

Chapter 13

Leukemic and Lymphoid Stem Cells

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Abstract Cancer cell heterogeneity is a feature of nearly all cancers and can be related to three major influences: (1) genetic and epigenetic heterogeneity due to clonal evolution/collapse; (2) microenvironmental influences; and (3) the underlying tissue hierarchy from which the tumor arises. Ongoing studies in whole genome sequencing and of the bone marrow, splenic and lymph node microenvironments demonstrate their contributions to tumor heterogeneity. In this chapter we will focus on the role of the hematopoietic hierarchy in blood cancer cellular heterogeneity; one of the most studied systems in mouse and human. Observations that myeloid and lymphoid malignancies harbor rare relatively quiescent therapy resistant cell populations date back over 30 years. Early studies in chronic myelogenous leukemia were consistent with a disease origin in the hematopoietic stem cell and subsequent studies have confirmed these findings. The publication by Bonnet et al. in 1994 offered the first prospective assessment of human cancer stem cell populations and established acute myeloid leukemia (AML) as a model system. In the intervening years, new technologies have allowed a continued reassessment of cancer stem cell populations in AML, myelodysplastic syndromes, multiple myeloma and acute lymphoblastic leukemia. While these studies have confirmed the existence of the rare cells capable of recapitulating the malignancy on transplantation, they have also identified considerable inter- and intra-patient heterogeneity with conflicting results on the ability to identify potential cancer stem cells using surface antigen profiles alone. Novel xenotransplantation models, whole genome sequencing and other technologies offer the tools to further refine this model in hematologic malignancies and develop rational therapies to target leukemia stem cells.

Keywords Hematopoietic stem cells • Leukemic stem cells • Pre-leukemic stem cells • Chronic myelogenous leukemia • Acute myelogenous leukemia • Acute lymphoblastic leukemia • Multiple myeloma • Myelodysplastic syndromes

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1 Introduction

Normal hematopoiesis relies on a highly regulated multi-tier hierarchy with a pool of rare, largely quiescent hematopoietic stem cells (HSCs) at the base (Rieger and Schroeder 2012; Morrison and Weissman 1994). Downstream are oligo-potent, lineage restricted progenitors with a capacity for robust proliferation in response to stress and terminally differentiated functional elements (Fig. 13.1). In hematopoiesis, the capacity for self-renewal is confined to mainly quiescent populations and is tightly regulated through cell autonomous and non-autonomous signals. Malignancies of the hematopoietic system are similarly heterogeneous and studies over several decades have consistently demonstrated that the capacity to repopulate the tumor on serial transplantation is confined to a rare population of cancer cells with the capacity to self-renew. Chronic phase CML is characterized by a limited mutation burden, a consistent leukemic stem cell (LSC) phenotype and an effective

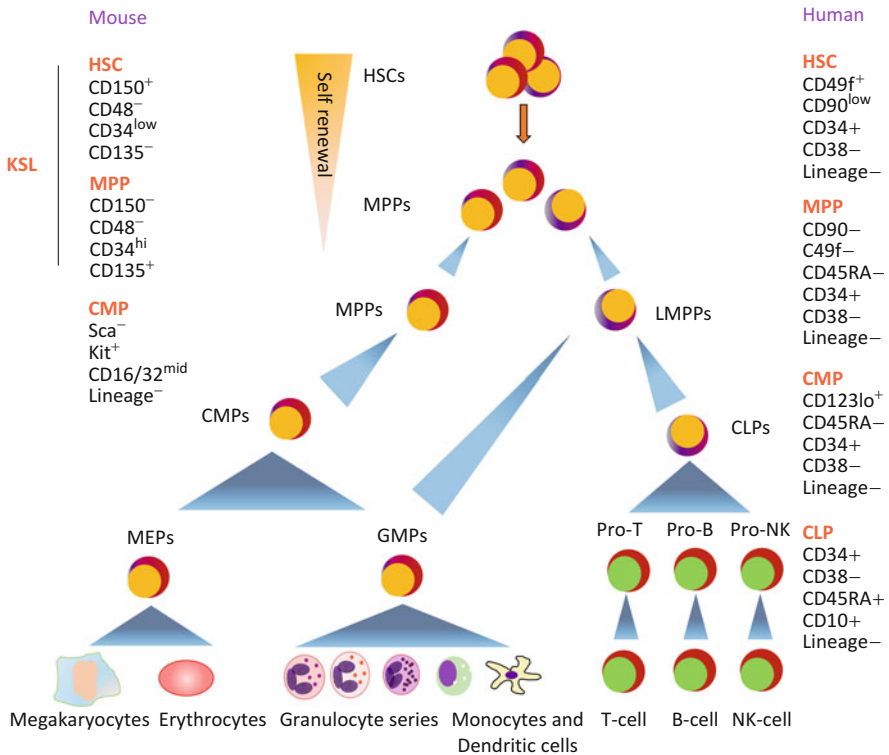


Fig. 13.1 Summary of the stages of normal hematopoiesis and their surface antigen phenotype(s) for HSCs and early progenitors for both mouse and human

therapy. In contrast, acute myelogenous leukemia (AML), myelodysplastic syndromes (MDS), B-cell acute lymphoblastic leukemia (B-ALL) and multiple myeloma (MM) result from multiple genetic and epigenetic events with increasing diversity in the cancer stem cell phenotypes. Studies of healthy aging donors as well as patients in remission have identified some of the potential early events and have identified possible limitations of targeting CSCs. Adding to the complexities, studies in human B-cell malignancies call into question the unidirectional nature of transitions between levels of the hierarchies.

2 Hematopoietic Stem Cells and Normal Hematopoiesis

2.1 Assays for the Study of Normal and Malignant Hematopoiesis

Study of normal and malignant hematopoiesis has been aided by a number of assays (Table 13.1). Commercially available standardized reagents and protocols permit comparison of results across labs. Historically suspension culture of HSC and early progenitor pools for more than a few days led to irreversible commitment and loss of stem cell function. Novel in vitro culture conditions extend the period of time prior to loss of HSC function and allow HSC expansion (Delaney et al. 2010). An adaption of the methylcellulose colony forming assays involves serial replating of methylcellulose colonies and serves as a surrogate marker for proliferative capacity with the number of replatings before CFU activity is extinguished related to self-renewal capacity. Cobblestone area forming (CAFC) and long term culture initiating cell (LTC-IC) assays allow the study of ST-HSC, MPPs and CMP/CLPs in vitro by co-culture with defined stroma. The readout for these assays is either scoring the number of CAFC beneath the stromal cell layer or transfer of hematopoietic cells into defined media methylcellulose to quantitate CFU activity. The CAFC assay has been adapted to allow large scale in vitro screening of potential LSC targeting molecules (Hartwell et al. 2013).

