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SPOTLIGHT REVIEW

Insights into the stem cells of chronic myeloid leukemia

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Chronic myeloid leukemia (CML) has long served as a paradigm for generating new insights into the cellular origin, pathogenesis and improved approaches to treating many types of human cancer. Early studies of the cellular phenotypes and genotypes represented in leukemic populations obtained from CML patients established the concept of an evolving clonal disorder originating in and initially sustained by a rare, multipotent, self-maintaining hematopoietic stem cell (HSC). More recent investigations continue to support this model, while also revealing new insights into the cellular and molecular mechanisms that explain how knowledge of CML stem cells and their early differentiating progeny can predict the differing and variable features of chronic phase and blast crisis. In particular, these emphasize the need for new agents that effectively and specifically target CML stem cells to produce non-toxic, but curative therapies that do not require lifelong treatments.
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Early evidence that chronic myeloid leukemia (CML) is a mutli-step clonal disease of HSC origin

As far back as 1951, Dameshek was advancing the concept that human CML originates in a HSC with a capacity to generate erythroid cells, granulocytes and platelets.[1] This was based on the observation that bone marrow cells from CML patients often showed an elevated proliferation of early recognizable cell types differentiating down all of these myeloid lineages even though the disease, itself, is characterized by the lineage-specific deregulated production of mature granulocytes. In the second half of the twentieth century, the Philadelphia (Ph) chromosome was discovered to be a signature feature of cells from CML patients[2] and, a few years later, was shown to be the cytogenetic outcome of a very specific exchange of DNA between chromosomes 9 and 22.[3] This rearrangement causes the 3′ end of the *ABL* gene to be juxtaposed to the 5′ end of the *BCR* gene resulting in the formation of a new *BCR–ABL* fusion gene that encodes an oncoprotein with constitutive tyrosine kinase activity.

The discovery of the Ph chromosome and its apparent restriction to CML was rapidly followed by the demonstration of the Ph chromosome (and later the associated *BCR–ABL* gene) in multiple lineages of myeloid cells.[4,5] This constituted the first direct evidence that CML is a clonal disorder, in addition to cementing the idea advanced by Dameshek that the clone originates in a normal HSC.[1] Studies of X-chromosome inactivation patterns in CML patients' cells subsequently confirmed the leukemic origin of the expanded granulomonocytic[6,7] compartment, as well as all of the circulating red blood cells, platelets and some B cells, but not T cells, in these patients.[6–8] Ultimately, recognition of the unique specificity of the *BCR–ABL* gene rearrangement in individual patients' cells provided the final definitive evidence that chronic phase CML is a multi-lineage clonal disease and the retrospective inference that it originates from a normal multipotent hematopoietic cell with long-term self-sustaining properties. Further support for the latter inference came from the documented durability of the chronic phase of the disease in many patients (typically 3–7 years) and the presumed genetic stability of chronic phase CML cells based on the lack of other cytogenetic alterations detected during that period. The first evidence of CML 'stem' cells with repopulating activity actually came by accident from a series of early clinical experiments in which several severely neutropenic recipients were transiently repopulated with Ph^+/*BCR–ABL*$^+$ cells following the infusion of unirradiated white blood cells obtained by leukapheresis from CML patients with high granulocyte counts.[9,10] What was not appreciated at the time was the co-mobilization of high numbers of primitive leukemic cells into the blood of such patients.[11–13]

However, continued cytogenetic follow-up of CML patients' cells showed that the oncogenic process in this disease is indeed one of clonal evolution. As discussed below, we now understand that this process is derivative of an acquired genomic instability that leads to the continuous acquisition of new genetic alterations and an accompanying selection of growth-advantaged subclones, the properties of which may differentially affect treatment response, as well as disease progression.

Evidence of a residual normal HSC population in CML patients was first provided from early studies of heavily treated patients.[14] Soon after, in the 1980's, we showed that a persisting and functionally intact, but suppressed, residual normal HSC population could be revealed in most patients when this compartment was selectively assessed.[15] These findings encouraged a broader search for therapeutic alternatives to allogeneic bone marrow transplantation that could take advantage of the possibility that these residual autologous HSCs might be sufficient to permanently re-establish normal hematopoiesis. Ultimately, this concept culminated in 'first-in-CML' studies demonstrating that the product of a shared oncogenic mutation could serve as a clinically useful therapeutic target.[16] This finding introduced the modern era of CML management in which inevitable death within 10 years because of ineligibility for an allogeneic transplant had previously been the rule for most patients. The finding that targeting the *BCR–ABL* oncoprotein could be highly effective also raised hopes that similarly tumor-specific treatments could be found for other malignancies. Then came the more recent sobering revelation that CML stem cells possess an innate relative resistance to *BCR–ABL*-targeted agents[17–21] and an unusual genomic instability.[19,22–24] These discoveries have, in turn, reactivated a search for newer

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agents that can be shown to kill CML stem cells.[25,26] In addition, they have highlighted the need for better models of the human disease that will allow a more accurate prediction of the clinical utility of newer agents.

Studies of CML patients and their cells have thus led the way in establishing the concept of malignancies as multi-step, clonal disorders that arise by the sequential acquisition of genetic (or epigenetic) alterations in cells with multipotent stem cell properties. A growing body of data suggests that these elements may be features of other cancers, albeit with sometimes substantive provisos and exceptions. These caveats recognize the significant differences being found in the mechanisms that regulate the turnover of undifferentiated cells in various tissues, the types of mutations or epigenetic changes required for their transformation (or that of their progeny), and the state of genetic/epigenetic evolution of a given tumor when it first becomes detectable.

