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The metabolically-modulated stem cell niche: a dynamic scenario regulating cancer cell phenotype and resistance to therapy

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This Perspective addresses the interactions of cancer stem cells (CSC) with environment which result in the modulation of CSC metabolism, and thereby of CSC phenotype and resistance to therapy. We considered first as a model disease chronic myeloid leukemia (CML), which is triggered by a well-identified oncogenetic protein (*BCR/Abl*) and brilliantly treated with tyrosine kinase inhibitors (TKi). However, TKi are extremely effective in inducing remission of disease, but unable, in most cases, to prevent relapse. We demonstrated that the interference with cell metabolism (oxygen/glucose shortage) enriches cells exhibiting the leukemia stem cell (LSC) phenotype and, at the same time, suppresses *BCR/Abl* protein expression. These LSC are therefore refractory to the TKi Imatinib-mesylate, pointing to cell metabolism as an important factor controlling the onset of TKi-resistant minimal residual disease (MRD) of CML and the related relapse. Studies of solid neoplasias brought another player into the control of MRD, low tissue pH, which often parallels cancer growth and progression. Thus, a 3-party scenario emerged for the regulation of CSC/LSC maintenance, MRD induction and disease relapse: the "hypoxic" versus the "ischemic" vs. the "acidic" environment. As these environments are unlikely constrained within rigid borders, we named this model the "metabolically-modulated stem cell niche."

Keywords: acidity, *BCR/Abl*, cancer stem cell, chronic myeloid leukemia, hypoxia, ischemia, leukemia stem cell, leukemia progenitor cell, minimal residual disease, neoplastic progression, resistance to imatinib-mesylate, stem cell niche

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The "Hypoxic" Stem Cell Niche and Stem Cell Maintenance and Cycling in "Hypoxia"

In the early '90, our laboratory elaborated on the stem cell niche (SCN) model proposed by Ray Schoefield in 1978 to define tissue sites dedicated to the maintenance of stem cell potential.[1] We addressed the effects of a low environmental oxygen tension on the maintenance of haematopoietic stem cells (HSC) in vitro. Overall, hematopoiesis was found markedly reduced in low oxygen (1% oxygen in incubation atmosphere, approximately corresponding to 10 μM O_2 and 7.6 mmHg) with respect to air. On the contrary, HSC endowed with marrow repopulation ability (MRA), but not less immature progenitors, are better maintained in low oxygen, where they are selected and enriched.[2] On the basis of these in vitro data, we proposed the existence in vivo of "hypoxic" HSC niches, where low oxygen tension would help HSC to proliferate as stem cells and maintain stem cell potential (self-renewal) while preventing their commitment to clonal expansion. This scenario implies that low oxygen tension is a physiological feature of SCN, i.e. that an environment which is "hypoxic" for the bulk of haematopoietic cells is actually "normoxic" for HSC.[3] Therefore, the term low oxygen will be used herein on to refer to the "hypoxic" conditions suitable for HSC maintenance, while the term *hypoxic SCN* will be maintained to indicate the tissue sites where this maintenance is ensured. Later studies of ours demonstrated that: (a) provided an appropriate combination of stem cell-active cytokines is added to cultures, HSC expansion can occur in low oxygen;[4] (b) in low oxygen, HSC have the option between cycling and quiescence, while the bulk of cell population is growth-arrested;[5] (c) in low oxygen (but not in air) stem cell potential is

markedly enhanced in cells which have undergone one replication cycle and is rapidly lost when cycling is sustained beyond the first cycle, indicating that low oxygen steers cycling of HSC toward self-renewal immediately after their rescue from quiescence and temporarily antagonizes clonal expansion.[6] The existence of physiologically "hypoxic" conditions in bone marrow (BM), as well as of hypoxic SCN in vivo, was later confirmed by others (for a review see ref. [7]).

