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Lalitha Nagarajan
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Acute Myelogenous Leukemia

Genetics, Biology and Therapy

 Springer

Editor

Lalitha Nagarajan
Department of Genetics
University of Texas MD
Anderson Cancer Center
Unit 1006
1515 Holcombe Boulevard
Houston TX 77030
USA
lnagaraj@mdanderson.org

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Contributors

Gheath Alatrash, DO, PhD Division of Cancer Medicine, University of Texas MD Anderson Cancer Center, Houston, TX, USA

Michael Andreeff, MD, PhD Department of Blood and Marrow Transplantation, University of Texas MD Anderson Cancer Center, Houston, TX, USA, mandreef@mdanderson.org

Gautam Borthakur, MD Department of Leukemia, The University of Texas MD Anderson Cancer Center, Houston, TX, USA, gborthak@mdanderson.org

George A. Calin, MD, PhD Departments of Experimental Therapeutics and Cancer Genetics, The University of Texas MD Anderson Cancer Center, Houston, TX, USA, gcalin@mdanderson.org

Bing Carter, PhD Section of Molecular Hematology and Therapy, Department of Stem Cell Transplantation and Cellular Therapy, The University of Texas MD Anderson Cancer Center, Houston, TX, USA

Michael E. Engel, MD, PhD Division of Pediatric Hematology/Oncology, Department of Pediatrics, Monroe Carell Jr. Children's Hospital at Vanderbilt, Vanderbilt-Ingram Cancer Center, Nashville, TN, USA, mike.engel@vanderbilt.edu

Elihu E. Estey, MD Department of Leukemia, The University of Texas MD Anderson Cancer Center, Houston, TX, USA

Zeev Estrov, MD Department of Leukemia, University of Texas MD Anderson Cancer Center, Houston, TX, USA, zertrov@mdanderson.org

Brunangelo Falini, MD Institute of Hematology, University of Perugia, Perugia, Italy, faliniem@unipg.it

Julie M. Fortier, MD Division of Oncology, Stem Cell Biology Section, Washington University School of Medicine, St. Louis, MO, USA

Timothy A. Graubert, MD Division of Oncology, Stem Cell Biology Section, Washington University School of Medicine, St. Louis, MO, USA, tgrauber@dom.wustl.edu

David Grimwade, MD, PhD Department of Medical and Molecular Genetics, King 's College London School of Medicine, London, UK, david.grimwade@genetics.kcl.ac.uk

Fabien Guidez, PhD Department of Medical and Molecular Genetics, King's College London School of Medicine, London, UK

Scott W. Hiebert, PhD Division of Pediatric Hematology/Oncology, Department of Pediatrics, Monroe Carell Jr. Children's Hospital at Vanderbilt, Vanderbilt-Ingram Cancer Center, Nashville, TN, USA

Jean-Pierre J. Issa, MD Department of Leukemia, University of Texas MD Anderson Cancer Center, Houston, TX, USA, jissa@mdanderson.org

Bharti Jasra, MD Departments of Experimental Therapeutics and Cancer Genetics, The University of Texas MD Anderson Cancer Center, Houston, TX, USA

Marina Konopleva, MD, PhD Section of Molecular Hematology and Therapy, Department of Leukemia, The University of Texas MD Anderson Cancer Center, Houston, TX, USA

Soheil Meshinchi, MD, PhD Clinical Research Division, Fred Hutchinson Cancer Research Center, Seattle, WA, USA

Anita R. Mistry, PhD Department of Medical and Molecular Genetics, King 's College London School of Medicine, London, UK

Jeffrey J. Molldrem, MD Department of Stem Cell Transplantation and Cellular Therapy, University of Texas MD Anderson Cancer Center, Houston, TX, USA, jmolldre@mdanderson.org

Beatrice U. Mueller, MD Department of Internal Medicine, University Hospital, Bern, Switzerland, beatrice.mueller@insel.ch

Lalitha Nagarajan, PhD Department of Molecular Genetics, University of Texas MD Anderson Cancer Center, Houston, TX, USA, lnagaraj@mdanderson.org

Milena S. Nicoloso, MD Departments of Experimental Therapeutics and Cancer Genetics, The University of Texas MD Anderson Cancer Center, Houston, TX, USA

Yasuhiro Oki, MD Department of Leukemia, University of Texas MD Anderson Cancer Center, Houston, TX, USA

Thomas Pabst, MD Department of Oncology, University Hospital, Bern, Switzerland

Nallasivam Palanisamy, PhD Michigan Center for Translational Pathology, University of Michigan Health System, Ann Arbor, MI, USA, nallasiv@med.umich.edu

Ismael Samudio, PhD Section of Molecular Hematology and Therapy, Department of Stem Cell Transplantation and Cellular Therapy, The University of Texas MD Anderson Cancer Center, Houston, TX, USA

Ellen Solomon, PhD Department of Medical and Molecular Genetics, King 's College London School of Medicine, London, UK

Derek L. Stirewalt, MD Clinical Research Division, Fred Hutchinson Cancer Research Center, Seattle, WA, USA, dstirewa@fhcrc.org

Peter J.M. Valk, PhD Department of Hematology, Erasmus University Medical Center Rotterdam, Rotterdam, The Netherlands, p.valk@erasmusmc.nl

