ are not enriched in AML populations following AraC cytoreduction. Panel C shows a Venn diagram outlining the process to select the top up-regulated protein-coding genes following AraC treatment. This list was further filtered to identify genes that also have drug-gene interactions as determined by gene set filtering using

the Drug Gene Interaction Database. A heat map shows expression levels of these 19 druggable genes across the samples shown in Panel A. Panel D shows that the 19 druggable gene targets are only transiently up-regulated by AraC exposure at the point of LRC emergence. At later timepoints of overt recurrence (>1 month following AraC), none of these genes remained over-expressed. Panel E shows the mean fluorescence intensity (MFI) of DRD2 protein expression within human leukemic CD45⁺CD33⁺ cells from the BM of xenografted mice treated with AraC (at Day 14 following 5 days at 50 mg/kg/day; subcutaneous delivery) or DRD2 antagonist TDZ (at Day 21 following 21 days at 22.5 mg/kg/day; intraperitoneal delivery). Each data point represents an individual mouse (diamonds, Patient 20; circles, Patient 23; squares, Patient 24) and the dotted line indicates the DRD2 MFI of vehicle-treated controls. ***P<0.001, by unpaired t-test. Panel F shows growth curves of Patient 19-xenografts representing vehicle controls, AraC treatment or AraC + TDZ treatment (AraC, subcutaneous delivery of 50 mg/kg/day; TDZ, intraperitoneal delivery of 22.5 mg/kg/day). Red and blue bars indicate the timing and duration of AraC and TDZ administration, respectively. *P<0.05, final BM leukemic blast levels between vehicle control versus AraC + TDZ treatment groups, by one-way ANOVA. Panel G shows that the addition of TDZ to AraC treatment reduces leukemic growth rates, calculated for individual mice based on growth curves presented in Panel F. Data are expressed as mean ± SE, **P<0.01 by unpaired t-test. Panel H shows secondary functional assays performed with human leukemic CD45⁺CD33⁺ cells recovered from the BM of xenografted mice treated with AraC or AraC + TDZ. To assay disease re-initiation capacity, equal numbers of human leukemic cells from Patient 20-xenografts were serially transplanted into secondary recipient mice, followed by analysis of leukemic chimerism in BM 6 weeks later. *P<0.05 by Fisher's exact test. Insufficient cells were available for secondary transplantation from xenografts of Patients 23 and 24, therefore all recovered human cells were dedicated to *in vitro* CFU assays. Data represent multiple CFU wells plated from BM pooled across multiple mice (Patient 23), or individual CFU wells plated from multiple independent mice (Patient 24). *P<0.05, by Fisher's exact test. Secondary assays were not performed with Patient 25-xenografts, as no human leukemic cells were detected in any of 7 mice treated with AraC + TDZ, whereas human leukemia persisted in 3/6 mice treated with AraC alone, "+"P= 0.07, Fisher's exact test. All data are expressed as mean ± SE.

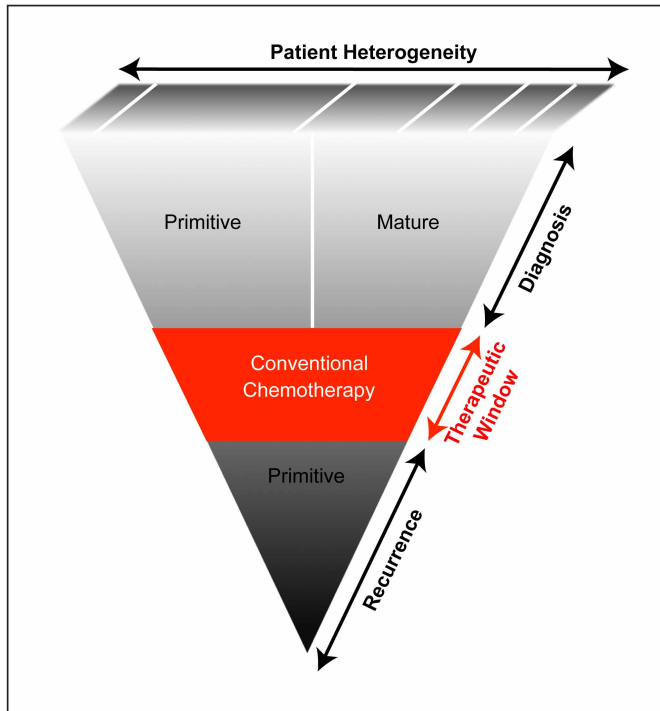
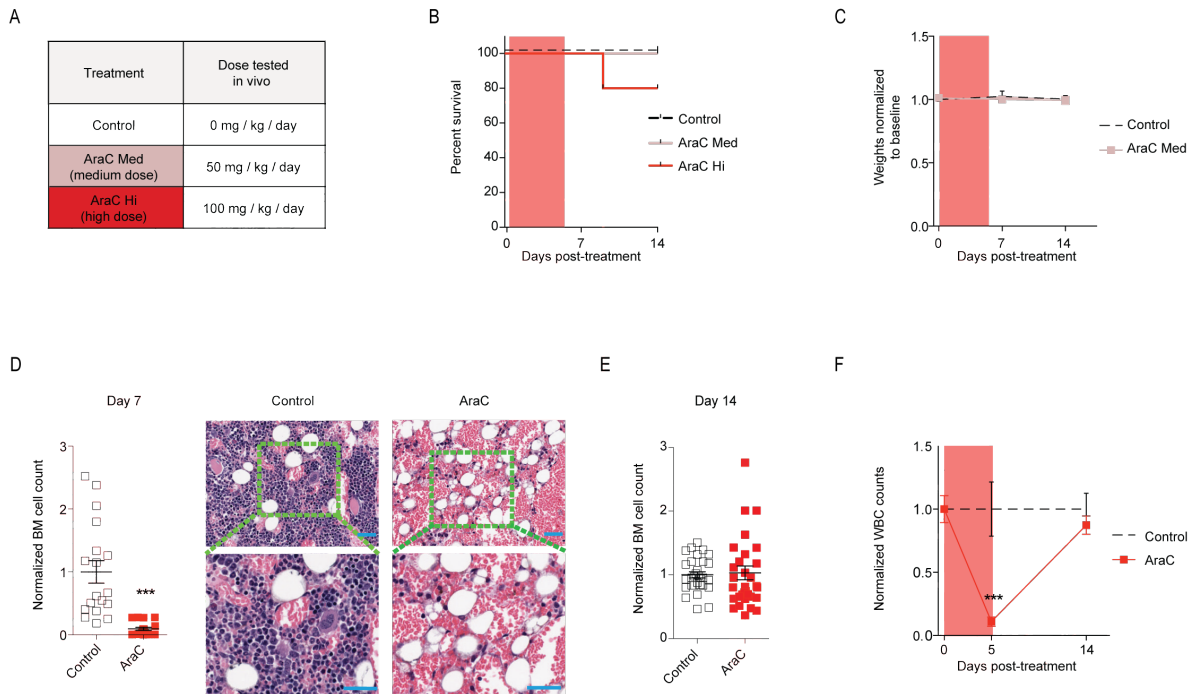


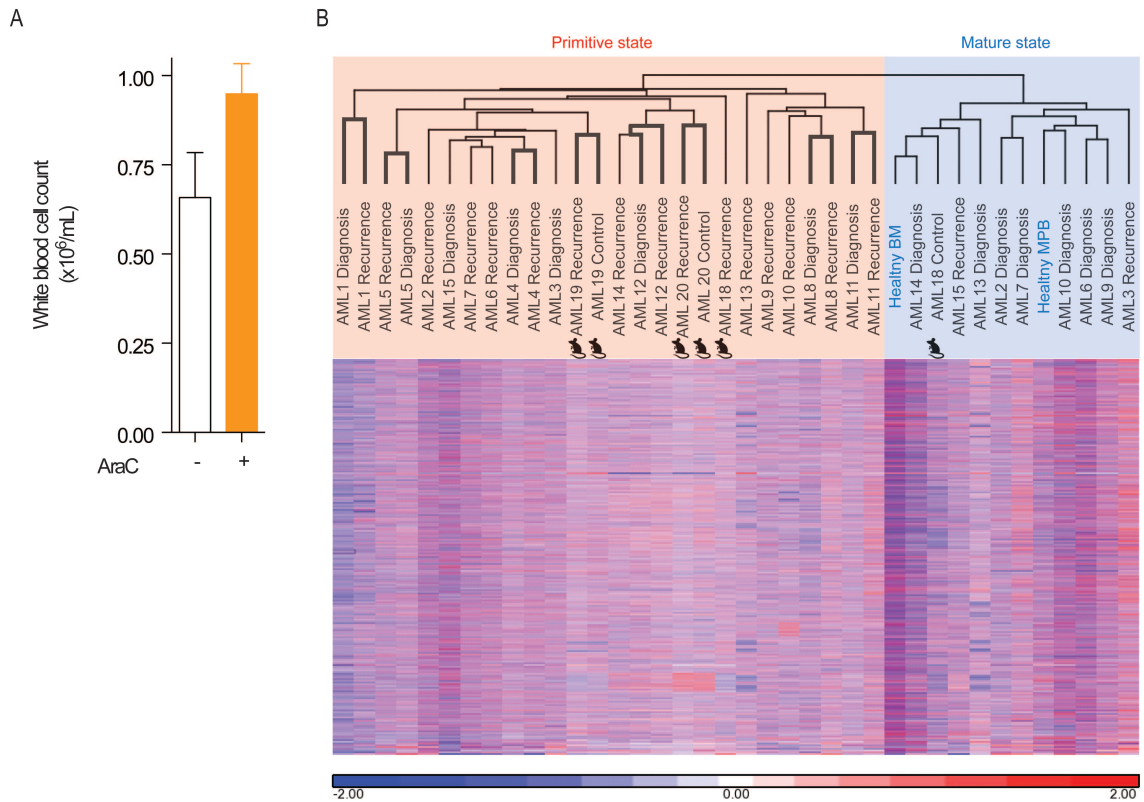
Figure 4.5. Model of the transcriptional events that define the AML recurrence process

At diagnosis, transcriptional profiling partitions genetically and phenotypically heterogeneous patients into two major subgroups defined by primitive or mature signatures of cellular differentiation. Conventional chemotherapy provokes the emergence of LRCs that re-establish leukemic disease, representing a sensitive therapeutic window for targeted intervention. Failure to eliminate LRCs leads to recurrent AML, which assumes homogeneously primitive transcriptional signatures among patients.



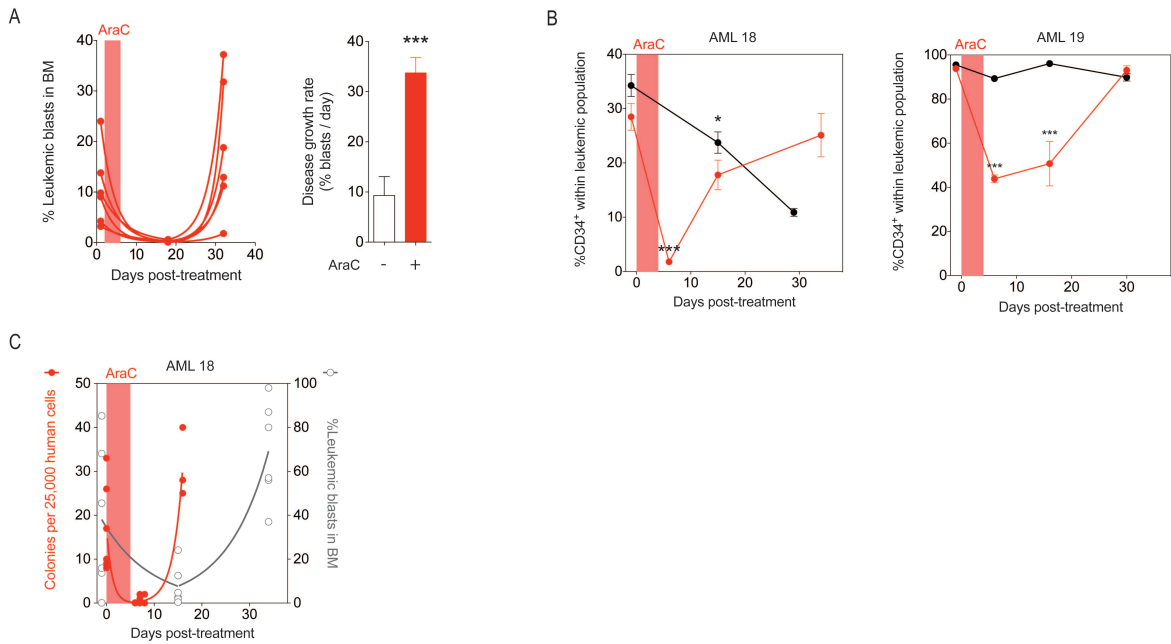
Supplementary Figure 4.1. Determination of the maximum tolerable dose of AraC *in vivo*

Panel A shows a summary of treatment groups receiving AraC at 50 and 100 mg/kg/day, indicated as AraC Med and Hi, respectively. Control-treated mice received saline (AraC 0 mg/kg). *In vivo* AraC regimens consisted of daily subcutaneous injections for 5 consecutive days. Panel B shows survival of non-transplanted NOD/SCID mice in response to AraC over a course of 14 days. The red bar indicates the timing and duration of AraC administration. Day 14 represents the experimental end point. Panel C shows longitudinal monitoring of mouse weight measured weekly for 14 days. The red bar indicates the timing and duration of AraC administration. AraC at the medium dose did not show deleterious effects on survival (Panel B) or weight and was therefore selected for subsequent *in vivo* experiments. Panel D (left) shows viable BM cell counts of AraC-treated mice, presented relative to saline controls on Day 7 post-treatment start, as a measure of BM cellularity. Each dot represents an individual mouse, across two independent experiments. Data are expressed as mean \pm SE by unpaired t-test, *** $P \leq 0.001$. Right panels show representative hematoxylin and eosin-stained BM sections of AraC versus control-treated mice on Day 7 post-AraC treatment start. Panel E shows recovery of BM viable cell counts of AraC-treated mice by Day 14 post-treatment. Panel F shows white blood cell (WBC) counts as a functional measure of BM output. Similar to the effect on BM cellularity, AraC induces a transient reduction of WBC counts. The red bar indicates the timing and duration of AraC administration. Data are expressed as mean \pm SE. *** $P < 0.001$ by two-way ANOVA.