Unique features of CML as a prototype stem cell-driven malignancy

Several features of human CML explain its premier role in laying the foundation of cancer stem cell biology. One is the simple fact that CML is, itself, a stem cell-driven leukemia. This means easy access to viable single-cell suspensions of malignant cells using relatively non-invasive methods to obtain primary tissue samples—a prerequisite for performing the types of quantitative analyses needed to interrogate stem cell populations with rigor and sensitivity. In contrast, experimentation with cells from most solid tumors depends on access to surgically removed specimens in which the tumor cell content is heterogeneously distributed and often difficult to obtain as a suspension of single cells that are still viable at the end of the dissociation process.

CML also differs from most other cancers in its usual presentation at an early stage in the oncogenic process when a clone of deregulated cells is being amplified, but they are still executing essentially normal differentiation programs. Nevertheless, the clone is already becoming numerically dominant and is destined to undergo further progression. Given the multistep and complex nature of the process of hematopoietic differentiation in normal individuals, surface phenotypes, transcriptional profiles and functional assays have proven to be critical to understand the earliest stages of hematopoietic differentiation when the cells of interest are rare and not morphologically distinguishable. The fact that chronic phase CML cells show various quantitative, but not qualitative, changes in their functional differentiation programs has facilitated the generation of data suggesting how clonal dominance is achieved in chronic phase CML. Such studies have also cast light on the molecular mechanisms involved, as well as providing clues as to the steps involved in the subsequent progression of the disease, and what may be required for future therapies to achieve cures. Last but not least, is the generally accepted exclusive association of the *BCR–ABL* mutation with CML and its complete penetrance in the clone. This has enabled the confident use of the Ph/*BCR–ABL* marker to specifically identify and quantify any CML patient's leukemic cells, regardless of the tissue from which they are obtained, their morphological appearance or other properties displayed.

Lessons from studies of the pathogenesis of CML

Definitive evidence of the origin of human malignancies requires prospective approaches for analyzing their cellular development. Such opportunities are obviously rare and CML is no exception to this general rule. It is, therefore, not surprising that our knowledge of the time course and nature of the steps that precede the diagnosis of chronic phase CML is sketchy and largely reliant on anecdotal events. In this regard, retrospective analyses of the time taken for the disease to appear in individuals previously exposed to an acute mutagenic event have provided the best information available on this topic. Such observations indicate an initial latent period of several years before most leukemic *BCR–ABL*$^+$ clones are expanded sufficiently to cause symptoms, albeit with a broad time distribution amongst individual patients.[27–29] This variable, but generally long, period preceding the detection of disease symptoms implies the existence of a correspondingly variable but, on average, long time required for the initially affected cell to generate a symptomatic *BCR–ABL*$^+$ clone.

One might speculate that the long latent period thus implicated is evidence of the need for additional mutations to occur before a subclone with chronic phase disease properties can develop. Consistent with such a hypothesis are two reports of *BCR–ABL* transcripts present at very low, but detectable, levels (in $<1\%$ of blood cells) in a majority of normal adults, but absent from newborns.[30,31] On the other hand, evidence of other mutations or epigenetic changes consistently contributing to the generation of chronic phase CML has not yet been obtained. The other argument against a two-hit hypothesis for chronic phase CML is provided by the experiments in which *BCR–ABL* expression has been introduced into primitive normal mouse[32] or human[33,34] hematopoietic cells using various viral transduction protocols. The efficiency and rapidity with which such forced expression of *BCR–ABL* can thereby produce transduced clones that recapitulate numerous features of chronic phase CML disease, suggests that additional genomic changes may not be required, at least when high levels of *BCR–ABL* expression are sustained.

Such gene transfer experiments have also been used to investigate the properties of clones generated by transducing cells at different stages of hematopoietic cell differentiation with *BCR–ABL*. The first such experiments using bone marrow from mice treated with 5-fluorouracil as the target cell population established the ability of forced overexpression of *BCR–ABL* to generate rapidly expanding multi-lineage clones in irradiated recipients. The typical outcome within 3–4 weeks after transplantation of the transduced cells is output of an excessive number of circulating granulocytes derived from transduced donor cells, a hypercellular bone marrow containing mainly myeloid cells at different stages of maturation, and extramedullary myelopoiesis in the spleen and liver accompanied by a massive and usually fatal infiltration of granulocytes in the lung.[35,36] However, the generation of a disease with these CML-like features appears to be dependent on restricting the transplant to transduced primitive cells. Thus, when unfractionated mouse bone marrow cells are transduced and transplanted, lineage-restricted lymphoid or macrophage malignancies are commonly produced.[35–37] More stringent dissection of the cell types able to regenerate a CML-like disease using transplants of highly purified transduced targets showed that neither purified common myeloid progenitors nor granulomonocytic progenitors were competent in contrast to upstream populations more enriched in HSCs.[38,39] These observations provide additional evidence supporting the HSC origin of CML in patients. They also argue against the theoretical possibility that *BCR–ABL* could be sufficient to initiate a self renewal program in downstream progenitors, in contrast to other types of genetic events that use this mechanism to initiate blast crisis

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or other clinically defined forms of $Ph^+/BCR–ABL^+$ acute leukemia.[39–41]

The variable, but generally long, phase that precedes the diagnosis of chronic phase CML also suggests a high variability in the precise stage of the disease when the diagnosis will be made. This variability is unquestionably further enhanced by the fact that more and more CML patients are being diagnosed by chance (before they have become symptomatic) as part of a routine check-up that reveals an elevated white blood cell count. Accordingly, we should expect a similarly increasing heterogeneity in the composition and properties of the leukemic cells in newly diagnosed CML patients. The idea that CML may be diagnosed in different individuals at varying, although usually early stages of the disease is an important one, as it affects how biological data are analyzed and how the results of clinical trials are interpreted.