Roel G.W. Verhaak, PhD Department of Hematology, Erasmus University Medical Center Rotterdam, Rotterdam, The Netherlands

The Leukemia Stem Cell

Zeev Estrov

Introduction and Historical Perspective

In a meeting held in the Charité Hospital in Berlin in 1909, Alexander Maximow postulated that all circulating blood cells arise from a single lymphocyte-like cell [68]. An almost identical hypothesis was proposed by Artur Pappenheim in 1917 [78]. This hypothesis was tested years later by numerous investigators who demonstrated that all hematopoietic cells arise from a single hematopoietic stem cell (HSC) [54, 75, 90, 116]. Our knowledge of the leukemogenic process has benefited from hematopoiesis research. Identification and characterization of the HSC led to the theory that leukemia is a stem cell disease, i.e., that leukemia arises from a neoplastic HSC.

As far as we know, leukemia has always existed. The first patient reported to exhibit symptoms most likely attributable to chronic lymphocytic leukemia was a 63-year-old Parisian lemonade salesman named Monsieur Vernis [108]. At first there was not much interest in this disease, and Armand Velpeau's report drew little attention. However, within a few years, other reports followed. The first cases of chronic myelogenous leukemia (CML) were reported by Donne and Craigie [30, 25], and the clinical characteristics of several patients with different forms of leukemia were published by John Hughes Bennett [5–9], who first used the term “leucocythemia.” The word ‘leukämie’ was coined by Rudolph Virchow who by then had described several patients with this disease [110–113]. Several decades passed before the morphological features of leukemia cells were defined and the pathophysiology of leukemia deciphered.

The stem cell concept has been applied to non-hematopoietic tissues. Embryonic stem cells and non-hematopoietic, tissue-specific stem cells have been isolated, characterized, and extensively studied [60]. Cells isolated from embryonic tissue, gametes, and fertilized eggs have been found to have

Z. Estrov (✉)

The Department of Leukemia, The University of Texas MD, Anderson Cancer Center,
Houston, TX, USA

e-mail: zestrov@mdanderson.org

totipotent and pluripotent capacities [47]. Although these two capacities are dissimilar, the cells received identical names from different investigators, and to avoid confusion a common terminology was agreed upon. According to the recently established nomenclature (Table 1), adult tissue-specific stem cells (such as HSCs) that are capable of generating and replacing terminally differentiated cells within their own tissue boundaries are termed multipotent (adult) stem cells.

Table 1 Stem cell nomenclature

<i>Totipotent stem cells:</i> The zygote (fertilized egg) (<i>totus</i> : entire). These cells have the capacity to give rise to every tissue associated with the embryo and, eventually, the adult
<i>Pluripotent stem cells:</i> The blastocyst (starting 4 days after fertilization) (<i>plures</i> : several). These cells can give rise to all tissue cells except the embryo's outer layer (the trophoblast) and the embryo-supporting placenta
<i>Multipotent stem cells:</i> Somatic (adult, tissue-specific) stem cells

The Hematopoietic Stem Cell

Concept and Definition

The hematopoietic system is thought to originate from multipotent HSCs capable of reproducing themselves (through a process termed “self-renewal”) and producing a hierarchy of downstream multilineage and unilineage progenitor cells that differentiate fully into mature cells [28, 58, 115]. The concept arose from studies exploring the formation of hematopoietic colonies in spleens of irradiated mice. It has been known since the late 1940s that exposing mice to ionizing radiation results in the generation of macroscopic hematopoietic spleen colonies [51]. In the early 1960s, Till and McCulloch [104] and their colleagues [3, 105] demonstrated that a spleen colony is generated from a single cell called a spleen colony-forming unit (CFU-S). It was demonstrated later that cells arising from a single CFU-S can rescue a lethally irradiated mouse, and subsequent limiting-dilution studies established that a single cell is capable of repopulating the entire hematopoietic system [54, 90, 72]. These studies defined the HSC as a cell that possesses self-renewal and clonogenic abilities and the capacity to differentiate into multiple lineages. HSCs with the capacity for both long-term and short-term repopulation of the mouse hematopoietic system have been identified. Similar studies with human bone marrow cells, performed in the severe combined immunodeficiency (SCID) mouse model, yielded similar results. HSCs are thought to be rare quiescent cells that, upon demand, give rise to progenitor cells (characterized *in vitro* and termed CFUs), which are destined to generate fully differentiated cells. The division of HSCs is thought to be either symmetric, producing two identical daughter cells that are either both stem cells or both progenitor cells, or

asymmetric, producing an HSC and a progenitor with diminished self-renewal capacity but with the ability to enact clonal expansion and maintain the circulating blood cell population [28, 74, 86, 110].