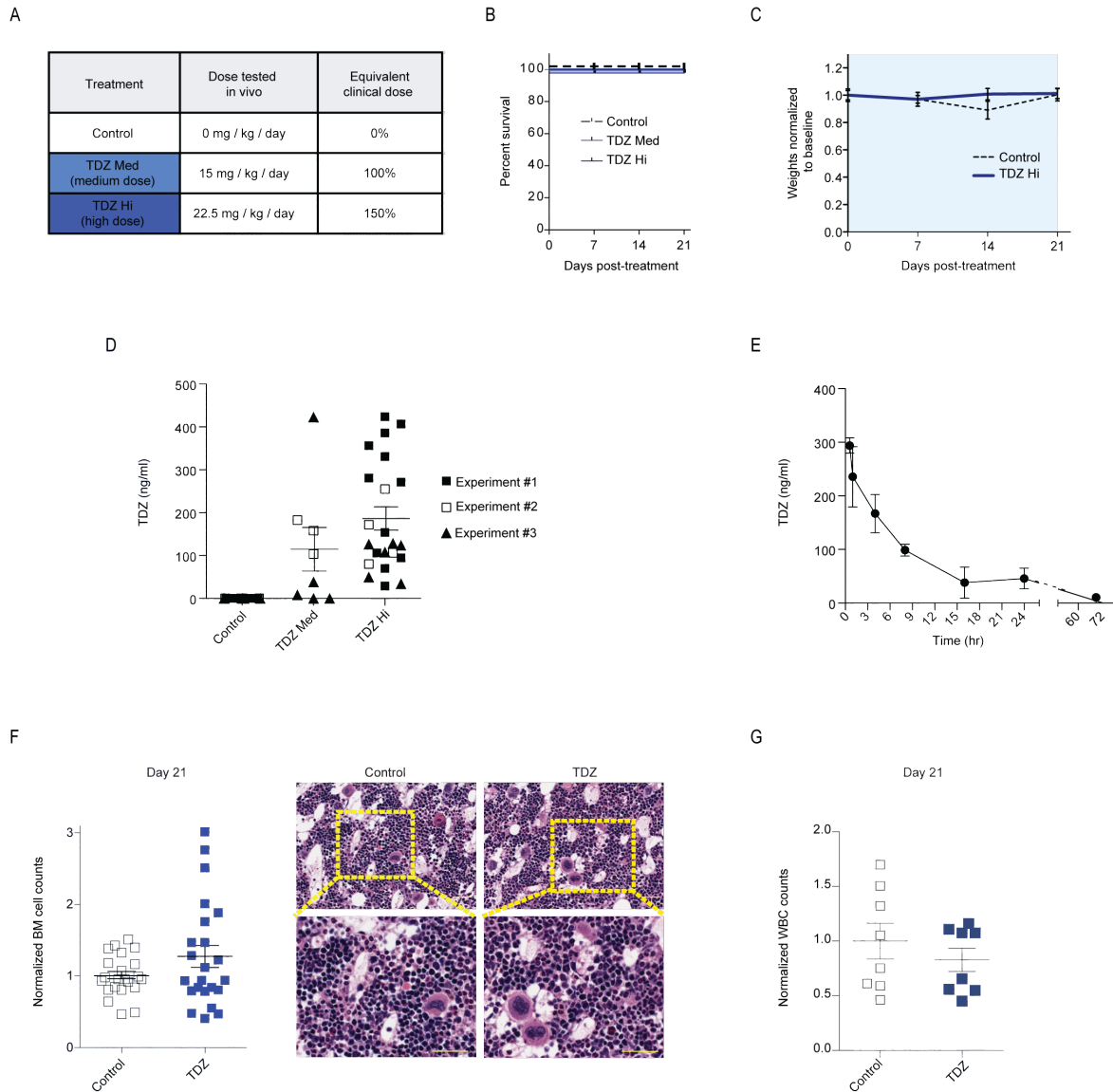
Supplementary Figure 4.2. Characterization of recurrence in AML-xenografts

Panel A shows restoration of white blood cell counts by Day 32 post-AraC treatment, as measured by image-based quantification of acridine orange fluorescence in whole blood. Data are expressed as mean \pm SE. * $P < 0.05$, by unpaired t-test. Panel B shows an unsupervised hierarchical global clustering analysis of xenografted AML samples (Patients 18-20) after recurrence post-AraC treatment or from saline treated controls, together with the human samples presented in Fig. 4.1B. Consistent with human patient analysis, leukemic xenografts either show a stably primitive state prior to and post- AraC treatment (Patients 19 and 20), or acquire a more primitive transcriptional signature upon recurrence following cytoreductive therapy (Patient 18). Bolded brackets highlight the AML patients with transcriptionally stable gene expression from diagnosis to recurrence.



Supplementary Figure 4.3. Phenotypic and functional AraC response dynamics in AML-xenografts

Panel A shows recurrence response dynamics following AraC therapy in mice xenografted with primary cells from Patient 19 (left). Data represent flow cytometry measurements of human leukemic chimerism from serial BM aspirates. The red bar indicates the timing and duration of AraC administration and each curve represents an individual mouse. On the right, the graph represents leukemic growth rates calculated for individual mice. Data are expressed as mean \pm SE. *** P <0.001, by unpaired t-test. Panel B shows longitudinal profiling of CD34⁺ cell frequencies within human leukemic populations measured by flow cytometry analysis of serial BM aspirates from xenografted mice. Red bars represent the timing and duration of AraC treatment. * P <0.05, *** P <0.001, relative to saline controls, by unpaired t-test. Panel C shows individual CFU wells plated from human CD45⁺CD33⁺ cells retrieved from xenograft BM, pooled across multiple mice at several time increments following AraC treatment (Patient 19). Separate cohorts of mice were sacrificed at each time point shown. Gray circles and curves represent the recovery kinetics of the bulk leukemic population, and the red bar indicates the timing and duration of AraC administration. All data are expressed as mean \pm SE.



Supplementary Figure 4.4. Determination of a clinically relevant dose of TDZ *in vivo*

Panel A shows a summary of TDZ treatment groups. Non-transplanted NOD/SCID mice were administered doses of 15 or 22.5 mg /kg/day that correspond to TDZ at medium dose (TDZ Med) and high dose (TDZ Hi), respectively. Control-treated mice received 30% captisol (TDZ vehicle). TDZ Med and Hi represent a weight-adjusted equivalent of 100% and 150% maximum clinical TDZ dose in humans (800mg/day³²). The *In vivo* TDZ regimen consisted of daily intraperitoneal injections for 21 consecutive days. Panel B shows mouse survival during the 21-day TDZ treatment period, with no adverse effects at any doses. Blue shading indicates the

timing and duration of TDZ delivery. Panel C shows the effect of *in vivo* TDZ treatment on mouse weight measured weekly for 21 days. Panel D shows that with TDZ-Hi dosing, murine plasma TDZ levels reach a clinically relevant range of plasma TDZ achievable in humans at steady states (200-2000 ng/ml³⁷). Therefore, TDZ Hi was selected for subsequent *in vivo* analyses. Panel E shows HPLC measurements of TDZ in murine plasma between 0-24 hr after TDZ injection at high dose. Plasma TDZ levels peaked at 1-hour post injection and declined to undetectable levels at 24 hours. Panel F shows that TDZ did not significantly alter the BM cellularity as illustrated by BM viable cell counts and representative hematoxylin and eosin-stained BM sections on Day 21. Each dot represents an individual mouse and the data are collected from 5 separate experiments. Panel G shows WBC counts, which similar to BM cellularity are unaffected by TDZ treatment. Data are represented as mean \pm SE.

Table 4.1. Clinical details of AML patient samples

ID	Disease stage	Tissue source	Cytogenetics / Molecular	%Blast Diagnosis	%Blast Relapse
1	Diagnosis / Relapse Pair	PB*	Normal / NA [#]	83	57
2	Diagnosis / Relapse Pair	PB	Normal / NA	13	56
3	Diagnosis / Relapse Pair	PB	Normal / NA	85	81
4	Diagnosis / Relapse Pair	PB	Normal / NA	83	83
5	Diagnosis / Relapse Pair	PB	Normal / NA	47	16
6	Diagnosis / Relapse Pair	PB	Normal / NA	46	77
7	Diagnosis / Relapse Pair	PB	Normal / NA	93	79
8	Diagnosis / Relapse Pair	PB	Normal / NA	75	98
9	Diagnosis / Relapse Pair	PB	Normal / NA	66	90
10	Diagnosis / Relapse Pair	PB	Normal / NA	70	67
11	Diagnosis / Relapse Pair	PB	Normal / NA	46	87
12	Diagnosis / Relapse Pair	PB	Normal / NPM1, FLT3-ITD	58	63
13	Diagnosis / Relapse Pair	BM** / PB	Add1,-3, del3 (q21), del5 (q13q33), -7, -10, add11, del12 (p11.2p13), add13, add16, t(7;17)(p13;p13), -18, +21	50	40
14	Diagnosis / Relapse Pair	BM / PB	Normal / NPM1, FLT3-ITD heterozygous	>70	94
15	Diagnosis	BM / PB	NA / None detected	78	76
16	Multiple over time	BM	Normal / FLT3-ITD	Fig 4.2A	Fig 4.2A
17	Multiple over time	BM	Normal / NA	Fig 4.2B	Fig 4.2B
18	Progressed from MDS	PB	inv(3)(q21q26.2), -7	47	
19	Diagnosis	PB	del(5) (q22q33), -7	89	
20	Diagnosis	PB	Normal / FLT3-ITD	68	
21	Diagnosis / post-induction Pair	BM / BM	Normal / NA	>50	>80
22	Diagnosis / post-induction Pair	PB	46-47,XX,del(5)(q13q33),del(13)(q12q14),+21,+22[cp26]	54	84
23	Diagnosis	BM	Normal / None detected	30	

24	Diagnosis	PB	NA / None detected	68	
25	Diagnosis	PB	NA / NA	92	
26	Progressed from MDS	PB	48,XY,+8,+13[8]/46,XY[5]	35	

* PB, peripheral blood

** BM, bone marrow

#NA, not available

Table 4.2. List of druggable up-regulated genes post-AraC

Gene Symbol	Full Gene Name	Fold change	p value
OR52J3	Olfactory Receptor, Family 52, Subfamily J, Member 3	1.75933	0.0111845
PLG	Plasminogen	1.59148	0.0015988
KRT6A	Keratin 6A	1.54608	0.0100511
MUC21	Mucin 21, Cell Surface Associated	1.45517	0.00904822
SLC2A2	Solute carrier family 2 (facilitated glucose transporter), member 2	1.43914	0.00358156
GPR119	G protein-coupled receptor 119	1.40593	0.0047744
S100A4	S100 calcium binding protein A4	1.36532	0.00567333
FASLG	Fas ligand (TNF superfamily, member 6)	1.3523	0.00942611
KCNJ10	Potassium inwardly-rectifying channel, subfamily J, member 10	1.34991	0.0134538
KCNA10	Potassium voltage-gated channel, shaker-related subfamily, member 10	1.32671	0.0123655
LEP	Leptin	1.32108	0.00545013
CMA1	Chymase 1, mast cell	1.31673	0.0146796
NALCN	Sodium leak channel, non selective	1.30772	5.39E-05
MC3R	Melanocortin 3 receptor	1.28131	0.037429
DRD2	Dopamine receptor D2	1.27927	0.0352181
TUBB2A	Tubulin, beta 2A class IIa	1.27229	0.0197661
TPO	Thyroid peroxidase	1.26242	0.0195282
GPX5	Glutathione peroxidase 5	1.26015	0.0242464
UGT2B10	UDP glucuronosyltransferase 2 family, polypeptide B10	1.25841	0.0299276

Supplementary Methods

Supplementary Method 1. Preparation of primary human hematopoietic samples

Primary AML patient and donor samples were provided by Juravinski Hospital and Cancer Centre, and London Health Sciences Centre (University of Western Ontario). The Labour and Delivery Clinic at the McMaster Children's Hospital provided cord blood samples. MNCs were prepared by density gradient centrifugation (Ficoll-Paque Premium; #17-5442-02; GE Healthcare) followed by red blood cell lysis using ammonium chloride solution (#07850; Stemcell Technologies). Lineage depletion of cord blood was carried out using a commercially available kit (#19309C, Stemcell Technologies) according to the manufacturer's instructions.

Supplementary Method 2. Murine recipients and Xenograft assays

Mice were bred and maintained at Stem Cell and Cancer Research Institute (SCCRI) animal barrier facility. For transplantation assays, 6-10 week old NOD/SCID mice were sublethally irradiated (200-350 Rads, using a ^{137}Cs γ -irradiator) 24 hours prior to intravenous transplantation of primary human samples^{8,10}.

Longitudinal *in vivo* monitoring of human leukemic chimerism was carried out by serial BM aspiration as previously described³⁸. Briefly, the anesthetized recipient mouse was placed in a supine position, the tibia and femur were flexed and the femur was punctured using a 28G ½" needle. 5-10 μl of BM cells were collected and the procedure was repeated at bi-monthly intervals on alternate femurs.

For assays of self-renewal, pooled BM cells from multiple xenografted primary recipients were serially transplanted intravenously into secondary animals at multiple cell doses normalized based on the number of injected human leukemia cells.

For secondary CFU assays, xenografted human leukemic cells were purified through fluorescence activated cell sorting (FACS) or by mouse cell exclusion using magnetic cell isolation (mouse CD45 and Ter119 microbeads, 130-052-301, and 130-049-901, Miltenyi Biotec) and subsequently seeded in methylcellulose at a range of 5000-50000 cells.

For drug delivery experiments, weekly weight measurements were used to ensure that appropriate dose per weight ratio was sustained throughout the treatment.

Supplementary Method 3. Fluorescence-activated cell sorting (FACS) and flow cytometry

Immunophenotyping for hematopoietic cell surface markers was carried out using a cocktail of conjugated antibodies (V450-CD45, APC-CD33, and PE-CD34 BD Biosciences). 7-aminoactinomycin D was used to discriminate live cells. When appropriate, fluorescence minus one (FMO) controls were used to optimize gating strategies for target cell populations. In experiments that required cell sorting, FACS-purified cell populations were routinely checked for purity. FACS sorting and flow cytometry were performed using FACS Aria II sorter and LSRII Cytometer (BD).

BD FACSDiva software was used for data acquisition and FlowJo software (Tree Star) was used for analysis.

Supplementary Method 4. RNA purification and Affymetrix analysis

RNA was isolated from healthy MNCs or FACS-purified leukemic blasts (based on side scatter and CD45 intensity³⁹) using a total RNA purification kit (Norgen biotek, ON, Canada) according to the manufacturer's instructions. Purified RNA was quantified on a Nanodrop 2000 Spectrophotometer (Thermo Scientific), and RNA integrity was assessed by a 2100 Bioanalyzer (Agilent Technologies).

Batch correction was performed on sources of technical variation (array technologies and/or isolation of human cells from patients versus xenografts). Expression values from all common gene symbols were used to create PCA plots. Pearson's correlation coefficient was used for hierarchical clustering to generate dendrograms. Gene set enrichment analysis (GSEA) was performed on normalized expression values of all common gene symbols between samples using GSEA software v2.1.0 (Broad Institute; Subramanian et al., 2005). Curated gene set (C2) and Gene Ontology (C5) gene set collections from Molecular Signatures Database (MSigDB) were used for GSEA analyses.

Supplementary Method 5. Image analysis

CFU images were acquired at 2x using Operetta High Content Screening (Perkin Elmer) by means of epi-fluorescence illumination and standard filter sets. Stitched whole well images were constructed in Columbus software (Perkin Elmer). Image

processing and analysis was performed using custom scripts in Acapella software (Version 3.1, Perkin Elmer).

Supplementary Method 6. Hematology analysis

Peripheral blood was collected from the superficial temporal vein and tail vein for WBC counts, measured using a Nexcelom Cellometer after acridine orange staining of diluted whole blood samples.

Chapter 5: Discussion

The LSC theory has advanced our understanding of AML to recognize that each cell in a leukemia population is not functionally equivalent, and that the elimination of LSCs should be prioritized over other cellular fractions of the disease (Dick, 2008). This new perspective has led to a general impression that tumor cells lacking self-renewal properties are relatively inert once initial cytoreductive debulking has been achieved. As a result, efforts to prevent relapse have become mainly focused on identifying selective targets to specifically distinguish LSCs from HSCs on a direct molecular level. This thesis offers experimental evidence that therapeutic LSC targeting can also be achieved by indirect means, via manipulation of the BM environment that houses them (**Chapters 2-3**). Additionally, it characterizes unique regenerative behaviour that emerges within leukemic populations under injury-specific conditions (**Chapter 4**). Strategic molecular targeting during these states can disable the regenerative response that drives leukemia recurrence. Together, these findings support the hypothesis that **LSCs display sensitivity to their external environment, and these interactions can be targeted in clinically meaningful ways**. Importantly, the new therapeutic opportunities that we have defined could not have been identified by regarding the leukemic hierarchy as an independent system.

The field is now adopting an ecological view of leukemia biology to understand the context-dependent behaviour of LSCs (**Figure 5.1**), and the work

presented here contributes to this emerging understanding. To date, only few studies have articulated tangible measures to target LSCs through their environmental interactions (Jin et al., 2006; Matsunaga et al., 2003; Nervi et al., 2009; Saito et al., 2010b), and our work expands this repertoire (**Figure 5.2**). Diversified approaches to eradicate LSCs will be critical to accomplish long-term disease control and recurrence prevention in AML.

5.1 Indirect targeting of LSC self-renewal

I began my examination of indirect LSC targeting by studying the Hh pathway, which has been associated with self-renewal processes in many tissues, through both autocrine and paracrine signaling mechanisms (Fabian et al., 2012; Lamm et al., 2002; Merchant and Matsui, 2010; Shin et al., 2011). Based on evidence of direct selective effects against LSC self-renewal in CML (Dierks et al., 2008; Zhao et al., 2009), Hh signaling inhibitors have been advanced to clinical trials for a range of hematological malignancies including AML (Jamieson et al., 2011; Merchant et al., 2010). However, unlike in CML (Dierks et al., 2008), our comprehensive transcriptional analysis of Hh pathway components was unable to clearly discriminate leukemic cells from healthy hematopoietic populations, regardless of the extent of subset purification (**Chapter 2**). Furthermore, *in vitro* small molecule antagonism of the pathway had no preferential effect against the clonogenic capacity of AML (**Chapter 2**) and ultimately could not explain the clinical benefits that have

been achieved by Hh pathway inhibition in AML patients to date (Jamieson et al., 2011).

