Chapter 6
Tissue "Hypoxia" and the Maintenance of Leukemia Stem Cells

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Contents

Abstract The relationship of the homing of normal hematopoietic stem cells (HSC) in the bone marrow to specific environmental conditions, referred to as the *stem cell niche* (SCN), has been intensively studied over the last three decades. These conditions include the action of a number of molecular and cellular players, as well as critical levels of nutrients, oxygen and glucose in particular, involved in energy production. These factors are likely to act also in leukemias, due to the strict analogy between the hierarchical structure of normal hematopoietic cell populations and that of leukemia cell populations. This led to propose that leukemic growth is fostered by cells endowed with stem cell properties, the leukemia stem cells (LSC), a concept readily extended to comprise the cancer stem cells (CSC) of solid tumors. Two alternative routes have been proposed for CSC generation, that is, the

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oncogenic staminalization (acquisition of self-renewal) of a normal progenitor cell (the "CSC in normal progenitor cell" model) and the oncogenic transformation of a normal (self-renewing) stem cell (the "CSC in normal stem cell" model). The latter mechanism, in the hematological context, makes LSC derive from HSC, suggesting that LSC share SCN homing with HSC. This chapter is focused on the availability of oxygen and glucose in the regulation of LSC maintenance within the SCN. In this respect, the most critical aspect in view of the outcome of therapy is the long-term maintenance of the LSC subset capable to sustain minimal residual disease and the related risk of relapse of disease.

Keywords Leukemia stem cell maintenance · Hypoxia · Oxygen · Stem cell niche

6.1 Introduction

The relationship of the homing of normal hematopoietic stem cells (HSC) in the bone marrow (BM) to specific environmental conditions, referred to as the "stem cell niche" (SCN), has been the object of intensive study over the last three decades. These conditions include the action of a number of molecular and cellular players as well as critical levels of nutrients, oxygen and glucose in particular, involved in energy production [1, 2]. These factors are likely to act also in leukemias. A strict analogy indeed emerged between the hierarchical structure of normal hematopoietic cell populations and that of leukemia cell populations [3]. This led to propose [4] that leukemic growth is fostered by cells endowed with stem cell properties, the leukemia stem cells (LSC), a concept readily extended to comprise the cancer stem cells (CSC) of solid tumors [5, 6]. More than one model exists to explain the relationship of CSC to their normal counterpart. Two alternative routes have been proposed for CSC generation [6], that is, the oncogenic staminalization (acquisition of self-renewal) of a normal progenitor cell (the "CSC in normal progenitor cell" model) and the oncogenic transformation of a normal (self-renewing) stem cell (the "CSC in normal stem cell" model). The latter mechanism, in the hematological context, makes LSC derive from HSC, an assumption which suggests that LSC share with HSC the homing behavior within SCN of BM [7, 8]. This chapter is focused on the role of the availability of oxygen and glucose in the regulation of LSC maintenance. In this respect, the most critical aspect in view of the outcome of therapy is the long-term maintenance of the LSC subset capable to sustain minimal residual disease (MRD) and the related risk of relapse of disease following successful induction of remission. The description of aspects of the regulation of LSC maintenance related to the symbiosis with stromal cells, to the action of soluble or cell surface/matrix-bound cytokines or growth factors, and to the modulation of intracellular signaling is left to other chapters of this book and/or to independent reviews.

6.2 Why Hypoxia Is Written "Hypoxia"

Tissue zones where stem cell potential is maintained are commonly referred to as "hypoxic" SCN [9], being "hypoxia" an inappropriate term in this context, as it is used to define in vitro or in vivo situations where O_2 concentration is lower than that of sea-level atmosphere (21% O_2). Indeed, O_2 concentration in normal tissues, even in the lung, is always significantly lower than 21% [10–13]. The papers we are referring to here provided convincing arguments for the use of the term "relatively (to 21%) low O_2 concentration" (to be shortened to "low O_2"), instead of "hypoxia," to define conditions corresponding to physiological tissue O_2 concentrations. Accordingly, any O_2 concentration typical of any tissue under physiological conditions should be considered as "*in situ* normoxia" [13]. On the other hand, the term hypoxia can be correctly used to refer to pathological situations where O_2 concentration is lower than that of the corresponding normal tissue. Therefore, it is important to point out that the concept of in situ normoxia applies to significantly different O_2 concentrations, between different tissues and different sites within the same tissue [13]. In BM, for instance, the normal overall O_2 concentration is lower than in most other tissues. BM cells are indeed physiologically distributed along a gradient of partial O_2 pressure (ppO_2) that ranges from around 5% O_2 close to blood vessels to about 0 ("anoxia") in the most distant regions. Thus, within BM, "normoxic" conditions correspond to O_2 concentrations as different as 5% from 0.1%, being even anoxia to be considered as in situ normoxia in some zones of BM [13].