Isolation of HSCs

Because it was thought that a single cell is capable of repopulating the hematopoietic system, several investigators have attempted to isolate HSCs by using phenotypic cell-surface markers associated with defined lineages and developmental stages of hematopoietic cells. Using high-speed multi-parameter flow cytometry, distinct cell populations were purified and collected for functional analysis. Several cell-surface proteins thought to be specific to primitive cells, such as CD34, CD133, and CD150, and combinations of cell-surface markers such as Lin⁻c-kit^{high}Thy^{low}Sca⁺ [76, 93, 103], Lin⁻Thy⁺CD34⁺CD38^{-/low}, or the SLAM family receptors [57] have been used to identify HSCs. Other techniques to isolate HSCs utilize cellular physical characteristics or enzymatic activity, such as the extrusion of Hoechst dye 33342 (like the activity of P-glycoprotein that is encoded by the *MDR* gene) identifying “side population cells” and the use of BODIPY aminoacetaldehyde to assess aldehyde dehydrogenase activity [39, 46, 56, 57, 96].

HSC Characterization

To test the stem cell characteristics of a cellular population, it is necessary to purify the cells to functional homogeneity and to demonstrate that every single purified cell is capable of reconstituting the entire hematopoietic system. In addition, it is necessary to show that every single cell of a phenotypically identical cellular population isolated from one animal is capable of reconstituting the hematopoietic system in another animal, i.e., that the stem cells that repopulated the hematopoietic system self-renew (Fig. 1). These goals cannot be reproducibly reached using any of the currently available stem cell fractionation techniques because not every cell of the phenotypically identical cell population is a functional stem cell. Rather, the end products of these fractionation assays are cells that are enriched with HSCs. Remarkably, some cells of the “stem cell-depleted” cellular fraction are also functional stem cells, as demonstrated in studies with CD34 fractionation. CD34 was the first HSC “marker,” and CD34 fractionation has been used clinically for HSC enrichment. Remarkably, hematopoietic stem cell transplantation has been successfully performed with a population of CD34-negative cells [120, 121], suggesting that functional HSCs are present within the CD34-negative cell fraction. Thus, a stem cell should be defined by its functional capacity, not by phenotype, surface marker expression, or other cellular characteristics.

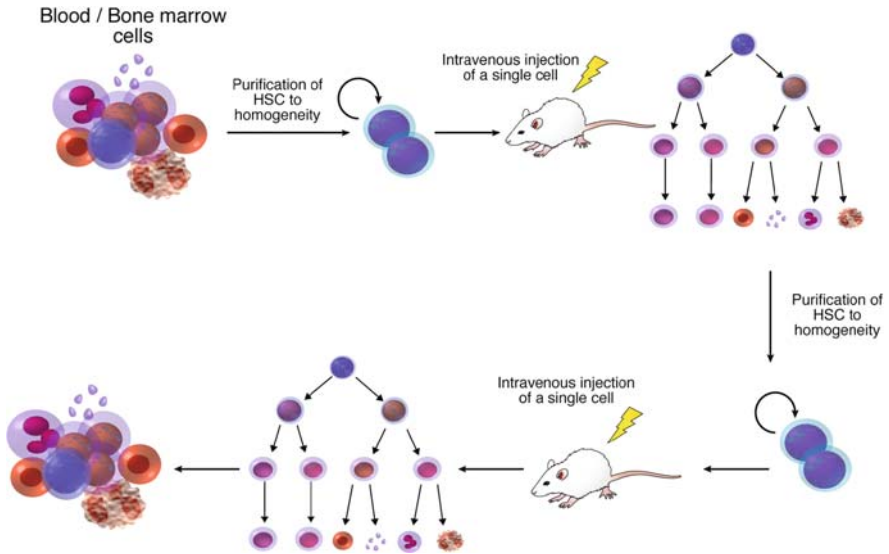


Fig. 1 Functional assessment of a purified population of HSCs. To confirm that fractionated cells are HSCs, every single cell of that homogenous population should have the capacity to repopulate the hematopoietic system in a lethally irradiated animal and, in addition, every single blood or bone marrow cell isolated from the irradiated animal by the same method should be capable of reconstituting all blood and bone marrow elements of an identical lethally irradiated animal (second-generation reconstitution)

The Hematopoietic Stem Cell Niche

Bone and bone marrow are intrinsically linked. Morphological and functional studies suggest that HSCs are located proximal to the endosteal surface of trabecular bone (reviewed in [71]). Several studies have demonstrated that osteoblast cells are required for this localization. Furthermore, HSCs express a calcium-sensing receptor. Stem cells lacking this receptor failed to localize to the endosteal niche and did not function normally after transplantation, highlighting the importance of the ionic mineral content of the bone itself and of the bone-derived matrix in the lodgment and retention of HSCs within the endosteal niche. However, HSCs function in the absence of osteoblasts. Extramedullary hematopoiesis is frequently found in patients with myeloproliferative disorders and in transgenic mice where osteoblast cells have been ablated, the marrow is aplastic, and extensive extramedullary hematopoiesis occurs. Thus, HSCs can survive and function in tissues that have no osteoblasts. The contributions of other cellular elements, such as stromal cells, osteoclasts, or perivascular cells, have been characterized. For example, it has been shown that HSCs can be recruited to a “vascular niche” that serves as an “extramedullary niche.” Experiments with parabiotic mice have demonstrated that HSCs circulate between the

blood and the bone marrow. Therefore, the existence of multiple types of HSC niches is not surprising. Whether different niches affect HSC function differently has yet to be determined.

The Leukemia Stem Cell

Cancer Stem Cell: Concept and Definition

The cancer stem cell hypothesis was proposed about 150 years ago by Rudolf Virchow and Julius Cohnheim, who argued that cancer results from the activation of dormant embryonic tissue remnants [23, 109].