The lack of a clear molecular distinction between HSCs and LSCs at the level of Hh signaling suggests that small molecule Hh inhibitors are unlikely achieving their clinical effects through direct selectivity. Furthermore, we reported mRNA and protein evidence that BM mesenchymal cells express high levels of Hh pathway receptors, unlike any of the hematopoietic populations examined (**Chapter 2**). This suggests that mesenchymal populations may be the primary Hh signal-transducing cells in BM, which could impact hematopoiesis indirectly. This hypothesis has since been reinforced by an independent study that was published in response to our work. This group showed that exogenous supplementation of Hh signals was found to assist healthy hematopoietic regeneration following irradiation-induced injury, but only in the presence of the *in vivo* BM niche (Drouet, 2013; Drouet et al., 2013). Together, these findings suggest that like in several other tissues, Hh ligands may represent important niche-acting signals that regulate homeostasis in BM (Fabian et al., 2012; Lamm et al., 2002; Peng et al., 2015; Shin et al., 2011). However, Hh-activated stromal cells have also been shown to provide support to malignant populations in solid tumors (Tian et al., 2009; Yauch et al., 2008) and may therefore also have targetable interactions with LSCs.

As a next step, it will be important to clarify in what way Hh inhibitors could be modulating mesenchymal BM cells to suppress LSCs. An important functional role

for this pathway has already been identified in mesenchymal progenitors, as Hh inhibition has been shown to promote cell fate commitment towards the adipocyte lineage (James et al., 2010; Suh et al., 2006). This property of adipogenesis induction is actually shared with another small molecule recently described to target AML in a non-cell-autonomous manner (Hartwell et al., 2013). Incidentally, loss of adipocytic differentiation is one of the niche-related changes that have been recently reported to accompany AML progression in BM (Hanoun et al., 2014; Medyouf et al., 2014) and adipocytes have been recently identified to be an important regulator of hematopoiesis (Naveiras et al., 2009). Therefore, these molecules may be achieving their effects by preserving the natural integrity of the BM niche, at the expense of leukemic expansion. This possibility should be further explored.

5.1.1 Future clinical perspectives: BM niche-targeted agents

The hypothesis that Hh inhibitors could counteract leukemic progression by restoring normal niche integrity is appealing, as correcting this type of pathology would likely benefit healthy hematopoietic cells as well. The BM microenvironment fundamentally guides the balance of HSC self-renewal versus differentiation (Mendelson and Frenette, 2014). As a result, deteriorated niche composition could reasonably influence the development of peripheral cytopenias commonly seen in AML patients (Estey and Döhner, 2006). In fact, several studies have reported that in leukemia-infiltrated BM, the differentiation of healthy murine HSPC populations becomes arrested in favour of widespread self-renewal (Cheng et al., 2015; Miraki-

Moud et al., 2013). It will be important to determine whether niche-targeted compounds may be able to correct the immature imbalance of the leukemic BM system as a whole, which could both restrict LSC self-renewal and allow healthy blood production to resume. Although these types of therapeutic strategies may not provide immediate alleviation of urgent circulating blast levels, they could be applied in a maintenance setting to achieve better symptom control and quality of life.

5.2 Common themes between HSCs and LSCs

In addition to similarities between HSCs and LSCs on the basis of morphogen signaling (**Chapter 2**), we have also identified surprisingly close parallels between HSC and LSC populations by comparative spatial and functional analysis (**Chapter 3**). When challenged to directly compete in BM repopulation assays, pressure from co-infused HSCs was successfully able to limit the BM colonization and self-renewal of LSC pools *in vivo* (**Chapter 3**). This observation has since been independently reproduced with a similar experimental design, using an engineered murine model of AML (Glait-Santar et al., 2015). Collectively, these data suggest that the preservation of the LSC state is dependent upon access to specialized BM niche sites that overlap with HSC requirements. Indeed, we confirmed that phenotypically primitive CD34⁺ leukemic cells spatially situate along endosteal BM surfaces (**Chapter 3**), mirroring the environmental conditions that support HSC localization (Guezguez et al., 2013). It is becoming clear that there is a diverse array of

specialized niches in BM, and the characteristics of an individual niche can provide a direct reflection of the nature of its inhabitant stem cells. Specifically, different subsets of healthy HSPCs have been described to occupy qualitatively distinct niches in BM, and CML-LSCs have been suggested to have divergent niche requirements relative to AML-LSCs as well (Ding and Morrison, 2013; Greenbaum et al., 2013; Guezguez et al., 2013; Hoggatt et al., 2013; Krause et al., 2013). In this context, the equivalence of HSC and AML-LSC niches is impressive, and suggests that the biology of LSCs resembles that of healthy HSCs on a fundamental level that may reflect their intrinsic qualities.

Similar to HSC exhaustion that occurs upon niche displacement, the failure of leukemia to thrive under conditions of restricted BM niche access suggests that LSCs cannot substitute alternative tissue sites to acquire their needed resources. This contrasts the common conception of cancer as versatile cells that can bypass the constraints of normal tissue development (Medema, 2013). The genetic plasticity of AML has been generally thought to allow leukemic cells to forge novel and unusual developmental trajectories. Consistent with this idea, unfamiliar patterns of antigen expression are often detected on the cell surface of leukemic blasts (Craig and Foon, 2008; Griffin and Löwenberg, 1986).

Despite these characterizations of leukemic cells as being developmentally aberrant, global transcriptional analysis has suggested that functionally validated populations of HSCs and LSCs share common transcriptional programs (Eppert et al.,

2011). Additionally, a recent study has provided an unprecedented large-scale analysis of cell surface proteins using single cell mass cytometry technology, revealing that antigen expression patterns are less disordered than expected in AML. Not only did multidimensional clustering indicate that leukemic subpopulations exhibited a limited diversity of cell surface phenotypes across AML patients, but it also suggested minimal divergence from normal paths of myeloid maturation (Levine et al., 2015). Overall, LSCs do not appear to be excused from the general boundaries of hematopoietic development, as there seems to be considerable molecular overlap between normal and malignant hematopoietic cells (**Chapter 2**) (Eppert et al., 2011; Levine et al., 2015), and the positioning of LSCs within the ecological context of BM tissue closely resembles that of HSCs (**Chapter 3**). These similarities may present challenges towards their selective molecular targeting, but suggest that LSC behavior may be more niche-dependent and predictable than currently perceived.

5.2.1 Future clinical perspectives: Novel HSCT conditioning procedures

On the basis of direct competition for shared BM niches between LSCs and HSCs, **Chapter 3** additionally provided preclinical xenograft data suggesting that G-CSF mobilization offers an effective tool to dislodge LSCs from protective BM sites. This enabled competitive niche replacement by healthy HSCs, in a cellular “musical chairs” that operated at the expense of LSC niche retention and self-renewal. This was achievable without toxic myeloablative conditioning measures, showing

promise as a new strategy that could allow reduced intensity of HSCT therapy. This is a critical goal, as HSCT remains the most effective curative therapy for AML disease (Hamilton and Copelan, 2012; Rowe, 2009) but is unavailable to many patients who are not predicted to tolerate the toxicity associated with high intensity preparative regimens.

A randomized controlled study of high risk AML patients has recently reported reduced relapse incidence and increased survival rates as a result of incorporating G-CSF into conditioning regimens for haploidentical allogeneic HSCT (Gao et al., 2014). Although the original motivation of G-CSF use in this study was to better sensitize leukemic cells to the preparative chemotherapy conditioning, in retrospect it was proposed that it could also have improved competitive HSC reconstitution, based on our work presented in **Chapter 3** (Abboud, 2014). Although it is not possible to dissociate chemosensitization from competitive repopulation effects in this instance, this study offers encouraging support for the investigational use of G-CSF in transplantation regimens and reinforces the safe nature of the approach. A more deliberate trial of mobilization-mediated competitive HSCT is warranted in a reduced intensity setting, which could ultimately allow a wider application of HSCT across AML patients who have achieved remission, particularly needed in high risk cytogenetic cases.

5.3 Unique properties of leukemic regeneration

Despite the molecular and functional similarities recognized between HSC and LSC subsets in **Chapters 2-3**, **Chapter 4** identified an important functional difference between leukemic regeneration and normal hematopoietic recovery, as assessed in a controlled xenograft setting. While leukemic cells were able to generate and sustain dramatic growth rates in response to cytoreductive AraC therapy, normal hematopoiesis rapidly reached a distinct plateau following the same cytotoxic challenge. It is not yet clear whether this functional divergence between healthy and malignant regeneration truly reflects any intrinsic differences at the stem cell level, or whether properties of the leukemic environment may contribute. In fact, the leukemic populations that resist AraC and re-establish disease appear to be more complex than originally anticipated. Current theories suggest that quiescent LSCs are selectively spared by chemotherapy and are independently responsible for seeding disease re-growth (Jordan et al., 2006). However, our data suggest that chemotherapy initially causes a massive suppression of phenotypically and functionally primitive cells, based on both xenograft modeling and human patient analysis (**Chapter 4**). Although the restoration of primitive populations was necessary for disease recurrence to occur, their initial suppression suggests that they may not represent particularly chemoresistant cells in the context of prolonged chemotherapeutic targeting. Instead, re-growth appears to be driven by the compensatory activation of any primitive cells that are fortunate to survive.

It is not disputed that in general, stem cells are mitotically dormant and would therefore be chemoprotected under steady states. However, cytoreductive challenge is likely to induce stem cell activation (Mendelson and Frenette, 2014), causing sensitization to any further chemotherapeutic attack. In patient settings, chemotherapy is rarely delivered as a single dose, and therefore our model realistically reflects sustained conditions of cytoreductive suppression over a several day period, in which dormant cell populations could become sensitized. A recent study similarly concluded that chemotherapy treatment actively recruits residual bladder tumor cancer stem cells (CSCs) into cell cycle (Kurtova et al., 2014), also suggesting temporary sensitivity to subsequent doses of treatment. However, upon chemotherapy withdrawal, this injury-provoked activation of CSCs fueled a tumor regenerative process that became central to the emergence of therapy resistance (Kurtova et al., 2014), reminiscent of the uncontrolled leukemic growth that we have observed following AraC treatment. Therefore, if CSC quiescence does not represent the primary limitation that is obstructing successful long-term therapy outcomes, it is critical to clearly understand the basis of therapy failure and disease re-growth that may be more important.

Towards the goal of characterizing the leukemic regenerative process, we have provided the first functional and molecular account of the changing leukemic cell dynamics that follow cytotoxic therapy treatment. Based on careful functional analysis of leukemic BM cells at multiple intervals following AraC administration, we

identified a key time point in which clonogenic cells massively expand prior to the overt recovery of leukemic disease (**Chapter 4**). We therefore performed comprehensive transcriptional profiling on leukemic cells retrieved at this unique stage, and revealed a novel gene signature of Leukemic Regenerating Cells (LRCs). The therapeutic value of this signature was demonstrated by proof-of-principle targeting of dopamine receptor D2 (DRD2), one of the gene products specifically upregulated among LRCs. In combination with AraC, DRD2 antagonism prevented the dramatic leukemic re-growth otherwise observed by delivering chemotherapy alone, offering a new strategy against recurrence in AML.

Several questions will be important to address as future extensions of this work. What provokes the activation of surviving leukemic precursors to reconstruct the disease? What are the specific cellular subsets that compose LRC populations, and how do they relate to healthy recovering BM cells? Although we have shown that LRC populations do contain cells capable of re-initiating disease in secondary recipients, their gene signatures do not reflect transcriptional states normally associated with LSCs (Eppert et al., 2011) (**Chapter 4**). Therefore, the molecular LRC signature that we defined may represent a unique cellular state of LSCs that specifically reflects their regenerative behaviour. Alternatively, LRCs may be a heterogeneous mixture of LSCs and non-renewing leukemic cells that play a regeneration-supporting role. This latter possibility could provide insight into the contrasting kinetics of leukemic versus healthy regeneration despite the similarities

between HSCs and LSCs otherwise described in this thesis (**Chapters 2 and 3**). Healthy BM microenvironments are known to coordinate rapid cellular proliferation followed by a disciplined return to homeostatic activity (Mendelson and Frenette, 2014). Contributions from injury-reactive leukemic cells could possibly skew these signals (Duan et al., 2014), resulting in disproportionate regenerative growth. Therefore, it will be valuable to dissociate whether or not heterogeneous cell subsets cooperate to promote leukemic recovery, in order to most deliberately target this process.

In many cancers, accelerated tumor repopulation represents an important source of therapy failure that requires further exploration for preventative approaches against recurrence (Kim and Tannock, 2005). We are the first to describe this phenomenon in AML, providing a new perspective to re-orient emphasis towards targeting unique regenerative properties of LRCs over approaches to chemosensitize quiescent cells (Saito et al., 2010b). We have demonstrated the value of this strategy through pre-clinical testing of novel combination chemotherapy regimens, and have identified avenues for further development based on dissecting the specific cellular interactions that drive leukemic regeneration.

5.3.1 Future clinical perspectives: Novel combination therapies

In addition to our encouraging demonstration that DRD2 inhibitor thioridazine (TDZ) is effective in combination with AraC, additional candidate agents remain to

be tested based on a wider examination of LRC-specific molecular signatures. As we observed beneficial effects of TDZ across a range of different AML patient samples, its effects do not appear to be restricted to specific genetic subtypes of disease. TDZ has already been advanced to a Phase I clinical trial in combination with AraC for relapsed or refractory AML (trial identifier #NCT02096289), demonstrating the direct clinical relevance of these findings. It will be necessary to more closely examine how TDZ impacts healthy regeneration, however no complications of prolonged cytopenia have been observed in recipient mice that received combination regimens. Combination chemotherapies designed to limit leukemic repopulation represent an appealing direction for induction therapy across diverse AML patient subtypes.

5.4 Spatial localization of primitive leukemia cells

Beyond the clinical applications that represent direct extensions of each chapter in this thesis, combined elements from multiple chapters have led to a new hypothesis towards the detection and treatment of AML in patients. The distinct regional preferences of LSCs (**Chapter 3**) suggests that they may exhibit spatial heterogeneity within BM that could be of diagnostic significance, particularly under conditions of minimum residual disease (MRD). In **Chapter 4**, our xenograft simulations of complete remission (CR) states were remarkable based on the complete absence of human leukemic cells in BM aspirates, despite the aggressive relapse that followed. Motivated by the observation that like healthy HSCs, CD34⁺

leukemic cells preferentially localize to trabecular bone areas and are rare in the central bone cavity (**Chapter 3**), we have since determined that in xenografts, aspirate sampling can fail to capture occult reservoirs of rare leukemic cells that are localized to highly trabeculated regions. It is possible that discrete spatial localization of residual cells could similarly contribute to MRD detection challenges in AML patients.

Our *in situ* imaging analysis of AML-infiltrated femurs has further suggested that LSC distribution may even be non-uniform with respect to their more mature progeny (**Chapter 3**). This interpretation could clarify the observation that AML cells collected from the majority of patients fail to produce leukemic grafts when transplanted into immune-deficient mice (Shlush et al., 2014). While there is evidence of better prognosis in these instances, most patients who lack functionally detectable LSCs still proceed to relapse (Monaco et al., 2004) despite the fact that relapse is thought to be an LSC-driven process. Efforts to explain this discrepant phenomenon by examinations of cell-intrinsic chemokine expression and BM-homing capacities of non-engrafting AML samples have been previously met without success (Pearce et al., 2006). The alternative possibility of localized regions of LSC enrichment is reminiscent of the spatial heterogeneity that is widely acknowledged to exist in solid tumors (Bedard et al., 2013; Gerlinger et al., 2012; Navin et al., 2011), but has been overlooked in leukemia due to the liquid nature of the tissue.

In contrast to former impressions of regular HSPC trafficking, recent insights have suggested that they instead appear rather stationary in BM unless disrupted by stimuli such as G-CSF mobilization (Cheung et al., 2013; Verovskaya et al., 2014). These conclusions were based on the use of genetic barcoding systems to track hematopoietic cells at the clonal level, in which an examination of barcodes within primitive populations suggested little clonal overlap between bone sites. Based on the emerging evidence for functional parallels between LSCs and HSCs (**Chapter 3**) (Glaït-Santar et al., 2015), it is plausible that primitive LSCs may exhibit similarly restricted motility in BM. In human patients, it has been genetically demonstrated that all leukemic subpopulations descend from a single founder clone as opposed to multifocal genetic origins of disease (Ding et al., 2012). The potential to examine similar conditions of disease initiation processes has not been well-captured by conventional experimental modeling of AML, in which leukemic cells are either introduced in a systemic manner by intravenous injection, or by establishing oncogenic mutations across the whole hematopoietic system in engineered mice. Instead, these possibilities will be best explored by monitoring distribution kinetics of leukemic subpopulations following localized intra-femoral delivery models.