Table 13.1 List of assays commonly employed in the study of normal and malignant hematopoiesis

Assay	Cell population applicable
Immune phenotyping	All levels
Serum free suspension culture	HSC, progenitor level
Methyl cellulose/CFU assay	Progenitor level: multipotent or lineage restricted
LTC-IC/cobblestone forming assay	ST-HSC, MPP, CMP, CLP
Syngeneic/xenotransplantation	Lt-HSC, ST-HSC, MPP
Limiting dilution analysis (LDA)	HSC or LSC frequency

2.2 *Identification of Normal Murine and Human Hematopoietic Stem Cells*

A major strength of studying normal hematopoietic stem cell populations in mice is the availability of congenic strains that allow tracking donor and recipient contribution to the peripheral blood, spleen and marrow following transplantation. The C57bl/6 strain remains the standard for studying hematopoiesis. The cumulative results of mouse transplantation studies have resulted in a defined surface antigen phenotype that permits the transplantation of a single cell with hematopoietic reconstitution (Kiel et al. 2005) (Fig. 13.1).

Early models for human AML xenotransplantation suffered from an inability to work with small cell numbers, the need for cytokine supplementation and the impact of residual immune function. Increasingly immune deficient strains of mice have revolutionized the study of both normal and malignant hematopoiesis (Ito et al. 2002; Shultz et al. 2005). The NOG/NSG and also the NOD/SCID $\beta 2m^{null}$ mice are among the most immune deficient strains of inbred laboratory mice described to date and demonstrate greater permissiveness in human tissue engraftment. These strains have been compared head to head with the NOD/SCID strain and permit engraftment of a greater percentage of primary AML patient samples as well as allow a higher level of engraftment. Direct transplantation of cells into the femur of the recipient further increases the efficiency of transfer (McKenzie et al. 2005). It is now possible to perform single cell xenotransplantation studies of normal human HSCs (Notta et al. 2011). Models attempting to maximize the capacity for human xenotransplantation studies in NOD, C57BL/6 and Balb/c strains are ongoing (Iwamoto et al. 2014). As xenotransplantation models improve results from prior LSC studies are being re-evaluated with regard to the impact of the assay on the observed phenotypes.

Application of the aforementioned assays has allowed careful delineation of the hematopoietic hierarchy in mouse and man. While an in depth review of normal hematopoiesis is outside the limits of this chapter several concepts are worth mentioning. First, the HSC pool size and makeup are tightly regulated through cell autonomous as well as extrinsic mechanisms many of which are still poorly understood (Fig. 13.1). There remains a mostly unexplained age dependent heterogeneity in the HSC pool with variation in the composition, kinetics, and progeny outputs when analyzed using clonal marking studies (Jordan and Lemischka 1990; Dick et al. 1985) or transplantation of highly purified populations (Uchida et al. 2003; Ema et al. 2005). Second, the assays employed to characterize stem cell and progenitor populations remain dependent upon the demonstration of a capacity for tissue regeneration following transplantation. The increasingly recognized heterogeneity of the populations in Fig. 13.1 and the instability of the available phenotypes have impaired efforts to circumvent the need for functional validation, i.e. transplantation.

3 The Role of Malignant Stem Cells in Myeloid Malignancies

3.1 *Leukemic Stem Cells in CML*

Chronic myeloid leukemia (CML) is a myeloproliferative disorder that can exist in three distinct phases, chronic phase, accelerated phase, and blast crisis, which is phenotypically identical to acute leukemia. The defining lesion of this disease, the Philadelphia chromosome (Ph), encodes the BCR/ABL proto-oncogene and results in a constitutively active protein tyrosine kinase product, p210BCR-ABL (Nowell and Hungerford 1960; Rowley 1973; de Klein et al. 1982). This event targets the normal HSC compartment resulting in inappropriate expansion of the granulocytic lineage. The LSC has been studied extensively in all phases of this disease as well as in the remission state i.e. minimal residual disease (MRD). Hence, CML is a near perfect paradigm for understanding the leukemia stem cell model.

In 1977, Philip Fialkow and colleagues provided some of the first data that CML is a clonal stem cell disease (Fialkow et al. 1977). They studied glucose-6-phosphate dehydrogenase (G-6-PD) isoenzymes in the granulocytes of eight women with CML, and utilized X-linked polymorphisms to compare the enzyme types found in skin cells, normal granulocytes, and CML granulocytes. They found that the patients were heterozygous at the X-linked G-6-PD locus in the skin cells, but homozygous for G-6-PD enzyme type in the CML cells, as well as in the erythrocytes, platelets, and macrophages. This data suggested that these cells were derived from a common stem cell, i.e. the normal HSC compartment. Since this initial discovery and the works of others, the CML stem cell phenotype in chronic phase has now been defined as CD34⁺CD38⁻CD90⁺Lin⁻Thy1⁺ (Ahuja et al. 1989; Jorgensen and Holyoake 2007; Eisterer et al. 2005; Holyoake et al. 1999).

Jamieson and colleagues evaluated samples from blast crisis CML to demonstrate that in CML blast crisis, secondary genetic events target the cell populations with surface antigen and gene expression profiles resembling a committed progenitor, CD34⁺, CD38⁺lineage⁻, and result in the acquisition of unlimited proliferative and self-renewal capacity, mostly through activation of the β -catenin-signaling pathway (Jamieson et al. 2004). In a subsequent report, Abrahamsson et al. (2009) further identified glycogen synthase kinase 3 β missplicing events as contributors to expansion of self-renewal capacity to the GMP population. Follow-up studies have also identified a role for other mutations and pathways in the transition from chronic phase CML to blast crisis.

The CML blast crisis model suggests that while the “first hit” (i.e. acquisition of the oncogene BCR-ABL), targets the normal HSC, secondary genetic events target the committed progenitor pool and result in unlimited proliferative and self-renewal potential. The secondary events that occur in blast crisis CML are the first clear evidence from human cancer that secondary events can instill the critical stem cell property of self-renewal in a population of cells that is normally non-self-renewing.