Interferon was the first treatment that consistently produced durable cytogenetic remissions in patients with chronic phase CML without the need for a rescue transplant.[42] Thus, before the mid 1980's, the management of most chronic phase CML patients with hydroxyurea or busulphan simply kept the clone size under control without affecting its subsequent course. Under these treatment conditions, progression from the chronic phase to the inevitably fatal blast phase occurred over a highly variable time frame, but with a reproducible median time after diagnosis of 5 years. Often, the onset of blast crisis was preceded by a 6–12-month period of more difficult disease control, referred to as the 'accelerated' phase.

Blast crisis

The hallmarks of blast phase CML are similar to those of other forms of human acute myeloid or lymphoid leukemia. These include a failure of the blast phase cells to differentiate and hence a rapid accumulation of non-functional cells in the marrow and blood. The variability in time separating the diagnosis of chronic phase disease and the onset of blast crisis predicts that a small but finite fraction of $BCR–ABL^+$ clones might progress so rapidly that blast crisis would occur even before the chronic phase cells had acquired clonal dominance. This would then give a clinical picture of '*de novo*' acute myeloid or B-lymphoid $Ph^+/BCR–ABL^+$ leukemia. It is interesting that this concept has been validated by the detection in some of these individuals of leukemic ($Ph^+/BCR–ABL^+$) myeloid cells capable of normal differentiation—thus providing evidence of the origin of the blast phase cells from a pre-existent, but incompletely expanded chronic phase clone.[43] Conversely, occasional chronic phase CML clones would be predicted (and have been found[44]) to show no evidence of progression for very long periods of time (14 years) with the result that some would actually die of other causes before any progression of their CML disease was detectable.

Progression to blast crisis involves the acquisition of secondary genetic changes that cause a block in the execution of normal differentiation programs necessary for mature blood cell formation. The most common of these genetic alterations are well established and known to be associated with other forms of acute lymphoid or myeloid leukemia. The superimposition of a differentiation blockade on cells already proliferating out of control provides a ready explanation for the rapidity with which non-functional blasts rapidly accumulate. However, it should be noted that the blast crisis of CML is usually associated with the generation of a subclone of cells that shows some myeloid (70%) or B-lymphoid (30%) features, but not both. Two models have been advanced to suggest how this might occur. One proposes that a chronic phase CML stem cell acquires additional mutations that variably block their further differentiation into one or more lineages, thereby leading to a skewed output of derivative blasts. The second proposes that the development of a blast crisis subclone results from the acquisition of additional mutation(s) by a member of one of the expanded downstream lineage-restricted progenitor compartments. The presence of clonal immunoglobulin gene rearrangements in lymphoid blast crisis and $Ph^+/BCR–ABL^+$ lymphoid leukemia has long supported the second explanation in these examples.[45] More recently, evidence of an origin of myeloid blast crisis in granulocyte-restricted chronic phase CML progenitors has also been reported.[40] It is interesting that these findings add to the accumulating experimental evidence that certain mutations are sufficient to reactivate or confer self-renewal activity upon cells in which such programs had previously been thought to be normally extinguished.[38,39,41,46–48]

The anatomy of primitive elements in chronic phase CML clones

CML progenitors

The preservation of 'normal' differentiation programs by cells within chronic phase CML clones provided initial confidence that assays developed to distinguish different stages of normal hematopoietic cell development might be similarly applicable to $Ph^+/BCR–ABL^+$ cells. Then through the quantification of the different compartments thus identified, important clues were gained about the molecular mechanisms leading to clonal dominance and a selective overproduction of granulocytes. The results of these studies showed that leukemic and normal progenitors of the same cell surface phenotype produce colonies of mature erythroblasts, granulocyte-macrophages or megakaryocytes (or mixtures of these) in growth factor-supplemented methyl cellulose (or agar) cultures with a similar distribution of colony sizes, morphologies and kinetics of growth and maturation. Indeed, to this day, the separate quantification of co-existing CML and normal progenitors (so-called colony-forming cells, CFCs) using short term *in vitro* colony assays depends on the additional application of methods to genotype the individual colonies obtained—either by cytogenetics, fluorescence *in situ* hybridization or reverse transcriptase–PCR.

The early results of using such approaches instantly revealed an extension of the picture postulated by Dameshek.[1] Not only are leukemic CFCs present in patients with chronic phase disease, their numbers on all lineages increase proportionately with the total size of the clone in both the blood and bone marrow.[49] Thus, by the time of diagnosis, these progenitor cells have already attained dominance within their respective compartments in most patients (Figure 1).[50] In addition, it is important to note that the leukemic progenitors are being spontaneously, continuously and proportionately mobilized into the blood. As a result, leukemic CFC concentrations in the blood that are 1000 to 10 000-fold above normal values can frequently be found in patients whose white blood cell counts have risen above 10^{11} per litre.