5.4.1 Future clinical perspectives: Spatial targeting of LSCs

Understanding the potential spatial heterogeneity of leukemia could clarify limitations associated with obtaining representative BM assessments during low levels of leukemic burden. However, the practical value of this hypothesis would be

dependent upon developing a strategy to better identify the presence of leukemic cells that may escape physical sample capture using traditional diagnostic aspirates from iliac crest sites. Based on evidence that leukemia locally remodels the BM niche (Schepers et al., 2013), confined regions of concentrated leukemic cells are likely to be associated with unique molecular or physical properties that could be detected by whole body imaging methods. In fact, an exploratory report of positron emission tomography BM imaging in human AML has already provided proof-of-principle evidence that this technique can detect localized sites of leukemic growth which were missed by routine aspirate sampling (Brierley et al., 2013). However, the relation of this finding to the most functionally relevant populations of LSCs remains to be examined. Molecular imaging of human cancer patients has not yet been used in conjunction with a detailed dissection of functional heterogeneity, which could advance our fundamental understanding of the disease and offer novel clinical approaches.

Knowledge of the locations of highly concentrated LSCs could ultimately enable spatially selective delivery of high-intensity therapeutics (Samant et al., 2009; Wong et al., 2006), which could both spare healthy HSPCs in non-infiltrated areas, and limit the formation of injured BM niches (Duan et al., 2014) that may contribute to excessive leukemic regeneration (**Chapter 4**). As most patients present with diffusely disseminated disease by the time symptoms emerge, this hypothesized approach would likely be most suitable in patients with minimally

progressed AML, or as a means of managing MRD in patients who have achieved remission. This suggestion provides a constructive strategy to approach therapy based on understanding localization properties that are similarly shared between LSCs and HSCs.

5.5 Concluding remarks

Although genetic mutations provide the necessary spark to ignite leukemic disease, the surrounding environment participates in a meaningful way to fuel its progression. Like other cancers, AML is considered to be an aging disease, and its increased incidence with age has been explained by the progressive accumulation of genetic lesions over time (Xie et al., 2014). However, aging programs distinctly involve the loss of tissue regulatory control, including the composition and function of the BM niche (Zhang et al., 2013), potentially contributing to a relaxed level of control over rogue hematopoietic clones. In fact, the niche can even directly drive dysplastic hematopoiesis as has been shown in genetic mouse models (Kode et al., 2014). These perspectives reinforce the hypothesis that the BM microenvironment plays an important participatory role in leukemia development, and it is important to understand these dynamics.

This thesis has revealed that the BM niche may be a targetable axis in AML, and that HSCs and LSCs share common BM niches. Additionally, it suggests that niche-induced quiescence may not be the primary limitation that prevents LSC eradication using conventional chemotherapeutic targeting. Instead, a dramatic

leukemic recovery process occurs which is out of proportion with healthy regeneration. A comprehensive understanding of the interactive processes that exist between HSCs, LSCs, and BM niche cells during pathological states will ultimately inform educated strategies for novel therapeutic interventions in AML. Evidence presented within this thesis offers several prospects towards this goal, including pharmacological targeting to preserve BM niche function under leukemic conditions, novel cell-based approaches to dissociate LSC-niche interactions, and combination therapies to prevent the reconstruction of functional leukemic hierarchies following cytoreduction. Other future directions could additionally involve molecular imaging to identify and target anatomical sites of highly concentrated LSC localization. These findings reinforce the importance of understanding diseased cells within their biological context, as it can present therapeutic opportunities that would be overlooked by conceptualizing LSCs in isolation. The past 20 years have cultivated a transformative movement to acknowledge and characterize the heterogeneous composition of both the leukemic hierarchy and the surrounding BM niche. It is now essential to integrate these insights towards a more comprehensive understanding of the BM as an interactive ecosystem under conditions of both health and disease.

Figures

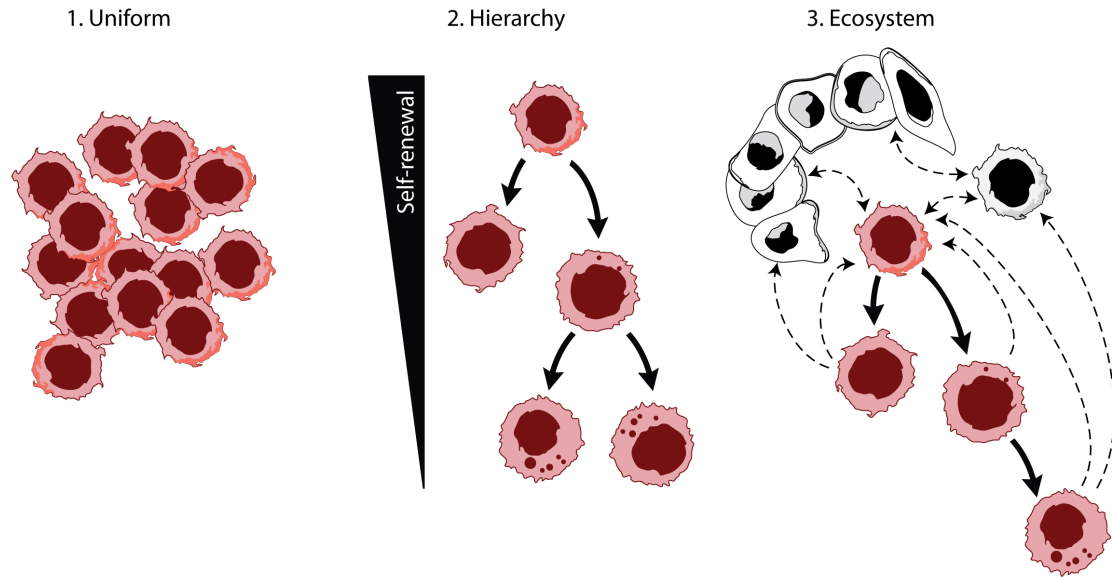


Figure 5.1. Progressively complex views of leukemia

Historically, all leukemic cells were viewed as equivalent, and cytotoxic chemotherapy was hoped to uniformly eliminate leukemic blasts. In the 1990's, a hierarchical model of leukemia was recognized, and attention became focused on identifying distinctive molecular qualities of LSCs to enable their preferential therapeutic targeting. This thesis has contributed to a new emerging view in which the leukemic hierarchy is part of a complex BM ecosystem, revealing multiple new angles to attack LSCs. Solid arrows indicate cellular ancestry, while dotted arrows indicate cell interactions. Coloured shading represents leukemic cells and grayscale shading represents healthy cell types.

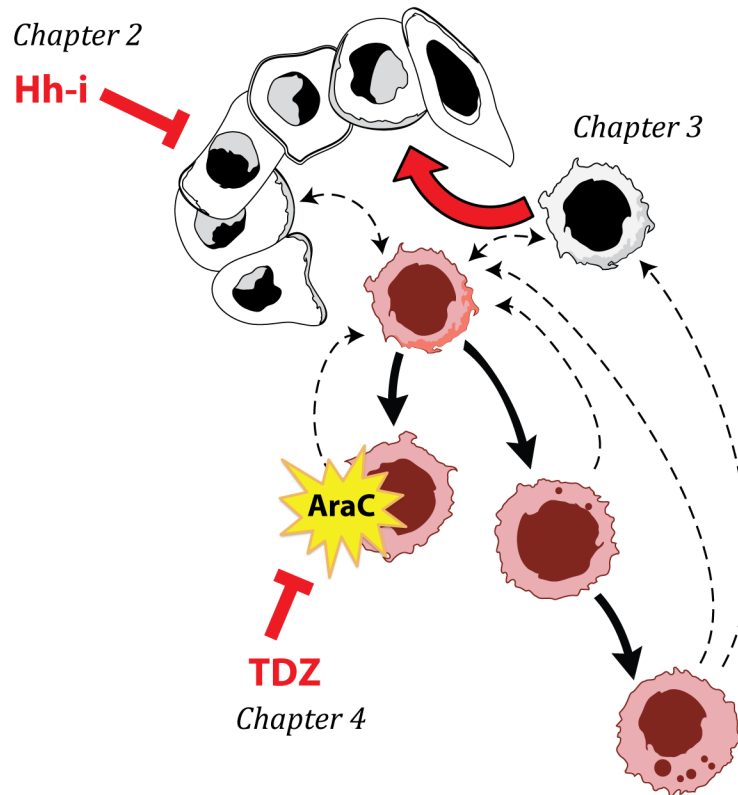


Figure 5.2. Summary of findings

Based on a view of LSCs as being interactive components of the BM environment, we have identified several novel avenues towards therapeutic targeting in AML. First, we have provided evidence to propose that LSCs could be modulated indirectly through the BM microenvironment using hedgehog pathway inhibitors (Hh-i; **Chapter 2**). We then described that healthy and leukemic stem cells share common BM niches, and that HSCs can compete to repopulate these sites at the expense of LSC self-renewal (**Chapter 3**). Finally, we provided a novel description of leukemic regeneration that follows treatment with AraC chemotherapy, and showed that we could suppress this early stage of leukemic recurrence using combined therapy with the DRD2 antagonist TDZ (**Chapter 4**). Solid arrows indicate cellular ancestry, while dotted arrows indicate cell interactions. Coloured shading represents leukemic cells and grayscale shading represents healthy cell types.

Appendix I: Bibliography

Abboud, C.N. (2014). Granulocyte colony-stimulating factor priming improves haploidentical stem cell transplantation in high-risk acute myeloid leukemia subsets: A new role for an old work horse. *Biology of Blood and Marrow Transplantation* *20*, 1873-1874.

Abramson, S., Miller, R.G., and Phillips, R.A. (1977). The identification in adult bone marrow of pluripotent and restricted stem cells of the myeloid and lymphoid systems. *Journal of Experimental Medicine* *145*, 1567-1579.

Agliano, A., Martin-Padura, I., Mancuso, P., Marighetti, P., Rabascio, C., Pruneri, G., Shultz, L.D., and Bertolini, F. (2008). Human acute leukemia cells injected in NOD/LtSz- scid/IL-2R γ null mice generate a faster and more efficient disease compared to other NOD/ scid-related strains. *International Journal of Cancer* *123*, 2222-2227.

Al-Hajj, M., Wicha, M.S., Benito-Hernandez, A., Morrison, S.J., and Clarke, M.F. (2003). Prospective identification of tumorigenic breast cancer cells. *Proceedings of the National Academy of Sciences* *100*, 3983-3988.

Andrews, R.G., Singer, J.W., and Bernstein, I.D. (1990). Human hematopoietic precursors in long-term culture: single CD34+ cells that lack detectable T cell, B cell, and myeloid cell antigens produce multiple colony-forming cells when cultured with marrow stromal cells. *Journal of Experimental Medicine* *172*, 355-358.

Arai, F., Hirao, A., Ohmura, M., Sato, H., Matsuoka, S., Takubo, K., Ito, K., Koh, G.Y., and Suda, T. (2004). Tie2/Angiopoietin-1 Signaling Regulates Hematopoietic Stem Cell Quiescence in the Bone Marrow Niche. *Cell* *118*, 149-161.

Arai, F., and Suda, T. (2007). Maintenance of quiescent hematopoietic stem cells in the osteoblastic niche. *Annals of the New York Academy of Sciences* *1106*, 41-53.

Arroyo, A.G., Yang, J.T., Rayburn, H., and Hynes, R.O. (1999). Alpha4 integrins regulate the proliferation/differentiation balance of multilineage hematopoietic progenitors in vivo. *Immunity* *11*, 555-566.

Ash, R.C., Detrick, D.A., and Zanjani, E.D. (1981). Studies of human pluripotential hemopoietic stem cells (CFU-GEMM) in vitro. *Blood* *58*, 309-316.

Avigdor, A., Goichberg, P., Shivtiel, S., Dar, A., Peled, A., Samira, S., Kollet, O., Hershkovich, R., Alon, R., Hardan, I., *et al.* (2004). CD44 and hyaluronic acid cooperate

with SDF-1 in the trafficking of human CD34+ stem/progenitor cells to bone marrow. *Blood* 103, 2981-2989.

Bakker, A.B.H., van den Oudenrijn, S., Bakker, A.Q., Feller, N., van Meijer, M., Bia, J.A., Jongeneelen, M.A.C., Visser, T.J., Bijl, N., Geuijen, C.A.W., *et al.* (2004). C-type lectin-like molecule-1: A novel myeloid cell surface marker associated with acute myeloid leukemia. *Cancer Research* 64, 8443-8450.

Baum, C.M., Weissman, I.L., Tsukamoto, A.S., Buckle, A.M., and Peault, B. (1992). Isolation of a candidate human hematopoietic stem-cell population. *Proceedings of the National Academy of Sciences* 89, 2804-2808.

Beachy, P.A., Karhadkar, S.S., and Berman, D.M. (2004). Tissue repair and stem cell renewal in carcinogenesis. *Nature* 432, 324-331.

Becker, A.J., McCulloch, E.A., and Till, J.E. (1963). Cytological demonstration of the clonal nature of spleen colonies derived from transplanted mouse marrow cells. *Nature* 197, 452-454.

Bedard, P.L., Hansen, A.R., Ratain, M.J., and Siu, L.L. (2013). Tumour heterogeneity in the clinic. *Nature* 501, 355-364.

Bennett, J.M. (1985). Proposed Revised Criteria for the classification of acute myeloid leukemia. *Annals of Internal Medicine* 103, 620-625.

Bennett, J.M., Catovsky, D., Daniel, M.T., Flandrin, G., Galton, D.A., Gralnick, H.R., and Sultan, C. (1976). Proposals for the classification of the acute leukaemias. French-American-British (FAB) co-operative group. *British Journal of Haematology* 33, 451-458.

Benveniste, P., Cantin, C., Hyam, D., and Iscove, N.N. (2003). Hematopoietic stem cells engraft in mice with absolute efficiency. *Nature Immunology* 4, 708-713.

Bhardwaj, G., Murdoch, B., Wu, D., Baker, D.P., Williams, K.P., Chadwick, K., Ling, L.E., Karanu, F.N., and Bhatia, M. (2001). Sonic hedgehog induces the proliferation of primitive human hematopoietic cells via BMP regulation. *Nature Immunology* 2, 172-180.

Bhatia, M., Wang, J.C., Kapp, U., Bonnet, D., and Dick, J.E. (1997). Purification of primitive human hematopoietic cells capable of repopulating immune-deficient mice. *Proceedings of the National Academy of Sciences* 94, 5320-5325.

Bloomfield, C.D., Shuma, C., Regal, L., Philip, P.P., Hossfeld, D.K., Hagemeyer, A.M., Garson, O.M., Peterson, B.A., Sakurai, M., Alimena, G., *et al.* (1997). Long-term survival of patients with acute myeloid leukemia: a third follow-up of the Fourth International Workshop on Chromosomes in Leukemia. *Cancer* 80, 2191-2198.

Bonnet, D., and Dick, J.E. (1997). Human acute myeloid leukemia is organized as a hierarchy that originates from a primitive hematopoietic cell. *Nature Medicine* 3, 730-737.

Bradley, T.R., and Metcalf, D. (1966). The growth of mouse bone marrow cells in vitro. *Australian Journal of Experimental Biology and Medical Sciences* 44, 287-300.

Brierley, C.K., Morgan, E.A., Sprague, J.R., Odejide, O.O., DeAngelo, D.J., and Steensma, D.P. (2013). An extreme example of focal bone marrow involvement in acute myeloid leukemia. *American Journal of Hematology* 88, 335-336.

Broxmeyer, H.E. (2005). Rapid mobilization of murine and human hematopoietic stem and progenitor cells with AMD3100, a CXCR4 antagonist. *Journal of Experimental Medicine* 201, 1307-1318.

Buick, R.N., Minden, M.D., and McCulloch, E.A. (1979). Self-renewal in culture of proliferative blast progenitor cells in acute myeloblastic leukemia. *Blood* 54, 95-104.

Bullinger, L., Döhner, K., Bair, E., Fröhling, S., Schlenk, R.F., Tibshirani, R., Döhner, H., and Pollack, J.R. (2004). Use of gene-expression profiling to identify prognostic subclasses in adult acute myeloid leukemia. *New England Journal of Medicine* 350, 1605-1616.