The CML stem cell has been studied in the minimal residual disease state as well. Targeted therapy with tyrosine kinase inhibitors (TKIs), such as Imatinib, Dasatinib, Nilotinib, induces complete cytogenetic responses {i.e. no evidence of the (Ph) chromosome by FISH analysis} in more than 80 % of patients (Druker et al. 1996, 2006). The BCR-ABL transcript can still be detected by RT-PCR in most patients, however. In patients with undetectable transcript levels, approximately 60 % of patients will relapse when TKI therapy is discontinued (Rousselot et al. 2007). These clinical observations suggest that a quiescent LSC exists in CML and is resistant to TKI therapy and thus represents a reservoir for relapse of the disease. Corbin and colleagues examined how the LSC and progenitor cells survive during Imatinib therapy and asked whether the LSC is BCR-ABL dependent or independent. Utilizing a series of phosphorylation assays; the group demonstrated that the CML stem cells are not dependent on BCR-ABL activity for survival, and persist in patients with CML despite prolonged treatment with a TKI (Corbin et al. 2011). This field of research is active and focused on identifying the critical pathways of the residual CML leukemia cells.

3.2 *Leukemic Stem Cells in AML*

In 1994, Lapidot and colleagues published their work characterizing the leukemia initiating capacity of a sub-population of leukemic blasts (Lapidot et al. 1994). Using a SCID xenotransplantation model, they demonstrated that the ability to transplant human AML into primary and secondary recipients was confined to a population of cells defined by the expression of CD34 and the lack of expression of CD38 and markers of lineage commitment. This paper was one of many by the Dick lab examining the potential applications of xenotransplantation to the study of normal and malignant hematopoiesis. In the two decades since the report, the original phenotype has been extended as new antigens capable of enriching for LSC activities have been identified (Table 13.2). These studies have focused primarily on expanding the original CD34⁺CD38⁻ phenotype.

Beginning in 2008, studies by the Bonnet lab called into question the reliance of the LSC field on the CD34⁺CD38⁻ phenotype (Taussig et al. 2008, 2010). First, Taussig et al. demonstrated that pre-treatment of unfractionated normal and AML bone marrow cells with antibodies against human CD38 impaired normal and leukemic engraftment in sub-lethally irradiated NOD-SCID recipients (Taussig et al. 2008). Pre-treatment of recipients with an antibody against the interleukin-2 receptor β chain (CD122) partially restored human engraftment through elimination of residual NK cell activity. Transplantation of CD34⁺CD38⁻ and CD34⁺CD38⁺ populations from seven AML samples into NSG or NOD/SCID/ β 2m^{null} mice pretreated with IVIG or anti-CD122 demonstrated LSC activity in CD34⁺CD38⁺ cells in all cases of AML demonstrating the impact of the model on the cancer stem cell phenotype.

Table 13.2 List of surface antigens reported to identify leukemic stem cell populations in patient samples

Antigen		Model	HSC	LSC	References
CD34	Cell-cell adhesion factor	NOD/SCID	+	+	Lapidot et al. 1994
CD123	IL-3 receptor alpha chain	NOD/SCID	-	+	Jordan et al. 2000
CLL-1	C-type lectin-like molecule 1	NOD/SCID	-	+	Bakker et al. 2004
CD33	Siglec-3	NOD/SCID	+	++	Taussig et al. 2005
CD47	Integrin-associated protein	NOG	+	++	Majeti 2011
CD25	Interleukin 2 receptor, alpha	NOG	-	+	Ishikawa et al. 2007
CD96	T-cell activated increased late protein	Rag2 ^{-/-} γc ^{-/-}	+	++	Hosen et al. 2007
CD32	Fc fragment of IgG, low affinity, IIa receptor	NOG	-	+	Ishikawa et al. 2007
CD45RA	Leukocyte common antigen (LCA)	NOD/SCID and NSG	-	+	Goardon et al. 2011
TIM3	Member of immunoglobulin superfamily	NSG	-	+	Jan et al. 2011

Leukemic blasts from patients with AML associated with mutations in the NPM1 (NPM1^{mut}) gene frequently lack surface expression of CD34. Taussig et al. examined the capacity of CD34⁺CD38⁻, CD34⁺CD38⁺, CD34⁻CD38⁺ and the CD34⁻CD38⁻ cells from patients with NPM1^{mut} AML to engraft in their xenotransplantation assay (Taussig et al. [2010](#)). LSC activity was demonstrated for either the CD34⁻CD38⁺, CD34⁻CD38⁻ or both populations. NPM1^{mut} cases expressing CD34 demonstrated LSC activity most frequently in the CD34⁺CD38⁺ population. For most cases LSC activity was present in multiple populations demonstrating both inter-patient as well as intra-patient heterogeneity in LSC phenotypes. Using LDA they assessed LSC frequency for the different populations within a single patient. For two patients, the LIC frequency was similar among phenotypically distinct LSC populations. In a third patient, three populations demonstrated similar LSC frequencies while LSCs were less frequent in the fourth, CD34⁻CD38⁺, population.

Sarry et al. ([2011](#)) and Eppert et al. ([2011](#)) likewise identified inter- as well as intra-patient heterogeneity in LSC surface antigen phenotype, although Eppert et al. ([2011](#)) found that LSC frequency was greatest in the CD34⁺CD38⁻ population compared to the CD34⁺CD38⁺ population. In all of the above studies, LDA assays confirmed that LSCs make up a rare population of cells in the samples.

Multiple LSC populations within a patient suggest that the LSC pool evolves in response to the addition of other genetic/epigenetic events or occurs as a response to therapy. Cytogenetics, whole genome sequencing and DNA methylation studies have documented changes in the makeup of the malignancy following relapse (Welch et al. [2012](#); Kroeger et al. [2008](#)). Our group carefully examined the impact

of the patients' clinical course on the LSC phenotype. Using either CD34 and CD38 or CD32 (CD34⁻ cases) and CD38, we characterized the LSC activity in the four populations defined by these antigens prior to therapy and following relapse using an NSG xenotransplantation model. LDAs of samples obtained prior to therapy and following relapse (Ho et al. 2013) identified a 9–90-fold expansion of functional LSC activity at relapse. There was considerable inter-patient as well as intra-patient heterogeneity in LSC surface antigen phenotypes from diagnostic samples. We demonstrated expansion of LSC activity at relapse to populations lacking LSC activity in the pre-treatment sample. The molecular basis for this evolution and how it relates to the cell of origin is a work in progress.