These findings reinforce the concept that chronic phase CML clones arise from and are sustained by HSCs whose genetically determined perturbation has little effect on any of the mechanisms that select (restrict), activate or execute the three major myeloid lineage differentiation programs but rather leads to a lineage non-specific amplification of the intermediate

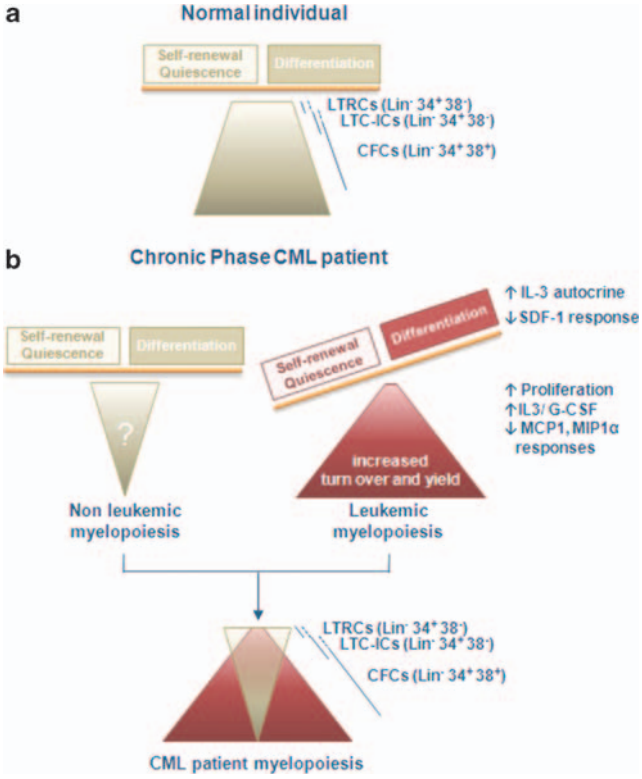

Figure 1 Schematic representation showing how leukemic myelopoiesis is differently deregulated at different stages of hematopoiesis in patients with chronic phase CML. (**a**) In normal adults (green) myelopoiesis is sustained throughout life by the regulated proliferative and differentiation activity of a large pool of HSCs (represented by the width of the top of the green trapezium). Production of mature myeloid blood cells (red blood cells, granulocytes and monocytes, and platelets; represented by the width of the bottom of the green trapezium) is accompanied by a successive series of cell divisions during which there is a progressive loss of proliferative potential and a restriction of differentiation potential to a single lineage. Cells within hematopoietic hierarchy can thus be distinguished by the proliferative and differentiation activity they display under conditions designed to optimally elicit these, either *in vivo* (where the most primitive cells are called long-term repopulating cells, LT-RCs) or *in vitro* (as long-term culture-initiating cells, LTC-ICs and CFCs). Surface markers, such as CD34 and CD38 are differentially expressed upon differentiation, progenitors being mostly $CD34^+CD38^+$ and HSCs exclusively $CD34^+CD38^-$. (**b**) In patients with chronic phase CML normal (green) and leukemic (brown) cell populations co-exist. In the stem cell compartment, normal HSCs (represented by the width of the top of the green trapezium) often outnumber the small numbers of their leukemic counterparts (represented by the narrow width of the top of the brown trapezium). However, current evidence suggests that the normal HSCs are outcompeted by the CML stem cells when these begin to differentiate, which the CML stem cells also attempt more frequently due to their somewhat higher turnover and increased probability of differentiation (see Figure 2). The autocrine secretion of IL-3 and G-CSF by primitive leukemic progenitors and their decreased sensitivity to antiproliferative stimuli, such as stromal cell-derived factor 1, MIP-1α and MCP-1, likely contribute to the observed growth advantage of leukemic myeloid progenitors and mature cells in patients resulting in their dominance of the peripheral blood and bone marrow of newly diagnosed CML patients with mature CML cells (enlarged base of brown trapezium).

committed progenitor pool. Thus, the selective excessive production of mature granulocytes characteristic of chronic phase CML is vested in the ability of the clone to produce an excessive number of all myeloid blood cell types and a very late-stage down-regulation of the nascent erythroid and megakaryocytic output potential of the leukemic CFCs.

CML stem cells

The application to blood and bone marrow samples from CML patients of additional assays developed to identify more primitive normal hematopoietic cells has enabled the similar detection of analogous leukemic elements in the earliest known compartments of hematopoietic cells. These include Ph^+/*BCR–ABL*$^+$ cells that can generate progeny CFCs for at least 6 weeks in cultures containing competent stromal feeder layers. The cells thus identified are commonly referred to as long-term culture-initiating cells (LTC-ICs).[13,51] In CML bone marrow samples, the frequency of such leukemic LTC-ICs is comparable to, or somewhat lower than, the frequency of LTC-ICs measured in bone marrow samples from normal subjects.[50,52] However, given the increased marrow cellularity of typical CML samples, the overall bone marrow content of LTC-ICs is likely to be the same as in normal subjects or perhaps slightly increased. On the other hand, most of the LTC-ICs detected are normal, in sharp contrast to the CFCs. And those that are leukemic are typically rare and often below the limit of detection.[50] This limit is determined by the number LTC-derived CFCs that can be obtained and individually genotyped. This constraint considerably restricts the sensitivity with which leukemic and normal LTC-ICs can be detected in many samples. LTC-ICs of both genotypes are also mobilized into the blood although at somewhat lower levels than the CFCs. As a result, blood samples from patients with high white blood cell counts are often the richest source of primitive chronic phase CML cells, even though the majority of those present are typically normal and this can only be determined by genotyping the colonies their progeny eventually generate.