6.3 The Low-Oxygen Stem Cell Niche in the Bone Marrow

The key feature of SCN is to enable HSC to proliferate without losing (or losing as slowly as possible) stem cell potential, i.e., to undergo the so-called self-renewal, which is a defining property of stem cells. Such a pattern of HSC proliferation is what sustains long-term maintenance of HSC, yet allowing their contribution to active hematopoiesis. The SCN concept emerged as a theoretical model [14], yet based on experimental ex vivo data indicating an uneven compartmentalization in BM of HSC and less immature hematopoietic progenitor cells (HPC), being HSC located preferentially close to the bone surface and HPC instead in proximity of the central sinus. This concept evolved later from the HSC/HPC contraposition to a model where different HSC subsets are distributed between "endosteal SCN," where the stem cell potential of HSC is maintained, and "vascular SCN," where HSC commitment to clonal expansion and differentiation is driven [15–17]. The relationship of SCN function to proximity to blood vessels is in keeping with a regulation based on nutrient supply, O_2 and glucose in particular [18, 19]. It emerged that SCN where HSC are physiologically long-term maintained are placed in BM zones where O_2 concentration is lower than in the surrounding tissue and that in situ normoxic conditions for HSC maintenance are ensured in sites at the lower extreme of ppO_2

gradient in BM [20]. It is this HSC-maintaining SCN that will be referred to hereafter with the acronym SCN, according to the "restrictive" original Schofield's definition [14].

The SCN model started to be enriched with mechanistic details in the early 1990s, on the basis of results of in vitro studies [21]. In murine BM cell cultures incubated in atmosphere at 1% O_2, HSC maintenance was found enhanced, while HPC were suppressed and the overall hematopoietic output markedly reduced. Thus, low ppO_2 emerged as a regulatory aspect of SCN function, and the concept of "hypoxic" SCN was introduced. An interesting aspect of this study was that pooled data obtained using a number of different progenitor/stem cell assays revealed a precise hierarchical "gradient" of resistance to low O_2, being the progenitor the more resistant, the higher its hierarchical level [21]. This means that an environment which is "hypoxic" for the bulk of hematopoietic cells and HPC is actually "normoxic" for HSC. Thus, the first mechanistic feature of SCN to emerge pointed to metabolic issues, and to the role of ppO_2 in particular, in the regulation of HSC maintenance. Similar data were then collected for human hematopoiesis and long-term repopulating HSC [22–24], and the existence of "hypoxic" SCN in vivo was later confirmed experimentally [9], leading to a complete systematization of the issue [13]. That said, it is necessary here to underscore a point relative to the abovementioned "gradient" of resistance to low ppO_2. To home selectively within SCN placed in tissue zones at the lowest ppO_2, HSC need to exhibit a metabolic profile that is not shared by HPC. This profile cannot simply consist of the compatibility with a generic tissue "hypoxia," which refers to a wide range of tissue O_2 concentrations. Either HSC or HPC exhibit indeed a "hypoxic" metabolic profile [25]. Thus, what qualifies HSC from HPC is that HSC (but not HPC) are capable to stand the lowest physiological ppO_2 and for extended times. The question invariably slides toward the upregulation of HIFα signaling, the best known driver of "cell adaptation to hypoxia." However, HIFα stabilization threshold is around 3% O_2 [26], and HIFα signaling is active in both HSC and HPC, so that it cannot sustain alone the critical metabolic differences between HSC and HPC or confer upon HSC all the features enabling their selective homing in low ppO_2 SCN. Only a small minority of cells where HIFα is stabilized is indeed capable to stand the low ppO_2 typical of SCN [9]. Thus, HIFα stabilization is a necessary, although not sufficient, condition for HSC maintenance, in keeping with what demonstrated for the maintenance of LSC [27] of chronic myeloid leukemia (CML).