3.3 *Cell of Origin in AML*

AML sequencing studies have demonstrated a median of 13 non-synonymous mutations per case (Cancer Genome Atlas Research 2013). How and where these mutations accumulate is an area of great interest. Miyamoto et al. isolated phenotypically distinct populations from patients with AML associated with t(8;21) following attainment of remission (Miyamoto et al. 2000). AML1/ETO transcripts were present in a fraction of stem cells, monocytes, and B-cells isolated from remission samples. Remission marrow cells were plated in methylcellulose and AML1/ETO transcripts were present in erythroid, granulocyte/macrophage, and megakaryocyte colonies. Thus, for AML associated with t(8;21), the cell of origin appears to be a stem cell population capable of giving rise to myeloid cells as well as B-cells. Recently, cell sorting and targeted mutation analysis were combined to investigate which stem or progenitor populations are targeted by specific mutations in AML (Shlush et al. 2014; Corces-Zimmerman et al. 2014; Jan et al. 2012). Following identification of each patient's leukemia-specific profile, Corces-Zimmerman et al. analyzed leukemic cells, T-cells, and HSCs (CD34⁺CD38⁻TIM3⁻CD99⁻) using targeted amplicon sequencing (Corces-Zimmerman et al. 2014). They confirmed the ability of isolated CD34⁺CD38⁻TIM3⁻CD99⁻ HSCs to establish multi-lineage engraftment of NSG recipients. Mutations occurring in the HSC populations were termed "Pre-leukemic mutations" and represented a subset of the patient's leukemia specific mutations. Leukemia-specific mutations not present in the HSC populations but present in the purified leukemic CD99⁺ TIM3⁺ cells were classified as "late events". Combining this data with that of an earlier study (Jan et al. 2012), they identified 74 mutations in 16 patients with AML; 49 % were preleukemic and 52 % were late events. Mutations overrepresented in the preleukemic group included DNA methylation, chromatin modification, and chromatin topology (IDH2, DNMT3A, ASXL1, and IKZF1) while mutations in FLT3, NPM1 and genes involved in activated signaling were overrepresented in the late event group. CD34⁺ cells from remission samples identified patient specific pre-leukemic mutations in remission CD34⁺ progenitors and their progeny. Shlush et al. reported similar findings in a separate cohort of patients (Shlush et al. 2014).

To determine the frequency of commonly occurring mutations in non-leukemic hematopoiesis, (Xie et al. 2014) analyzed data from The Cancer Genome Atlas (TCGA) database. They selected patients with 11 different tumor types but no prior history of a hematologic malignancy or treatment. 2728 individuals were analyzed and a final list of 77 mutations in 58 cases was identified. Sixty-four of the events were in 19 genes including DNMT3A, TET2, JAK2, ASXL1, TP53, SF3B1, BCORL1, ASXL2 and SH2B3. Comparing this dataset with the TCGA datasets for myeloproliferative disorders, MDS, AML and chronic lymphocytic leukemia (CLL); DNMT3A, JAK2, TET2, ASXL1, TP53 and SF3B1 were consistently mutated in the TCGA blood dataset and at least two of the other datasets. The authors argued that these mutations were likely to be early events in the initiation of hematologic malignancies. Mutations of IDH1, RUNX1, NRAS, PHF6 were identified in the AML, MPN and MDS datasets but not in the TCGA blood dataset. CEBPA, WT1, PTPN11, KIT, SMC1A, and SMC3 were frequently mutated in the TCGA AML dataset but not in the TCGA blood dataset. These mutations were surmised to be later events.

These studies support a model in which early mutations alter the “landscape” of the HSC pool (Corces-Zimmerman et al. 2014). This altered HSC pool is able to give rise to both myeloid and lymphoid progeny although skewed. Later events in the HSC or its progeny further modify the cell state and activate cell signaling resulting in the ability of cycling malignant progenitor populations to self-renew and completing leukemic transformation (Fig. 13.2). These studies rely on the deconstruction of the tumor and depend on an intact relationship between a cell’s surface antigen profile in cancer with that in normal hematopoiesis. With the advent of improved methods to target gene expression to normal cord blood stem cell populations and current xenotransplantation models the tools are in place to prospectively build human leukemias in vivo (Moriya et al. 2012; Chou et al. 2011). IPS technology provides an alternative approach to reverse engineering leukemia (Liu et al. 2014).

3.4 Lessons from Murine Models of Leukemia

Murine models of leukemia allow researchers to take full advantage of the extensive knowledge base of murine normal hematopoiesis and the power of murine genetics. As mutations in human AML are identified, they are modeled in the mouse. A complete review of murine models for leukemia is beyond the scope of this effort. We will highlight two stories where these systems were employed to address key concepts in LSC biology.

Early studies of LSCs in CML and AML suggested that the HSC pool was the primary target for early events with additional events in progenitors resulting in full transformations. Studies in mice have examined the potential of leukemia initiating events to transform HSCs as well as non-self-renewing progenitors. In 2006 Krivtsov et al. initiated acute leukemia in mice by targeting the expression of the