The notion that leukemia stem cells (LSCs) might exist emerged from experiments performed in the early 1970s showing that only a subset of leukemia cells was capable of in vitro proliferation [70, 73, 98]. These in vitro studies, together with the in vivo studies described below, suggested that among the entire population of leukemia cells there are rare cells with the potential for self-renewal that drive the expansion of the leukemic clone. These LSCs are thought to exhibit characteristics similar to those of normal HSCs, with the exception that LSCs do not necessarily differentiate into different lineages. The current dogma is that all leukemia cells are derived from LSCs and that the descendents of these cells are clones.

It is thought that LSCs, like normal HSCs, give rise to differentiated daughter progenitor cells (termed CFU-Ls) that differentiate into leukemic blasts that lose their self-renewal capacity (Fig. 2). However, defects in the cellular machinery of CFU-Ls usually eliminate their ability to differentiate fully into morphologically and phenotypically mature cells. As a result, the leukemic cell population consists of undifferentiated and variably differentiated leukemia cells. The

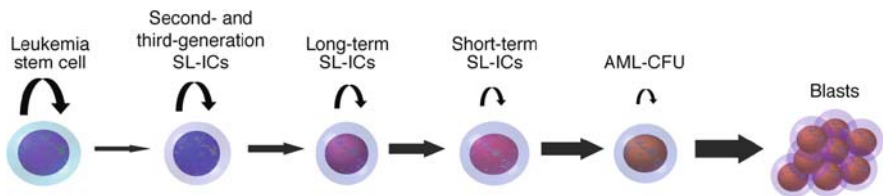


Fig. 2 Proposed hierarchal differentiation of LSCs: Rare LSCs with self-renewal capacity (*curved arrows*) give rise to SCID leukemia-initiating cells (SL-ICs) capable of initiating leukemia in second- and third-generation mice. These SL-ICs give rise to long-term SL-ICs capable of repopulating the marrow of identical second-generation mice. The short-term SL-ICs are capable of repopulating the marrow of the injected animal and give rise to AML colony-forming units (AML-CFU) that have a low rate of self-renewal. During this hierarchal differentiation process, the proliferation capacity (*straight arrows*) of the leukemic cells increases (adopted from [49] and [83]). This model is based on the assumption that human leukemia cells that engraft in SCID and NOD/SCID mice carrying LSC characteristics

degree of differentiation of leukemia cells has been used to identify their lineage (for example, myeloid versus lymphoid). Indeed, the current clinical classification of leukemias is based on the presence of “differentiation markers” in the leukemia blasts [10, 107].

Models of Leukemogenesis

The currently accepted paradigm of leukemogenesis rests on the theory that leukemia arises from a single cell and is maintained by a small population of LSCs [37, 49, 69]. It is thought that normal HSCs themselves can undergo leukemic transformation. First, in normal HSCs, the machinery for self-renewal is already activated, and second, stem cells persist for a long time; therefore, the opportunity to accumulate mutations in these cells is greater than that in mature, short-lived cell types. Nonetheless, restricted progenitors could potentially be transformed, either by acquiring mutations that cause them to self-renew or by inheriting existing mutations from the preleukemic stem cells and then acquiring additional mutations themselves. Indeed, Jamieson et al. [53] reported that in chronic myelogenous leukemia, the LSC is the differentiated progenitor CFU-granulocyte-macrophage (CFU-GM).

Two models of leukemogenesis have long been proposed. According to the stochastic model, leukemia consists of a homogenous population of immature cells and a few cells that can either self-renew or proliferate in a stochastic manner [61, 86, 104]. In contrast, in the hierarchy model, leukemia consists of a heterogeneous population, within which only a small percentage of LSCs generate leukemia clones and sustain the disease. Recently, a third model was proposed [83]. According to this model, mature leukemia cells can de-differentiate and regain functional LSC capacity [53]. Whereas the first two models hold that only a few immature cells sustain leukemia, the third model allows that mature cells can regain self-renewal capacity. One might envision leukemia as a disease in which the phenotypically mature and immature neoplastic cells, characterized by genomic instability, multiply and form new leukemic clones selected to expand based on their proliferation and survival capacity. Such cells might at times either be quiescent or be proliferate; and during this process, they may differentiate or self-renew without adhering to the “rules” of the normal hematopoietic system.

LSC Phenotype and Function

The phenotypic and functional properties of normal HSCs have been used to study LSCs [15, 29, 42] (Wissman 2000). Classic studies of human acute myeloid leukemia (AML) have shown that AML consists of a heterogeneous population of cells with a small percentage of quiescent (non-cycling) cells [22, 114], of

which only 0.1–1% harbor the capacity to initiate leukemia when injected into SCID or non-obese diabetic (NOD)/SCID mice [15, 62]. In most AML subtypes, except for promyelocytic leukemia (AML-M3), the cells capable of transplanting leukemia in NOD/SCID mice have been shown to have a $CD34^+CD38^-$ or $CD34^+CD38^{+low}$ phenotype similar to that of normal HSC, whereas more mature $CD34^+CD38^+$ leukemic blasts failed to engraft [12, 15]. Using clonal tracking of retroviral-transduced normal and leukemic cells in NOD/SCID mice, it was demonstrated that both normal and LSC compartments were composed of individual stem cell classes that differed in their repopulating and self-renewal capacities [44, 49]. These elegant studies demonstrated that a fractionated population of phenotypically identical human leukemia cells engrafting in NOD/SCID mice consists of cells with different capacities. Some cells, termed NOD/SCID leukemia-initiating cells (SL-ICs), could be harvested and, when injected into a second and third generation of identical mice, engrafted and induced leukemia. A “marker” to determine whether a certain cell is a short-term or long-term SL-IC has not been identified.