One of the mechanisms operating within the SCN to sustain hematopoiesis while making HSC lose stem cell potential as slowly as possible consists in the regulation of balance between HSC quiescence and cycling. It was therefore crucial to establish whether low O_2 modulates this balance. The issue was addressed using murine BM cell cultures incubated at 1% O_2 and two different strategies, i.e., determining the suppression of cycling HSC following treatment with 5-fluoro-uracil (5FU) [28] and the coupling between mitotic history and maintenance of stem cell potential [29]. It was found that, after 5 days in low O_2, 1/3 of HSC are induced to quiescence (5FU-resistant), while 2/3 are 5FU-sensitive, suggesting that low ppO_2 of SCN in vivo is compatible with the cycling of a substantial proportion of HSC [28]. It also emerged that one replication cycle at 1% O_2 boosts stem cell potential and that this

effect is lost when cycling is sustained for more than one cycle at 1% O_2 or cells are incubated in "normoxia" or in the presence of interleukin-3. This indicates that HSC self-renewal occurs immediately upon HSC recruitment from quiescence to cycling, provided this happens in low O_2, suggesting that low ppO_2 of SCN in vivo is a crucial factor for the maintenance of stem cell potential of HSC, helping HSC to proliferate as stem cells (self-renewal), and preventing their commitment to clonal expansion. On the contrary, stem cell potential is typically lost when proliferation is extensively stimulated, such as in the presence of IL-3 or relatively high ppO_2 [29].

While all the earlier studies of the effects of low O_2 on HSC maintenance were carried out incubating cells at 1% O_2 (or even higher O_2%), the question emerged of whether O_2 concentrations lower than 1%, detectable in vivo [10, 11, 13, 20], should be considered to play a regulatory role in SCN. An affirmative answer was obtained in vitro, showing that at 0.1% O_2 not only HSC survival but also HSC cycling, a crucial aspect of SCN function, is maintained, although induction of quiescence is favored [30, 31]. Actually, O_2 concentrations ranging between 0.1% and 1.0% were classified even as "moderate hypoxia" [26]. A direct measurement of O_2 concentration in BM showed that ppO_2 is around 10 mmHg, equivalent to 1.3% O_2, within the vascular SCN [32], the SCN type where ppO_2 is believed to be relatively high. This suggests that ppO_2 of endosteal SCN should be well below the 1% O_2 concentration used for the earlier studies in vitro of the effects of low O_2 on HSC maintenance. The compatibility of O_2 concentrations as low as 0.1–0.2% with SCN function led to the use these concentrations for the studies of leukemias summarized below.

6.4 Low Oxygen and "Oncogene Suppression" in Leukemias

Working with CML stabilized cell lines or primary explants, it was shown that the culture in atmosphere at 0.1% O_2 for relatively long incubation times (7–10 days) markedly reduced cell bulk with respect to time zero, while in the residual population stem cell potential was integrally maintained on a per-cell basis. This parallels strictly what observed for normal hematopoiesis. A crucial outcome of these experiments was that incubation in low O_2 also completely suppressed $BCR/Abl_{protein}$, the product of the fusion oncogene responsible for CML pathogenesis, but not BCR/Abl_{mRNA} [33]. Consequently, the CML cell subset capable to stand incubation in low O_2 is independent of BCR/Abl for persistence in culture but remains genetically leukemic (Figs. 6.1 and 6.2). Thus, when BCR/Abl-independent cells were transferred to growth-permissive incubation conditions ("normoxia"), their stem cell potential was exploited, as expected, via $BCR/Abl_{protein}$ re-expression, which is capable to ensure a maximal expansion of cell population. It is extremely relevant that the suppression of driver oncogenic proteins ("oncogene suppression") following cell incubation in low O_2 is not restricted to CML (Fig. 6.1) but is extended to other types of leukemias [34, 35], such as murine Friend's erythroleukemia (MEL; erythropoietin receptor and glycoprotein 55), human acute myeloblastic leukemia (AML; AML1/ETO), and human acute promyelocytic leukemia (APL; PML/