Selection of Leukemia Stem and Progenitor Cell Subsets in Low Oxygen

On the basis of all above, we investigated on the effects of low oxygen on different types of leukemia cell populations. Incubation of murine erythroleukemia (MEL) cells in low oxygen markedly reduced cell bulk with respect to time zero,[8] in keeping with what observed for normal hematopoiesis. The maintenance of stem cell potential in the few MEL cells capable to stand low oxygen was determined by the culture-repopulation ability (CRA) assay, an in vitro method to determine MRA.[5,9] The CRA assay is based on cell transfer from primary cultures where the experimental treatment is carried out (i.e., incubation in low oxygen) to non-selective, growth-permissive secondary cultures (i.e. incubated in air). Cells surviving incubation in low oxygen were capable of repopulating secondary cultures efficiently, although with a kinetics significantly delayed with respect to that exhibited by equal numbers of cells transferred from control primary cultures incubated in air. Nevertheless, once repopulation started, its kinetics was identical to that obtained with control cells and reached identical peak values. Moreover, when 5-fluorouracil (5FU) was added to primary cultures following cell selection in low oxygen, the repopulation of secondary cultures by low oxygen/5FU-resistant cells (around 1% of the number plated in primary cultures) was delayed further, but, again, exhibited kinetics and peak values identical to those obtained with low oxygen-resistant/5FU-sensitive cells. These results indicated that stem cell potential is maintained integrally in MEL cells after their selection in low oxygen or suppression of their cycling fraction, although this potential is exploited with different kinetics, once conditions permissive for clonal expansion are established.[8] It is worth pointing out here that the quiescent (and thereby 5FU-resistant) leukemia cell subset is believed to contain the high-end of leukemia stem cell (LSC) compartment, in particular the LSC sustaining minimal residual disease (MRD), which eventually determines relapse of leukemia in patients where therapy has been successful in inducing remission.

Overall, the results summarized above indicated that: (1) different leukemia cell subsets, including quiescent LSC, can be individually selected in primary cultures incubated in low oxygen; (2) the outcome of this selection can be verified by determining the repopulation kinetics of secondary growth-permissive cultures (drug-free, incubated in air) of CRA assays, being the more delayed this repopulation, the higher the hierarchical level of selected cells.[9] As these experiments were carried out using a stabilized cell line, it emerged that an appropriate manipulation of culture environment can reveal a marked phenotypical heterogeneity within a clonal cell population. On this basis, leukemia cell lines appeared suitable to investigate on the behavior and response to drug treatments of different LSC as well as leukemia progenitor cell (LPC) subsets.

Low Oxygen and Selection of Chronic Myeloid Leukemia Cells Insensitive to Therapy

Incubation in low oxygen of chronic myeloid leukemia (CML) cells of stabilized lines or primary explants completely suppresses $BCR/Abl_{protein}$, the fusion protein responsible for CML pathogenesis. Cells selected in low oxygen, which are independent of *BCR/Abl* signaling for persistence in culture, remain however genetically leukemic, as they re-express $BCR/Abl_{protein}$ if cultures are shifted to incubation in air. When the stem cell potential of CML cells selected in low oxygen is measured by CRA assay, they repopulate the secondary cultures incubated in air according to a delayed, LSC-type kinetics. These findings have a tremendous impact on CML cell resistance to imatinib-mesylate (IM; Gleevec®), the prototype of tyrosine kinase inhibitors (TKi) used for CML therapy. Indeed, the stem cell potential of cells selected in low oxygen is completely insensitive to treatment with IM, because its molecular target is suppressed in LSC, while full sensitivity to IM is rescued in the LSC progeny where $BCR/Abl_{protein}$ is re-expressed during incubation in non-selective secondary cultures.[10-12]

These results led to link IM-resistance to reversible $BCR/Abl_{protein}$ suppression and this suppression, in turn, to the suitability of LSC to home in the hypoxic SCN we had found to ensure HSC maintenance. This was a previously undescribed mechanism of insensitivity to IM which is appropriately referred to as *refractoriness*. Indeed, the term *primary resistance* should be reserved to a genetically-determined (and therefore irreversible) insensitivity to IM already present in CML cells before the treatment starts, and the term *secondary resistance* to that induced in a cell subset by later *BCR/Abl* mutations in IM-treated patients. The capacity of LSC to nest in HSC niches was later demonstrated.[13,14]

That the environment-induced LSC insensitivity to IM we described is not a genetically-blocked event, but a phenotypical adaptation has the following important consequences. (1) All LSC, and not just an LSC subset (following subset-restricted mutations), are potentially insensitive to TKi targeting *BCR/Abl*, being stem cells inherently adaptable to low oxygen. (2) It is predictable that even the next generations of *BCR/Abl*-active TKi, despite their enhanced action on CML cell bulk,[15] will be ineffective in suppressing low oxygen-adapted LSC. (3) Phenotypical, rather than genetical changes explain easily the clinical finding that relapse of disease upon IM discontinuation (which releases pressure against the $BCR/Abl_{protein}$-dependent clonal expansion of LSC) is very often sustained by a cell population expressing wild-type *BCR/Abl*, i.e., occurs in patients who have not developed secondary resistance to IM and therefore respond well to the

reintroduction of IM after relapse.[16,17] (4) Our data are perfectly in keeping with the observation that TKi-insensitive LSC express *BCR/Abl*, being this expression usually determined by FISH or PCR instead of measuring protein levels.[18-20] (5) Our model provides a conceptually simple way to get out of the controversy as to whether TKi actually inhibit *BCR/Abl* kinase in *BCR/Abl*-positive CML cells responsible for MRD.[21-23]