Transplant experiments to detect CML stem cells with *in vivo* repopulating activity have lagged behind *in vitro* studies. One reason for this is the very low frequency in the blood or marrow of CML cells with *in vivo* repopulating activity, as predicted by the LTC-IC findings; hence the reported use of very large innocula to detect any of such *in vivo* activity. A second reason is the fact that adequately immunodeficient long-lived mice to serve as suitable hosts have only recently become available. Both sub-lethally irradiated SCID and NOD/SCID mice retain sufficient endogenous natural killer cell activity to eliminate injected human short-term repopulating cells even though those with long-term activity may survive[53] and it may be that chronic phase CML cells have a generalized heightened sensitivity to natural killer activity compromising further their ability to perform in these mice. Thus, although a substantial leukemic population can be readily regenerated from transplanted blast crisis CML patients' cells, this is not true with chronic phase CML cell transplants. In the latter case, the output of leukemic (Ph^+/*BCR–ABL*$^+$) cells in SCID or NOD/SCID mice is usually either very low or at best transient with normal human cells invariably assuming dominance when any reconstitution is detected beyond the first 6–8 weeks post transplant.[54–57] Using the more highly natural killer-deficient NOD/SCID-β2-microglobulin-null mice as hosts, we found that CML cells with both short and longer term reconstituting activity could be detected in some chronic phase CML patients' samples.[12] However, in none of these studies was evidence of an expanding population of chronic phase leukemic cells noted. The generation of interleukin-2 (IL-2) receptor-γc-null mice on the NOD/SCID (NSG)[58] or RAG2-null[41,59] background provides a significant

further improvement in the frequency of normal human repopulating cells detectable, in the corresponding level of hematopoietic reconstitution obtainable and in the durability of the reconstituted populations that can be followed. Our initial experience using NSG mice as hosts of CML cells has validated the utility of these mice for characterizing chronic phase CML stem cells able to sustain leukemic cell output for many months (I. Sloma and C. Eaves, unpublished observations).

CML cells with LTC-IC or *in vivo* repopulating activity also display the same surface marker profiles as their normal counterparts. In fact, to this date, no reproducible distinguishing surface marker has been documented that allows the differential isolation of CML and normal stem cells with equivalent functional read-outs. CML stem cells are negative for lineage markers (Lin–) including those present on granulocytes (CD15 and CD66b), moncytes and macrophages (CD14), erythroid cells (glycophorin A and CD71), megakaryocytes (CD41 and CD61), natural killer cells (CD56 and CD16) or T- (CD3, CD4 and CD8) or B-lymphocytes (CD19, CD20 and CD38). They also express the same markers that are present on or in primitive normal human hematopoietic cells (CD34, Thy-1/CD90, CD133 and aldehyde dehhydrogenase activity) and display a similar ability to efflux Rhodamine-123.[13,51,60–62] The only exception to these shared phenotypes is explained by the slightly higher proportion of very primitive CML cells that are in cycle.[63] Accordingly, as for CML CFCs, their more primitive precursors can be specifically identified as leukemic and then characterized only through the application of genotyping approaches to directly sorted subsets of cells with highly enriched functionally defined activities, or to the amplified progeny of functionally defined primitive cells.

This point is particularly important because the most primitive cells are extremely rare elements and, as noted, the Ph/*BCR–ABL*$^+$ components are usually outnumbered many-fold by their normal counterparts, in sharp contrast to the prevalence of leukemic cells in all of the later progenitor and terminally differentiating compartments.[13,50,52] This is also true for phenotypes, such as the CD34$^+$CD38$^-$ subset that is highly enriched for LTC-ICs and repopulating cells. However, even this phenotype rarely contains the most primitive types of functionally defined cells at purities of $>30\%$ due to the contaminating presence of more differentiated cell types that can share the same phenotype. Because the latter vary from sample to sample, the relative frequency of primitive CML cells as defined by more stringent functional criteria is not well predicted by the prevalence of Ph$^+$/*BCR–ABL*$^+$ CD34$^+$CD38$^-$ cells.

Mechanisms of deregulated primitive cell output in chronic phase CML

The fidelity with which the chronic phase CML clones recapitulate the complete hierarchy of normal hematopoiesis (except for the production of T-lineage cells) is consistent with the assumed origin and maintenance of CML clones by leukemic stem cells that are phenotypically and functionally similar to the normal HSC pool. At the same time, it is important to note that neither the predominance of all leukemic myeloid lineages nor the excessive output of granulocytes and monocytes that are both characteristic of chronic phase CML clones result directly from changes in chronic phase CML stem cell behavior. Rather these disease-associated features are due to biological changes that deregulate the output of hematopoietic cells at later stages of differentiation.