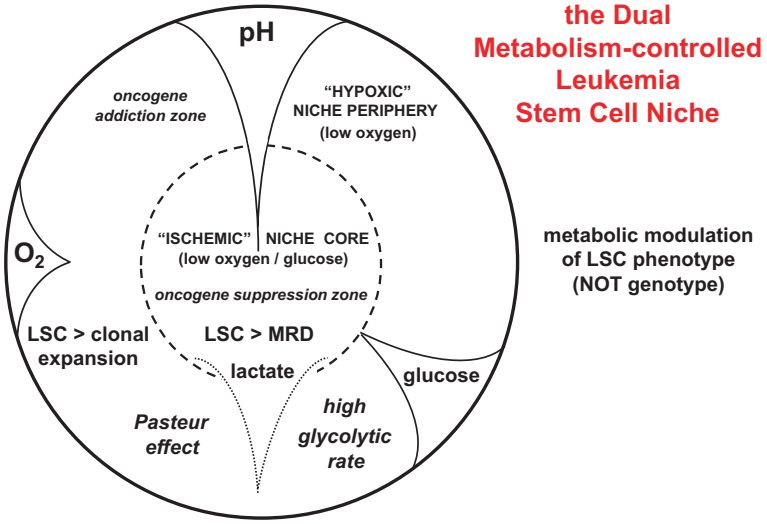

Fig. 6.1 Schematic representation of the "Dual Metabolism-controlled CML Stem Cell Niche," as hypothesized on the basis of published data and hypotheses [41, 49–51, 66, 86]. The "hypoxic" stem cell niche (SCN) periphery, where ppO_2 is low but glucose concentration is relatively high, allows the expression of the oncogenic protein (maintenance of oncogene addiction), steering leukemia stem cells (LSC) to clonal expansion. In the "ischemic" SCN core, characterized by both O_2 and glucose shortage, the oncogenic protein is suppressed, boosting LSC self-renewal and maintanance of minimal residual disease (MRD)

RARα). In all leukemias tested, oncogene suppression is relatively slow (3–7 days after the beginning of incubation in low O_2), indicating that the phenomenon cannot be considered as a direct consequence of HIFα stabilization, which is triggered within few minutes of cell transfer to low O_2 [9]. Nevertheless, HIFα activity has been shown necessary for the maintenance of LSC of CML [27]. As for the downstream, effector mechanisms of oncogene suppression in low O_2, they belong to a complex panel which includes transcriptional, translational, and posttranslational events [36–39]. It is important to point out that the downregulation of these processes occurring in low O_2 is not a generalized phenomenon and that the block of protein synthesis does not apply to a number of factors, many of which are apparently important for cell maintenance under metabolic restriction [39, 40].

A key point of the scenario summarized above is that oncogene suppression occurring in low O_2 does not correlate with a loss of stem cell potential, at least in the case of CML [33, 41] and MEL [34] cells. This points to the existence of a leukemia cell subset endowed with LSC properties and capable to survive and cycle (34 and Tanturli M, Cheloni G, Bono S and Poteti M, unpublished data) in the absence of oncogenic signaling (Fig. 6.1). The selection of such an LSC subset under metabolic pressure suggests that maintained oncogenic stimulation makes LSC unfit to home the SCN, due to the detrimental effects of growth-promoting

stimuli in a growth-limiting environment. On the contrary, oncogene suppression would enable LSC to persist as such within the SCN, without risking to be pushed by oncogenic signaling to commitment to clonal expansion and differentiation. Thus, the loss of “oncogene addiction” [42–44] would be a necessary step of LSC adaptation to SCN (Fig. 6.1). In other words, in order to earn their long-term maintenance within the SCN, LSC are actually required to revert to a normal, HSC-like phenotype. On the other hand, when the homeostatic balance within the LSC compartment shifts from the maintenance of stem cell potential to commitment to clonal expansion and then differentiation, LSC would shift from the core to the periphery SCN environment, which is permissive for the expression of the oncogenic protein and the boosting of clonal expansion by oncogenic signaling. Under this perspective, two points are worth being underscored: (a) oncogene suppression is not a genetically blocked event but a fully reversible phenotypical adaptation, like the one proposed to describe the relationship between HSC and HPC [45]; (b) the withdrawal of oncogenic signaling in LSC appears functionally equivalent to that of cytokine signaling in HSC [3]. With respect to (b), it is to note that BCR/Abl-induced growth factor independence of hematopoietic cells is mediated at least in part by IL3 signaling [46], which indeed impairs HSC maintenance in low O_2 [29].