Calvi, L.M., Adams, G.B., Weibrecht, K.W., Weber, J.M., Olson, D.P., Knight, M.C., Martin, R.P., Schipani, E., Divieti, P., Bringham, F.R., *et al.* (2003). Osteoblastic cells regulate the haematopoietic stem cell niche. *Nature* 425, 841-846.

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

Casanova-Acebes, M., Pitaval, C., Weiss, L.A., Nombela-Arrieta, C., Chèvre, R., A-González, N., Kunisaki, Y., Zhang, D., Van Rooijen, N., Silberstein, L.E., *et al.* (2013). Rhythmic modulation of the hematopoietic niche through neutrophil clearance. *Cell* 153, 1025-1035.

Champlin, R., Khouri, I., Anderlini, P., Gajewski, J., Kornblau, S., Molldrem, J., Shimoni, A., Ueno, N., and Giralt, S. (2001). Nonmyeloablative preparative regimens for

allogeneic hematopoietic transplantation. *Bone Marrow Transplantation* 27 Suppl 2, S13-22.

Cheng, H., Hao, S., Liu, Y., Pang, Y., Ma, S., Dong, F., Xu, J., Zheng, G., Li, S., Yuan, W., *et al.* (2015). Leukemic marrow infiltration reveals a novel role for Egr3 as a potent inhibitor of normal hematopoietic stem cell proliferation. *Blood* 126, 1302-1313.

Cheson, B.D., Bennett, J.M., Kopecky, K.J., Büchner, T., Willman, C.L., Estey, E.H., Schiffer, C.A., Doehner, H., Tallman, M.S., Lister, T.A., *et al.* (2003). Revised recommendations of the International Working Group for Diagnosis, Standardization of Response Criteria, Treatment Outcomes, and Reporting Standards for Therapeutic Trials in Acute Myeloid Leukemia. *Journal of Clinical Oncology* 21, 4642-4649.

Cheung, A.M.S., Nguyen, L.V., Carles, A., Beer, P., Miller, P.H., Knapp, D.J.H.F., Dhillon, K., Hirst, M., and Eaves, C.J. (2013). Analysis of the clonal growth and differentiation dynamics of primitive barcoded human cord blood cells in NSG mice. *Blood* 122, 3129-3137.

Civin, C.I., Strauss, L.C., Brovall, C., Fackler, M.J., Schwartz, J.F., and Shaper, J.H. (1984). Antigenic analysis of hematopoiesis. III. A hematopoietic progenitor cell surface antigen defined by a monoclonal antibody raised against KG-1a cells. *Journal of Immunology* 133, 157-165.

Clutterbuck, R.D., Hills, C.A., Hoey, P., Alexander, P., Powles, R.L., and Millar, J.L. (1985). Studies on the development of human acute myeloid leukaemia xenografts in immune-deprived mice: comparison with cells in short-term culture. *Leukemia Research* 9, 1511-1518.

Colmone, A., Amorim, M., Pontier, A.L., Wang, S., Jablonski, E., and Sipkins, D.A. (2008). Leukemic cells create bone marrow niches that disrupt the behavior of normal hematopoietic progenitor cells. *Science* 322, 1861-1865.

Cook, K.M., Sifri, Z.C., Baranski, G.M., Mohr, A.M., and Livingston, D.H. (2013). The role of plasma granulocyte colony stimulating factor and bone marrow dysfunction after severe trauma. *Journal of American College of Surgeons* 216, 57-64.

Coombs, C.C., Tavakkoli, M., and Tallman, M.S. (2015). Acute promyelocytic leukemia: where did we start, where are we now, and the future. *Blood Cancer J* 5, e304.

Corey, S.J., Minden, M.D., Barber, D.L., Kantarjian, H., Wang, J.C.Y., and Schimmer, A.D. (2007). Myelodysplastic syndromes: the complexity of stem-cell diseases. *Nature Reviews Cancer* 7, 118-129.

Costello, R.T., Mallet, F., Gaugler, B., Sainy, D., Arnoulet, C., Gastaut, J.A., and Olive, D. (2000). Human acute myeloid leukemia CD34+/CD38- progenitor cells have decreased sensitivity to chemotherapy and Fas-induced apoptosis, reduced immunogenicity, and impaired dendritic cell transformation capacities. *Cancer Research* 60, 4403-4411.

Craddock, C.F., Nakamoto, B., Andrews, R.G., Priestley, G.V., and Papayannopoulou, T. (1997). Antibodies to VLA4 integrin mobilize long-term repopulating cells and augment cytokine-induced mobilization in primates and mice. *Blood* 90, 4779-4788.

Craig, F.E., and Foon, K.A. (2008). Flow cytometric immunophenotyping for hematologic neoplasms. *Blood* 111, 3941-3967.

Czechowicz, A., Kraft, D., Weissman, I.L., and Bhattacharya, D. (2007). Efficient Transplantation via Antibody-Based Clearance of Hematopoietic Stem Cell Niches. *Science* 318, 1296-1299.

Dalerba, P., Cho, R.W., and Clarke, M.F. (2007). Cancer Stem Cells: Models and Concepts. *Annual Review of Medicine* 58, 267-284.

Dexter, T.M., Allen, T.D., Lajtha, L.G., Schofield, R., and Lord, B.I. (1973). Stimulation of differentiation and proliferation of haemopoietic cells in vitro. *Journal of Cellular Physiology* 82, 461-473.

Dexter, T.M., Spooner, E., Toksoz, D., and Lajtha, L.G. (1980). The role of cells and their products in the regulation of in vitro stem cell proliferation and granulocyte development. *Journal of Supramolecular Structure* 13, 513-524.

Dick, J.E. (2008). Stem cell concepts renew cancer research. *Blood* 112, 4793-4807.

Dierks, C., Beigi, R., Guo, G.-R., Zirlik, K., Stegert, M.R., Manley, P., Trussell, C., Schmitt-Graeff, A., Landwerlin, K., Veelken, H., *et al.* (2008). Expansion of Bcr-Abl-positive leukemic stem cells is dependent on hedgehog pathway activation. *Cancer Cell* 14, 238-249.

Ding, L., Ley, T.J., Larson, D.E., Miller, C.A., Koboldt, D.C., Welch, J.S., Ritchey, J.K., Young, M.A., Lamprecht, T., McLellan, M.D., *et al.* (2012). Clonal evolution in relapsed acute myeloid leukaemia revealed by whole-genome sequencing. *Nature* 481, 506-510.

Ding, L., and Morrison, S.J. (2013). Haematopoietic stem cells and early lymphoid progenitors occupy distinct bone marrow niches. *Nature* *495*, 231-235.

Döhner, H., Estey, E.H., Amadori, S., Appelbaum, F.R., Büchner, T., Burnett, A.K., Dombret, H., Fenaux, P., Grimwade, D., Larson, R.A., *et al.* (2010). Diagnosis and management of acute myeloid leukemia in adults: recommendations from an international expert panel, on behalf of the European LeukemiaNet. *Blood* *115*, 453-474.

Dominici, M., Le Blanc, K., Mueller, I., Slaper-Cortenbach, I., Marini, F., Krause, D., Deans, R., Keating, A., Prockop, D., and Horwitz, E. (2006). Minimal criteria for defining multipotent mesenchymal stromal cells. The International Society for Cellular Therapy position statement. *Cytotherapy* *8*, 315-317.

Dominici, M., Rasini, V., Bussolari, R., Chen, X., Hofmann, T.J., Spano, C., Bernabei, D., Veronesi, E., Bertoni, F., Paolucci, P., *et al.* (2009). Restoration and reversible expansion of the osteoblastic hematopoietic stem cell niche after marrow radioablation. *Blood* *114*, 2333-2343.

Doulatov, S., Notta, F., Laurenti, E., and Dick, J.E. (2012). Hematopoiesis: A human perspective. *Cell Stem Cell* *10*, 120-136.

Driessens, G., Beck, B., Caauwe, A., Simons, B.D., and Blanpain, C. (2012). Defining the mode of tumour growth by clonal analysis. *Nature* *488*, 527-530.

Drouet, M. (2014). Determining rational target for in vivo hedgehog gene therapy. *Experimental Hematology* *42*, 159-160.

Drouet, M., Garrigou, P., Peinnequin, A., and Hérodin, F. (2014). Short-term sonic-hedgehog gene therapy to mitigate myelosuppression in highly irradiated monkeys: hype or reality? *Bone Marrow Transplantation* *49*, 304-309.

Duan, C.-W., Shi, J., Chen, J., Wang, B., Yu, Y.-H., Qin, X., Zhou, X.-C., Cai, Y.-J., Li, Z.-Q., Zhang, F., *et al.* (2014). Leukemia propagating cells rebuild an evolving niche in response to therapy. *Cancer Cell* *25*, 778-793.

Duman-Scheel, M., Weng, L., Xin, S., and Du, W. (2002). Hedgehog regulates cell growth and proliferation by inducing Cyclin D and Cyclin E. *Nature* *417*, 299-304.

Dzierzak, E., and Speck, N.A. (2008). Of lineage and legacy: the development of mammalian hematopoietic stem cells. *Nature Immunology* *9*, 129-136.

Eliasson, P., and Jonsson, J.I. (2010). The hematopoietic stem cell niche: low in oxygen but a nice place to be. *Journal of Cellular Physiology* 222, 17-22.

Ellis, S.L., Grassinger, J., Jones, A., Borg, J., Camenisch, T., Haylock, D., Bertoncello, I., and Nilsson, S.K. (2011). The relationship between bone, hemopoietic stem cells, and vasculature. *Blood* 118, 1516-1524.

Eppert, K., Takenaka, K., Lechman, E.R., Waldron, L., Nilsson, B., van Galen, P., Metzeler, K.H., Poepl, A., Ling, V., Beyene, J., *et al.* (2011). Stem cell gene expression programs influence clinical outcome in human leukemia. *Nature Medicine* 17, 1086-1093.

Estey, E., and Döhner, H. (2006). Acute myeloid leukaemia. *Lancet* 368, 1894-1907.

Estey, E.H. (2011). How to manage high-risk acute myeloid leukemia. *Leukemia* 26, 861-869.

Estey, E.H. (2012). Acute myeloid leukemia: 2012 update on diagnosis, risk stratification, and management. *Am J Hematol* 87, 89-99.

Fabian, S.L., Penchev, R.R., Jacques, B.S., Rao, A.N., Sipilä, P., West, K.A., McMahon, A.P., and Humphreys, B.D. (2012). Hedgehog-Gli pathway activation during kidney fibrosis. *The American Journal of Pathology* 180, 1441-1453.

Fausser, A.A., and Messner, H.A. (1978). Granuloerythropoietic colonies in human bone marrow, peripheral blood, and cord blood. *Blood* 52, 1243-1248.

Felipe Rico, J., Hassane, D.C., and Guzman, M.L. (2012). Acute myelogenous leukemia stem cells: From Bench to Bedside. *Cancer Letters* 338, 4-9

Ford, C.E., Hamerton, J.L., Barnes, D.W., and Loutit, J.F. (1956). Cytological identification of radiation-chimaeras. *Nature* 177, 452-454.

Forman, S.J., and Rowe, J.M. (2013). The myth of the second remission of acute leukemia in the adult. *Blood* 121, 1077-1082.

Fujisaki, J., Wu, J., Carlson, A.L., Silberstein, L., Putheti, P., Larocca, R., Gao, W., Saito, T.I., Lo Celso, C., Tsuyuzaki, H., *et al.* (2011). In vivo imaging of Treg cells providing immune privilege to the haematopoietic stem-cell niche. *Nature* 474, 216-219.

Gao, J., Graves, S., Koch, U., Liu, S., Jankovic, V., Buonamici, S., El Andaloussi, A., Nimer, S.D., Kee, B.L., Taichman, R., *et al.* (2009). Hedgehog signaling is dispensable for adult hematopoietic stem cell function. *Cell Stem Cell* 4, 548-558.

Gao, L., Wen, Q., Chen, X., Liu, Y., Zhang, C., Gao, L., Kong, P., Zhang, Y., Li, Y., Liu, J., *et al.* (2014). Effects of priming with recombinant human granulocyte colony-stimulating factor on conditioning regimen for high-risk acute myeloid leukemia patients undergoing human leukocyte antigen-haploidentical hematopoietic stem cell transplantation: A multicenter randomized controlled study in Southwest China. *Biology of Blood and Marrow Transplantation* 20, 1932-1939.

Garrido, S.M., Appelbaum, F.R., Willman, C.L., and Banker, D.E. (2001). Acute myeloid leukemia cells are protected from spontaneous and drug-induced apoptosis by direct contact with a human bone marrow stromal cell line (HS-5). *Experimental Hematology* 29, 448-457.

Gerlinger, M., Rowan, A.J., Horswell, S., Larkin, J., Endesfelder, D., Gronroos, E., Martinez, P., Matthews, N., Stewart, A., Tarpey, P., *et al.* (2012). Intratumor heterogeneity and branched evolution revealed by multiregion sequencing. *New England Journal of Medicine* 366, 883-892.

Gillet, J.-P., Calcagno, A.M., Varma, S., Marino, M., Green, L.J., Vora, M.I., Patel, C., Orina, J.N., Eliseeva, T.A., Singal, V., *et al.* (2011). Redefining the relevance of established cancer cell lines to the study of mechanisms of clinical anti-cancer drug resistance. *Proceedings of the National Academy of Sciences* 108, 18708-18713.

Glait-Santar, C., Desmond, R., Feng, X., Bat, T., Chen, J., Heuston, E., Mizukawa, B., Mulloy, J.C., Bodine, D.M., Larochelle, A., *et al.* (2015). Functional Niche Competition Between Normal Hematopoietic Stem and Progenitor Cells and Myeloid Leukemia Cells *Stem Cells* 33, 3635-3642.

Godley, L.A., and Larson, R.A. (2008). Therapy-related myeloid leukemia. *Seminars in Oncology* 35, 418-429.

Grassinger, J., Haylock, D.N., Williams, B., Olsen, G.H., and Nilsson, S.K. (2010). Phenotypically identical hemopoietic stem cells isolated from different regions of bone marrow have different biologic potential. *Blood* 116, 3185-3196.

Gratwohl, A., Pasquini, M.C., Aljurf, M., Atsuta, Y., Baldomero, H., Foeken, L., Gratwohl, M., Bouzas, L.F., Confer, D., Frauendorfer, K., *et al.* (2015). One million haemopoietic stem-cell transplants: a retrospective observational study. *The Lancet Haematology* 2, e91-e100.

Greenbaum, A., Hsu, Y.-M.S., Day, R.B., Schuettpelz, L.G., Christopher, M.J., Borgerding, J.N., Nagasawa, T., and Link, D.C. (2013). CXCL12 in early mesenchymal progenitors is required for haematopoietic stem-cell maintenance. *Nature* 495, 227-230.

Griffin, J.D., and Löwenberg, B. (1986). Clonogenic cells in acute myeloblastic leukemia. *Blood* *68*, 1185-1195.

Guan, Y., Gerhard, B., and Hogge, D.E. (2003). Detection, isolation, and stimulation of quiescent primitive leukemic progenitor cells from patients with acute myeloid leukemia (AML). *Blood* *101*, 3142-3149.

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

Guezguez, B., Campbell, C.J.V., Boyd, A.L., Karanu, F., Casado, F.L., Di Cresce, C., Collins, T.J., Shapovalova, Z., Xenocostas, A., and Bhatia, M. (2013). Regional localization within the bone marrow influences the functional capacity of human HSCs. *Cell Stem Cell* *13*, 175-189.

Guzman, M.L., Neering, S.J., Upchurch, D., Grimes, B., Howard, D.S., Rizzieri, D.A., Luger, S.M., and Jordan, C.T. (2001). Nuclear factor-kappaB is constitutively activated in primitive human acute myelogenous leukemia cells. *Blood* *98*, 2301-2307.

Hamilton, B.K., and Copelan, E.A. (2012). The Role of Hematopoietic Stem Cell Transplantation in the Treatment of Acute Myeloid Leukemia. *Stem Cells* *30*, 1581-1586.

Hanahan, D., and Weinberg, R.A. (2000). The Hallmarks of Cancer. *Cell* *100*, 57-70.

Hanahan, D., and Weinberg, R.A. (2011). Hallmarks of Cancer: The Next Generation. *Cell* *144*, 646-674.