Our model predicts that LSC of CML adapted to low oxygen are independent, and deprived, of the selective advantage represented by *BCR/Abl* signaling, i.e., are not oncogene-addicted.[24] Such an advantage is useful to maximize the expansion of cell population when environmental conditions are permissive, but not to enhance the maintenance of LSC responsible for MRD when clonal expansion is restrained. Indeed, what is crucial for LSC maintenance is not the availability of oncogenic growth-promoting signals, but the capacity of cycling in low oxygen, i.e., a property that HSC physiologically exhibit and is functional to steer mitotic activity toward self-renewal (see above). In this respect, one can envision LSC adapted to low oxygen as revertant to a normal phenotype. That LSC of CML cycle in low oxygen is indicated by their sensitivity to 5FU (unpublished data).

An extremely important consequence of LSC cycling under conditions where LSC are refractory to TKi is that cycling fosters neoplastic progression while disease remains at a subclinical level. Indeed, cycling is necessary for transmission of mutations to progeny.[25] This is what we define *dynamic maintenance of MRD*, a phenomenon which we believe it is much better suited than LSC quiescence to explain the combination of insensitivity to TKi with liability to neoplastic progression.[9]

Glucose Shortage and Treatment-Insensitive CML Cells

We deepened the effects of low oxygen on $BCR/Abl_{protein}$ expression and LSC selection in CML cell populations by varying, at time zero of incubation, cell density and glucose concentration in culture.[11] While some *BCR/Abl*-dependent growth was possible in low oxygen under non-limiting glucose concentrations, glucose exhaustion from culture medium emerged as the critical condition for the reduction of total cell number with respect to time zero, as well as for $BCR/Abl_{protein}$ suppression and the consequent acquisition of cell refractoriness to IM. Long-sustained oxygen/glucose shortage has been shown to occur in vivo within neoplastic masses, even to induce cell death in the bulk of tumor.[26,27] Within normal BM, the hypoxic SCN represent ideal sites to get close to glucose exhaustion, due to the enhanced glucose consumption rate in low oxygen (the Pasteur effect). We therefore hypothesized that glucose is constantly at extremely low concentrations within the core of hypoxic SCN, which we defined, perhaps inappropriately, "ischemic" SCN.[9] The latter would represent the site of BCR/Abl-independent LSC self-renewal and of dynamic maintenance of IM-resistant MRD (**Fig. 1**). On the other hand, within the poorly oxygenated areas surrounding the "ischemic" SCN core, $BCR/Abl_{protein}$ expression would be maintained and LSC progeny would undergo clonal expansion, exhibiting a *BCR/Abl*-dependent competitive advantage over normal hematopoiesis. The consequent high rate of glucose consumption in peripheral areas of SCN would contribute to maintain glucose shortage in the core and thereby the functional compartmentalization of SCN.

The above scenario impacts on the "alternative" models proposed for the origin of Cancer Stem Cells (CSC): the *CSC in normal stem cell* and the *CSC in progenitor cell models*.[28] We believe both models fit CML biology, being the former adequate to describe $BCR/Abl_{protein}$-negative LSC capable of *BCR/Abl*-independent self-renewal (like normal HSC) and the latter $BCR/Abl_{protein}$-positive LSC where self-renewal is sustained by *BCR/Abl* signaling (**Fig. 2**). This view is well in keeping with the *chiaroscuro* model proposed for a reversible transition between normal haematopoietic stem and progenitor cell phenotypes.[29] The CML cells with a relatively low sensitivity to IM described in some studies are likely to be a mixture of $BCR/Abl_{protein}$-negative and -positive LSC.[18-23] These phenotypical differences are obviously restricted to CML, but the dual metabolically-regulated CSC model we propose may apply equally well to solid