6.5 The Dual “Hypoxic/Ischemic” LSC Niche in Chronic Myeloid Leukemia

When the metabolic consequences of incubation of CML cells in low O_2 were addressed, it was glucose consumption from culture medium to emerge as the crucial condition for the reduction of total cell number with respect to time zero, as well as for $BCR/Abl_{protein}$ suppression. By varying time zero cell density and glucose concentration in cultures incubated in low O_2, it was established that the kinetics of this suppression is strictly related to that of glucose exhaustion [41]. Deepening the issue, it was shown that the lack of glucose is capable to drive $BCR/Abl_{protein}$ suppression independently of O_2 shortage [40]. Thus, glucose availability emerged as a converging trait of the metabolic control of $BCR/Abl_{protein}$ expression, suggesting that it is severe energy restriction in general, rather than low O_2 in particular, to trigger the $BCR/Abl_{protein}$ suppression machinery that qualifies the SCN environment (Figs. 6.1 and 6.2). If this is the case, why do we focus on low O_2 environments? For two reasons: (a) because the lower solubility of O_2 when compared to glucose, affecting their diffusion in tissues, makes O_2 shortage precede glucose shortage physiologically while getting away from blood vessel [47]; (b) because the enhanced glucose consumption rate (the Pasteur effect) typical of cell metabolism at low ppO_2 makes of low O_2 a powerful modulator of glucose availability. The time lapse between the onset of glucose shortage and that of O_2 shortage (a) well explains the markedly delayed kinetics of $BCR/Abl_{protein}$ suppression with respect to that of HIFα stabilization. Point (b) is affected by HIFα stabilization itself, glucose exhaustion being more

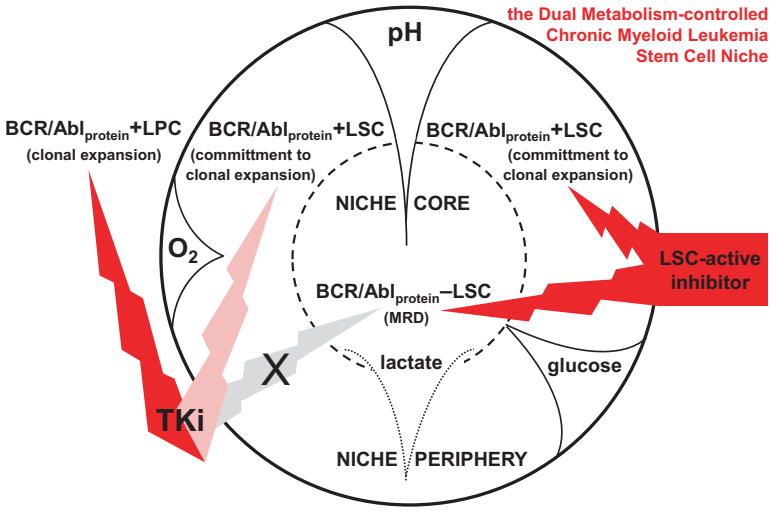

Fig. 6.2 Schematic representation of the "Dual Metabolism-controlled CML Stem Cell Niche," as hypothesized on the basis of published data and hypotheses [41, 49–51, 66, 86]. The "hypoxic" stem cell niche (SCN) periphery, where ppO_2 is low but glucose concentration is relatively high, allows $BCR/Abl_{protein}$ expression, making BCR/Abl-dependent self-renewal of leukemia stem cells (LSC) sensitive to tyrosine kinase inhibitors (TKi) active on BCR/Abl signaling (pink bolt). In the "ischemic" SCN core, characterized by both O_2 and glucose shortage, $BCR/Abl_{protein}$ suppression and BCR/Abl-independent self-renewal of LSC maintain minimal residual disease (MRD) protected from TKi action (grey bolt/X). On the other hand, TKi reach maximal activity outside SCN (red bolt on the left) against leukemia progenitor cells (LPC) engaged in CML clonal expansion (100% in replication). When $BCR/Abl_{protein}$ is suppressed, the question of the presence or the absence of primary or secondary *BCR/abl* mutations determining BCR/Abl resistance to TKI is irrelevant. Many non-TKi, LSC-active inhibitors act irrespective of $BCR/Abl_{protein}$ expression or suppression, thus succeeding in targeting both MRD and clonal expansion (red bolts on the right)

efficiently driven in low ppO_2 zones than in zones at higher ppO_2 where HIFα is not stabilized. The role of HIFα in the control of leukemic growth is reviewed in [48].

What summarized above led to define two different nutrient-restricted SCN environments, the low O_2 and the low O_2/glucose, playing distinct roles in the biology of leukemias (Fig. 6.1). These two SCN environments can be referred to, respectively, as the "hypoxic" and the "ischemic" SCN zones [41, 49–51]. The term "ischemic" sounds as inappropriate as "hypoxic" in the context of SCN. Indeed, "ischemia" refers to in vivo (but not in vitro) pathological occurrences where the tissue is damaged as a consequence of nutrient shortage, whereas in the SCN this shortage has a precise regulative role. A permanently insufficient glucose diffusion, affecting cell viability, has been shown in solid neoplasias [47] and is likely to characterize also the hyperplastic BM of leukemias, CML in particular. A simple model drawing the relationship between the two SCN zones predicts the onset of ischemia-like conditions within the core of hypoxic SCN [49–51]. In the SCN core, glucose would get close to exhaustion due to the combined effects of the high rate of consumption (in the SCN periphery) and the scarce diffusion (higher distance from