Hanoun, M., Zhang, D., Mizoguchi, T., Pinho, S., Pierce, H., Kunisaki, Y., Lacombe, J., Armstrong, S.A., Dührsen, U., and Frenette, P.S. (2014). Acute myelogenous leukemia-induced sympathetic neuropathy promotes malignancy in an altered hematopoietic stem cell niche. *Cell Stem Cell* *15*, 365-375.

Hao, Q.L., Shah, A.J., Thiemann, F.T., Smogorzewska, E.M., and Crooks, G.M. (1995). A functional comparison of CD34 + CD38- cells in cord blood and bone marrow. *Blood* *86*, 3745-3753.

Harris, N.L., Jaffe, E.S., Diebold, J., Flandrin, G., Muller-Hermelink, H.K., Vardiman, J., Lister, T.A., and Bloomfield, C.D. (1999). World Health Organization classification of neoplastic diseases of the hematopoietic and lymphoid tissues: report of the Clinical

Advisory Committee meeting-Airlie House, Virginia, November 1997. *Journal of Clinical Oncology* 17, 3835-3849.

Harrison, D.E. (1980). Competitive repopulation: a new assay for long-term stem cell functional capacity. *Blood* 55, 77-81.

Hartwell, K.A., Miller, P.G., Mukherjee, S., Kahn, A.R., Stewart, A.L., Logan, D.J., Negri, J.M., Duvet, M., Järås, M., Puram, R., *et al.* (2013). Niche-based screening identifies small-molecule inhibitors of leukemia stem cells. *Nature Chemical Biology* 9, 840-848.

Haylock, D.N., and Nilsson, S.K. (2005). Stem cell regulation by the hematopoietic stem cell niche. *Cell Cycle* 4, 1353-1355.

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

Hofmann, I., Stover, E.H., Cullen, D.E., Mao, J., Morgan, K.J., Lee, B.H., Kharas, M.G., Miller, P.G., Cornejo, M.G., Okabe, R., *et al.* (2009). Hedgehog Signaling Is Dispensable for Adult Murine Hematopoietic Stem Cell Function and Hematopoiesis. *Cell Stem Cell* 4, 559-567.

Hoggatt, J., Mohammad, K.S., Singh, P., Hoggatt, A.F., Chitteti, B.R., Speth, J.M., Hu, P., Poteat, B.A., Stilger, K.N., Ferraro, F., *et al.* (2013). Differential stem- and progenitor-cell trafficking by prostaglandin E2. *Nature* 495, 365-369.

Hooper, A.T., Butler, J.M., Nolan, D.J., Kranz, A., Iida, K., Kobayashi, M., Kopp, H.G., Shido, K., Petit, I., Yanger, K., *et al.* (2009). Engraftment and reconstitution of hematopoiesis is dependent on VEGFR2-mediated regeneration of sinusoidal endothelial cells. *Cell Stem Cell* 4, 263-274.

Hooper, J.E., and Scott, M.P. (2005). Communicating with hedgehogs. *Nature Reviews Molecular Cell Biology* 6, 306-317.

Hope, K.J., Jin, L., and Dick, J.E. (2004). Acute myeloid leukemia originates from a hierarchy of leukemic stem cell classes that differ in self-renewal capacity. *Nature Immunology* 5, 738-743.

Horton, S.J., and Huntly, B.J.P. (2012). Recent advances in acute myeloid leukemia stem cell biology. *Haematologica* 97, 966-974.

Hosen, N., Park, C.Y., Tatsumi, N., Oji, Y., Sugiyama, H., Gramatzki, M., Krensky, A.M., and Weissman, I.L. (2007). CD96 is a leukemic stem cell-specific marker in human acute myeloid leukemia. *Proceedings of the National Academy of Sciences* *104*, 11008-11013.

Ichikawa, Y., Pluznik, D.H., and Sachs, L. (1966). In vitro control of the development of macrophage and granulocyte colonies. *Proceedings of the National Academy of Sciences* *56*, 488-495.

Ishikawa, F., Yoshida, S., Saito, Y., Hijikata, A., Kitamura, H., Tanaka, S., Nakamura, R., Tanaka, T., Tomiyama, H., Saito, N., *et al.* (2007). Chemotherapy-resistant human AML stem cells home to and engraft within the bone-marrow endosteal region. *Nature Biotechnology* *25*, 1315-1321.

James, A.W., Leucht, P., Levi, B., Carre, A.L., Xu, Y., Helms, J.A., and Longaker, M.T. (2010). Sonic Hedgehog influences the balance of osteogenesis and adipogenesis in mouse adipose-derived stromal cells. *Tissue Engineering Part A* *16*, 2605-2616.

Jamieson, C., Cortes, J.E., Oehler, V., Bacarani, M., Kantarjian, H.M., Papayannidis, C., Rice, K.N., Zhang, X., Shaik, N., Courtney, R., *et al.* (2011). Phase 1 Dose-Escalation Study of PF-04449913, An Oral Hedgehog (Hh) Inhibitor, in Patients with Select Hematologic Malignancies. *ASH Annual Meeting Abstracts* *118*, 424.

Jan, M., Chao, M.P., Cha, A.C., Alizadeh, A.A., Gentles, A.J., Weissman, I.L., and Majeti, R. (2011). Prospective separation of normal and leukemic stem cells based on differential expression of TIM3, a human acute myeloid leukemia stem cell marker. *Proceedings of the National Academy of Sciences* *108*, 5009.

Jin, L., Hope, K.J., Zhai, Q., Smadja-Joffe, F., and Dick, J.E. (2006). Targeting of CD44 eradicates human acute myeloid leukemic stem cells. *Nature Medicine* *12*, 1167-1174.

Jin, L., Lee, E.M., Ramshaw, H.S., Busfield, S.J., Peoppl, A.G., Wilkinson, L., Guthridge, M.A., Thomas, D., Barry, E.F., Boyd, A., *et al.* (2009). Monoclonal antibody-mediated targeting of CD123, IL-3 receptor α chain, eliminates human acute myeloid leukemic stem cells. *Cell Stem Cell* *5*, 31-42.

Jordan, C.T., Guzman, M.L., and Noble, M. (2006). Cancer stem cells. *New England Journal of Medicine* *355*, 1253-1261.

Jordan, C.T., Upchurch, D., Szilvassy, S.J., Guzman, M.L., Howard, D.S., Pettigrew, A.L., Meyerrose, T., Rossi, R., Grimes, B., Rizzieri, D.A., *et al.* (2000). The interleukin-3

receptor alpha chain is a unique marker for human acute myelogenous leukemia stem cells. *Leukemia* 14, 1777-1784.

Joseph, C., Quach, J.M., Walkley, C.R., Lane, S.W., Lo Celso, C., and Purton, L.E. (2013). Deciphering hematopoietic stem cells in their niches: a critical appraisal of genetic models, lineage tracing, and imaging strategies. *Cell Stem Cell* 13, 520-533.

Kamel-Reid, S., and Dick, J.E. (1988). Engraftment of immune-deficient mice with human hematopoietic stem cells. *Science* 242, 1706-1709.

Katayama, Y., Battista, M., Kao, W.M., Hidalgo, A., Peired, A.J., Thomas, S.A., and Frenette, P.S. (2006). Signals from the sympathetic nervous system regulate hematopoietic stem cell egress from bone marrow. *Cell* 124, 407-421.

Kelly, P.N., Dakic, A., Adams, J.M., Nutt, S.L., and Strasser, A. (2007). Tumor growth need not be driven by rare cancer stem cells. *Science* 317, 337-337.

Khan, I., Altman, J.K., and Licht, J.D. (2012). New strategies in acute myeloid leukemia: redefining prognostic markers to guide therapy. *Clinical Cancer Research* 18, 5163-5171.

Kiel, M.J., Iwashita, T., Yilmaz, O.H., Terhorst, C., and Morrison, S.J. (2005). SLAM family receptors distinguish hematopoietic stem and progenitor cells and reveal endothelial niches for stem cells. *Cell* 121, 1109-1121.

Kikushige, Y., Shima, T., Takayanagi, S.-i., Urata, S., Miyamoto, T., Iwasaki, H., Takenaka, K., Teshima, T., Tanaka, T., Inagaki, Y., *et al.* (2010). TIM-3 Is a promising target to selectively kill acute myeloid leukemia stem cells. *Cell Stem Cell* 7, 708-717.

Kim, J.J., and Tannock, I.F. (2005). Repopulation of cancer cells during therapy: an important cause of treatment failure. *Nature Reviews Cancer* 5, 516-525.

Kiuru, M., Hidaka, C., Hubner, R.-H., Solomon, J., Krause, A., Leopold, P.L., and Crystal, R.G. (2009). Sonic hedgehog expands diaphyseal trabecular bone altering bone marrow niche and lymphocyte compartment. *Molecular Therapy* 17, 1442-1452.

Kode, A., Manavalan, J.S., Mosialou, I., Bhagat, G., Rathinam, C.V., Luo, N., Khiabani, H., Lee, A., Murty, V.V., Friedman, R., *et al.* (2014). Leukaemogenesis induced by an activating β -catenin mutation in osteoblasts. *Nature* 506, 240-244.

Konopleva, M., Konoplev, S., Hu, W., Zaritskey, A.Y., Afanasiev, B.V., and Andreeff, M. (2002). Stromal cells prevent apoptosis of AML cells by up-regulation of anti-apoptotic proteins. *Leukemia* 16, 1713-1724.

Krause, D.S., Fulzele, K., Catic, A., Sun, C.C., Dombkowski, D., Hurley, M.P., Lezeau, S., Attar, E., Wu, J.Y., Lin, H.Y., *et al.* (2013). Differential regulation of myeloid leukemias by the bone marrow microenvironment. *Nature Medicine* *19*, 1513-1517.

Krebsbach, P.H., Kuznetsov, S.A., Bianco, P., and Robey, P.G. (1999). Bone marrow stromal cells: Characterization and clinical application. *Critical Reviews in Oral Biology and Medicine* *10*, 165-181.

Krivtsov, A.V., Twomey, D., Feng, Z., Stubbs, M.C., Wang, Y., Faber, J., Levine, J.E., Wang, J., Hahn, W.C., Gilliland, D.G., *et al.* (2006). Transformation from committed progenitor to leukaemia stem cell initiated by MLL–AF9. *Nature* *442*, 818-822.

Krönke, J., Bullinger, L., Teleanu, V., Tschürtz, F., Gaidzik, V.I., Kühn, M.W.M., Rücker, F.G., Holzmann, K., Paschka, P., Kapp-Schwörer, S., *et al.* (2013). Clonal evolution in relapsed NPM1-mutated acute myeloid leukemia. *Blood* *122*, 100-108.

Kruger, J.A., Kaplan, C.D., Luo, Y., Zhou, H., Markowitz, D., Xiang, R., and Reisfeld, R.A. (2006). Characterization of stem cell-like cancer cells in immune-competent mice. *Blood* *108*, 3906-3912.

Kunisaki, Y., Bruns, I., Scheiermann, C., Ahmed, J., Pinho, S., Zhang, D., Mizoguchi, T., Wei, Q., Lucas, D., Ito, K., *et al.* (2013). Arteriolar niches maintain haematopoietic stem cell quiescence. *Nature* *502*, 637-643.

Kurtova, A.V., Xiao, J., Mo, Q., Pazhanisamy, S., Krasnow, R., Lerner, S.P., Chen, F., Roh, T.T., Lay, E., Ho, P.L., *et al.* (2014). Blocking PGE2-induced tumour repopulation abrogates bladder cancer chemoresistance. *Nature* *517*, 209-213.

Lamm, M.L.G., Catbagan, W.S., Laciak, R.J., Barnett, D.H., Hebner, C.M., Gaffield, W., Walterhouse, D., Iannaccone, P., and Bushman, W. (2002). Sonic hedgehog activates mesenchymal Gli1 expression during prostate ductal bud formation. *Developmental Biology* *249*, 349-366.

Lapidot, T., Pflumio, F., Doedens, M., Murdoch, B., Williams, D.E., and Dick, J.E. (1992). Cytokine stimulation of multilineage hematopoiesis from immature human cells engrafted in SCID mice. *Science* *255*, 1137-1141.

Lapidot, T., Sirard, C., Vormoor, J., Murdoch, B., Hoang, T., Caceres-Cortes, J., Minden, M., Paterson, B., Caligiuri, M.A., and Dick, J.E. (1994). A cell initiating human acute myeloid leukaemia after transplantation into SCID mice. *Nature* *367*, 645-648.

Larochelle, A., Vormoor, J., Hanenberg, H., Wang, J.C.Y., Bhatia, M., Lapidot, T., Moritz, T., Murdoch, B., Xiao, X.L., Kato, I., *et al.* (1996). Identification of primitive human hematopoietic cells capable of repopulating NOD/SCID mouse bone marrow: Implications for gene therapy. *Nature Medicine* 2, 1329-1337.

Larson, R.A. (2007). Etiology and management of therapy-related myeloid leukemia. *American Society of Hematology Education Program*, 1, 453-459.

Lassailly, F., Foster, K., Lopez-Onieva, L., Currie, E., and Bonnet, D. (2013). Multimodal imaging reveals structural and functional heterogeneity in different bone marrow compartments: functional implications on hematopoietic stem cells. *Blood* 122, 1730-1740.

Lemoli, R.M., and D'Addio, A. (2008). Hematopoietic stem cell mobilization. *Haematologica* 93, 321-324.

Levine, J.H., Simonds, E.F., Bendall, S.C., Davis, K.L., Amir, E.-A.D., Tadmor, M.D., Litvin, O., Fienberg, H.G., Jager, A., Zunder, E.R., *et al.* (2015). Data-driven phenotypic dissection of AML reveals progenitor-like cells that correlate with prognosis. *Cell* 162, 184-197.

Lin, T., Jones, R.J., and Matsui, W. (2009). Cancer stem cells: Relevance to SCT. *Bone Marrow Transplantation* 43, 517-523.

Lo Celso, C., Fleming, H.E., Wu, J.W., Zhao, C.X., Miake-Lye, S., Fujisaki, J., Cote, D., Rowe, D.W., Lin, C.P., and Scadden, D.T. (2009). Live-animal tracking of individual haematopoietic stem/progenitor cells in their niche. *Nature* 457, 92-96.

Lord, B.I., Testa, N.G., and Hendry, J.H. (1975). The relative spatial distributions of CFUs and CFUc in the normal mouse femur. *Blood* 46, 65-72.

Löwenberg, B., and De Zeeuw, H.M. (1979). A method for cloning T-lymphocytic precursors in agar. *American Journal of Hematology* 6, 35-43.

Lymperi, S., Ersek, A., Ferraro, F., Dazzi, F., and Horwood, N.J. (2011). Inhibition of osteoclast function reduces hematopoietic stem cell numbers in vivo. *Blood* 117, 1540-1549.

Majeti, R., Becker, M.W., Tian, Q., Lee, T.L., Yan, X., Liu, R., Chiang, J.H., Hood, L., Clarke, M.F., and Weissman, I.L. (2009). Dysregulated gene expression networks in human acute myelogenous leukemia stem cells. *Proceedings of the National Academy of Sciences* 106, 3396-3401.

Marie, J.P., Izaguirre, C.A., Civin, C.I., Mirro, J., and McCulloch, E.A. (1981). Granulopoietic differentiation in AML blasts in culture. *Blood* *58*, 670-674.

Martelli, M.P., Pettirossi, V., Thiede, C., Bonifacio, E., Mezzasoma, F., Cecchini, D., Pacini, R., Tabarrini, A., Ciurnelli, R., Gionfriddo, I., *et al.* (2010). CD34+ cells from AML with mutated NPM1 harbor cytoplasmic mutated nucleophosmin and generate leukemia in immunocompromised mice. *Blood* *116*, 3907-3922.

Matsunaga, T., Takemoto, N., Sato, T., Takimoto, R., Tanaka, I., Fujimi, A., Akiyama, T., Kuroda, H., Kawano, Y., Kobune, M., *et al.* (2003). Interaction between leukemic-cell VLA-4 and stromal fibronectin is a decisive factor for minimal residual disease of acute myelogenous leukemia. *Nature Medicine* *9*, 1158-1165.

Matsuzaki, Y., Kinjo, K., Mulligan, R.C., and Okano, H. (2004). Unexpectedly efficient homing capacity of purified murine hematopoietic stem cells. *Immunity* *20*, 87-93.

Mayani, H., Flores-Figueroa, E., and Chávez-González, A. (2009). In vitro biology of human myeloid leukemia. *Leukemia Research* *33*, 624-637.