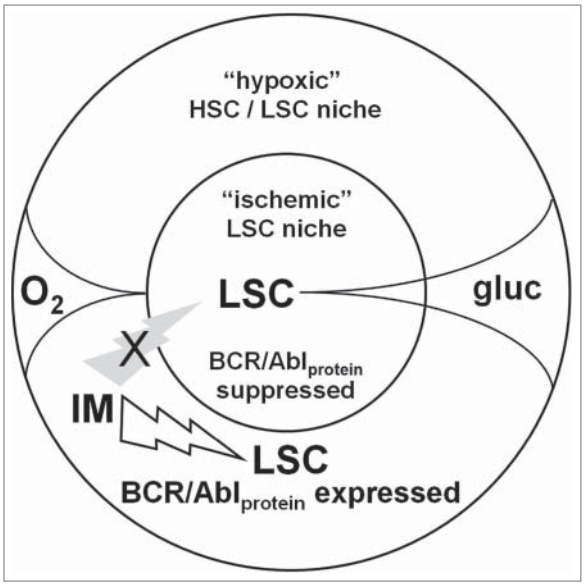

Figure 1. Homing of immature CML cell subsets within the "hypoxic" or the "ischemic" stem cell niche. Oxygen (O_2) and glucose (gluc) concentrations decrease from the external to the internal niche areas. Glucose shortage in the area where oxygen is exhausted drives $BCR/Abl_{protein}$ suppression. LSC: leukemia stem cell; IM: imatinib-mesylate; white bolt: IM effective; gray bolt / X: IM ineffective.

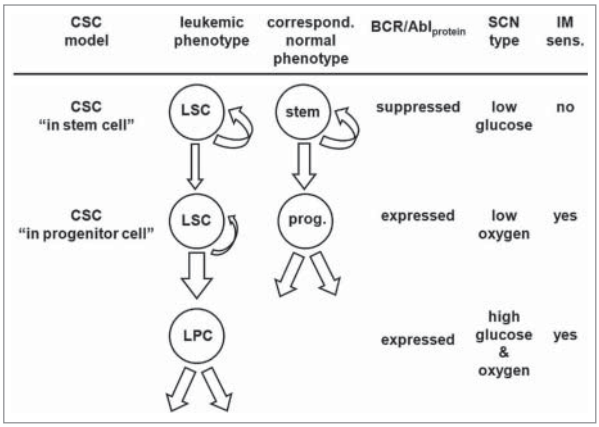

Figure 2. "Metabolic" modulation of LSC phenotypes in CML. Relationship of different models for the generation of CSC from normal immature cells (see ref. 28) to the expression of *BCR/Abl* protein in LSC subsets and to their preferential homing in different tissue environments. CSC: cancer stem cell; LSC: leukemia stem cell; LPC: non-stem leukemia progenitor cell; stem: stem cell; prog.: progenitor cell; SCN: stem cell niche; IM sens.: sensitivity to Imatinib-mesylate; curved arrows: self-renewal; single straight arrows: hierarchical top-down phenotype shift; double straight arrows: clonal expansion (symmetric division). Leukemic phenotypes: the width of single arrows reflects the different behavior which likely characterizes the different LSC subsets. Note that the phenotype correspondence (correspond.) between LSC and HSC does not necessarily imply that the latter are hosted in low-glucose tissue areas (the issue is not addressed in this paper).

tumors, where an abnormal and insufficient vasculature often determines a reduced oxygen and nutrient supply.[26,27]

Is There a Role for an "Acidic" Leukemia/Cancer Stem Cell Niche?

Although supported by a number of experimental data, the cancer SCN model we described above disregards an aspect, low pH, which is a common feature of poorly oxygenated tissue areas. In these areas, low pH is due to the high lactate and H^+ concentrations generated via the enhanced glycolysis which is necessary to maintain energy levels.[30] Actually, tumor cells rely on glycolysis for energy production even when there is enough oxygen to support mitochondrial function; this phenomenon is known as *aerobic glycolysis* or *Warburg effect*. Activation of glycolysis is a necessary requirement for rapidly expanding cell populations, as glycolysis produces energy quickly (although less efficiently than respiration), but also provides building blocks necessary to the synthesis of macromolecules. This means that the expansion of cell population would be compromised if glycolysis were inhibited just because there is enough oxygen around to drive cell respiration.[31,32] Thus, the acidification of tumor cell masses is due not only to poor blood perfusion and/or diffusion across cancer tissue, but also (and often perhaps mainly) to an intrinsic property of proliferating cancer cells. Actually, we believe that acidification usually precedes the onset of "hypoxic" conditions, as it depends on glucose availability rather than oxygen shortage. The presence of acidic, but oxygenated, regions has been indeed demonstrated in tumor xenografts.[33] Overall, in cancer cell masses, the effects of low pH may be independent of those of low oxygen.