blood vessels of SCN core than SCN periphery). While HIFα is surely stabilized in both the SCN core and SCN periphery, it is most probably the HIFα-dependent metabolic reprogramming of the latter which conditions the asset and function of SCN core. It is worth pointing out here that the existence of ischemia-like conditions emerged in normal BM from studies of the role of blood perfusion, rather than O_2 diffusion, in the control of hematopoiesis and in the hierarchical distribution of HSC in BM. These studies showed that the most primitive HSC reside in BM zones where blood perfusion is lowest, implying that HSC maintenance within the SCN is supported by a strongly reduced supply of nutrients as well as of blood-borne soluble factors [52, 53]. The capacity of LSC to nest in HSC niches has been also demonstrated [54, 55].

The dual "hypoxic/ischemic" SCN delineated above impacts LSC modeling in CML. As mentioned in the *Introduction*, Reya et al. summarized two alternative models for CSC generation, the cancerization of a normal stem cell or the staminalization of a normal progenitor cell, meaning that cells exhibiting either the stem or the progenitor cell phenotype may long-term sustain cancer growth [6]. The identification of two possible "metabolism-driven" phenotypes for LSC of CML led us to propose that, short of being alternative to each other, *both* models fit with CML biology, i.e., that two LSC subsets with different functional roles coexist in CML (Fig. 6.2). The "stem cell LSC model" seems indeed adequate to describe a BCR/$Abl_{protein}$-negative LSC subset capable of BCR/Abl-independent self-renewal, while the "progenitor cell LSC model" suits an LSC subset where BCR/$Abl_{protein}$ is expressed and self-renewal is sustained by BCR/Abl-dependent signaling [49–51]. It is worth reminding here that CML and MEL include an LSC subset capable to cycle in the absence of oncogenic signaling (Tanturli et al. unpublished data; [34]). It is reasonable to think that this subset cycles more slowly than that where oncogenic signaling is active, as it is deprived of the growth advantage oncogenic signaling confers. Such an advantage, however, while being useful to maximize clonal expansion whenever possible, would be highly detrimental to the long-term maintenance of LSC. The dual CML stem cell compartment described above is well in keeping with the view of CML as a stem cell-derived but progenitor cell-driven disease [56], if one transposes the concepts of "stem" and "progenitor" cells to the two different LSC subsets described above.

6.6 The LSC Niche and the Refractoriness of MRD to Treatment in CML

The studies where the response of CML to low O_2 was initially characterized also addressed the effects of treatment with imatinib mesylate (IM; Gleevec®), the inhibitor of the constitutive enzymatic activity of BCR/Abl which is the prototype of tyrosine kinase inhibitors (TKi) used for CML therapy. As expected on the basis of what explained above, cells where BCR/$Abl_{protein}$ had been reversibly suppressed

following incubation at 0.1% O_2 were resistant to treatment, obviously because devoid of the molecular target of IM [33, 41]. The crucial aspect of such an outcome is that the maintenance of CML stem cell potential was completely insensitive to IM (Fig. 6.2). This insensitivity is appropriately referred to as "refractoriness," while the term "primary resistance" should be reserved to genetically determined (and therefore irreversible) insensitivity to IM already present before the treatment starts, and the term "secondary resistance" to the irreversible insensitivity acquired by a cell subset via mutations under treatment pressure. It is worth discussing resistance to IM under the light of the SCN model delineated in the previous section.

Metabolic pressure within the SCN controls LSC sensitivity or refractoriness to IM as a phenotypical adaptation, rather than a genetically blocked event, depending on whether LSC reside within the "hypoxic" or the "ischemic" SCN zones where $BCR/Abl_{protein}$ is expressed or suppressed, respectively (Fig. 6.2). The concept of resistance to IM as a phenotypical change is in keeping with the marked phenotypical heterogeneity of CML cells as for BCR/Abl expression and sensitivity to IM [57, 58] and with the fact that relapse of disease upon discontinuation of IM most often occurs without signs of development of mutation-driven secondary resistance to IM, so that the relapsed patient responds well to the reintroduction of IM [59]. Accordingly, the TKi-resistant CML progenitor cells shown to be BCR/Abl-positive by FISH or PCR [60–62] are likely to be BCR/Abl_{mRNA}-positive/$BCR/Abl_{protein}$-negative. This is a conceptually simple way to resolve the controversy of whether TKi inhibit or not BCR/Abl kinase in LSC [60–63], by concluding that TKi are always effective but LSC do not respond due to the lack of the TKi molecular target. The presence of $BCR/Abl_{protein}$-negative LSC also explains easily the lack of LSC-suppressive effects of not only IM but also "second-generation" BCR/Abl-active TKi, despite their enhanced action on CML cell bulk [64, 65]. The ineffectiveness of IM as well as dasatinib on LSC was confirmed using CML cells, from stabilized lines or explanted from patients, kept at 0.1% O_2 for incubation times sufficient to drive glucose exhaustion from culture medium [66]. In this scenario, it was predicted that even next generations of BCR/Abl-active TKi will be useless to suppress LSC [35, 50, 51].