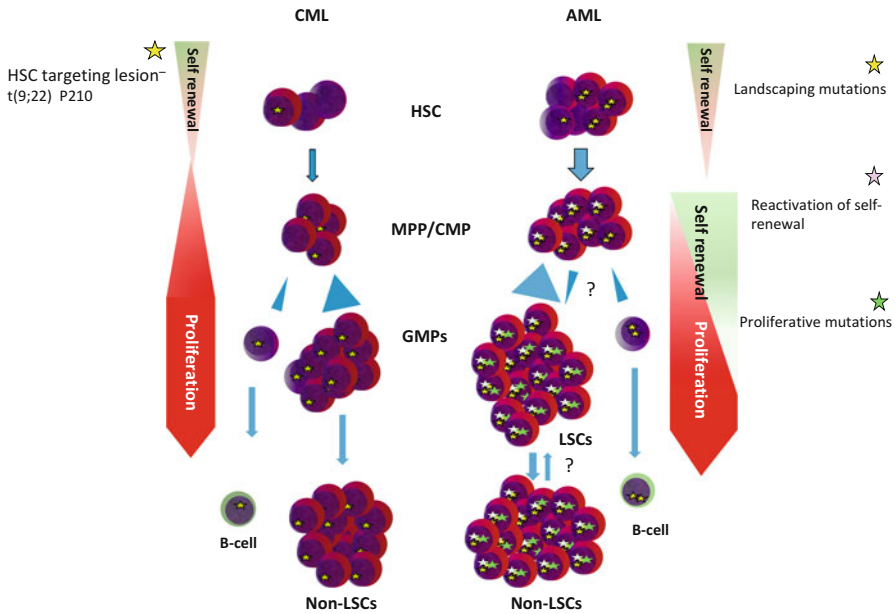


Fig. 13.2 *Model for chronic and acute myeloid leukemia.* (a) Chronic myelogenous leukemia is the result of t(9;22) targeting the quiescent HSC. The LSC is a functioning HSC able to give rise to both myeloid and lymphoid progeny with expansion of the myeloid progenitor pools and a granulocytic predominance. Inhibition of the BCR-ABL fusion product restores normal hematopoiesis but does not eradicate the LSC. (b) AML. Early genetic events alter the “landscape” of the quiescent HSC pool without completing leukemic transformation. The altered HSC pool is able to give rise to both myeloid and lymphoid progeny although skewed. Later events in the progeny activate self-renewal and cell signaling resulting in the ability of cycling malignant progenitor populations to self-renew and completing the leukemic transformation. As the disease progresses, more committed progenitor populations acquire the potential for self-renewal. It has yet to be determined if these states are inter-exchangeable in human disease as demonstrated by Nolan et al. in a murine model

MLL-AF9 fusion product to GMPs (Krivtsov et al. 2006). They demonstrated that the LSCs possess an immunophenotype and gene expression profile similar to that of normal GMPs. A subset of genes (363) in the MLL-AF9 LSC signature overlap with the expression profile of normal murine HSCs and the expression of profiles of human MLL associated AML. Subsequent studies have employed this model to refine the critical events in MLL mediated leukemia as well as identify novel therapeutics (Krivtsov et al. 2013; Wang et al. 2010; Hanoun et al. 2014; Miller et al. 2013). A similar capacity to activate a self-renewal program in progenitor populations has been demonstrated for the MLL-ENL and MOZ-TIF2 fusion products (Huntly et al. 2004; Cozzio et al. 2003). This capacity is not shared among all driver mutations BCR-ABL, FIt3 ITD mutations as well as the HOXA9-MEIS1 fusion product are not capable of fully transforming normal progenitors. Interestingly, MLL-AF9, when driven under the endogenous MLL promoter is unable to transform GMP populations.

Data from human studies supports the presence of multiple distinct LSC populations in patients with AML. Gibbs et al. (2012) applied Cytof technology to characterize LSCs populations in a murine model for AML driven by the HoxA9-Meis1 fusion. Three distinct LSC populations capable recapitulating the original immunophenotype on transplantation were identified, Lin⁻kit⁺, Gr1⁺kit⁺ and Lym⁺kit⁺. Detailed Cytof analyses of recipient marrow following transplantation of these independent LSC populations demonstrated shared signaling networks. The authors concluded that their data was not consistent with a unidirectional process of differentiation and that stemness may reflect a cellular state that exists independently of surface antigen definition.

3.5 *Stem Cells in Myelodysplastic Syndromes*

The myelodysplastic syndromes are a heterogeneous group of diseases characterized by bone marrow failure and a variable risk for transformation to acute leukemia. Intermediate and high risk MDS represent pre-leukemic states and are treated as such. MDS is characterized by recurrent cytogenetic and molecular abnormalities and sequencing efforts have identified overlap of the mutational spectrum in MDS with AML (Bejar et al. 2011). Recognized as a stem cell disorder early on (Raskind et al. 1984), clonal involvement of the HSC pool and its progeny is supported by studies in which HSC and early progenitors demonstrate the presence of patient specific genetic events (Nilsson et al. 2002; Tehranchi et al. 2010; Will et al. 2012). HSC and progenitor populations from primary patient samples demonstrate changes in the size of the HSC, CMP, CMP and MEP pools (Will et al. 2012). Studies evaluating the impact of therapy on the HSC pool in patients with MDS have established that despite clinical responses to lenalidomide or 5-azacytidine, including remission, the malignant HSC pool remains untargeted and serves as a reservoir for relapse (Will et al. 2012; Craddock et al. 2013; Tehranchi et al. 2010). Until recently, functional assessment of clonal HSCs and progenitors from patient samples has been hampered due to difficulties in achieving sustained engraftment of clonal hematopoiesis immune deficient mice (Nilsson et al. 2000, 2002). Two recent reports demonstrate sustained clonal engraftment of recipient mice following co-transplantation of sorted hematopoietic cells with bone marrow stromal cells via intrafemoral injection (Muguruma et al. 2011; Kerbaay et al. 2004).

With the identification of disease driving genetic events in MDS, the development of novel murine models for MDS that mirror human disease is also likely to help define the role of fully transformed stem cell populations in MDS. Early murine models for MDS were mainly limited to the less common CMML category while recent models demonstrate a phenotype more representative of the breadth of MDS (Barlow et al. 2010; Moran-Crusio et al. 2011). A recent model in which expression of the Dicer gene was targeted in bone marrow osteoblasts highlighted the role of the bone marrow microenvironment in the pathophysiology of MDS (Raaijmakers et al. 2010). These models will allow the investigation of the functional cancer stem cell populations in MDS.