BCR–ABL expression

Although there are some reports of reduced *BCR–ABL* expression in some very primitive CML cells,[64,65] our experience and that of others has been the opposite; that is, *BCR–ABL* transcripts (and active oncoprotein) are found at the highest levels in the most primitive cells and consistently in all of them.[20,40,66] However, the *consequences* of *BCR–ABL* expression lead to diverse effects in different cell types. For example, *BCR–ABL* expression appears to have a direct and immediate ability to specifically activate an autocrine IL-3 and granulocyte colony-stimulating factor loop in primitive CML cells that, in turn, stimulates the stem cells to differentiate (rather than to self-renew). However, in their immediate downstream progeny, increased exposure to IL-3 and granulocyte colony-stimulating factor causes a rapid and deregulated lineage non-specific expansion of leukemic CFC numbers. The normal HSC production of normal CFCs is thereby greatly diluted and unable to compete for the external cues required for their continued growth and maturation. Ultimately this results in the progeny of the CML CFCs becoming dominant throughout the entire hematopoietic system in the terminal compartments. Hence, even when the final output of cells of a particular lineage is reduced (for example, as is frequently the case for red blood cell production), all of the cells are, nevertheless, usually found to be derived from the leukemic clone (Figure 1).[6,7] At the same time, direct assessment of more primitive populations typically reveals the presence of a persistent, even predominant, normal (polyclonal) stem cell compartment.[67,68] On the other hand, if the bulk of the leukemic cells are killed by treatments that are either non-specific (for example, intensive chemotherapy[69]) or specific (for example, tyrosine kinase inhibitors that target the *BCR–ABL* oncoprotein[70,71]), the residual normal HSCs, being the more numerous, are stimulated to reinitiate normal hematopoiesis. This can then produce a full cytogenetic, as well as a hematologic remission, the duration of which will depend on the effectiveness and duration of the anti-leukemic treatment applied.

Altered proliferation control

The slow expansion of leukemic stem cell numbers and the larger and more rapid expansion of later cell types are associated with (and likely due to) an increase in the proportion of these cells that are actively proliferating. The most definitive measurements of the proportion of leukemic or normal CFCs and LTC-ICs in S-phase have been obtained using treatments that specifically kill cycling cells and that can then be coupled with functional assays for stem or progenitor activity. Common examples of this approach involve exposing the cells of interest in the living state to high specific activity ^{3}H-thymidine or other relatively cell cycle-specific chemotherapeutic agents for varying periods of time and then assaying the treated cells for retained or lost stem or progenitor activity. In normal adult marrow, where the vast majority of the most primitive hematopoietic cells are located, almost all of these are quiescent and hence are not immediately affected by short term treatments.[72] In contrast, successively later stages of differentiation, identified as the cells that produce progressively smaller colonies, show a correspondingly progressive increase in the proportion of their members that are already proliferating *in vivo*. Thus, by the time hematopoietic cells normally begin to be morphologically identifiable, all appear to be proliferating and the onus for compartment control normally shifts to an almost exclusive reliance on mechanisms that control apoptosis.[43] It is interesting that, although many intermediate types of

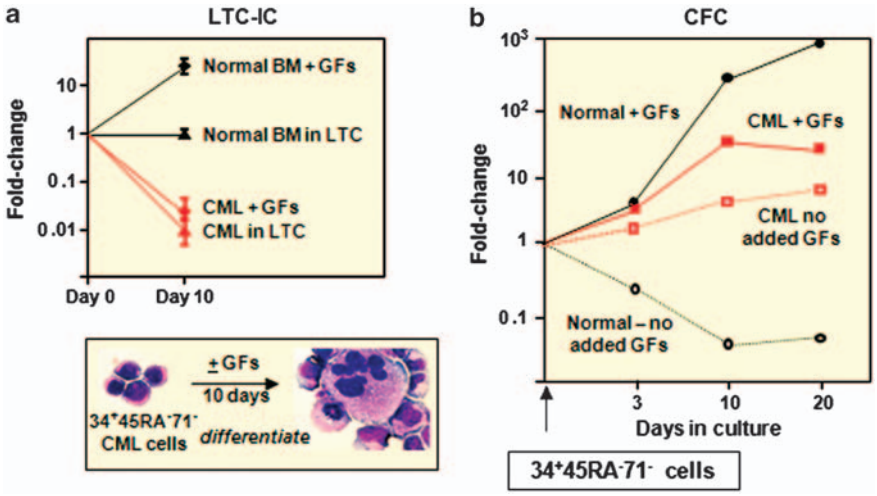

Figure 2 *In vitro* primitive CML cells can divide and differentiate autonomously but self-renew more poorly than primitive normal cells. (**a**) CML stem cell self-renewal defect CML LTC-IC numbers decrease rapidly and dramatically both when cultured in suspension in serum-free medium supplemented with human recombinant growth factors (+GFs = SF, Flt3L, IL-3, IL-6 and G-CSF) or in serum-supplemented medium on a stromal feeder layer (LTC = established from normal human marrow). Under these same conditions, normal LTC-IC numbers expand several-fold or are maintained. The loss of CML LTC-ICs in such cultures has been related to an increased propensity for their rapid differentiation (lower left box) at the expense of executing self-renewal divisions. (**b**) Altered control of CML progenitor proliferation and apoptosis—Normal primitive hematopoietic cells ($CD34^+CD45RA^-CD71^-$) are dependent on specific exogenous GFs to suppress the activation of apoptotic mechanisms. Thus, when these cells are cultured in the absence of these GFs, CFCs numbers drop 10- to 100-fold within 10 days. In contrast, analogous subsets of CML cells incubated under the same conditions proliferate and transiently expand the total input CFC number.