Low extracellular pH (pHe) in a tumor is associated with poor prognosis,[34] resistance to radio- and chemo-therapy[35] and increased mutation rate.[36] Low pHe also promotes the rate of P-glycoprotein-dependent transport across plasma membrane, leading to reduced retention, and thereby cytotoxic efficacy, of chemotherapeutic drugs (a feature believed typical of CSC). Accordingly, the normalization of extracellular acidity may increase the chemosensitivity of tumor cells.[37] We recently found that in melanoma cells low pHe promotes epithelial/mesenchymal transition (EMT), reduces proliferation rate and increases resistance to pro-apoptotic agents via the NF-κB pathway.[38] Thus, one can hypothesize that in tumor cells acidity favors the selection of the stem cell phenotype, which is often associated to EMT. In glioma cells, indeed, acidity, independently of oxygen shortage, promotes self-renewal, via mechanisms which involve HIF-2α but are overall still unclear.[39]

The low pHe of tumors derives from the fact that the increase of intracellular lactate and H^+ concentrations due to sustained glycolysis could be lethal for cancer cells if not kept under control. Therefore, an additional adaptive feature of cancer cells is the overexpression and increased activity of cell surface transporters which raise intracellular pH (pHi) via the extrusion of lactate and H^+ and the consequent lowering of pHe.[40,41] High pHi contributes to maintain glycolysis-driven proliferation, while low pHi inhibits glycolysis and activates oxidative metabolism.[42-44] It has been reported that a shift toward low pHe is associated with reduced glucose consumption and lactate production, promoting oxidative metabolism.[45] The pressure pH exerts on cells is powerful, as it has been shown that at low pHe oxidative metabolism is induced even in the presence of high glucose concentrations, which enforce instead glycolysis at normal pHe (Crabtree effect).

Overall, the above information points to a linkage between acidity and oxidative metabolism independently of glucose availability. When glucose is limited or its consumption is reduced due to low pH, an AMPK-dependent arrest of anabolic pathways occurs and metabolism is redirected to fatty acid oxidation and oxidative phosphorylation. Consequently, most available substrates are consumed to maximize energy production, rather than to sustain anabolic pathways critical to cell proliferation, in keeping with what hinted to at the beginning of this chapter.[46] Such a metabolic asset seems to suit CSC very well, which more frequently than other cancer cells just require energy levels sufficient to survive, rather than those necessary for clonal expansion. Accordingly, mitochondria are necessary for HSC to preserve their stemness and in particular to control the balance between

self-renewing cell division and symmetrically committed cell division.[47] Thus, CSC would be characterized by oxidative metabolism and reduced oxygen/glucose consumption, and would be hosted in areas at low pHe and glucose availability (**Fig. 3**).

Lagadinou et al. reported that LSC-enriched acute myeloid leukemia cell populations over-expressing BCL-2 produce oxygen radicals as by-products of low-level oxidative phosphorylation, and that in these cells BCL-2 inhibition reduces oxidative metabolism and selectively eradicates quiescent LSC.[48] LSC were defined indeed unable to utilize glycolysis when mitochondrial respiration is inhibited, indicating in turn that mitochondrial respiration is active in these cells and necessary to drive energy production from glycolysis. Therefore, as summarized in **Figure 3**, a low oxidative state[49] (combined with a low glycolytic rate) is likely to characterize LSC specifically among cells capable to stand low pH generated by cells at high glycolytic rate. On the other hand, metformin, an oral anti-diabetic drug, was shown to target primary T-ALL cell populations enriched in LSC,[50] which also suggests that LSC require oxidative metabolism.[51]

Going back to the metabolic compartmentalization within the SCN, one can hypothesize that peripheral SCN areas are primarily characterized by a high rate of glucose consumption and glycolysis (by the oncogene-addicted cell bulk) rather than by the exhaustion of available oxygen and the consequent secondary enhancement of glycolysis via the *Pasteur effect*. Thus, the complete coverage of energy needs by glycolysis in peripheral SCN areas would lead to spare oxygen and make it available in the SCN core to LSC/CSC relying on oxidative metabolism (**Fig. 3**). In keeping with this hypothesis, we found that melanoma cells grown for 24h at low pH (6.7–6.9) in air maintain 100% viability even after 48 h of transfer to 1% oxygen but do not survive when transferred to 0.3% oxygen (unpublished data), according to the observation that at least 0.5% oxygen is required to keep oxidative metabolism going.[52,53] On this basis, the term acidic SCN is perhaps more appropriate than "ischemic" SCN to define the sites where the more immature LSC/CSC subset is maintained.