Other combinations of cell-surface markers [75] and other techniques, including the extrusion of Hoechst dye 33342, have been used to identify LSCs [16, 39, 56]. Several studies suggest that HSCs and LSCs share some cell-surface markers but not others. For example, HSCs and LSCs both express CD34, CD71, and HLA-DR. However, Thy-1 (CD90) and c-Kit (CD117) are expressed on HSCs but not on LSCs, and CD123 the interleukin-3 receptor- α are expressed on LSCs but not on HSCs [12, 13, 55]. Remarkably, cytogenetically abnormal LSCs have been found in the $CD34^+CD90^+$ populations in several patients with AML, and in rare cases, $CD34^-$ cells as well as $CD34^+$ cells have successfully engrafted and initiated human leukemia in mice [17, 85, 102]. In acute promyelocytic leukemia, unlike in other myeloid leukemias, the characteristic translocation has been observed in $CD34^+CD38^+$ but not in $CD34^+CD38^-$ cells [15, 41].

The NOD/SCID mouse studies described above suggest that AML, like normal hematopoiesis, is indeed organized as a hierarchy, which is initiated and maintained by an LSC that gives rise to SL-ICs with short-term and long-term repopulating capacities that, in turn, give rise to cells with abnormal differentiation programs, leading ultimately to the production of blasts and abnormally differentiated leukemia cells [28] (Fig. 2). However, Taussig et al. [101] showed recently that cells expressing well-established mature myeloid markers (CD33 or CD13) could function as SL-ICs, thereby questioning the validity of surface marker expression as a predictor of stem cell function. Taussig’s data agree with previous studies that have demonstrated that cells devoid of self-renewal capacity, such as committed progenitors and mature cells, could be targets for leukemic transformation. For example, activation of promoter elements of several mature myeloid-specific human genes (like MRP8, CD11b, or cathepsin G) induces human leukemias in transgenic mouse models [18, 52, 119]. Recently, Cozzio et al. [23] showed that the leukemic fusion gene MLL-ENL, which results from the t(11;19) translocation,

induced the same type of leukemia whether transduced into HSCs, common myeloid progenitors (CMPs), or granulocyte-macrophage progenitors (GMPs). Furthermore, the fusion gene MOZ-TIF2 has also recently been shown to contribute to the transformation of both HSCs and committed myeloid progenitors. Thus, the activation of an appropriate oncogene in a mature hematopoietic cell could transform the cell into an LSC with self-renewal capacity. In contrast, So et al. [92], using a different fusion partner of MLL, GAS7, showed that only the transduction of murine HSC, and not CMP or GMP, resulted in the production of mixed-lineage leukemias in mice. Therefore, a universal phenotype for LSCs may not exist, and patient-to-patient variation in cell-surface protein expression may be the rule.

Of note, a similar heterogeneity exists in HSC gene expression: genes thought to form a stem cell gene signature have been identified; however, dissimilar data suggest that the existence of such a signature may be premature [50, 80, 82]. Because stem cell-specific properties, such as self-renewal, quiescence, and proliferation, are not governed by genes that are specific to stem cells, it has been proposed that there is a “stem cell state” rather than a “stem cell portrait” in the hematopoietic system [125]. The “stem cell state” may represent a transient and potentially reversible state that cells can assume in response to the correct trigger. Using a similar concept, one can argue that there is an “LSC state” rather than an “LSC phenotype,” a concept that is supported by the well-described lineage “infidelity” in human leukemias [91]. Whereas defining the sets of conditions that pertain to the LSC state may allow the development of strategies to eliminate LSCs or render them inconsequential, the identification of specific genes and surface markers expressed solely by LSCs may be difficult or impossible.

Taken together, these data suggest that LSCs should be characterized by function rather than by phenotype and be referred to as “leukemia-initiating cells.” It is unclear whether studies performed using SCID or NOD/SCID mouse models reflect normal human physiology and/or pathophysiology. Thus, the overall conclusion that can be drawn from those studies is that the capability of a leukemia cell to initiate and sustain leukemia in humans, regardless of its phenotype, physical characteristics, or function in immune-deficient mice, is the most important biological feature of the cells that we refer to as LSCs.