CFCs are normally found in the blood along with some of their more primitive precursors, cell-cycle analyses reveal these to be quiescent.[43]

When similar measurements are applied to primitive CML cells, the overall trend is a pronounced increase in the proliferative activity of all cells detectable as CFCs[43] and some increase in the proliferative activity of LTC-ICs, regardless of their location in marrow or blood.[63] The autocrine IL-3/granulocyte colony-stimulating factor mechanism documented to be active in virtually every proliferating primitive ($CD34^+$) CML cell[66,73] provides a candidate mechanistic explanation for both the increased cycling and viability of CML CFCs as both of these cytokines are known to stimulate these activities in normal CFCs. In addition, primitive CML cells show a reduced insensitivity to the specific stromal cell-derived chemokines that can contribute to the decreasing degrees of quiescence exhibited by primitive normal cells *in vivo* and in the LTC system (that is, stromal cell-derived factor 1, SDF-1; monocyte chemotactic protein 1, MCP-1; and macrophage inflammatory protein 1 α, MIP-1α[74–76]). Thus in combination, an increased mobilization of primitive CML cells and an increased proliferative activity would be predicted outcomes.[43] Moreover, primitive CML cell responses to transforming growth factor-β, another major inhibitor of primitive normal hematopoietic cell proliferative activity,[77–79] do not appear to be perturbed.[80] Thus it has been inviting to speculate that this latter mechanism may be a key regulator of the quiescent status of the most primitive CML cells. Data for CML cells with *in vivo* repopulating activity has been more difficult to generate; likely because these more primitive cells are so rare. In addition, for the proportion that might be proliferating, detection could be further compromised by the same mechanisms that prevent normal (long-term) HSCs in the $S/G_2/M$-phases of the cell cycle from engrafting.[53,63]

Altered control of apoptosis

Normal primitive hematopoietic cells are also dependent on specific exogenous growth factors to suppress the activation of apoptotic mechanisms that would otherwise lead to their death. Thus, when these cells are cultured in the absence of these, both total cell and CFCs numbers drop 10- to 100-fold within 10 days.[81] In contrast, analogous subsets of CML progenitors incubated under the same conditions proliferate and expand the total input CFC numbers.[81] This is also attributable to the autocrine secretion of IL3 and granulocyte colony-stimulating factor by all types of primitive CML cells,[66] albeit at decreasing levels as they differentiate.[20]

Altered control of CML stem cell self-renewal

Growing evidence indicates that the differentiation and self-renewal fate outcomes of proliferating HSCs can be influenced and, under some conditions determined, by extrinsic cues. At the same time, the cell must have set up an intrinsic state to enable it to act like a stem cell both in terms of its 'poised' chromatin structure and in terms of the regulatory machinery it must have in place to sustain a *status quo* situation or initiate change towards lineage program selection and activation.[82] Again, a lack of sources of primary CML stem cells that can be obtained in sufficiently pure form has inhibited progress in delineating the pathways that mediate altered CML stem cell behavior. Nevertheless, even limited studies *in vitro* have long suggested that CML cells may actually display an intrinsically determined reduced self-renewal potential when compared with well stimulated primitive normal cells (Figure 2) and that this may explain why CML stem cells are poorly competitive in both *in vitro* and *in vivo* 'experiments'.[54,56,83,84]

We have further shown that exposure of normal HSCs (LTC-ICs) to excessive IL-3 can inhibit the self-renewal-enhancing action of slightly lower concentrations of IL-3 when provided in combination with high concentrations of Steel factor and Flt3-ligand.[85] Thus, it is very tempting to speculate that the autocrine secretion of IL-3 by the most primitive CML cells may induce the inhibitory effect elicited by exposure to excessive IL-3 in their normal counterparts. This hypothesis is also supported by studies performed using a recently reported

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'improved' mouse model of CML. In this model, *BCR–ABL* expression is driven by the *Scl* promoter to more closely mimic the very high expression of *BCR–ABL* in CML stem cells and its progressive decrease upon differentiation.[20,86] Other interesting auxiliary possibilities to suggest an altered CML self-renewal behavior have come from studies of additional genetically engineered mouse models of CML.[86,87] These latter studies have identified different genes (*B catenin, Shh, Pml, Alox5 and Foxo*) that are essential for CML propagation in recipients.[88–93] The challenge now with all of these findings is to determine the extent to which they are relevant to primary CML in humans and to which they can be exploited to design better prognostic or therapeutic approaches.

Related to the poor maintenance (that is, rapid loss) of primary human CML stem cells *in vitro* and difficulties encountered in demonstrating their expansion *in vivo*, is the growing recognition that these cells are becoming decreasingly available to investigators. Thus, we have embarked on studies to examine whether the factors used to successfully amplify primitive normal HSCs can be applied to CML stem cells without causing disease progression. Preliminary results suggest that this approach may indeed be fruitful.[94]

Properties that affect responses to *BCR–ABL*-targeted therapeutics

The rapid and dramatic responses attained in CML patients treated with imatinib mesylate (IM) introduced a new era of genomic-driven approaches to cancer drug development. At the same time, initial clinical experience with IM has also revealed a number of unanticipated results that have dampened the utility of this agent and have led to new findings about CML stem cells that may likewise have broader significance in the oncology field.