3.6 *Molecular Characterization of LSCs*

While there have been a large number of reports applying gene expression analysis to characterize samples from large cohorts of patients with AML, only a few have studied the malignant stem cell populations (Ishikawa et al. 2007; Guzman et al. 2001b; Goardon et al. 2011; Gentles et al. 2010; Majeti et al. 2009; Eppert et al. 2011). Guzman et al (2001b) examined the expression levels of 1400 genes related to cancer and apoptosis in leukemic CD34⁺CD38⁻ cells from primary patient samples and demonstrated aberrant expression of DAPK and IRF-1. Studies that followed applied microarray technology to compare leukemic CD34⁺CD38⁻ cells to leukemic CD34⁺CD38⁺ cells (Ishikawa et al. 2007), leukemic CD123⁺CD34⁺CD38^{low} Lineage⁻ cells to normal HSCs (Majeti et al. 2009; Gentles et al. 2010) and finally functionally defined LSC and normal HSC populations to leukemic populations lacking LSC activity (Eppert et al. 2011). Two of these signatures were shown to have prognostic significance (Eppert et al. 2011; Gentles et al. 2010). These differing approaches have yielded varying results with limited overlap but form a database for future queries. The LSC signature generated by Eppert et al. was enriched for an HSC signature. They also compared their LSC signature with data sets derived from embryonic stem cell as well as hematopoietic stem and progenitor populations. Their LSC signature was positively correlated with published HSC signatures and negatively correlated with more differentiated cell signatures. Majeti et al. compared normal HSCs to leukemic CD123⁺CD34⁺ CD38^{low} Lineage⁻ cells and identified the *Adherens junction*, *Ribosome*, *Regulation of actin cytoskeleton*, *Tight junction* and *Focal adhesion* pathways (KEGG) as the top five dysregulated pathways in leukemic progenitors. With the advent of new platforms to characterize miRNA levels as well as DNA methylation changes, integration of these datasets with functional data will be critical.

3.7 *Therapeutic Targeting of LSCs in Myeloid Malignancies*

Two decades have passed since publication of the study by Lapidot et al. (1994) that launched the stem cell model for AML. While there is agreement in the need to identify agents capable of eradicating the malignant stem cell population; increasing recognition of the heterogeneity of the LSC pool in and between patients complicates the design and implementation of LSC targeted therapies. The study of normal stem cell biology has identified a number of shared pathways that are responsible for self-renewal including sonic hedgehog, Wnt, Notch, and BMI1. These pathways are known to be necessary for malignant stem cell function in multiple tumor types. Efforts to target these pathways are in different phases of translation to the clinic.

A second approach to targeting cancer stem cells is to identify surface proteins present on cancer stem cell populations that are lacking or expressed at a lower level

on normal stem cell and progenitor populations (Table 13.2). Anti-CD33 therapy has been in the clinical arena for over a decade; while there is a benefit to a small subset of patients; this approach overall has been disappointing (Burnett et al. 2011). Similar early phase clinical efforts for CD123, CLL1 are ongoing as are pre-clinical studies targeting CD44, CD47, TIM-3 and IL1RAP (Majeti 2011; Askmyr et al. 2013; Kikushige and Miyamoto 2013). In addition to standard monoclonal antibody therapy, Bi-specific T-cell engagers (BiTEs) and CAR-T efforts using C33, CD123 and LeY antigens are in the pre-clinical and early clinical stages (Aigner et al. 2013; Ritchie et al. 2013; Gill et al. 2014; Dutour et al. 2012). A major limitation of the LSC antigen targeting approach is that none of the currently published LSC antigens appear to be both present on all of the LSC populations in a patient while lacking on normal HSCs and/or progenitors.

A third approach is to target unique cancer stem cell dependencies. The development of mutation specific therapies is one such approach although most mutations are limited to a small fraction of patients except for NPM1 and FLT3 mutations. An early study demonstrated constitutive NF Kappa β activity in LSCs as compared to normal HSCs (Guzman et al. 2001a). This has been confirmed and a recent study demonstrated that NF Kappa β activity was common to murine and human LSC populations and associated with expansion of the LSC pool (Kagoya et al. 2014). An ongoing Children's Oncology Group trial is investigating the benefits of adding bortezomib to standard induction therapy. In 2005 Guzman et al. published a follow-up study in which they characterized the ability of a natural product, parthenolide, to selectively eradicate LSC populations (Guzman et al. 2005). Initially selected for its ability to inhibit NF Kappa β signaling (Hehner et al. 1999), subsequent studies have identified alternative activities of this class of compounds including redox balance, heat shock protein response, proteasome signaling and glycolysis (Pei and Jordan 2012; Pei et al. 2013). Subsequent studies have confirmed the utility of this class of compounds in targeting the above pathways and identified other molecules with similar effects or that synergize with parthenolide (Lagadinou et al. 2013; Dai et al. 2010; Hassane et al. 2008, 2010).

4 The Role of Malignant Stem Cells in B-cell Malignancies

4.1 Normal B-cell Development

In the current model for B-cell development, multi-potent progenitors undergo a cell fate decision giving rise to a multipotent lymphoid progenitor that then give rise to either B-cells or T-cells with the early pro-B-cell the first stage of B-cell development (Fig. 13.1). Early B-cell development occurs in the bone marrow followed by exodus to the lymph organs. Following selection and maturation in the germinal centers of primary and secondary lymph organs, B-cells return to the bone marrow. Developmental stage can be identified using the expression of specific

surface antigens and the rearrangement status of Ig H and L chains. CD34⁺CD10⁺CD19⁻ define the common lymphoid progenitors while pro-B-cells are defined as CD34⁺CD10⁺CD19⁺ and pre-B-cells are CD34⁻CD10⁺CD19⁺. Rearrangement of the VDJ H chain locus is characteristic of pro-B-cells while expression of a pre-BCR, composed of IgH chains and surrogate L chains marks the pre-B-cell population (Hystad et al. 2007). Memory B-cells, plasmablasts and mature plasma cells are similarly defined by their surface antigen expression profile (Memory B-cell: CD27⁺CD20⁺CD45⁺⁺IL6R⁻CD138⁻CD38^{dim}; Plasmablast: CD20⁻CD38⁺⁺CD45⁺⁺IL6R⁺⁺CD138⁻ and Mature plasma cells: CD20⁻CD38⁺⁺CD45^{dim}IL6R⁺CD138⁺⁺).

Cells of the myeloid lineage including erythrocytes, megakaryocytes, and granulocytes have well defined and frequently quite short half-lives while a subset of mature B-cell populations are long lived. This is the basis for immunologic memory allowing for a more rapid antigen specific response following re-exposure by rapid expansion of memory B-cells. In 2006, Luckey et al. (2006) analyzed the expression profiles of naïve, effector and memory T-cells as well as naïve, germinal center, memory B-cells and plasma cells. Transcripts augmented in memory cell populations compared to naïve and effector cell populations were enriched in HSCs and lost following commitment. Likewise, transcripts down regulated in memory cell populations were down regulated in HSCs and increased with differentiation.