Molecular Pathways Regulating HSCs and LSCs

The current dogma is that self-renewal is the hallmark property of stem cells in both normal and neoplastic tissues. Therefore, the molecular pathways that regulate this self-renewal capacity have been studied extensively. Several genes, transcription factors, and cell-cycle regulators modulate the self-renewal, proliferation, and differentiation of HSCs [95, 123]. The genes SCL, GATA-2,

LMO-2, and AML-1 (also known as CBFA2 or RUNX1) govern the transcriptional regulation of early hematopoiesis, and the deregulation of these genes through chromosomal aberrations plays a key role in leukemogenesis. For example, the gene encoding the transcription factor SCL is the most frequent target of chromosomal rearrangements in children with T-cell acute lymphoblastic leukemia [4]. SCL is normally expressed in HSCs and immature progenitors and is downregulated during differentiation, and its abnormal activation might initiate malignant transformation [64]. Similarly, abnormal activation of AML-1, a gene that is required for normal hematopoiesis, as a result of translocation t(8;21) and the generation of the fusion protein AML-ETO is thought to be leukemogenic [67, 77]. The fusion protein AML-ETO has been shown to induce stem cell self-renewal [26]. However, increased self-renewal is of no apparent pathogenic consequence, presumably because secondary mutations are necessary for the expression of the leukemic phenotype [26].

Other transcription factors such as the Homeobox (Hox) genes, which include HoxB4, and the Wnt-signaling pathway have well-described roles in regulating the self-renewal and differentiation of HSCs [85, 124]. HoxB4 is abundantly expressed in HSCs, plays a role in HSC expansion, and declines as terminal differentiation proceeds [88]. Of note, deregulated expression of Hox family members such as HoxA9 is commonly observed in AML [38, 63]. The Wnt-signaling pathway, whose activity is critical to the development of several organs, plays an important role in the regulation of hematopoietic stem and progenitor cell function [85, 94]. Overexpression of β -catenin, a downstream activator of the Wnt signaling pathway, expands the HSC pool [86], and activation of the Wnt pathway increases the expression of transcription factors and cell-cycle regulators important in HSC renewal, such as HoxB4 and Notch-1 [31, 85].

The Notch/Jagged pathway modulates extracellular regulatory signals controlling HSC fate [2]. Members of the Notch family have critical roles in keeping HSCs in an undifferentiated state and may act as gatekeepers for factors governing self-renewal and lineage commitment [81]. Of interest, the gene encoding the Notch receptor is rearranged by recurrent chromosomal translocations in some patients with T-cell acute lymphoblastic leukemia [32]. Transcription factors and cell-cycle regulators associated with oncogenesis, such as Bmi-1 and Sonic hedgehog (Shh), regulate the proliferation HSCs and LSCs [99, 106]. Bmi-1, a member of the Polycomb family [65], is expressed in normal and leukemic HSCs and regulates self-renewal by modulating the activity of genes governing proliferation, survival, and lineage commitment [66, 79]. Although direct evidence for the role of Shh in the regulation of self-renewal is lacking, *in vitro* studies demonstrated HSC self-renewal activity of Shh in combination with various growth factors [11]. Recent data suggest that PTEN (phosphatase and tensin homologue deleted on chromosome ten), a negative regulator of the phosphatidylinositol 3 kinase (PI3K) pathway, has essential roles in restricting the activation of HSCs, in lineage fate determination, and in the prevention of leukemogenesis [122]. Furthermore, it has been shown recently that PTEN dependence could distinguish HSCs from LSCs [118].

Taken together, these data suggest that differential expression of several transcription factors controls the fate of HSCs and plays a critical role in the determination of self-renewal, differentiation, and lineage commitment. These pathways are under the control of various intracellular stimuli as well as cytokines and stromal factors from adjacent cells in the bone marrow. Further studies of these transcription factors in HSCs and the mechanisms causing their deregulation are likely to provide us with better targets for therapy.

Cellular HSC and LSC Regulators

Within the hematopoietic microenvironment, early progenitors are thought to be maintained and compartmentalized in a specific location in the endosteal lining of the bone cavities. However, extramedullary niches have also been described, as discussed above. Extramedullary disease is occasionally found in several leukemias. Although it is well established that the leukemogenic process is bone marrow derived, it is not clear whether, as in normal HSCs, an LSC niche exists.

Normal hematopoietic and leukemia cells proliferate in response to several cytokines and hematopoietic growth factors [34, 84]. Thus, the regulation of normal and leukemic hematopoiesis is the result of multiple processes involving cell–cell and cell–extracellular matrix interactions and the actions of specific growth factors and other cytokines as well as intrinsic modulators of hematopoietic development. Despite some important progress, the genetic and cellular factors that influence stem cells to differentiate into developmentally restricted progenitor cells or to self-renew to replace cells that have become committed to differentiation are still poorly understood.

Clinical Significance

LSCs, Drug Resistance, and Relapse

Despite the development of several new agents that effectively reduce the tumor burden in patients with leukemia, relapse continues to be the most common cause of death, particularly in patients with AML. Therefore, most, if not all, patients who attain complete remission still harbor disease-sustaining cells. Because these residual leukemia cells cannot be detected by conventional techniques (such as light microscopy), the term minimal residual disease (MRD) is used to describe them [33]. Current efforts directed at detecting and quantifying MRD are based on the assumption that eradication of disease-sustaining cells will improve treatment outcomes [35, 100]. The question of why neoplastic cells with disease-sustaining capacity survive chemotherapy has prompted extensive research in recent decades.