SCN zones where LSC are capable to cycle slowly and self-renew independently of BCR/Abl signaling and are therefore protected from TKi action represent ideal sites for the long-term maintenance of treatment-resistant MRD of CML (Fig. 6.2). Such an outline facilitates MRD modeling, because it prevents us from restricting MRD to quiescent LSC, although there is no doubt that quiescence boosts resistance to treatment when drugs generically active against cycling cells are used. What we call a "dynamic maintenance" of MRD [49–51] appears indeed better suited than LSC quiescence to explain the combination of refractoriness to TKi with liability to neoplastic progression. First, the finding that cycling of HSC in low O_2 fosters self-renewal rather than clonal expansion [29] suggests that the same may apply to LSC. A relatively high stem cell potential of the mutated CML subclone stored in the SCN before relapse of disease reaches the clinical level may be behind the often very rapid and aggressive course of relapse once it is triggered. Second, neoplastic progression while disease remains at a subclinical level is best explained if LSC

cycle, because cell cycling is necessary to transmit mutations to progeny, which are undoubtedly frequent in the genetically unstable CML cells.

The process leading from MRD to relapse of disease in CML can be summarized hypothetically as follows: (a) BCR/Abl-independent, IM-insensitive self-renewal within the “ischemic” SCN core would maintain (MRD) non-mutated or mutated LSC; (b) migration out of the SCN core or (transient?) increase of glucose supply therein would make LSC switch to conditions typical of the “hypoxic” SCN periphery, where ppO_2 is still very low but glucose concentration is higher, which allow the rescue of $BCR/Abl_{protein}$ expression; (c) BCR/Abl-dependent signaling in the SCN periphery would enhance LSC self-renewal and enable commitment to clonal expansion; and (d) a move of LSC to better oxygenated BM zones (the “vascular” niches) would drive clonal expansion and differentiation (if any), making relapse of disease emerge at the clinical level (Fig. 6.2). Of course, from phase (b) on, CML growth returns to be TKi-sensitive in the majority of cases, so that there is risk of relapse only in the absence of therapy [59]. The majority of relapsed patients indeed does not present mutations, or, if it does, the mutations are incapable of conferring secondary TKi resistance. It is likely that the shift from chronic to accelerated to blast clinical phase of CML is founded on the above biological frame.

6.7 To Exploit the Metabolic Control of LSC Compartment for Therapeutic Purposes

The long-standing use for cancer therapy of drugs most of which are cell cycle-specific antiblastic agents obviously leads to trace back treatment-resistant MRD to quiescence, to quiescent stem cells in particular. Furthermore, especially in the hematopoietic field, quiescence is a substantial component within the SCN of the mechanisms protecting stem cells from premature commitment to clonal expansion and differentiation [14]. Therefore, the combination of treatments capable of inducing (stem) cell exit from quiescence with cycle-specific antiblastic agents has been proposed to try to suppress MRD [67]. A more advanced approach to the therapy of neoplasias is based on the use, instead of drugs acting generically against cycling cells, of selective inhibitors of disease-specific targets, such as BCR/Abl in CML, with obvious advantages in terms of reduced toxicity on normal cells (therapeutic index). In theory, such an approach should be also active on quiescent cells sustaining MRD, as neoplastic cells may depend on oncogenic signaling not only for proliferation but also survival (oncogene-addicted cells). An opposite, less optimistic perspective springs from the SCN model we proposed for CML, which also applies to MEL. In these leukemias, the loss of oncogene addiction driven under metabolic pressure makes not only survival but also self-renewal independent of oncogenic signaling (see above). Thus, the pool of cells resistant to drugs targeting the oncogenic protein may be larger than the quiescent LSC subset, to include LSC capable to cycle in the absence of oncogenic signaling. Therefore, to push LSC to exit from

quiescence would not be equivalent to make them sensitive to treatment. In this case, a possible alternative strategy would be to combine the targeting of the oncogenic protein with treatments capable to prevent or revert its suppression. In CML, either the treatment with specific drugs [68] or the interference with the metabolic adaptation of LSC to the SCN (Poteti M et al., manuscript in preparation) was shown to prevent $BCR/Abl_{protein}$ suppression following incubation at 0.1% O_2.