4.2 *The Role of Leukemic Stem Cells in B-cell ALL*

B-cell Acute Lymphoblastic leukemia (B-ALL) arises in an early B-cell progenitor (Teitell and Pandolfi 2009) from the accumulation of genetic events by hematopoietic stem cells and/or progenitor cells. Early immunoglobulin rearrangement studies demonstrated that a quarter of early B-ALL cases contain more than two IgH rearrangements. Sequential analyses of a few patients found that the patient specific pattern of IgH gene rearrangement may change over the course of the disease (Wright et al. 1987). TEL-AML1 B-ALL is a rare disease and cases of in-utero transfer of a pre-leukemic clone between twins have been reported. When one twin presents with B-ALL, the other twin may harbor the preleukemic clone. Hong et al. demonstrated that CD34⁺CD38⁻CD19⁺ leukemic cells from four patients with TEL-AML1 B-ALL were capable of transplanting the leukemia in mice (Ma et al. 1998). The peripheral blood of the healthy twin of one patient contained found very rare circulating TEL-AML1 positive CD34⁺CD38⁻CD19⁺ pre-leukemic cells. Castor et al. (2005) examined the involvement of CD34⁺CD38⁻CD19⁻ and CD34⁺CD38⁻CD19⁺ cells from children and adults with B-ALL for presence of the TEL-AML1, P190 BCR-ABL and P210 BCR-ABL translocations. TEL-AML1 and p190BCR-ABL involved the B-cell progenitor population but not the CD34⁺CD38⁻CD19⁻ HSC pool while p210BCR-ABL cases involved both populations. Xenotransplantation studies revealed that only the CD34⁺CD38⁻CD19⁺ cells from TEL-AML1, P190 BCR-ABL and p210BCR-ABL cases gave rise to B-ALL

upon transplantation. CD34⁺CD38⁻CD19⁻ cells gave rise to normal reconstitution regardless of the type of B-ALL. This suggests that for these pediatric (TEL-AML1 and p190BCR-ABL) and adult (p210BCR-ABL) B-ALL, the cell of origin may differ but the functional LSC has a conserved phenotype, that of a committed B-cell progenitor. Cobaleda et al. (2000) isolated leukemic cells from B-ALL samples associated with t(9;22). In contrast to the study by Castor et al. (2005), only CD34⁺CD38⁻ cells were capable of engrafting NOD/SCID mice. Cox et al. (2004) employed a series of in vitro assays as well as xenotransplantation to characterize the surface antigen profile of B-ALL leukemia initiating cells. LSC activity was restricted to the CD34⁺CD10⁻ and CD34⁻CD19⁻ populations. This study did not include B-ALL associated with a t(9;22) or t(4;11).

The above studies relied on NOD/SCID assay and studies in AML have shown the potential impact of this assay on the phenotypes of LSC population(s) identified. Rau et al. (2014) undertook an extensive study of primary ALL samples using an NSG model with intrafemoral transplantation. The patients represented three distinct ALL risk groups. Despite using a number of surface antigens, they were unable to enrich for LSC activity. All populations demonstrated similar engraftment frequencies as well as kinetics of engraftment. LDA of the sorted populations demonstrated similar frequencies for LSC activity regardless of surface antigen phenotype. Their data was consistent with the lack of a hierarchy in acute B lymphoblastic leukemia and suggest a non-hierarchical model to account for tumor heterogeneity.

Efforts are underway to improve human B-ALL LSC models using cord blood stem cell targeting and xenotransplantation. Barabe et al. (2007) targeted the expression of the MLL-ENL and MLL AF9 fusion transcripts in primary cord blood progenitor cells. Recipient mice developed both B-ALL and AML that was transferrable into secondary recipients. Retroviral insertion analyses and IgH locus analyses demonstrated that the B-ALL recipients contained differing rearrangements of the IgH locus. 40 % of the cases were consistent with transformation of an early hematopoietic progenitor. Using serial transplantation to model disease progression, they showed that the contribution of clones with unarranged IgH loci diminished with passage while alternative LSC populations arising from more mature B-cell progenitors maintained the disease.

4.3 Myeloma Stem Cells

Multiple myeloma (MM) belongs to a spectrum of diseases that includes monoclonal gammopathy of undetermined significance (MGUS), smoldering myeloma and symptomatic myeloma. MGUS is present in approximately 3 % of the general population 50 years of age and older with a risk of transformation to multiple myeloma of 1 % per year. In 2009, it was demonstrated that in up to 75 % of myeloma patients a detectable M-protein was identifiable 8 or more years prior to diagnosis (Weiss et al. 2009). This is consistent with a clonal process maintained by a population(s)

of cells that are long lived. Immunoglobulin sequencing studies in patients with MM demonstrates the presence of extensive hypermutation without intraclonal variation consistent with the development of the malignancy at the post-germinal center B-cell.

Myeloma was one of the cancer types in which tumor heterogeneity was initially assessed. In 1968, Bergsagel and Valeriote characterized the capacity of a murine plasma cell line to initiate a malignancy in recipient syngeneic mice (Bergsagel and Valeriote 1968). 3×10^4 malignant plasma cells were required to form at least one colony in the spleen of recipient mice. They used this model to show that most of the plasma cells were cycling and sensitive to vinblastine, a cell cycle dependent chemotherapeutic agent. In 1977, these findings were reproduced using human myeloma cells. More recent efforts have employed two different approaches to address heterogeneity in plasma cell tumors; NOD/SCID and NSG xenotransplantation models such as those noted above or models in which human (SCID-hu) or rabbit (SCID-rab) bone fragments are implanted directly into immunocompromised mice followed by injection of the myeloma cells into the implant.

In 1998, (Yaccoby et al. 1998) employed a SCID-Hu xenotransplantation model for studying primary MM samples. $CD38^{++}CD45^{-}$ plasma cells engrafted the implanted human bone whereas the plasma cell depleted cells did not. In normal B-cell development, terminal plasma cell differentiation is characterized by loss of CD45 and increasing CD38 expression. Hosen et al. reported CFU activity as well sustained engraftment in the SCID-rab model for $CD138^{-}CD19^{-}CD38^{++}$ cells (Hosen et al. 2012). $CD19^{+}$ B-cells lacked CFU activity and engraftment potential. $CD138^{+}$ cells engrafted in a subset of the cases.