Normal HSCs are mostly quiescent and, because of that, protected from cell-cycle-dependent insults. Similarly, LSCs are thought to be quiescent [42] and, therefore, resistant to endogenous or exogenous apoptotic stimuli [27, 59]. Several studies have provided data that support this theory, including one by Guzman et al. [45], which demonstrated that as much as 96% of the LSC population, as defined by the phenotype $CD34^+/CD38^-/CD123^+$, was in the G0 phase of the cell cycle. Thus, the current dogma that the resting status of the putative LSCs protects them from the commonly used cell-cycle-specific chemotherapeutic agents ignores the fact that non-cell-cycle-specific agents are routinely used to treat all forms of leukemia.

Resistance to drugs and toxins by cells in the quiescent phase is thought to be mediated through the expression of ATP-associated transporters [27]. High levels of ATP-binding cassette transporters have been found in both normal and cancer stem cells but not in lineage-committed progenitor cells [20, 40, 89]. Because of this property, the efflux of fluorescent dyes such as Hoechst 33342 was thought to isolate HSCs [36, 39]. Thus, quiescent LSCs inherently possessing drug resistance mechanisms or acquiring them through mutations [27] are thought to survive chemotherapy and sustain the disease [1, 42, 45, 48, 86, 103]. Remarkably, this theory has been generally accepted, despite data showing that HSC may be non-quiescent [117], that ATP-associated transporters are present in mature non-quiescent cells [87], and that drug and dye efflux can be found in cells that do not exhibit stem cell capacity [19, 97].

It is likely that secondary events, such as the development of mutations, further contribute to the intrinsic resistance properties of LSCs. Quiescent LSCs and non-quiescent leukemia-sustaining cells may carry the initial mutations leading to genomic instability resulting in secondary mutagenic events that contribute to the development of a more resistant phenotype. Alternatively, random secondary mutations or mutations occurring as a result of selective pressure caused by therapy may contribute to disease progression and drug resistance. This has been seen in patients with CML treated with imatinib mesylate, in whom mutations of the ATP-binding site of BCR-ABL are well documented [21].

LSCs and Strategies to Cure Leukemia

As discussed above, relapse continues to be the most frequent cause of treatment failure, particularly in AML. The clinical benefits of early detection of MRD remain uncertain and are under investigation. The most commonly used assay to identify residual cells expressing a leukemia-specific molecular abnormality is polymerase chain reaction (PCR). This technique has several limitations, however, including false-positive and false-negative results, lack of standardization among laboratories, and insufficient sensitivity (i.e., the inability to detect clinically significant MDR that results in clinical relapse). More

importantly, PCR cannot distinguish LSCs from their terminally differentiated progeny, i.e., it cannot distinguish between leukemia-sustaining cells and clinically insignificant MRD [35, 83, 100].

Rather than MRD “detection and monitoring,” characterization of the molecular and biologic features of the cells that initiate and maintain leukemia is the essential step in the development of novel strategies to cure this disease. These cells have dissimilar morphologic features, no specific phenotype, no specific cell-surface or molecular markers, and they may have various degrees of abnormal maturation and may or may not be quiescent. They also may or may not fit the current definition of an LSC. Function is the only common denominator of these cells. Leukemia-sustaining cells may be different in different subtypes of leukemia and may vary from patient to patient because of the large variety of mutagenic events that initiate and drive this disease. Whether or not the cells that initiate and sustain leukemia fit the current agreed-upon features of “stem cells,” these are the cells on which we should concentrate our efforts.

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Epigenetic Mechanisms in AML – A Target for Therapy

Yasuhiro Oki and Jean-Pierre J. Issa

Abstract Epigenetics refers to a stable, mitotically perpetuated regulatory mechanism of gene expression without an alteration of the coding sequence. Epigenetic mechanisms include DNA methylation and histone tail modifications. Epigenetic regulation is part of physiologic development and becomes abnormal in neoplasia, where silencing of critical genes by DNA methylation or histone deacetylation can contribute to leukemogenesis as an alternative to deletion or loss-of-function mutation. In acute myelogenous leukemia (AML), aberrant DNA methylation can be observed in multiple functionally relevant genes such as *p15*, *p73*, *E-cadherin*, *ID4*, *RAR β 2*. Abnormal activities of histone tail-modifying enzymes have also been seen in AML, frequently as a direct result of chromosomal translocations. It is now clear that these epigenetic changes play a significant role in development and progression of AML, and thus constitute important targets of therapy. The aim of targeting epigenetic effector protein or “epigenetic therapy” is to reverse epigenetic silencing and reactivate various genes to induce a therapeutic effect such as differentiation, growth arrest, or apoptosis. Recent clinical studies have shown the relative safety and efficacy of such epigenetic therapies.