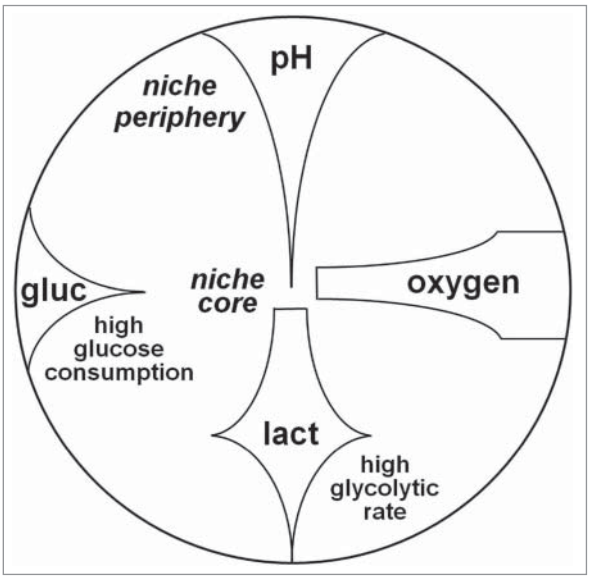

Figure 3. Oxidative metabolism survives within the "acidic" core of stem cell niches. Glucose (gluc) is exhausted in peripheral niche areas due to the high rate of glycolysis, resulting in the production of lactate (lact), which diffuses to, and lowers pH in, the niche core (where pH reaches the lowest levels). Oxygen is spared in the "glycolytic" periphery and let free to diffuse to the core, where it contributes to lactate metabolism and the relative production of energy. According to this hypothesis, niche periphery would home oxygen-independent CSC and niche core glucose-independent/lactate-dependent CSC.

Of relevance to the cohabitation of "glycolytic" and "oxidative" subsets of immature cancer cells within the different SCN regions is the *metabolic symbiosis* demonstrated by Sonveaux et al.[54] In this scenario, lactate produced by "glycolytic" cells is taken up by "oxidative" cells, which use lactate via the tricarboxylic acid cycle as their main substrate for mithocondrial activity. This is the so-called *reverse Warburg effect*, which has been shown to characterize the metabolic symbiosis between breast or prostate cancer cells and cancer-associated fibroblasts.[55,56] Whether the reverse Warburg effect applies to leukemia cells within the SCN of BM remains to be demonstrated. However, the consumption of lactate and its processing via an oxidative metabolic pathway provides a likely explanation as to how LSC/CSC can manage to produce enough energy within the SCN core following glucose exhaustion in the periphery. In this light, acidity may be crucial to define the SCN core conditions, rather than per se, in that it characterizes the environment where lactate is supplied. The concept of metabolic symbiosis renders borders within the niche difficult to define (compare **Figure 3** to **Figure 1**). This conclusion is in keeping with other information challenging the model of location-dependent stem cell regulation within tissues. It is considered very possible indeed that LSC/CSC are capable to bounce flexibly and reversibly between a lactate-producing and a lactate-consuming state,[57] as suggested by a report that cancer cells which are at low oxygen at one moment may be well oxygenated an hour later and viceversa.[58] Further support to the view that cells with different metabolic profiles may cohabitate in the same environment came from recent work indicating that the "hypoxic" phenotype of HSC is cell-specific rather than location-dependent.[59]

Concluding Remarks

All above points to a functional compartmentalization of different LSC/CSC subsets which is based on their metabolic asset, rather than to a location-centered compartmentalization related to the availability of oxygen or glucose per se in discrete SCN zones. In this respect, it is

mandatory to note that our conclusions derive from the combination of information relative to leukemias and solid tumors, which commands to cross-confirm experimentally the data we referred to. As far as CML is concerned, we can be reasonably sure that the conditions we established in vitro for *BCR/Abl* protein suppression reflect those enabling the maintenance of IM-resistant MRD in vivo. However, experiments directed to the metabolic characterization of this phenomenon are still going on in our laboratory. With respect to other neoplastic diseases, including leukemias and solid tumors, for which the molecular oncogenesis is less well characterized, we believe that the homing of an LSC/CSC subset within the SCN core is invariably paralleled by the suppression of all endogenous stimuli to extensive proliferation and clonal expansion.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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