A perhaps more straightforward approach to suppress MRD maintained under conditions where oncogenic signaling is suppressed is represented by the targeting of mechanisms driving the overall LSC adaptation to these conditions (rather than those specifically related to oncogene suppression, discussed in the previous paragraph). As those conditions are likely to consist of a low O_2/glucose environment, HIFα appears an obvious potential therapeutic target (see text above and, as examples, [69–71]), and HIFα targeting was tested. Echinomycin was the first HIFα inhibitor to be used to treat leukemias. The drug, capable to block the DNA-binding activity of HIFα, was found to target human AML and LSC in particular in a xenogeneic model in vivo, reducing leukemia burden and extending mouse survival, as well as, more importantly, suppressing disease development following transplantation into secondary recipients. Equally important is the finding that echinomycin does not interfere with HSC self-renewal, thus exhibiting a high therapeutic index [72, 73]. In murine APL models driven by both PML-RARα and PLZF-RARα, the HIF-1α transcription antagonist EZN-2968 or the HIF-1α inhibitor EZN-2208 are capable alone of debulking leukemia and prolonging mouse survival and, when administered in combination with all-trans retinoic acid, suppressing LSC and eradicating leukemia [71, 74]. Furthermore, L-ascorbic acid inhibits HIF-1α transcription and reduces growth of APL and CML cell lines [75]. Finally, acriflavine, which decreases HIF transcriptional activity by inhibiting α/β dimerization, reduces growth and LSC maintenance in cultures incubated at low O_2 of CML cell lines and cells explanted from a number of patients as well as in *BCR/abl*-induced mice used as a stem cell-driven disease model [66].

An alternative strategy to the block of HIF-dependent LSC adaptation to SCN consists of taking advantage of low O_2 to activate prodrugs which then target LSC already adapted to SCN. TH-302 is a "hypoxia"-activated prodrug that is specifically cytotoxic in low O_2 via multiple mechanisms and is capable to reduce leukemia burden and to prolong recipient survival in murine models of human AML [76]. Similar results were obtained by treating acute lymphoblastic leukemia (ALL) cells with the "hypoxia"-activated DNA cross-linking agent PR-104; in a phase I/II trial study, PR-104 was shown effective in the treatment of AML or ALL patients, even refractory to standard therapy or in relapse [77, 78].

As explained above, HIFα is a necessary, but not sufficient, mediator of LSC maintenance. Indeed, other critical regulators of stem cell compartment unrelated to HIFα signaling, or more in general to the metabolic control of LSC homing within the SCN, have been identified as potential therapeutic targets. The product of the arachidonate-5-lipoxygenase (5-LO) gene (alox5) is one of these regulators, and Zileuton, a specific 5-LO inhibitor, induces depletion of short-term LSC and multipotent progenitor cells of CML, likely by blocking the commitment of long-term

LSC, in the murine CML model mentioned above [79, 80]. Zileuton also impairs stem cell function in polycythemia vera [81]. Likewise, alox15 inhibition by PD146176 causes LSC depletion and prevents CML initiation [82]. Omacetaxine (formerly homoharringtonine) markedly reduces both LSC and leukemia bulk in vitro and in vivo, also when *BCR/abl* is mutated [83], exemplifying a general advantage of non-BCR/Abl-targeting drugs. The hedgehog signaling inhibitors cyclopamine or GDC-0449 target LSC in CML [84]. BMS-214662 induces apoptosis of both proliferating and quiescent LSC of CML via mechanism not associated with the farnesyltransferase-inhibitory activity of the drug [85]. Finally, the inhibition of MEK5/ERK5 pathway reduces growth and LSC maintenance in cultures incubated at low O_2 of CML cell lines and cells explanted from a number of patients and in the murine CML model mentioned above [86]. Very often, normal HSC were found much less sensitive than LSC to these treatments. The vast array of new options for leukemia therapy summarized above, once its translatability to clinical practice is verified, induces a reasonable hope to succeed in suppressing LSC and MRD in patients affected by a number of different leukemias, thus aiming at cure rather than care.

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