Introduction

Carcinogenesis is a multistep process at the molecular level [64], driven by genetic alterations such as gene mutation and deletion, resulting in activation of oncogenes or inactivation of tumor suppressor genes [64]. Epigenetic changes have also been shown to play a significant role in the malignant transformation of cells [84, 12]. Epigenetics refers to a stable, mitotically perpetuated regulatory mechanism of gene expression without an alteration of the gene coding

J.-P.J. Issa (✉)

Department of Leukemia, University of Texas MD Anderson Cancer Center,
Houston, TX, USA

e-mail: jissa@mdanderson.org

sequence. Epigenetic mechanisms include DNA methylation and histone tail modifications such as acetylation and methylation [84, 12]. Epigenetic changes can lead to carcinogenesis by silencing critical genes [84, 12]. DNA methylation is very stable and maintained once established, except in special states such as embryogenesis [146, 81]. Histone modifications are more dynamic biochemical changes in the context of expression regulation [146, 81] but are also involved in stable gene silencing. Another, more flexible mechanism of epigenetic regulation is through small regulatory non-coding antisense RNAs, which can achieve transcriptional or posttranscriptional gene silencing [9], although the role of these processes in carcinogenesis is unknown. Over the past decades, alterations in DNA methylation and histone modifications in leukemogenesis have been well described and are now recognized as targets of therapy for AML and other hematological malignancies.

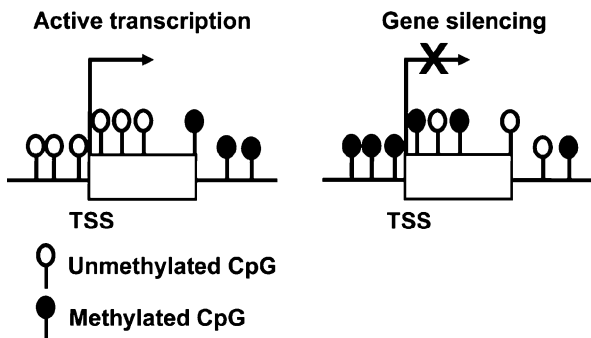
DNA Methylation and DNA Methyltransferase (DNMT)

The addition of a methyl-group to cytosine forming 5-methylcytosine in DNA has genetic and epigenetic effects on cellular development, differentiation, and carcinogenesis [84, 12]. In mammalian DNA, cytosine methylation is restricted to cytosine followed by guanosine (the CpG dinucleotide) [84, 12]. DNA methylation is accomplished by DNA methyltransferases (DNMTs), which catalyze the covalent addition of a methyl group to the 5' position of cytosine from a donor *S*-adenosylmethionine [67]. Three different proteins, DNMT1, DNMT3A, and DNMT3B, have been shown to have DNA methyltransferase catalytic activity in mammalian cells [123, 122, 9]. In general, DNMT1 serves as a maintenance DNMT, while DNMT3A and 3B serve as *de novo* DNMTs introducing methyl groups to previously unmethylated CpG sites [123, 122]. DNMT3L, another type of DNMT, does not have catalytic activity but has been identified as a stimulator of the catalytic activity of DNMT3A and DNMT3B [21, 147, 11]. Once DNA methylation is established in a CpG nucleotide, it is maintained after cell division through the activity of DNMTs, which localize to replication foci to work on newly synthesized hemi-methylated DNA [132, 102]. Recent studies suggest that DNA methylation status is determined via complex mechanisms where DNMTs interact with each other and with other proteins to induce DNA methylation [134, 53]. The major target of DNA methylation in normal mammalian cells is repeated transposable sequences, but it also plays a key role in imprinting and X chromosome inactivation in women [84, 12].

CpG sites are rare in the human genome relative to their predicted frequency, presumably because they were eliminated during evolution through C to T mutations of methylcytosine [13]. On the other hand, the human genome contains small regions with clusters of CpG sites, called "CpG islands," where the frequency of CpG is higher than expected [13]. About half of all human

genes have CpG islands in their promoter regions, and these are not usually methylated in normal tissues, regardless of the transcriptional status of the gene. Methylation in a CpG island is associated with changes in chromatin organization and consequent repression of gene transcription (Fig. 1). One mechanism by which gene silencing is achieved is that of methylation of cytosine residues in CpG dinucleotides triggers the binding of methyl-binding proteins to DNA, which attracts histone deacetylases and histone methylases that eventually modify the structure of histones into a condensed chromatin state [119]. Condensation of the chromatin prevents specific transcription factors or DNA-dependent RNA polymerase from having access to the promoter region to cause gene silencing [84, 12]. Histone H3 lysine 9 (H3K9) methylation appears to trigger further DNA methylation through a feedback loop, thus reinforcing gene silencing [7, 149]. Since CpG methylation is maintained after cell division, gene silencing by DNA methylation is also maintained and is essentially stable once it is established. It can be only reversed physiologically and reset in early embryogenesis [116]. About half of human genes do not have CpG islands in their promoters. In these cases, DNA methylation can mark the silenced state but can be reversed physiologically by activation of gene expression [16]. The extent to which non-CpG island methylation plays a role in carcinogenesis remains unclear.

Fig. 1 DNA methylation and gene silencing. Each circle indicates CpG sites, *filled circle* being methylated CpG, *open circle* unmethylated CpG, and *white box* indicates exon 1. TSS, transcription start site.



DNA Methylation in AML

Cancers have altered patterns of DNA methylation. The global DNA methylation level is often decreased in malignant cells. Simultaneously, hypermethylation also occurs in specific regions of the genome [84, 12]. Hypomethylation was initially postulated as a mechanism of carcinogenesis through activation of oncogenes [50]. It is also known that hypomethylation is associated with chromosomal instability *in vitro*, and this may play a role in carcinogenesis [24]. On the other hand, aberrant DNA hypermethylation can clearly contribute to