Matsui and colleagues used magnetic bead enrichment/depletion approaches to obtain $CD138^{+}CD34^{-}$ and $CD138^{-}CD34^{-}$ bone marrow cells from 24 MM patients. $CD138^{+}CD34^{-}$ cells were unable to form colonies in vitro while the $CD138^{-}CD34^{-}$ cells generated colonies of morphologically mature plasma cells expressing CD138. Engraftment of NOD/SCID mice by myeloma was observed only with $CD138^{-}CD34^{-}$ cells which gave rise to $CD138^{+}$ cells in the recipient mice. MM samples depleted of CD19, CD45 and CD20 cells lacked colony forming potential consistent with a memory B-cell-like progenitor. They refined the surface antigen profile of myeloma propagating populations in a follow-up report, $CD138^{-}CD27^{+}CD19^{+}$, and demonstrated that this population was resistant to lenalidomide, bortezomib and cyclophosphamide; agents commonly used to treat patients with MM (Matsui et al. 2004; Matsui et al. 2008).

In 2013, Chaidos and colleagues addressed the disagreement as to the surface antigen phenotype of MM cells responsible for tumor maintenance (Chaidos et al. 2013). They extensively characterized the surface antigen profiles of MM blood and marrow samples coupled with IgH CDR3 characterization. They found that the MM clone did not include pre-germinal center B-cells but was comprised of a mixture of mature $CD19^{+}$ B-cells (resting memory B-cells), plasmablasts, and $CD138^{low}$ and $CD138^{+}$ plasma cells. They also identified a $CD138^{-}$ population they termed the pre-plasma cell (pre-PC). They sorted and transplanted $CD19^{+}$ B-cells, Pre-PCs and PCs from MM patients into sub lethally irradiated NSG mice. Recipients of $CD138^{+}$

PCs displayed BM engraftment in 9 of 12 cases. Negatively enriched Pre-PCs also demonstrated engraftment while none of the mice receiving CD19⁺ cells showed evidence of engraftment. They proposed a model in which myeloma-stem cell activity was confined to two interconvertible populations of MM cells distinguishable only by the level of expression of CD138. It is unclear to what degree the heterogeneous results reported relate to clinical features of the samples studied, the approach to isolating populations (negative selection vs positive selection) or the different models employed for xenotransplantation assays. Alternatives to a reliance on surface antigen profiling have employed sorting based on ALDH expression or Hoechst 33,342 staining (Matsui et al. 2004). Additional studies will be necessary to further clarify the exact nature of the myeloma initiating cell using a uniform and standardized set of isolation approaches and xenotransplantation model.

4.4 Targeting Malignant Stem Cells in B-cell ALL and Myeloma

Similar to other cancers, pathways regulating normal stem cell self-renewal have been examined in B-ALL and MM. The SHH pathway is active in MM stem cells and early pre-clinical efforts have demonstrated some efficacy (Agarwal et al. 2014; Peacock et al. 2007).

CD19 is expressed on the cell of origin and the LSC for many cases of B-cell ALL and its expression is carried through late into B-cell development (see above). There is general excitement about several novel approaches to targeting CD19 in B-ALL and other B-cell malignancies. Monoclonal antibodies against CD19 have been conjugated to antineoplastic agents as well as to a single-chain variable region capable of binding to the CD3 T-cell receptor. These agents have shown activity in early phase trials and are now being tested in the upfront setting. Likewise, CAR-T-cells targeting CD19 have had success in relapsed B-ALL (Maude et al. 2014; Davila et al. 2014). These efforts have been expanded to most B-cell malignancies. The observation that MM propagating cells express CD20 led to a small clinical trial employing Rituximab, a monoclonal antibody against CD20 (Moreau et al. 2007). This trial demonstrated little efficacy for this approach in patients and this approach has not moved forward. Antibodies targeting CD138, present on some MM stem cell phenotypes but not others, has been studied and is currently undergoing phase 3 testing.

5 Conclusion and Future Directions

In 2011, Hanahan and Weinberg updated their treatise on the hallmarks of cancer which include sustaining proliferative signaling, evading growth suppressors, resisting cell death and enabling replicative immortality (self-renewal) (Hanahan and

Weinberg 2011). These properties are shared by normal cells during normal development and homeostasis where they are compartmentalized and tightly regulated. Although initial studies in AML and CML demonstrated an overlap of the cancer stem cell phenotype with that of the normal HSC; subsequent studies have shown that in cancer the capacity for self-renewal can expand to less quiescent progenitor populations. As the disease progresses, the cancer stem cell phenotypes in AML, MM and ALL resemble late progenitors as “stemness” moves further out into the hierarchy. It will be critical to define the cancer specific mechanisms driving this expansion as targeting these pathways may restore control of self-renewal without restricting self-renewal in normal populations. Recently, constitutive activation of the nuclear factor-kappa B (NF- κ B) pathway was shown to expand functional LSC activity as assessed by LDA (Kagoya et al. 2014). As additional agents capable of targeting key pathways involved in self-renewal become available, studying their impact on all compartments in cancer and normal tissue will be critical.

Whole genome sequencing studies have outlined the genetic space for most hematologic malignancies. Interestingly, studies of normal appearing hematopoietic stem cells in the diagnosis and remission samples from patients with AML have identified cells retaining a capacity for multi-lineage differentiation with disease specific mutations. These may represent residual pre-leukemic stem cells with overlap of these mutations with those identified in peripheral blood and marrow samples from individuals without a hematologic malignancy. The frequency and nature of mutations in individuals without a hematologic malignancy is tightly associated with aging as are the diagnoses of AML, MDS and Myeloma. These findings will need to be validated in aged healthy individuals with follow-up analyses to ensure they affect a long-lived population. We will require a greater understanding of how these mutations alter the landscape of the stem cell pool prior to accumulating additional genetic events. A frequent statement in reviews and articles on cancer stem cells is that CSCs represent a reservoir for relapse hence the interest in identifying and phenotyping these populations. How to effectively target a pre-leukemic stem cell pool that may be indistinguishable from normal HSCs will likely serve as a task for the next decade. As demonstrated so well by chronic phase CML, it may not be necessary to eradicate pre-leukemic HSCs as long as there are effective therapies for their progeny.

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