McCulloch, E.A. (1983). Stem cells in normal and leukemic hemopoiesis (Henry Stratton Lecture, 1982). *Blood* *62*, 1-13.

McCune, J.M., Namikawa, R., Kaneshima, H., Shultz, L.D., Lieberman, M., and Weissman, I.L. (1988). The SCID-hu mouse: Murine model for the analysis of human hematolymphoid differentiation and function. *Science* *241*, 1632-1639.

Medema, J.P. (2013). Cancer stem cells: The challenges ahead. *Nature Cell Biology* *15*, 338-344.

Medvinsky, A., and Dzierzak, E. (1996). Definitive hematopoiesis is autonomously initiated by the AGM region. *Cell* *86*, 897-906.

Medyouf, H., Mossner, M., Jann, J.-C., Nolte, F., Raffel, S., Herrmann, C., Lier, A., Eisen, C., Nowak, V., Zens, B., *et al.* (2014). Myelodysplastic cells in patients reprogram mesenchymal stromal cells to establish a transplantable stem cell niche disease unit. *Cell Stem Cell* *14*, 824-837.

Mendelson, A., and Frenette, P.S. (2014). Hematopoietic stem cell niche maintenance during homeostasis and regeneration. *Nature Medicine* *20*, 833-846.

Méndez-Ferrer, S., and Frenette, P.S. (2007). Hematopoietic stem cell trafficking: Regulated adhesion and attraction to bone marrow microenvironment. *Annals of the New York Academy of Sciences* *1116*, 392-413.

Mendez-Ferrer, S., Lucas, D., Battista, M., and Frenette, P.S. (2008). Haematopoietic stem cell release is regulated by circadian oscillations. *Nature* *452*, 442-447.

Méndez-Ferrer, S., Michurina, T.V., Ferraro, F., Mazloom, A.R., Macarthur, B.D., Lira, S.A., Scadden, D.T., Ma'ayan, A., Enikolopov, G.N., and Frenette, P.S. (2010). Mesenchymal and haematopoietic stem cells form a unique bone marrow niche. *Nature* *466*, 829-834.

Merchant, A., Joseph, G., Wang, Q., Brennan, S., and Matsui, W. (2010). Gli1 regulates the proliferation and differentiation of HSCs and myeloid progenitors. *Blood* *115*, 2391-2396.

Merchant, A.A., and Matsui, W. (2010). Targeting hedgehog -- A cancer stem cell pathway. *Clinical Cancer Research* *16*, 3130-3140.

Meyers, C.A., Albitar, M., and Estey, E. (2005). Cognitive impairment, fatigue, and cytokine levels in patients with acute myelogenous leukemia or myelodysplastic syndrome. *Cancer* *104*, 788-793.

Mikkola, H.K.A., and Orkin, S.H. (2006). The journey of developing hematopoietic stem cells. *Development* *133*, 3733-3744.

Miller, P.H., Cheung, A.M.S., Beer, P.A., Knapp, D.J.H.F., Dhillon, K., Rabu, G., Rostamirad, S., Humphries, R.K., and Eaves, C.J. (2012). Enhanced normal short term human myelopoiesis in mice engineered to express human-specific myeloid growth factors. *Blood* *121* e1-e4.

Miraki-Moud, F., Anjos-Afonso, F., Hodby, K.A., Griessinger, E., Rosignoli, G., Lillington, D., Jia, L., Davies, J.K., Cavenagh, J., Smith, M., *et al.* (2013). Acute myeloid leukemia does not deplete normal hematopoietic stem cells but induces cytopenias by impeding their differentiation. *Proceedings of the National Academy of Sciences* *110*, 13576-13581.

Monaco, G., Konopleva, M., Munsell, M., Leysath, C., Wang, R.-Y., Jackson, C.E., Korbling, M., Estey, E., Belmont, J., and Andreeff, M. (2004). Engraftment of acute myeloid leukemia in NOD/SCID mice is independent of CXCR4 and predicts poor patient survival. *Stem Cells* *22*, 188-201.

Moore, M.A., and Metcalf, D. (1973). Cytogenetic analysis of human acute and chronic myeloid leukemic cells cloned in agar culture. *International Journal of Cancer* *11*, 143-152.

Mullighan, C.G., Phillips, L.A., Su, X., Ma, J., Miller, C.B., Shurtleff, S.A., and Downing, J.R. (2008). Genomic analysis of the clonal origins of relapsed acute lymphoblastic leukemia. *Science* 322, 1377-1380.

Murashige, N., Kami, M., and Takaue, Y. (2003). G-CSF priming in acute myelogenous leukemia. *New England Journal of Medicine* 349, 2071-2072

Nagasawa, T., Hirota, S., Tachibana, K., Takakura, N., Nishikawa, S., Kitamura, Y., Yoshida, N., Kikutani, H., and Kishimoto, T. (1996). Defects of B-cell lymphopoiesis and bone-marrow myelopoiesis in mice lacking the CXC chemokine PBSF/SDF-1. *Nature* 382, 635-638.

Nakada, D., Oguro, H., Levi, B.P., Ryan, N., Kitano, A., Saitoh, Y., Takeichi, M., Wendt, G.R., and Morrison, S.J. (2014). Oestrogen increases haematopoietic stem-cell self-renewal in females and during pregnancy. *Nature* 505, 555-558.

Naveiras, O., Nardi, V., Wenzel, P.L., Hauschka, P.V., Fahey, F., and Daley, G.Q. (2009). Bone-marrow adipocytes as negative regulators of the haematopoietic microenvironment. *Nature* 460, 259-263.

Navin, N., Kendall, J., Troge, J., Andrews, P., Rodgers, L., McIndoo, J., Cook, K., Stepansky, A., Levy, D., Esposito, D., *et al.* (2011). Tumour evolution inferred by single-cell sequencing. *Nature* 472, 90-94.

Nervi, B., Ramirez, P., Rettig, M.P., Uy, G.L., Holt, M.S., Ritchey, J.K., Prior, J.L., Piwnicka-Worms, D., Bridger, G., Ley, T.J., *et al.* (2009). Chemosensitization of acute myeloid leukemia (AML) following mobilization by the CXCR4 antagonist AMD3100. *Blood* 113, 6206-6214.

Nickels, E.M., Soodalter, J., Churpek, J.E., and Godley, L.A. (2013). Recognizing familial myeloid leukemia in adults. *Therapeutic Advances in Hematology* 4, 254-269.

Ninomiya, M., Abe, A., Katsumi, A., Xu, J., Ito, M., Arai, F., Suda, T., Ito, M., Kiyoi, H., Kinoshita, T., *et al.* (2006). Homing, proliferation and survival sites of human leukemia cells in vivo in immunodeficient mice. *Leukemia* 21, 136-142.

Nombela-Arrieta, C., Pivarnik, G., Winkel, B., Canty, K.J., Harley, B., Mahoney, J.E., Park, S.Y., Lu, J., Protopopov, A., and Silberstein, L.E. (2013). Quantitative imaging of haematopoietic stem and progenitor cell localization and hypoxic status in the bone marrow microenvironment. *Nature Cell Biology* 15, 533-543.

Notta, F., Doulatov, S., Laurenti, E., Poepl, A., Jurisica, I., and Dick, J.E. (2011). Isolation of single human hematopoietic stem cells capable of long-term multilineage engraftment. *Science* 333, 218-221.

Olson, T.S., Caselli, A., Otsuru, S., Hofmann, T.J., Williams, R., Paolucci, P., Dominici, M., and Horwitz, E.M. (2013). Megakaryocytes promote murine osteoblastic HSC niche expansion and stem cell engraftment after radioablative conditioning. *Blood* 121, 5238-5249.

Pabst, C., Krosl, J., Fares, I., Boucher, G., Ruel, R., Marinier, A., Lemieux, S., Hébert, J., and Sauvageau, G. (2014). Identification of small molecules that support human leukemia stem cell activity ex vivo. *Nature Methods* 11, 436-442.

Parkin, B., Ouillette, P., Li, Y., Keller, J., Lam, C., Roulston, D., Li, C., Shedden, K., and Malek, S.N. (2013). Clonal evolution and devolution after chemotherapy in adult acute myelogenous leukemia. *Blood* 121, 369-377.

Pearce, D.J., Taussig, D., Zibara, K., Smith, L.-L., Ridler, C.M., Preudhomme, C., Young, B.D., Rohatiner, A.Z., Lister, T.A., and Bonnet, D. (2006). AML engraftment in the NOD/SCID assay reflects the outcome of AML: Implications for our understanding of the heterogeneity of AML. *Blood* 107, 1166-1173.

Peng, T., Frank, D.B., Kadzik, R.S., Morley, M.P., Rathi, K.S., Wang, T., Zhou, S., Cheng, L., Lu, M.M., and Morrisey, E.E. (2015). Hedgehog actively maintains adult lung quiescence and regulates repair and regeneration. *Nature* 526, 578-582.

Petit, I., Szyper-Kravitz, M., Nagler, A., Lahav, M., Peled, A., Habler, L., Ponomaryov, T., Taichman, R.S., Arenzana-Seisdedos, F., Fujii, N., *et al.* (2002). G-CSF induces stem cell mobilization by decreasing bone marrow SDF-1 and up-regulating CXCR4. *Nature Immunology* 3, 687-694.

Pike, B.L., and Robinson, W.A. (1970). Human bone marrow colony growth in agar-gel. *Journal of Cellular Physiology* 76, 77-84.

Plaks, V., Kong, N., and Werb, Z. (2015). The cancer stem cell niche: How essential is the niche in regulating stemness of tumor cells? *Cell Stem Cell* 16, 225-238.

Pluznik, D.H., and Sachs, L. (1965). The cloning of normal "mast" cells in tissue culture. *Journal of Cellular Physiology* 66, 319-324.

Pollyea, D.A., Gutman, J.A., Gore, L., Smith, C.A., and Jordan, C.T. (2014). Targeting acute myeloid leukemia stem cells: a review and principles for the development of clinical trials. *Haematologica* 99, 1277-1284.

Porwit, A., and Saft, L. (2011). The AML–MDS interface—leukemic transformation in myelodysplastic syndromes. *Journal of Hematopathology* 4, 69-79.

Protin, U., Schweighoffer, T., Jochum, W., and Hilberg, F. (1999). CD44-deficient mice develop normally with changes in subpopulations and recirculation of lymphocyte subsets. *Journal of Immunology* 163, 4917-4923.

Quintana, E., Shackleton, M., Sabel, M.S., Fullen, D.R., Johnson, T.M., and Morrison, S.J. (2008). Efficient tumour formation by single human melanoma cells. *Nature* 456, 593-598.

Ramasamy, R., Lam, E.W.F., Soeiro, I., Tisato, V., Bonnet, D., and Dazzi, F. (2007). Mesenchymal stem cells inhibit proliferation and apoptosis of tumor cells: impact on in vivo tumor growth. *Leukemia* 21, 304-310.

Rouchi, A.H., and Mahdavi-Mazdeh, M. (2015). Regenerative medicine in organ and tissue transplantation: Shortly and practically achievable? *International Journal of Organ Transplantation Medicine* 6, 93-98.

Rowe, J.M. (2009). Optimal induction and post-remission therapy for AML in first remission. *American Society of Hematology Education Program*, 396-405.

Rowe, J.M., and Tallman, M.S. (2010). How I treat acute myeloid leukemia. *Blood* 116, 3147-3156.

Saito, Y., Kitamura, H., Hijikata, A., Tomizawa-Murasawa, M., Tanaka, S., Takagi, S., Uchida, N., Suzuki, N., Sone, A., Najima, Y., *et al.* (2010a). Identification of Therapeutic Targets for Quiescent, Chemotherapy-Resistant Human Leukemia Stem Cells. *Science Translational Medicine* 2, 17ra19.

Saito, Y., Uchida, N., Tanaka, S., Suzuki, N., Tomizawa-Murasawa, M., Sone, A., Najima, Y., Takagi, S., Aoki, Y., Wake, A., *et al.* (2010b). Induction of cell cycle entry eliminates human leukemia stem cells in a mouse model of AML. *Nature Biotechnology*, 28, 275-280.

Samant, R.S., Fox, G.W., Gerig, L.H., Montgomery, L.A., and Allan, D.S. (2009). Total scalp radiation using image-guided IMRT for progressive cutaneous T cell lymphoma. *The British Journal of Radiology* 82, e122-125.

Sanchez, P.V., Perry, R.L., Sarry, J.E., Perl, A.E., Murphy, K., Swider, C.R., Bagg, A., Choi, J.K., Biegel, J.A., Danet-Desnoyers, G., *et al.* (2009). A robust xenotransplantation model for acute myeloid leukemia. *Leukemia* 23, 2109-2117.

Sarry, J.-E., Murphy, K., Perry, R., Sanchez, P.V., Secreto, A., Keefer, C., Swider, C.R., Strzelecki, A.-C., Cavelier, C., Récher, C., *et al.* (2011). Human acute myelogenous leukemia stem cells are rare and heterogeneous when assayed in NOD/SCID/IL2R γ -deficient mice. *Journal of Clinical Investigation* *121*, 384-395.

Sausville, E.A., and Burger, A.M. (2006). Contributions of human tumor xenografts to anticancer drug development. *Cancer Research* *66*, 3351-3354.

Sawyers, C.L., Gishizky, M.L., Quan, S., Golde, D.W., and Witte, O.N. (1992). Propagation of human blastic myeloid leukemias in the SCID mouse. *Blood* *79*, 2089-2098.

Scadden, D.T. (2014). Nice neighborhood: Emerging concepts of the stem cell niche. *Cell* *157*, 41-50.

Schepers, A.G., Snippert, H.J., Stange, D.E., van den Born, M., van Es, J.H., van de Wetering, M., and Clevers, H. (2012). Lineage tracing reveals Lgr5+ stem cell activity in mouse intestinal adenomas. *Science* *337*, 730-735.

Schepers, K., Pietras, E.M., Reynaud, D., Flach, J., Binnewies, M., Garg, T., Wagers, A.J., Hsiao, E.C., and Passegué, E. (2013). Myeloproliferative Neoplasia Remodels the Endosteal Bone Marrow Niche into a Self-Reinforcing Leukemic Niche. *Cell Stem Cell* *13*, 285-299.

Schofield, R. (1978). The relationship between the spleen colony-forming cell and the haemopoietic stem cell. *Blood Cells* *4*, 7-25.

Semerad, C.L., Christopher, M.J., Liu, F., Short, B., Simmons, P.J., Winkler, I., Levesque, J.-P., Chappel, J., Ross, F.P., and Link, D.C. (2005). G-CSF potently inhibits osteoblast activity and CXCL12 mRNA expression in the bone marrow. *Blood* *106*, 3020-3027.

Shih, A.H., and Levine, R.L. (2011). Molecular biology of myelodysplastic syndromes. *Semin Oncol* *38*, 613-620.

Shin, K., Lee, J., Guo, N., Kim, J., Lim, A., Qu, L., Mysorekar, I.U., and Beachy, P.A. (2011). Hedgehog/Wnt feedback supports regenerative proliferation of epithelial stem cells in bladder. *Nature* *472*, 110-114.

Shiozawa, Y., Pedersen, E.A., Havens, A.M., Jung, Y., Mishra, A., Joseph, J., Kim, J.K., Patel, L.R., Ying, C., Ziegler, A.M., *et al.* (2011). Human prostate cancer metastases target the hematopoietic stem cell niche to establish footholds in mouse bone marrow. *Journal of Clinical Investigation* *121*, 1298-1312.

Shlush, L.I., Zandi, S., Mitchell, A., Chen, W.C., Brandwein, J.M., Gupta, V., Kennedy, J.A., Schimmer, A.D., Schuh, A.C., Yee, K.W., *et al.* (2014). Identification of pre-leukaemic haematopoietic stem cells in acute leukaemia. *Nature* *506*, 328-333.

Shultz, L.D., Schweitzer, P.A., Christianson, S.W., Gott, B., Schweitzer, I.B., Tennent, B., McKenna, S., Mobraaten, L., Rajan, T.V., and Greiner, D.L. (1995). Multiple defects in innate and adaptive immunologic function in NOD/LtSz-scid mice. *The Journal of Immunology* *154*, 180-191.

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

Siminovitch, L., McCulloch, E.A., and Till, J.E. (1963). The distribution of colony-forming cells among spleen colonies. *Journal of Cellular Physiology* *62*, 327-336.