The major clinical limitations encountered with IM therapy of CML are as follows. First, to be effective, IM must be given continuously for at least several years, and its discontinuation almost always results in a rapid reappearance of large numbers of leukemic cells. Second, even in treated patients, 10–20% of patients in early chronic phase and up to 40% with advanced phase disease will either fail to achieve an initial response or will develop an early relapse and 1–3% or will progress very rapidly into BC. Accumulating evidence has now shown that very primitive CML cells in the chronic phase are relatively unresponsive to IM and, in fact, to many tyrosine kinase inhibitors both *in vitro* and *in vivo*.[17,18,95] These rare leukemic cells also share in their possession of multiple unique features that would be expected to promote both common and acquired mechanisms of resistance to *BCR–ABL*-targeted therapeutics.[19,20,22,96–100] The latter include the greatly increased expression of *BCR–ABL* in primitive ($CD34^+CD38^-$) CML cells (a >100-fold increase in transcripts and a 3–10-fold increase in protein and kinase activity) that appears to be independent of the cycling status of the cells.[19,20,40,62,101] These cells also display a selectively low and high level of expression of various transporters critical for obtaining useful levels of the drug intracellularly. For example, *OCT1*, the gene that encodes a key transporter required for IM uptake,[102,103] is expressed at extremely low levels in primitive CML cells.[20,98] Similarly, *ABCB1* (*MDR*) and *ABCG2*, two genes that encode key transporters that efflux many drugs are expressed at selectively elevated levels on primitive CML cells.[20,104,105]

Heightened genomic instability has long been thought to be a feature of CML and in the last decade quantitative measurements have now provided definitive evidence that the *BCR–ABL* fusion gene markedly enhances the genomic instability in hematopoietic cells.[106–108] This property is mediated in concert with elevated levels of reactive oxygen species-dependent DNA damage that affect the genome broadly, thereby promoting the generation of IM-resistant derivatives.[24,109] The rapid generation of *BCR–ABL* mutants in primary CML cells *in vitro* extends recent findings from similar experiments with *BCR–ABL*-transduced murine BaF3 cells[110] and adult human bone marrow cells.[111] It is of note that the frequency of mutants seen in primary CML cells appears to be higher in the most primitive leukemic cells than in the mature elements.[22] Although the explanation for this remains to be established, it is possible that this might be caused by the elevated levels of *BCR–ABL* oncoprotein in the more primitive cells, and/or an enhanced accumulation of mutant cells in the most primitive leukemic cell compartments that are characterized by a slower turnover. Regardless of the underlying mechanisms, it is important to note that many of these parameters, in particular, the biological (survival) response of $CD34^+$ CFCs to IM *in vitro*, *OCT1* expression, IM uptake and the frequency of *BCR–ABL* kinase domain mutations in the $CD34^+$ population may be useful predicators of clinical IM responses.[103,112–114]

Searching for new strategies to eradicate CML stem cells

The recognized innate, as well as acquired, mechanisms that make CML stem cells relatively insensitive to IM (and other tyrosine kinase inhibitors) has prompted considerable interest in developing strategies to target these cells more effectively. Two such lines of activity have involved global gene expression analyses and the identification of downstream partners essential for maximum *BCR–ABL* oncoprotein activity. These have reinforced early evidence of activation of the JAK/STAT, PI3K/AKT, RAS/MAPK and NFκB pathways in the primitive CML cells.[115–122] These studies have also identified differentially expressed genes involved in the regulation of DNA repair, cell cycle control, cell adhesion, homing, transcription factors and drug metabolism.[117,120–123] Potential new therapeutic targets include several recently identified regulators of CML stem and progenitor self-renewal and proliferation; for example, promyelocytic leukemia protein, β-catenin, various RNA binding proteins and members of the sonic hedgehog pathway.[89–91,97,124,125] Another candidate target identified is CXCR4, the chemokine receptor thought to be involved in normal stem cell localization in the marrow and found to promote the survival of quiescent CML progenitors.[126] Another report has suggested that combining histone deacetylase inhibitors (for example, LAQ824) with IM may be more effective in targeting quiescent CML stem cells than IM alone by inhibiting several genes important to the regulation of HSC maintenance and survival.[26] More recently, evidence has been found to suggest that targeting autophagy, a process that allows cells to adapt to environmental stresses, might also allow CML stem and progenitor cells to be effectively targeted.[127] Other potential strategies include induction of protein phosphatase-2A activation by FTY720[128,129] and exposure of CML stem cells to the farnesyltransferase inhibitor (BMS-214662).[25,130] In addition, we have been pursuing a potential strategy based on targeting JAK2 to inhibit the activity of a biologically important multi-molecular complex that we have recently shown it forms with AHI-1, a novel signalling intermediate encoded by the *Abelson helper integration site 1 (AHI-1)* gene and the *BCR–ABL* oncoprotein.[131,132]

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Conclusion

Studies of CML continue to provide new insights into the complex process of malignant transformation and disease progression and the rigor, creativity and patience required to achieve progress. We can expect that further investigative focus of this leukemia will bring improved therapeutic options to those who remain at risk for fatal outcomes and that additional insights of broader applicability and benefit will also be forthcoming from such studies.

Conflict of interest

The authors declare no conflict of interest.

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SPOTLIGHT