Singh, S.K., Hawkins, C., Clarke, I.D., Squire, J.A., Bayani, J., Hide, T., Henkelman, R.M., Cusimano, M.D., and Dirks, P.B. (2004). Identification of human brain tumour initiating cells. *Nature* *432*, 396-401.

Somervaille, T.C.P., and Cleary, M.L. (2006). Identification and characterization of leukemia stem cells in murine MLL-AF9 acute myeloid leukemia. *Cancer Cell* *10*, 257-268.

Southam, C.M., and Brunschwig, A. (1961). Quantitative studies of autotransplantation of human cancer. Preliminary report. *Cancer* *14*, 971-978.

Spangrude, G.J., Heimfeld, S., and Weissman, I.L. (1988). Purification and characterization of mouse hematopoietic stem cells. *Science* *241*, 58-62.

Stone, R.M. (2013). Acute myeloid leukemia in first remission: To choose transplantation or not? *Journal of Clinical Oncology* *31*, 1262-1266.

Suh, J.M., Gao, X., McKay, J., McKay, R., Salo, Z., and Graff, J.M. (2006). Hedgehog signaling plays a conserved role in inhibiting fat formation. *Cell Metabolism* *3*, 25-34.

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

Taussig, D.C., Vargaftig, J., Miraki-Moud, F., Griessinger, E., Sharrock, K., Luke, T., Lillington, D., Oakervee, H., Cavenagh, J., Agrawal, S.G., *et al.* (2010). Leukemia-initiating cells from some acute myeloid leukemia patients with mutated nucleophosmin reside in the CD34(-) fraction. *Blood* 115, 1976-1984.

Tavor, S. (2004). CXCR4 regulates migration and development of human acute myelogenous leukemia stem cells in transplanted NOD/SCID mice. *Cancer Research* 64, 2817-2824.

Terstappen, L.W., Huang, S., Safford, M., Lansdorp, P.M., and Loken, M.R. (1991). Sequential generations of hematopoietic colonies derived from single nonlineage-committed CD34+CD38- progenitor cells. *Blood* 77, 1218-1227.

Tian, H., Callahan, C.A., DuPree, K.J., Darbonne, W.C., Ahn, C.P., Scales, S.J., and de Sauvage, F.J. (2009). Hedgehog signaling is restricted to the stromal compartment during pancreatic carcinogenesis. *Proceedings of the National Academy of Sciences* 106, 4254-4259.

Till, J.E., and McCulloch, E.A. (1961). A direct measurement of the radiation sensitivity of normal mouse bone marrow cells. *Radiation Research* 14, 213-222.

Trowbridge, J.J., Scott, M.P., and Bhatia, M. (2006). Hedgehog modulates cell cycle regulators in stem cells to control hematopoietic regeneration. *Proceedings of the National Academy of Sciences* 103, 14134-14139.

van Rhenen, A., van Dongen, G.A.M.S., Kelder, A., Rombouts, E.J., Feller, N., Moshaver, B., Walsum, M.S.v., Zweegman, S., Ossenkoppele, G.J., and Jan Schuurhuis, G. (2007). The novel AML stem cell associated antigen CLL-1 aids in discrimination between normal and leukemic stem cells. *Blood* 110, 2659-2666.

Vardiman, J.W., Harris, N.L., and Brunning, R.D. (2002). The World Health Organization (WHO) classification of the myeloid neoplasms. *Blood* 100, 2292-2302.

Vardiman, J.W., Thiele, J., Arber, D.A., Brunning, R.D., Borowitz, M.J., Porwit, A., Harris, N.L., Le Beau, M.M., Hellström-Lindberg, E., Tefferi, A., *et al.* (2009). The 2008 revision of the World Health Organization (WHO) classification of myeloid neoplasms and acute leukemia: rationale and important changes. *Blood* 114, 937-951.

Vargaftig, J., Taussig, D.C., Griessinger, E., Anjos-Afonso, F., Lister, T.A., Cavenagh, J., Oakervee, H., Gribben, J., and Bonnet, D. (2011). Frequency of leukemic initiating cells does not depend on the xenotransplantation model used. *Leukemia* 26, 858-860.

Verovskaya, E., Broekhuis, M.J.C., Zwart, E., Weersing, E., Ritsema, M., Bosman, L.J., van Poele, T., de Haan, G., and Bystrykh, L.V. (2014). Asymmetry in skeletal distribution of mouse hematopoietic stem cell clones and their equilibration by mobilizing cytokines. *Journal of Experimental Medicine* 211, 487-497.

Walasek, M.A., van Os, R., and de Haan, G. (2012). Hematopoietic stem cell expansion: Challenges and opportunities. *Annals of the New York Academy of Sciences* 1266, 138-150.

Wang, J.C., Doedens, M., and Dick, J.E. (1997). Primitive human hematopoietic cells are enriched in cord blood compared with adult bone marrow or mobilized peripheral blood as measured by the quantitative in vivo SCID-repopulating cell assay. *Blood* 89, 3919-3924.

Williams, D.A. (1993). Ex vivo expansion of hematopoietic stem and progenitor cells-robbing Peter to pay Paul? *Blood* 81, 3169-3172.

Wilson, A., Laurenti, E., Oser, G., van der Wath, R.C., Blanco-Bose, W., Jaworski, M., Offner, S., Dunant, C.F., Eshkind, L., Bockamp, E., *et al.* (2008). Hematopoietic stem cells reversibly switch from dormancy to self-renewal during homeostasis and repair. *Cell* 135, 1118-1129.

Winkler, I.G., Sims, N.A., Pettit, A.R., Barbier, V., Nowlan, B., Helwani, F., Poulton, I.J., Van Rooijen, N., Alexander, K.A., Raggatt, L.J., *et al.* (2010). Bone marrow macrophages maintain hematopoietic stem cell (HSC) niches and their depletion mobilizes HSCs. *Blood* 116, 4815-4828.

Wong, J.Y.C., Liu, A., Schultheiss, T., Popplewell, L., Stein, A., Rosenthal, J., Essensten, M., Forman, S., and Somlo, G. (2006). Targeted total marrow irradiation using three-dimensional image-guided tomographic intensity-modulated radiation therapy: an alternative to standard total body irradiation. *Biology of Blood and Marrow Transplantation* 12, 306-315.

Xavier, A.C., Ge, Y., and Taub, J.W. (2009). Down syndrome and malignancies: a unique clinical relationship: A paper from the 2008 william beaumont hospital symposium on molecular pathology. *The Journal of Molecular Diagnostics* 11, 371-380.

Xie, M., Lu, C., Wang, J., McLellan, M.D., Johnson, K.J., Wendl, M.C., McMichael, J.F., Schmidt, H.K., Yellapantula, V., Miller, C.A., *et al.* (2014). Age-related mutations associated with clonal hematopoietic expansion and malignancies. *Nature Medicine* 20 1472-1478.

Xie, Y., Yin, T., Wiegnaebe, W., He, X.C., Miller, D., Stark, D., Perko, K., Alexander, R., Schwartz, J., Grindley, J.C., *et al.* (2009). Detection of functional haematopoietic stem cell niche using real-time imaging. *Nature* 457, 97-101.

Yamazaki, S., Ema, H., Karlsson, G., Yamaguchi, T., Miyoshi, H., Shioda, S., Taketo, M.M., Karlsson, S., Iwama, A., and Nakauchi, H. (2011). Nonmyelinating Schwann cells maintain hematopoietic stem cell hibernation in the bone marrow niche. *Cell* 147, 1146-1158.

Yauch, R.L., Gould, S.E., Scales, S.J., Tang, T., Tian, H., Ahn, C.P., Marshall, D., Fu, L., Januario, T., Kallop, D., *et al.* (2008). A paracrine requirement for hedgehog signalling in cancer. *Nature* 455, 406-410.

Zhang, G., Li, J., Purkayastha, S., Tang, Y., Zhang, H., Yin, Y., Li, B., Liu, G., and Cai, D. (2013). Hypothalamic programming of systemic ageing involving IKK- β , NF- κ B and GnRH. *Nature* 497, 211-216.

Zhang, J., Niu, C., Ye, L., Huang, H., He, X., Tong, W.G., Ross, J., Haug, J., Johnson, T., Feng, J.Q., *et al.* (2003). Identification of the haematopoietic stem cell niche and control of the niche size. *Nature* 425, 836-841.

Zhao, C., Chen, A., Jamieson, C.H., Fereshteh, M., Abrahamsson, A., Blum, J., Kwon, H.Y., Kim, J., Chute, J.P., Rizzieri, D., *et al.* (2009). Hedgehog signalling is essential for maintenance of cancer stem cells in myeloid leukaemia. *Nature* 458, 776-779.

Zheng, J., Huynh, H., Umikawa, M., Silvany, R., and Zhang, C.C. (2011). Angiopoietin-like protein 3 supports the activity of hematopoietic stem cells in the bone marrow niche. *Blood* 117, 470-479.

Zipori, D. (2014). Self-renewal, induced proliferation, and autonomous cell growth represent distinct modes of cell multiplication: Relevance to the cancer stem Cell theory. In *Cancer Stem Cells*, V.K. Rajasekhar, ed. (Wiley Blackwell).

Zou, Y.R., Kottmann, A.H., Kuroda, M., Taniuchi, I., and Littman, D.R. (1998). Function of the chemokine receptor CXCR4 in haematopoiesis and in cerebellar development. *Nature* 393, 595-599.

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Appendix III: List of Scientific Publications

Published Refereed Papers

Boyd, A. L., Salci, K. R., Shapovalova, Z., McIntyre, B. A. S., & Bhatia, M. (2013). Nonhematopoietic cells represent a more rational target of in vivo hedgehog signaling affecting normal or acute myeloid leukemia progenitors. *Experimental Hematology*. doi:10.1016/j.exphem.2013.05.287

Guezguez, B., Campbell, C. J. V., **Boyd, A. L.**, Karanu, F., Casado, F. L., Di Cresce, C., Collins, T. J., Zhapovalova, Z., Xenocostas, A., Bhatia, M. (2013). Regional localization within the bone marrow influences the functional capacity of human HSCs. *Cell Stem Cell*, 13(2), 175–189. doi:10.1016/j.stem.2013.06.015

Boyd, A. L., & Bhatia, M. (2014). Bone marrow localization and functional properties of human hematopoietic stem cells. *Current Opinion in Hematology*, 21(4), 249–255. doi:10.1097/MOH.0000000000000055

Boyd, A. L., Campbell, C. J. V., Hopkins, C. I., Fiebig-Comyn, A., Russell, J., Ulemek, J., Foley, R., Leber, B., Xenocostas, A., Collins, T., Bhatia, M. (2014). Niche displacement of human leukemic stem cells uniquely allows their competitive replacement with healthy HSPCs. *Journal of Experimental Medicine*, 211(10), 1925–1935. doi:10.1016/j.ymthe.2004.07.029

Benoit, Y. D., Guezguez, B., **Boyd, A. L.**, & Bhatia, M. (2014). Molecular pathways: epigenetic modulation of Wnt-glycogen synthase kinase-3 signaling to target human cancer stem cells. *Clinical Cancer Research : an Official Journal of the American Association for Cancer Research*, 20(21), 5372–5378. doi:10.1158/1078-0432.CCR-13-2491

Boyd, A. L., & Bhatia, M. (2015) Playing musical chairs with bone marrow transplantation to eliminate leukemia stem cells, *Molecular & Cellular Oncology*, 2:3, e988480, DOI: 10.4161/23723556.2014.988480

Manuscript under review

Boyd, A. L.*, Aslostovar, L.*, Fiebig-Comyn, A., Shapovalova, Z., Almakadi, M., Reid, J., Casado, C. L., Salci, K. R., Xenocostas, A., Foley, R., Leber, B., and Bhatia, M. Cellular and Molecular Targeting of Recurrence in Acute Myeloid Leukemia.

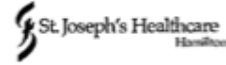
Manuscripts awaiting submission

Benoit, Y. D.*, Mitchell, R. R.*, Risueño, R. M.*, Schnerch, A., **Boyd, A. L.**, Aslostovar, L. May, L., Nakanishi, M., Shapovalova, Z., Dang, U. M., Eguchi, M., Graham, M., Fiebig-Comyn, A., Jones, M., Xenocostas, A., Trus, M. R., Leber, B., Collins, T. J., Szabo, E., Bhatia, M. Sam68-mediated disruption of CBP/ β -catenin neoplastic transcriptional programming allows selectivity against human cancer stem cells

Aslostovar, L., Benoit, Y. D., **Boyd, A. L.**, Casado, F. L., Reid, J., Tanasijevic, B., Siddiqui, A. H., Mitchell, R. R., Jamie McNicol, J. and Bhatia, M. Immunotargeting of Dopamine Receptor 1 Induces Functional Differentiation of Leukemic Progenitors

*Equal contribution

Appendix IV: Ethics Approvals



Hamilton Integrated Research Ethics Board RENEWAL FORM *Review of an Active Study (to be completed by HIREB Chair only)*

REB Project #: 08-042-T

Principal Investigator: Dr. Michael Trus

Project Title: Stem Cell and Cancer Research Institute-Live Cell Bank

Approved for Continuation

Approved conditional on changes noted in "Conditions" section below

Type of Approval:

Full Research Ethics Board

Research Ethics Board Executive

REB Approval Period: Approval period covers January 17-2015 to January 17-2016

New Enrolment Suspended

Suspended pending further review

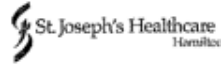
Conditions:

The Hamilton Integrated Research Ethics Board operates in compliance with and is constituted in accordance with the requirements of: The Tri-Council Policy Statement on Ethical Conduct of Research Involving Humans; The International Conference on Harmonization of Good Clinical Practices; Part C Division 5 of the Food and Drug Regulations of Health Canada, and the provisions of the Ontario Personal Health Information Protection Act 2004 and its applicable Regulations; for studies conducted at St. Joseph's Hospital, HIREB complies with the health ethics guide of the Catholic Alliance of Canada.


Suzette Salama PhD., Chair
Raelene Rathbone, MB BS, MD, PhD, Chair

2/4/2015
Date of REB Meeting

All Correspondence should be addressed to the HIREB Chair(s) and forwarded to:
HIREB Coordinator
293 Wellington St. N, Suite 102, Hamilton ON L8L 6E7
Tel. 905-521-2100 Ext. 42013 Fax: 905-577-8378



**Hamilton Integrated Research Ethics Board
RENEWAL FORM**

Review of an Active Study (to be completed by HIREB Chair only)

REB Project #: 08-330

Principal Investigator: Dr. Mickie Bhatia

Project Title: Characterization of Mesodermal Factors in Hematopoietic Stem Cell

Approved for Continuation

Approved conditional on changes noted in "Conditions" section below

Type of Approval:

Full Research Ethics Board

Research Ethics Board Executive

REB Approval Period: Approval period covers July 15-2015 to July 15-2016

New Enrolment Suspended

Suspended pending further review

Conditions:

The Hamilton Integrated Research Ethics Board operates in compliance with and is constituted in accordance with the requirements of: The Tri-Council Policy Statement on Ethical Conduct of Research Involving Humans; The International Conference on Harmonization of Good Clinical Practices; Part C Division 5 of the Food and Drug Regulations of Health Canada, and the provisions of the Ontario Personal Health Information Protection Act 2004 and its applicable Regulations; for studies conducted at St. Joseph's Hospital, HIREB complies with the health ethics guide of the Catholic Alliance of Canada.



Suzette Salama PhD., Chair
Raelene Rathbone, MB BS, MD, PhD, Chair

7/8/2015

Date of REB Meeting

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University Veterinarian's Signature: *Kallan Duff* Date: *April 3/13* AREB Chair's Signature: *B. Peregath* Date: *April 3/13*

Principal Investigator's Signature: *[Signature]* Date: *MAR 21/2013*

Prepared By:
Name:
Email Address:

Refer to the Guide for Preparation of AUPs for assistance with completing this form (available at http://www.fhs.mcmaster.ca/healthresearch/areb_forms.html)

Section 1 Project Title

Title:

Type of Project: Research New Project Research Pilot Study Other

Teaching* Ongoing Project

*Attach Teaching Addendum available at http://www.fhs.mcmaster.ca/healthresearch/areb_forms.html.

Section 2 Principal Investigator

Last Name: First Name:

Title: Department:

Business Phone: Home Phone:

Emergency Phone: Pager Number:

Laboratory Room #: Laboratory Phone:

Institutional Email:

Mailing Address:

PI Training: OR AH Inj An Gas An SS IM TN EP BL Bnd Bio

Experience and/or other training regulatory update: