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Energy shortage regulates BCR-Abl expression in Chronic Myeloid Leukemia cells via transcriptional/post-transcriptional control

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Abstract

Low oxygen tension is a critical aspect of the metabolic milieu of stem cell niches. It has been hypothesized that low oxygen tensions in stem cell niches *in vivo* offer a selective advantage to the maintenance of Hematopoietic Stem Cells (HSC), being HSC, but not less immature progenitors, well suited to stand low oxygen tensions. It was indeed shown in our laboratory that resistance to severe hypoxia *in vitro* defines hierarchical levels within hematopoietic populations.

The cancer stem cell (CSC) hypothesis postulates that tumor cells are hierarchically organized with respect to tumor growth initiation and maintenance. The source of CSC is not entirely clear, and may differ depending on the specific disease. Some experimental results are consistent with the idea that CSC can derive from normal stem cells that have undergone oncogenic transformation. In contrast, it is possible that malignant non-stem progenitor cells acquire the capacity of self-renewal. In addition, tumors have an elevated rate of glucose uptake and consumption through glycolysis, that offers a growth advantage to cancer cells under a hypoxic environment.

Chronic Myeloid Leukemia (CML) is a clonal myeloproliferative disorder driven by the BCR-Abl oncogenic fusion protein with constitutive kinase activity, which drives autonomous cell survival and growth.

It was also shown in our laboratory that stabilized and cloned leukemia cell lines are highly heterogeneous, to comprise cells with a hypoxia-adapted "stem" phenotype as well as others with a hypoxia-sensitive "progenitor" phenotype, suggesting that these phenotypes are not genetically "frozen", but flexibly, and reversibly, expressed. Thus, leukemia cell lines emerged as suitable models to assess independently the sensitivity to chemoterapy of the "stem" or the "progenitor" leukemia cell phenotypes to chemotherapy, provided an experimental system capable to select the one from the other (such as incubation in hypoxia or in glucose starvation for different times) is established.

BCR-Abl protein suppression emerged as a crucial feature of CML cells adaptation to hypoxia. Furthermore, in hypoxia, while surviving leukemia cells are generally growth-arrested, Leukemia Stem Cells (LSC) are in large part cycling,

indicating that cycling in hypoxia is a specific property of LSC. Thus, hypoxia-selected LSC, while remaining genetically leukemic, are phenotypically independent from BCR-Abl signaling. The suppression of BCR-Abl, molecular target of the current CML therapy, combined with the capacity of cycling within the hypoxic niches, makes of hypoxia-adapted LSC the most likely candidate to sustain treatment-insensitive Minimal Residual Disease (MRD) of CML.

The main target of this study was to characterize molecular mechanisms regulating BCR-Abl expression in blast crisis CML cell lines (K562 and KCL22). We found that hypoxia strongly inhibits the overall growth of CML cell populations and suppresses both the expression and phosphorylation of BCR-Abl protein and transcription of BCR-abl mRNA. These pointed to a dual action of hypoxia as far as BCR-Abl expression in concerned, transcriptional and/or post-transcriptional.

To test whether hypoxia *per se* is capable of driving the selection of CML cells, or it additional environmental/metabolic factors are required, such as glucose availability, we maintained cells in normoxia in the absence or the presence of glucose for 14 days and compared the data with those obtained in hypoxia: glucose shortage reduced cell survival and downregulated BCR-Abl protein but, unlike hypoxia, did not affect BCR-abl mRNA levels.

The effect of environmental/metabolic factors on LSC were determined using the Culture Repopulating Ability (CRA) assay, where the time-dependent selection of LSC in hypoxic/glucose-starved cultures is estimated following cell transfer to secondary standard cultures where the expansion of population is allowed. Results allowed us to confirm our hypothesis that energy shortage (hypoxia or glucose deprivation) represents the condition selecting LSC cells, BCR-Abl-negative.

Introduction

Hematopoiesis and Hematopoietic Stem Cells

The Hierarchy of Hematopoietic Cells

All blood cells are generated from a common hematopoietic stem cell (HSC) through an extremely dynamic process called hematopoiesis or blood cell formation.

The generation of definitive or adult-type HSC during development occurs in the aorta-mesonephros-gonad region (AGM) of the embryo, and more recently there have been reports that may indicate that definitive HSC are also found in the yolk sac and later in placenta. The fetal liver is seeded with HSC from the AGM and they are expanded there and generate a large amount of progeny cells. Postnatally, hematopoiesis takes place in the BM where the HSCs as well as a complex mix of dividing and maturing cells of different lineages can be found.

The process of hematopoiesis can be described as hierarchical with the rare HSCs at the top of the hierarchy giving rise first to progenitors and then to precursors with single lineage commitment and ending in terminally differentiated mature cells of various lineages. In between the HSC and terminally differentiated cells, there is a continuum of progenitors at different stages, which, depending on certain stimuli, can divide and progress towards certain lineages (**Figure I**). It is generally believed that hematopoietic development divides at an early stage into a myeloid and a lymphoid branch. The lymphoid branch gives rise to B, T and natural killer cells, while the myeloid branch differentiates to all other cell types including erythrocytes. Indeed, two distinct progenitor populations with either lymphoid or myeloid restricted potential, the so-called common lymphoid progenitors (CLP) and common myeloid progenitors (CMP), have been isolated from mouse BM.

HSCs are pluripotent and should therefore be able to give rise to all hematopoietic lineages. They are operationally defined as cells that can completely reconstitute a recipient following BM ablation. Similarly, they must have the capacity to self-renew to give rise to other stem cells. A distinction is often made between long-term repopulating HSCs (LT-HSCs) and short-term repopulating HSCs

(ST-HSCs). The only way to test these criteria is through BM transplantation experiments into lethally irradiated recipients (**Larsson J. et al. 2005**).

Although the HSC can only be absolutely defined through BM transplantation assays, advances in cell sorting have allowed near purification of murine HSC.

Early committed progenitor populations, depicted in **Figure 1**, can also be purified. HSC are highly enriched in a population that is negative for lineage markers (Lin^-) and positive for Sca1 and c-Kit (LSK cells). c-Kit, which is the receptor for stem cell factor (SCF), has a wider expression pattern than Sca1, marking most multipotent progenitors (MPPs). Sca1 and c-Kit are often used together for positive selection of HSCs from Lin^- cells.

The LSK cells contain LT-HSC, ST-HSC and MPPs, which cannot repopulate recipients. Further enrichment for LT-HSC has been demonstrated by including negative selection for the CD34 marker. Indeed, LSK $CD34^-$ cells contain a very high proportion of HSCs and isolated single LSK $CD34^-$ cells have been transplanted and have given full long-term hematopoietic reconstitution. A further separation between LT-HSC, ST-HSC and MPP has recently been made by showing that repopulating HSC express the thrombopoietin (Tpo) receptor (c-mpl), whereas MPP do not, and MPPs express Flt3, whereas pluripotent repopulating HSC do not (**Bryder D. et al. 2006**).

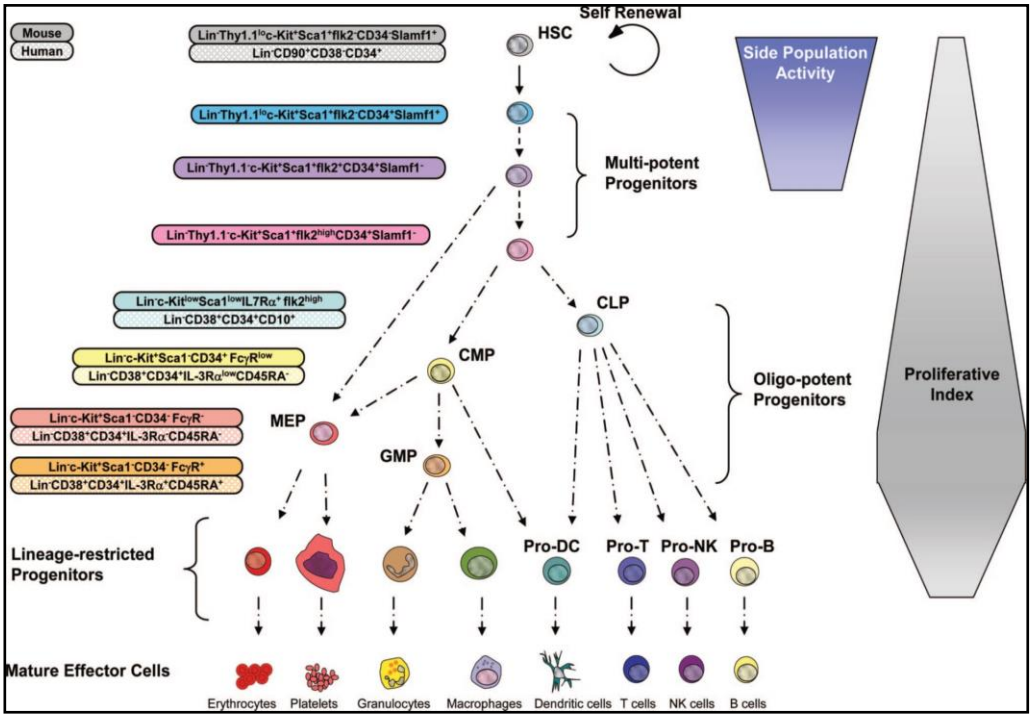


Figure I. Model of the hematopoietic developmental hierarchy. Self-renewing HSCs reside at the top of the hierarchy, giving rise to a number of multipotent progenitors. Multipotent progenitors give rise to oligo-potent progenitors including the CLP, which gives rise to mature B lymphocytes, T lymphocytes, and natural killer (NK) cells. The common myeloid progenitor (CMP) gives rise to granulocyte-macrophage progenitors, which differentiate into monocytes/macrophages and granulocytes, and megakaryocyte/erythrocyte progenitors, which differentiate into megakaryocytes and erythrocytes. Both CMPs and CLPs have been proposed to give rise to dendritic cells. The cell surface phenotype of many of these cell types is shown for the murine and human systems (**Bryder D. et al. 2006**).

Regulation of Hematopoiesis

HSC behaviour and function are governed by two intimately entangled entities. One is the gene expression pattern in the cell and the other is the combination of external signals within the hematopietic microenvironment. Transcription factors are internal signals that regulate gene expression, while external signals are mediated by cell-cell interactions, cell-extracellular matrix (ECM) interactions and soluble growth factors. Signals from internal and external regulatory factors decide whether HSCs are maintained in quiescence, proliferate, undergo apoptosis, or migrate out of the BM space (**Larsson J. et al. 2005**).

The acquisition of genetic and epigenetic alterations leads to deregulation of these processes, including the onset of uncontrolled growth and the development of hematological diseases such as leukaemia **(Kennedy A. et al. 2008)**. Understanding the cellular and molecular controls of normal blood cell development makes it possible to answer questions about the origin and treatment of these diseases **(Sachs L. 1996)**.

The Hematopoietic Stem Cell Niche

Hematopoietic Stem Cells Reside in Bone Marrow Niches

HSCs, which reside in BM, proliferate and differentiate to supply blood components to the body. In order to maintain the homeostasis blood homeostasis, HSCs are regulated tightly by a specific microenvironment called “stem cell niche”. The niche is composed of a vast milieu of cellular and humoral factors. Two different

niche environments, called "osteoblastic" and "vascular" niches have been identified to alternatively regulate HSC maintenance, clonal expansion and exhaustion. It is debated wheter the two different niches are physically separated - or represent different states transiently induced in a single niche by competing stimuli. The periosteal region of the BM is indeed a complex structure where HSCs are influenced not only by osteoblasts but also by vasculature and other stromal structures. (**Nakamura-Ishizu A. et al. 2013**).

Evidence for a Hypoxic Nature of the Hematopoietic Stem Cell Niche

While it is widely assumed that direct contact to the supportive osteoblasts and stromal cells regulate HSCs within the endosteal niche, the overall nature of the environment was found essential for the maintenance and protection of HSCs. Considering that BM niches harboring quiescent HSCs are located at distance of several cell diameters from blood vessels, such as the thin-walled sinusoids, the physiological condition of the niche environment appears to be a relatively low oxygen tension and a limited nutrient supply (**Eliasson P. et al. 2010**).

The ability to isolate and culture stem cells in vitro has greatly advanced the understanding of the role of the niche in some stem cell systems. Like all other cells, stem cells were typically cultured under ambient oxygen tensions, with very little attention paid to the metabolic milieu of the niche in which they normally resided *in vivo*. This practice led researchers to focus on identifying growth factors and signaling proteins, failing to explore the equally important metabolic factors of the niche (**Scadden D.T. et al. 2006**).

Later, studies carried out by altering oxygen concentrations in the incubation atmosphere showed that oxygen tension is a regulator of adult as well as embryonic and adult stem cells (**Cipolleschi M.G. et al. 1993**, **Eliasson P. et al. 2010, Panchision D.M. et al. 2009**, **Silvan U. et al. 2009**) and fueled the hypothesis that low oxygen tension is a critical aspect of the metabolic milieu of stem cell niches (**Cipolleschi M.G. et al. 1993**). Furthermore, the identification of previously undescribed stem cells in compartments known to be notoriously hypoxic, as in the

case of the kidney medulla/papilla, continues to drive the excitement behind this hypothesis (**Oliver J.A. et al. 2004**).

Indeed, adult tissues experience a wide range of oxygen tensions that are considerably different from the inhaled ambient oxygen tensions of 21% (160 mmHg) (**Figure II**). The partial pressure of oxygen (pO_2) of inspired air progressively decreases after it enters the lungs and as it travels in the blood throughout the body. By the time it reaches organs and tissues, pO_2 levels have dropped to 2%-9% (14–65 mm Hg) (**Brahimi-Horn M.C. et al. 2007**). This pressure is a drastic departure from oxygen tensions in ambient air that are typically considered ‘‘normoxic’’ by conventional standards of cell-culture practice.

It has been hypothesized that low oxygen tensions in stem cell niches offers a selective advantage to HSC mainteinance, being HSC, but not less immature progenitors, well suited to stand low oxygen tensions (**Cipolleschi M.G. et al. 1993**). Indeed, growing cells that undergo aerobic metabolism are subject to some degree of oxidative stress through the generation of reactive oxygen species that can damage DNA. This risk is supported by direct evidence that mouse embryonic fibroblasts accumulate more mutations and senesce faster when cultured under 20% O_2 than cells cultured under 3% O2 (**Busuttil R.A. et al. 2003**). Thus, by residing in anatomical compartments at relatively low oxygen tensions (in the range of 1%-9%), HSCs may escape this damage together with growth pressure (**Figure II**).

In addition, hypoxia has been shown to activate molecular pathways typical of stem cells, such as Oct4 and Notch. Finally, oxygen tensions as low as 1% appears to decrease proliferation and maintain ESC pluripotency, while higher oxygen tensions (3%-5%) appear to maintain pluripotency with no effect on proliferation (**Mohyeldin A. et al. 2010**).

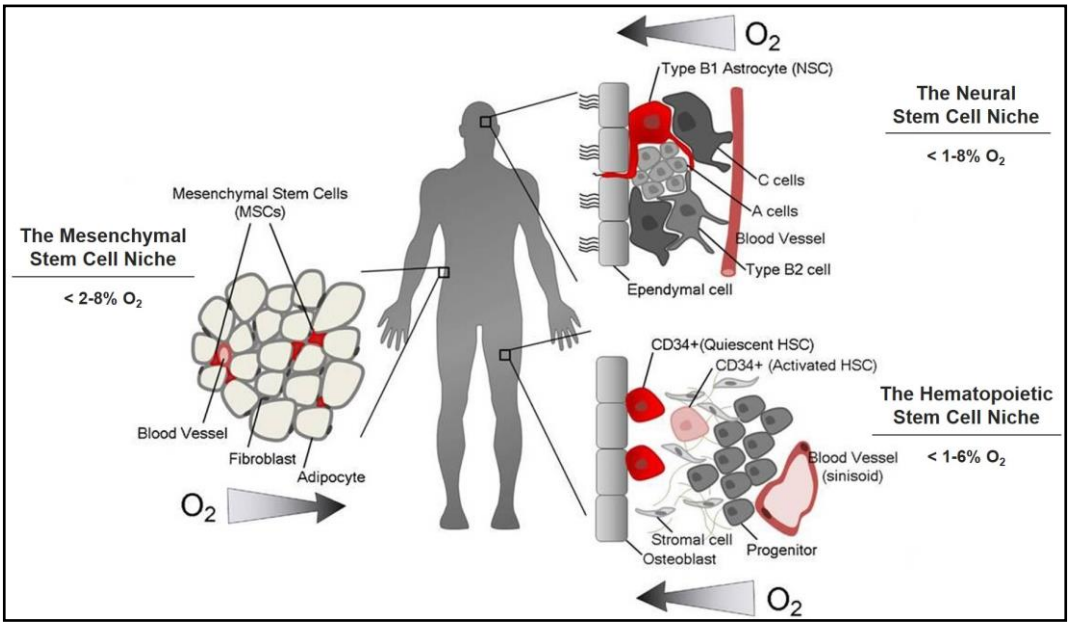


Figure II. Oxygen Tension Measurements in Various Stem Cell Compartments. Currently available data are reported for hematopoietic, mesenchymal, and neural stem cells in their designated niches: the bone marrow, adipose tissue, and the subventricular zone (SVZ), respectively. Red cells represent HSCs, MSCs, and NSCs. Although direct measurements from the SVZ have never been performed, measurements of oxygen tension as low as 0.55% O_2 in various areas of the brain suggest that gradients of oxygen may exist in the SVZ (**Mohyeldin A. et al. 2010**).

Hypoxic Niche for Cancer Stem Cells

The cancer stem cell hypothesis postulates that tumor cells are hierarchically organized with respect to tumor growth initiation and maintenance. CSCs, known also as tumor-initiating cells, have now been isolated from several human cancers, including leukemias, breast, brain melanoma, colon, and pancreatic cancer, and the presence of CSC in tumor cell populations correlates strongly with tumor recurrence and treatment failure (**Mohyeldin A. et al. 2010**).

The characterization of CSC niche and its regulators has been object of intense research. Initial reports on CSCs argued for an intimate relationship with tumor vasculature, suggesting a perivascular niche like that seen in the Neural Stem Cell Niche, NSC (**Figure 3**) (**Calabrese C. et al. 2007**).

Besides the well-established perivascular niche, CSC have recently been proposed to reside in a second niche type, further away from vasculature and, as a consequence, more hypoxic (**Li Z. et al. 2009**). Hypoxic microenvironments within tumors have long been appreciated to be a product of aberrant vasculature and due to

a rapidly dividing tumor mass that outstrips its vascular supply (**Figure III**) (**Pouysségur et al. 2006**). Zones of necrosis can readily be seen in rapidly dividing cancers, and hypoxia has been associated with treatment resistance, local invasion, and poor clinical outcome (**Keith B. et al. 2007**). In addition, the undifferentiated phenotype of solid tumors seen often in neuroblastoma, breast, and cervical cancers strongly correlates with tumor hypoxia (**Mohyeldin A. et al. 2010**).

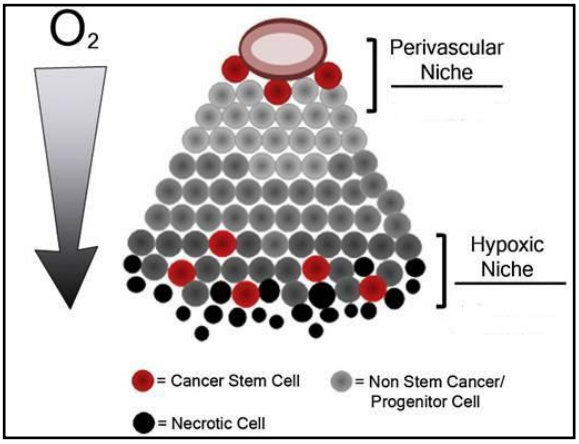


Figure III. Cancer Stem Cell Niche. (**Mohyeldin A. et al. 2010**)

CSC typically represent a small fraction of the total tumor, can be enriched on the basis of cell-surface marker expression, and generate serially transplantable tumors in recipient immunodeficient mice. Some CSC also express ABC glycoprotein transporters at the cell surface, a trait shared with normal HSC. These transporters effectively pump out vital dyes, resulting in a characteristic unlabeled "side-population" of cells detected in flow cytometry plots. Unfortunately, these transporters also eliminate chemotherapeutic drugs, thereby promoting the multidrug resistance (MDR) observed in a large number of cancer cell lines.

The source of CSC is not entirely clear, and may differ depending on the specific disease. Some experimental results are consistent with the idea that CSC can derive from normal stem cells that have undergone oncogenic transformation, as described for human AML leukemic stem cells (**Huntly B.J.P. et al. 2005**). In contrast, it is possible that malignant progenitor cells, or even differentiated cells, acquire the capacity of self-renewal (**Krivtsov A.V. et al. 2006**).

Within the high cell density typical of tumors, the microenvironmental restrictions which have just been described produce a fierce clonal competition, in which the staminal pool is given an advantage by its resistance to hypoxia. In these regions the neoplastic population is continuously exposed to the effects of hypoxic/ischemic environments with the following consequences: (a) selection of hypoxia-resistant clones, either arising spontaneously in the course of neoplastic progression, or driven by hypoxia itself, which is capable of acting as a potent gene modulator and is a major source of genetic instability in tumor populations; and (b) the creation of hypoxic niches, in which cancer cells selected for their adaptation to hypoxia can survive indefinitely in the dormant state, resisting both radio- and chemo-therapy. It therefore seems imperative to eradicate these cancer pockets and to pursue an active search for treatments attempting to prevent the ultimate mechanism of cell adaptation to hypoxia (**Olivotto M. et al. 2008**).

The predilection of cancer cells for anaerobic glucose metabolism

Compared to non-malignant tissues, tumours in general have a high rate of glucose uptake accompanied by elevated glucose consumption through glycolysis (**Gatenby R.A. et al. 2004**). In the 1850s, Louis Pasteur showed that aerating yeast, a facultative anaerobe, leads to an increase in growth but a decrease in fermentation and glucose consumption, the "Pasteur effect". So for biomass production yeast are incubate in oxygenated conditions but for ethanol production oxygen is made limiting.

The initial recognition that cancer cells exhibit atypical metabolic characteristics can be traced back to the pioneering work of Otto Warburg over the first half of the twentieth century. In the presence of oxygen, most normal tissues metabolize glucose to pyruvate through glycolysis, and then completely oxidize a large fraction of the generated pyruvate to carbon dioxide in the mitochondria through the tricarboxylic acid (TCA) cycle and theoxidative phosphorylation (OxPhos). Under anaerobic conditions, normal cells redirect glycolytic pyruvate away from mitochondrial oxidation and instead largely reduce it to lactate. The fundamental paradigm

stemming from Warburg's studies was that in contrast to normal cells, tumors metabolized glucose to lactate even under aerobic conditions, despite this process being by far less efficient: 38 versus 2 ATP molecule per molecule of glucose (**Figure IV**). This seemingly paradoxical phenomenon, called the "Warburg effect", has been observed in several tumor types and often occurs in parallel with a marked increase in glucose uptake and consumption (**Brahimi-Horn M.C. et al. 2007**, **Cantor J.R. et al. 2012**).

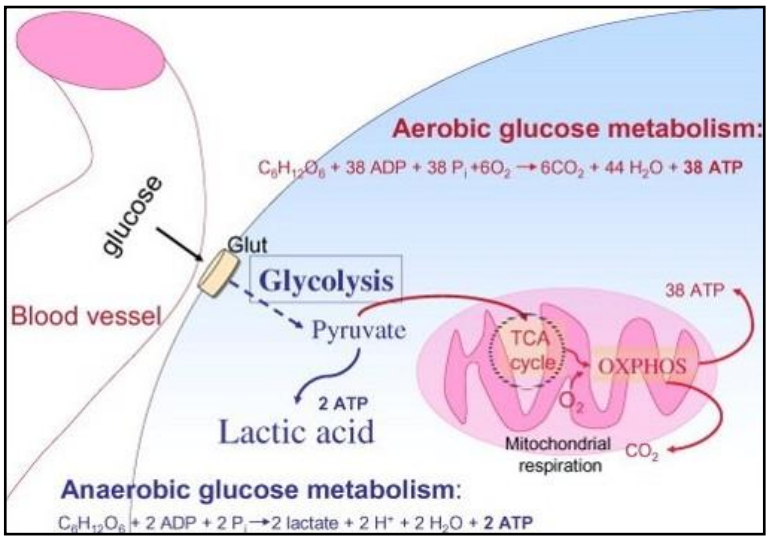


Figure IV. Tumour cells switch from aerobic to anaerobic glucose metabolism despite the lower energy yield. Non-malignant and malignant cells take-up glucose from the blood stream via membrane glucose transporters (Glut) and metabolize glucose through the glycolytic pathway to form pyruvate. Pyruvate in non-malignant cells enters the mitochondrial tricarboxylic acid cycle (TCA) and oxygen-requiring oxidative phosphorylation (OxPhos) to produce CO_2 and 38 molecules of ATP. In contrast malignant cells convert pyruvate to lactic acid to produce two molecules of ATP (**Brahimi-Horn M.C. et al. 2007**).

It is only now that the global mechanism behind the cancer cell predilection for glucose metabolism to lactic acid is starting to be understood and that the hypoxia-inducible factors (HIF) are surfacing as one of the major actors. HIF acts basically in two ways: promoting anaerobic glucose metabolism and repressing or rendering more efficient mitochondrial respiration. The genes coding for certain glucose transporters and for enzymes of the glycolytic pathway are among the many genes that are directly up-regulated by HIF. The conversion of pyruvate to lactic acid is also enhanced by up-regulation of the enzyme lactate dehydrogenase A (LDH-A),

the enzyme that catalyses pyruvate conversion to lactic acid (**Fantin V.R. et al. 2006**).

Cancer cells prefer to use aerobic glycolysis for ATP production while still retaining the function of OxPhos for the following reasons:

- Glycolysis is more suitable for cancer growth. Since proliferation of cancer tissues is faster than normal tissues, it not only needs energy, but also metabolic intermediates for the biosynthesis of macromolecules. Many intermediates from glycolysis and the TCA cycle can be used to synthesize macromolecules, such as nucleic acids, lipids and proteins, required for cancer growth (**Zheng J. 2012**).

- A too efficient productions of ATP (via cell respiration) may not be a good thing for cancer cells. If cancer cells use glucose with high efficiency, ADP is massively converted to ATP. The high concentration of ATP will inhibit phosphofructokinase 1 (PFK1), the rate-limiting enzyme in glycolysis and pyruvate kinase 1 (PK1), and glycolysis will be inhibited. Inhibited glycolysis is unfavorable for cancer cell growth. Although glycolysis yields less ATP than OxPhos, the speed of ATP generation in the former is quicker than in the latter. Generally speaking, rapidly proliferating tissues such as cancer and embryonic tissues rely more on glycolysis for ATP production, whereas differentiating tissues rely primarily on OxPhos (**Berridge M.V. et al. 2010**, **Vander Heiden M.G. et al. 2009**).

- Glycolysis offers a growth advantage to cancer cells under a hypoxic environment. Glycolysis produces lactate which is released into the extracellular space, leading to an acidic microenvironment which enhances the invasion and metastasis of cancer cells (**Gatenby R.A. et al. 2008**, **Vaupel P. 2010**).

- Due to the decrease of mitochondrial OxPhos, less reactive oxygen species (ROS) are generated, which are cytotoxic to cancer cells (**Zheng J. 2012**).

Increasing evidences show that oncogenes such as Ras and c-Myc, and the protein kinase Akt, which promote cell survival are also responsible for the peculiar cancer cell metabolism.

c-Myc was found to induce up-regulation of the glucose transporter GLUT1 and several glycolytic enzymes as well as LDH-A.

The tumour suppressor p53 on the other hand maintains cells in normal aerobic mitochondrial metabolism by slowing glycolysis. Regulation of glycolysis occurs

though the induction by p53 of the TP53-induced glycolysis and apoptosis regulator (TIGAR) gene that decreases the level of fructose-2,6-bisphosphate resulting in inhibition of glycolysis and through negative regulation of the glycolytic enzyme phosphoglycerate mutase (PGM).

Repression of pyruvate metabolism through mitochondrial respiration provides a mitochondrial bypass mechanism that promotes anaerobic glucose metabolism but also spares oxygen. This occurs though HIF activation of the expression of pyruvate dehydrogenase kinase 1 (PDK1) that inhibits pyruvate dehydrogenase (PDH), the enzyme that converts pyruvate to acetyl-CoA for entry into the TCA cycle. LDH-A knockdown not only suppresses lactate production in tumour cells but increases oxygen consumption and OxPhos activity, thereby tipping back the glycolysis/respiration balance (**Brahimi-Horn M.C. et al. 2007**).

Chronic Myeloid Leukemia

Chronic Myeloid Leukemia (CML) is a clonal myeloproliferative disorder characterized by the Philadelphia (Ph) chromosome, which results from t(9;22) (q34;q11) balanced reciprocal translocation. The molecular consequence of the Ph chromosome is the generation of the BCR-abl oncogene that encodes for the chimeric BCR-Abl oncoprotein, with constitutive kinase activity that promotes the growth advantage of leukemic cells (**Figure V**) (**Cilloni D. et al. 2012**).

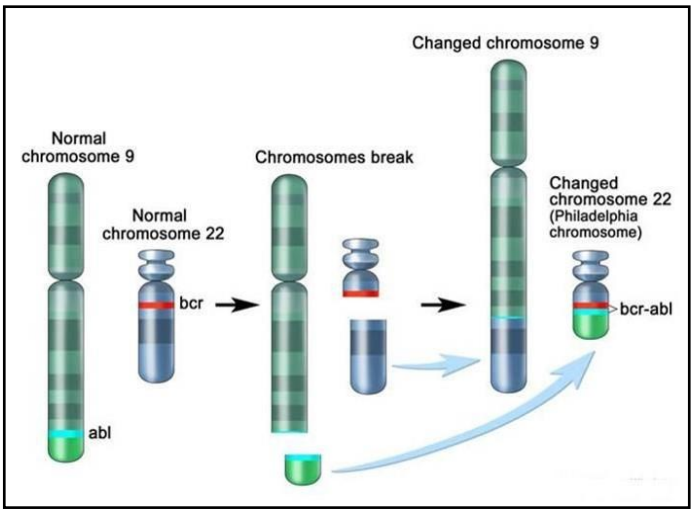


Figure V. BCR-Abl translocation in Chronic Myeloid Leukemia. (www.cancer.gov).

The deregulated tyrosine kinase activity of BCR-Abl has been shown to be necessary and sufficient to maintain the leukemia phenotype of CML (**Cilloni D. et al. 2012**). The constitutive activation of the Abl tyrosine kinase is a primary event in the genesis of CML, as shown by the retrovirally mediated insertion of a human BCR-abl gene into murine hematopoietic stem cells and the creation of BCR-abl transgenic mice (**Daley G.Q. et al. 1990**). This represents a critical issue in the effort to design molecular therapies.

Natural hystory of CML

CML is characterized by biphasic (and sometimes triphasic) course. The disease is usually recognized in a relatively indolent chronic phase (CP) that may continue for several years. CP inevitably undergoes progression to an "accelerated phase" (AP) and then the so-called "blast crisis" (BC), which are distinguished by the overproduction of relatively immature cells (**Figure VI**). In most cases, CP can be suppressed with imatinib mesylate (IM), a tyrosine kinase inhibitor (TKI) active on the BCR-Abl protein (**Cortes J. 2004**).

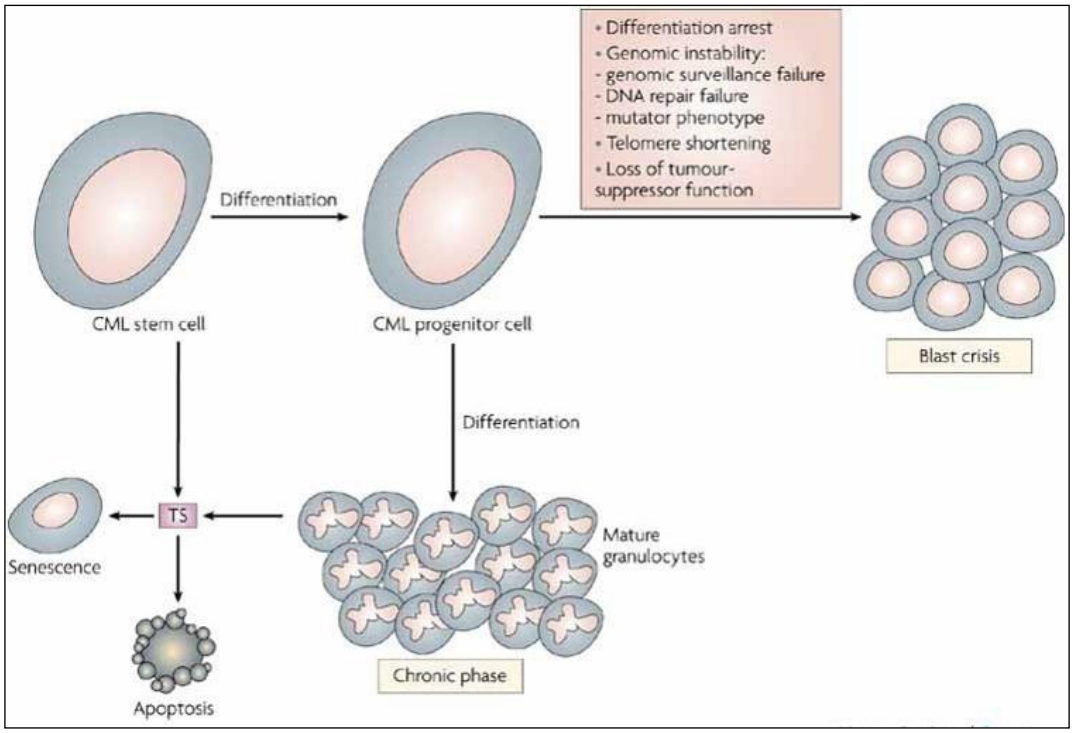


Figure VI. Disease progression in Chronic Myeloid Leukemia. In chronic phase (CP), the bulk of leukemic stem cells remain capable of undergoing differentiation, leading to the excessive production of mature granulocytes. In the advanced disease, differentiation is blocked and disease is sustained by the proliferation of immature blasts. Deleterious genetic events (inset) are believed to accumulate within stem and progenitor cells of the leukemic clone until there are sufficient secondary mutations to drive the transition from chronic phase to advanced disease. These include: an increase in genomic instability through interference with genomic surveillance and DNA-repair proteins and a progressive telomere shortening. In CP cells essential tumour-suppressor (TS) proteins remain functional and allow cells to undergo replicative senescence or apoptosis. However, in advanced phase blasts there is evidence that TS function has been lost (**Melo J.V. et al. 2007**).

However, long interruption or discontinuation of IM treatment is not recommended, even in patients with complete molecular remission, because IM does not kill quiescent leukemia stem cells (LSC), which thus persist in a majority of patients and may cause disease relapse. The resistance to IM that develops in a portion of CP patients is most often caused by a mutation in the kinase domain abolishing binding of IM to BCR-abl or by the amplification of BCR-abl gene. Moreover, advanced-stage CML responds poorly to any therapy including IM. LSC are cancer stem cells (CSCs) which are therefore typically characterized by the ability to self-renew, thereby representing a cell subset of oncogenic cells capable of initiating and propagating disease. Therefore, to cure patients with CML, LSC need to be eradicated (**Gangemi R. et al. 2009**). Hence, immunization against CSC markers might be an important method for CSC elimination. In fact, the only long-lasting remissions of CML are achieved by immunotherapy associated with HSC transplantation or IFN-α treatment (**Pinilla I.J. et al. 2009**). In BC, another type of self-renewing cell different from typical LSC has been identified: these cells can be defined "staminalized" granulocyte-macrophage progenitors and are responsible for the rapid expansion of the blast cell population (**Jamieson C.H.M. et al. 2004**).

BCR-Abl as a direct cause of genetic damage and instability

During the progression of CML, genomic instability of leukemic cells leads to the accumulation of mutations including chromosomal abnormalities found in about 80% of CML patients. These genetic changes result in the generation of malignant cells with activated alternative oncogenic pathways and thus independent of the transformation potential of the BCR-Abl protein (**Smahel M. 2012**).

The effects of BCR-Abl on cell signaling have been well documented (**Melo J.V. et al. 2007**). These effects include increased proliferation through the activation of *ras*, increased transcriptional activity via STAT recruitment, decreases in apoptosis through activation of PI3K/AKT and changes in adhesion and phosphorylation of cytoskeletal proteins. While the contribution of these pathways in initiating CML is clear, it is not clear wheter these pathways drive progression from chronic-phase to advanced-phase disease (**Skorski T. 2002**).

BCR-Abl is believed to cause genomic instability directly. When considering the effects of BCR-Abl on genetic instability, one must confront a seeming paradox: cell lines with constitutively activated kinases, such as BCR-Abl, accumulate more DNA damage than similar cell lines without such a constitutive activation, yet cell lines with activated kinases repair DNA damage faster. However, the combination of more DNA damage and more repair activity may lead to less exact repair. Furthermore, since BCR-Abl also up-regulates antiapoptosis genes bcl-2 and bcl-xL, and thus causes G2/M delay, the stage is set to accumulate DNA damage without the mechanisms to eliminate these cells (**Majsterek I et al. 2002**, **Slupianek A. et al. 2002**). Bcr-Abl itself can also cause DNA damage by increasing reactive oxygen species (ROS); these ROS can lead to DNA base-pair transversions (GC→TA) and transitions (GC→AT) (**Sattler M. et al. 2000**). Taken together, BCR-Abl dependent genetic instability may lay the groundwork for chromosomal aberrations, mutations, and changes in gene expression that hallmark progression.

An increase in BCR-Abl level is seen in advanced-phase CML, and this increased activity affects several cellular functions. Blast phase is paralleled by an increase in both BCR-abl mRNA and protein levels. This increase is not extreme (approximately 3-fold) but appears associated with an increase in activation of signaling (as evidenced by increased Crkl phosphorylation), and *in vitro* changes of clonality, growth factor independence, proliferation, and block in apoptosis (**Barnes D.J. et al. 2005**, **Modi H. et al. 2007**).

Since BCR-Abl level is associated with progression, a logical question is whether this phenomenon also plays a role in imatinib resistance, since blast crisis is relatively resistant to TKI therapy. However, the relationship of increased BCR-Abl levels to imatinib resistance is unclear. The culture of cell lines in the presence of

imatinib to induce the development of resistance to the drug led to suggest that higher BCR-Abl levels are associated with a shorter time to the development of abl point mutations. On the contrary, the use of a model of human CD34+ cells transduced with Bcr-Abl led to suggest that high Bcr-Abl expression is associated with increased sensitivity to imatinib, suggesting that these cells were "addicted" to high levels of BCR-Abl (**Barnes D.J. et al. 2005**, **Modi H. et al. 2007**). Of note is that the mechanism of increased Bcr-Abl mRNA and protein levels in progressive disease is unclear; although some cases of blast crisis have multiple copies of the Ph chromosome, such abnormalities are not the rule.

If BCR-Abl causes genomic instability, it is logical that unchecked BCR-Abl will inevitably yield further genomic changes. But why did the Ph chromosome arise in the first place? Were there genetic lesions in the stem cell that created poor DNA maintenance even *before* the acquisition of Ph, facilitating the incidence of the disease? Two observations suggest a stem cell disorder preceding the acquisition of Ph. First, the BCR-abl transcript can be found in the peripheral blood of more than 25% of normal "older" (>55 years) individuals (**Bose S. et al. 1998**) Such findings could be either from the increased instability associated with aging, or accumulation of genotoxic insults over time. The second interesting finding is that clonal abnormalities in Ph^- cells are seen in approximately 5% of patients with chronic-phase CML treated with imatinib, despite the maintenance of Complete Cytogenetic Response Rate, CCyR (**Bumm T. et al. 2003**, **O'Dwyer M.E. et al. 2003**). This occurrence suggests that more primitive stem cells, before those acquiring the Ph, have some genetic instability, and when imatinib selects against the Ph clone, these cells gain a competitive advantage (**Radich J.P. 2007**).

The functions of the translocation partners

The human *abl* gene, which is a proto-oncogene with omology to the v-*abl* oncogene carried by the Abelson murine leukemia virus (A-MuLV), has been mapped to a region of 225 kb on chromosome 9 and it is expressed as either a 6- or a 7-kb mRNA transcript, with alternatively spliced first exons, exons 1b and 1a,

respectively, spliced to the common exons 2-11. Exon 1b is approximately 200 kb 5' of exon 1a (**Chissoe S.L. et al. 1995**).

It encodes a 145-kDa non-receptor tyrosine kinase protein ubiquitously expressed, with 2 isoforms arising from alternative splicing of the first exon **(Deininger M.W.N. et al. 2000)**.

The normal Abl protein is distributed in both the nucleus and cytoplasm of cells and can shuttle between the two compartments **(Ren R. 2005)**, and it binds specifically to DNA, suggesting that this may be critical to its normal biological function. In contrast, the chimeric p210 BCR-Abl and other Abl transforming proteins are only present in the cytoplasm, and lack the ability to bind DNA **(Clarkson B. et al. 2003)**.

Several structural domains can be defined within the protein (**Figure VII**). Three SRC homology domains (SH1-SH3) are located toward the NH_2 terminus. The SH1 domain carries the tyrosine kinase function, whereas the SH2 and SH3 domains allow for interaction with other proteins. Proline-rich sequences in the center of the molecule can, in turn, interact with SH3 domains of other proteins, such as Crk. Toward the end, nuclear localization signals and the DNA-binding and actin-binding motifs are found.

Several fairly diverse functions have been attributed to Abl, and the emerging picture is complex. Thus, the normal Abl protein is involved in the regulation of cell cycle, in the cellular response to genotoxic stress, and in the transmission of information about the cellular environment through integrin signaling.

Overall, it appears that the Abl protein serves a complex role as a cellular module that integrates signals from various extracellular and intracellular sources and that influences decisions relative to cell cycle and apoptosis **(Deininger M.W.N. et al. 2000)**.

Mice with a homozygous disruption of the *abl* gene -- either through a null mutation or a deletion of the carboxy-terminal third of the protein -- are variably affected, but phenotypes include an increased incidence of perinatal mortality, lymphopaenia and osteoporosis. abl-null mice are also smaller, with abnormal head and eye development **(Ren R. 2005)**.

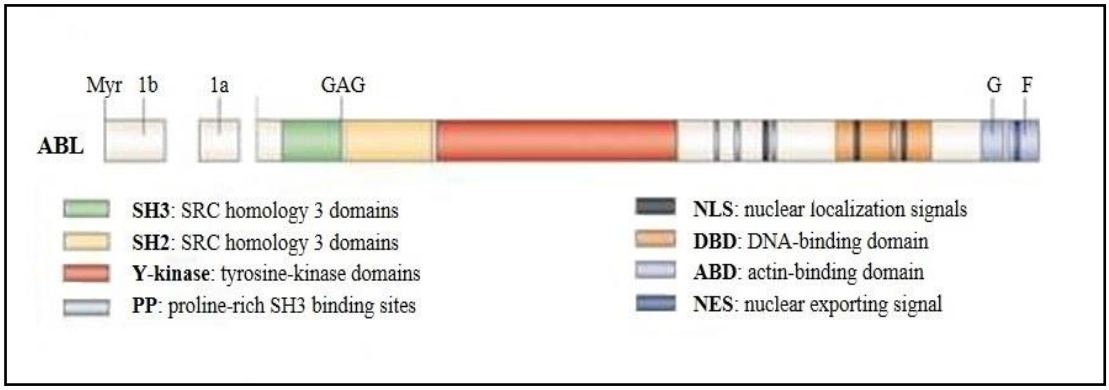


Figure VII. Structure of the Abl protein. Two isoforms of Abl (human types 1a and 1b) are generated by alternative splicing of the first exon, one of them (1b) contains a myristoylation modification site (Myr). Apart from the alternatively spliced sequences, the aminoterminal half of Abl contains tandem SRC homology 3 (SH3), SH2 and the tyrosine-kinase (Y-kinase) domains. These domains can assemble into an auto-inhibitory structure, in which the SH3 and SH2 domains function as a 'clamp' that holds the kinase in the 'off' state. In Abl1b, the myristoyl group at the extreme end of the amino-terminal segment also binds to the tyrosine-kinase domain and functions as a 'latch' that keeps the SH3–SH2 clamp in place. In its carboxy-terminal region, Abl contains four proline-rich SH3 binding sites (PPs), three nuclear localization signals (NLSs), one nuclear exporting signal (NES), a DNA-binding domain (DBD), and an actin-binding domain (ABD). This actin-binding domain contains binding sites for both monomeric (G) and filamentous (F) forms of actin. The points in Abl that fuses with BCR and GAG (for v-Abl) are indicated **(Ren R. 2005)**.

The *Breakpoint Cluster Region (BCR)* gene maps to a region of about 135 kb on chromosome 22 and contains 23 exons. The first intron separating exons 1 and 2 was initially shown to span a distance of 68 kb but is now known to include two additional exons (an alternative exon 1 and an alternative exon 2).

Two transcripts, 4.5- and 7.0-kb long, have been found, which apparently encode for the same, cytoplasmatic 160-kDa protein that, like Abl, is ubiquitously expressed (**Laurent E. et al. 2001**).

The 5' untranslated region of the *BCR* gene is important because the fusion gene created by the Ph translocation is placed under the regulation of the *BCR* promoter (**Muller A. et al. 1989**, **Zhu Q.S. et al. 1990**, **Shah et al. 1991**). A region of ~ 1-kb upstream of the transcription start site was demonstrated to be the principle site of promoter activity.

Within this region, a CAAT box (conserved sequence upstream of start point of transcription, which is recognized by transcription factors) at position 2644 as well as an inverted CAAT sequence at position 2718 have been localized. However, neither *in vitro* nor *in vivo* studies suggest that these sequences are key factors for transcriptional regulation of the *BCR* gene.

There is also a TATA box (conserved sequences in the promoter that specify the position at which transcription is initiated) 120 bp downstream of the CAAT box. This TATA box, TTTAA, is also accompanied by the consensus sequence TCATCG, required for 5' capping of the transcript.

DNA footprinting and gel retardation assays have also been used to discover potential sites for DNA/protein interaction, which might reflect transcription factor activity. Several sites were found including ones containing the consensus sequence GGGCCGG (as well as the inverted sequence) for the SP1 transcription factor. A unique sequence AGGGCCTCAGTTTCCCAAAAGGCA, for which no binding protein is known, was also detected. Deletion of that site (*versus* other potential binding sites) resulted in a significant decrease of promoter activity in BCR-abl-transfected cells (**Zhu Q.S. et al. 1990**, **Shah N.P. et al. 1991**).

The 5' untranslated region of the *BCR* gene is also very rich in GC content, which makes up 80% of the nucleotides within this stretch of the DNA (**Hariharan I.K. et al. 1987**, **Lifshitz B et al. 1988**). Within this GC-rich region is a segment 18 nucleotides long at sites 2376 to 2393, containing the sequence GCGGCGGCGGCGGCGGCG with its inverted repeat 363 bp down-stream. These sequences are apt to form secondary stem and loop structures, with a possible role in translational regulation (**Lifshitz B et al. 1988**), and are common to many housekeeping genes. Other possible start sites upstream of the normal AUG codon have been found; however, these have short open reading frames because there are stop codons downstream of these sites. It has been suggested that these short transcripts could also play a role in regulating protein translation (**Muller A. et al. 1989**).

At the protein level, several structural motifs can be delineated (**Figure VIII**). The first N-terminal exon encodes a serine-threonine kinase. The only substrates of this kinase identified so far are Bap-1, a member of the 14-3-3 family of proteins, and possibly BCR itself. A coiled-coil domain at the N-terminus of BCR allows dimer formation in vivo. The center of the molecule contains a region with *dbl*-like and pleckstrin-homology (PH) domains, that stimulate the exchange of guanidine triphosphate (GTP) for guanidine diphosphate (GDP) on Rho guanidine exchange factors, which in turn may activate transcription factors such as NF-kB. The C-

terminus has GTPase activity for Rac, a small GTPase of the Ras superfamily that regulates actin polymerization and the activity of an NADPH oxidase in phagocytic cells. In addition, BCR can be phosphorylated on several tyrosine residues, especially tyrosine 177, which binds Grb-2, an important adapter molecule involved in the activation of the Ras pathway **(Deininger M.W.N. et al. 2000)**.

BCR-deficient mice develop normally, although their neutrophils have been shown to produce excess levels of oxygen metabolites following their activation.

Depending on the precise breakpoints in the translocation and RNA splicing, different forms of BCR-Abl protein with different molecular weights (p185 BCR-Abl, p210 BCR-Abl and p230 BCR-Abl) can be generated in patients, associated with Acute Lymphoblastic Leukemia (ALL), Chronic Myeloid Leukemia (CML) and a milder form of CML, respectively **(Ren R. 2005)**.

Three breakpoint regions within the *BCR* genome are responsible for generating the predominant BCR-Abl fusion proteins. The minor breakpoint cluster (*m-bcr*) spans 54-kb and results in an e1a2 7.0 mRNA that generates $p190^{BCR\text{-}Abl}$. The major breakpoint cluster (*M-bcr*) spans 5.8 kb and results in either a b2a2 or b3a2 8.5 kb mRNA producing $p210^{BCR\text{-}Abl}$. A third breakpoint located at the 3' end of the gene (µ-*bcr*) generates a e19a2 9.0 kb mRNA forming $p230^{BCR\text{-}Abl}$ (**Wong S. et al. 2004**).

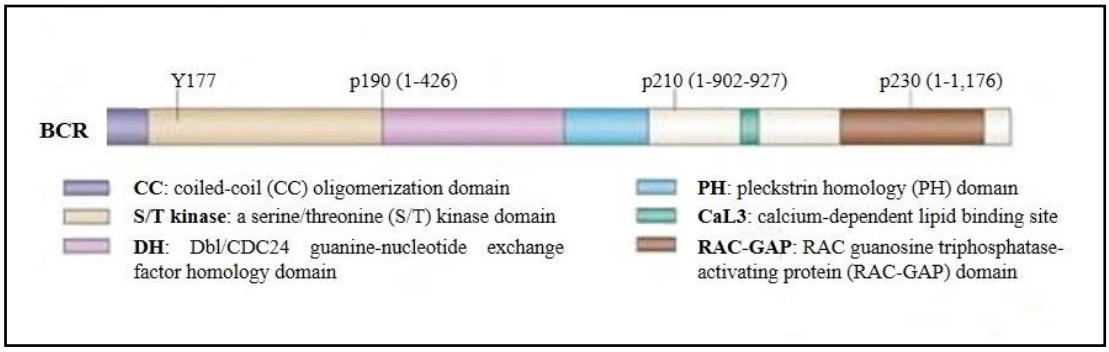


Figure VIII. Structure of the BCR protein. BCR contains a coiled-coil (CC) oligomerization domain, a serine/threonine (S/T) kinase domain, a Dbl/CDC24 guanine-nucleotide exchange factor homology (DH) domain and a pleckstrin homology (PH) domain, a putative calcium-dependent lipid binding site (CaLB) and a RAC guanosine triphosphatase-activating protein (RAC-GAP) domain. BCR also contains binding sites for growth factor receptor-bound protein 2 (GRB2) at tyrosine 177 (Y177), as well as for the GRB10, 14-3-3 and the Abl proteins, through its SH2 domain. p185, p210 and p230 indicate the points at which BCR most commonly fuses to Abl. The number of amino acids in each form are indicated in parentheses **(Ren R. 2005)**.

Interaction between BCR and BCR-Abl

In Ph+ cells, BCR-Abl phosphorylates BCR on tyrosine residues (Y177) reducing its kinase activity. It is know that the overexpression of BCR in Ph+ cells produces a phosphoserine form of BCR, which inhibits the oncogenic effects of BCR-Abl (**Wu Y. et al. 1999**). In fact, BCR becomes resistant to tyrosine phosphorylation in BCR-Abl cells when its expression is in molar excess. In this condition, the excess of BCR protein is mostly in the phosphoserine form and reduces the phosphotyrosine content of BCR-Abl, strongly inhibiting its oncogenic activity (**Wu Y. et al. 1999**, **Lin F. et al. 2001**). This phosphoserine form of BCR is also predominant after overexpression of BCR in soft agar clones of the CML K562, a Ph+ cell line, containing an inducible BCR gene. As a consequence, these clones have reduced ability to induce extramedullary leukemia (**Lin F. et al. 2001**).

On the basis of these results, it has been proposed that the BCR protein plays two roles in CML (**Figure IX**) (**Arlinghaus R.B. 2002**). In the tyrosine phosphorylated form, BCR would be neutralized as an inhibitor of BCR-Abl effects and would serve as an important facilitator of BCR-Abl-induced leukemia, possibly in the form of a heterotetramer structure with BCR-Abl (**McWhirter J.R. et al. 1993**). On the other hand, in the serine/threonine-phosphorylated form, BCR would function as an inhibitor of BCR-Abl oncogenic ability.

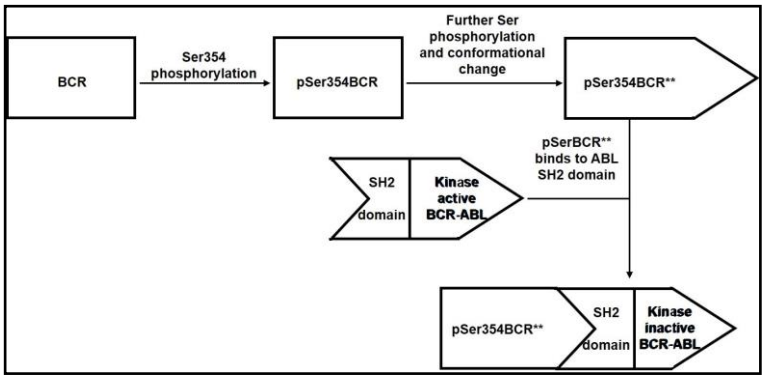


Figure IX. Model describing the interaction of phosphoserine BCR with the SH2 domain of BCR-Abl. The model illustrates a two-step serine phosphorylation process and subsequent conformational change of BCR involving serine 354. Following phosphorylation of serine 354, a unique structural form of BCR is produced by further serine phosphorylation. This form is identied as pSer BCR**. The pSer BCR** structure is proposed to bind to a site within the SH2 domain of the Abl sequence causing perturbation of the catalytic domain, which leads to reduced tyrosine kinase activity. In essence the data predict a two site model for the SH2 domain. One site binds phosphotyrosine, and a second site binds phosphoserine BCR (**Arlinghaus R.B. 2002**).

It has proposed that BCR may play a role in generating the myeloid phenotype caused by BCR-Abl in CML patients and may be an important player in the chronic phase of CML by down-modulating BCR-Abl. That BCR-Abl expression increases that of BCR protein was shown in TonB210 cells, originated from a clone of murine IL-3-dependent hematopoietic BaF3 cells, in which BCR-Abl expression is controlled by a tetracyclineinducible promoter and BCR is stably transduced by lentivirus infection (**Perazzona B. 2008**). After imatinib treatment of TonB210 cells, the levels of BCR-Abl and, surprisingly, of the BCR protein decreased, indicating that the tyrosine kinase function of BCR-Abl is required to up-regulate BCR protein expression. In addition, withdrawal of doxycycline also reduced BCR-Abl and BCR protein levels, confirming that BCR-Abl is required for the increased expression of the BCR protein. In BaF3 and 32D cells, BCR was expressed at extremely low levels, but the treatment with the proteasome inhibitor calpain inhibitor I restored BCR expression. Forced expression of BCR-Abl in BCR-transduced cells restored high expression of BCR protein, confirming that BCR-Abl is required for preventing degradation of the BCR protein.

Taken together these findings indicate that BCR-Abl upregulate BCR expression by interfering with proteasome-mediated degradation of the BCR protein (**Perazzona B. 2008**).

The BCR-Abl oncogenetic pathway

Abnormal interactions between the BCR-Abl oncoprotein and other cytoplasmic molecules lead to the disruption of key cellular processes. Examples include the perturbation of the Ras mitogen-activated protein kinase (MAPK) pathway leading to increased proliferation, the Janus-activated kinase (JAK)-STAT pathway leading to impaired transcriptional activity, and the phosphoinositide 3-kinase (PI3K)/AKT pathway resulting in increased apoptosis (**Figure X**) (**Cilloni D. et al. 2012**).

The phosphorylation of BCR Tyr177 is essential for BCR-Abl mediated leukemogenesis, and its mutation largely abolishes GRB2 binding and diminishes BCR-Abl induced Ras activation (**Zhang X. et al. 2001**). The latter results from the interaction of BCR-Abl with other cytoplasmic proteins, which function as adaptor

molecules, thus creating multiprotein signaling complexes. The BCR-Abl/GRB2 complex recruits Son of Sevenless (SOS), which is constitutively associated with the GRB2 SH3 domain. In turn, the BCR-ABL/GRB2/SOS complex stimulates conversion of the inactive GDP-bound form of Ras to its active GTP-bound state and the activation of the scaffold adapter GRB2-associated binding protein 2. As a consequence, the GRB2/GAB2/SOS complex causes constitutive activation of the Ras downstream pathway, thereby activating the MAPK ERK1/2, which results in abnormal cell proliferation. In addition, this complex activates the PI3K/AKT pathway **(Ren R. 2005)**, which promotes survival by suppressing the activity of the forkhead O (FOXO) transcription factor, and enhances cell proliferation by inducing p27 proteosomal degradation and mTOR activation. In addition, BCR-Abl, through PI3K/AKT/FOXO4 and finally through upregulation of mTOR, potently blocks important cellular processes, such as autophagy. BCR-Abl may activate PI3K by more than one pathway, because Crk and Crkl have also been shown to connect BCR-Abl with PI3K (**Cilloni D. et al. 2012**). Once activated, PI3K activates AKT kinase, which serves as a key downstream effector by exerting many cellular effects through the phosphorylation of downstream substrates that regulate the apoptotic machinery, as Bad, caspase 9, Mdm2, and Ask1, resulting in prolonged survival and expansion of the abnormal clone (**Franke T.F. et al. 1997**).

Key transcription factors are involved in BCR-Abl signaling. Among these a key role is played by STAT1 and STAT5 (Signal Transducer and Activation of Transcription), which are constantly active in BCR-Abl positive cell lines and in primary cells from CML patients, contributing to the induction of cytokine independence. In normal cells, nuclear translocation of STATs occurs exclusively after cytokine binding to receptors and is mediated by activation of the receptor-associated JAK kinases. By contrast, in CML, STATs seem to be activated in a JAK-independent manner through a direct association of STAT SH2 domains with phosphorylated tyrosines on BCR-Abl. Activation of STAT5 is at least in part responsible for protection from programmed cell death through the upregulation of the antiapoptotic molecule Bcl-xL together with the inactivation of the proapoptotic molecule BAD by AKT (**Cilloni D. et al. 2012**).

Another postulated nuclear target of the transforming activity of the BCR-Abl protein is the protooncogene Myc, which is expressed at a high level in CML cells. Myc activation seems to be independent of the RAS pathway but directly upregulated by the Abl SH2 region (**Cilloni D. et al. 2012**). Several lines of evidence indicate that Myc is often overexpressed in blast crisis compared with the chronic phase, thus linking Myc to progression. Inhibition of c-Myc *in vitro* with antisense oligonucleotides or dominant-negative constructs can inhibit BCR-Abl transformation or leukemogenesis (**Sawyers C.L. et al. 1992**).

Despite the seemingly endless expansion of the list of pathways that are activated by BCR-Abl, and the increasing complexity that is being revealed in these pathways, it seems that all of the transforming functions of BCR-Abl depend on its tyrosine kinase activity (**Lugo T.G. et al. 1990**). This precondition has an incredible intrinsic clinical potential with regard to the development of more sophisticated targeted therapies.

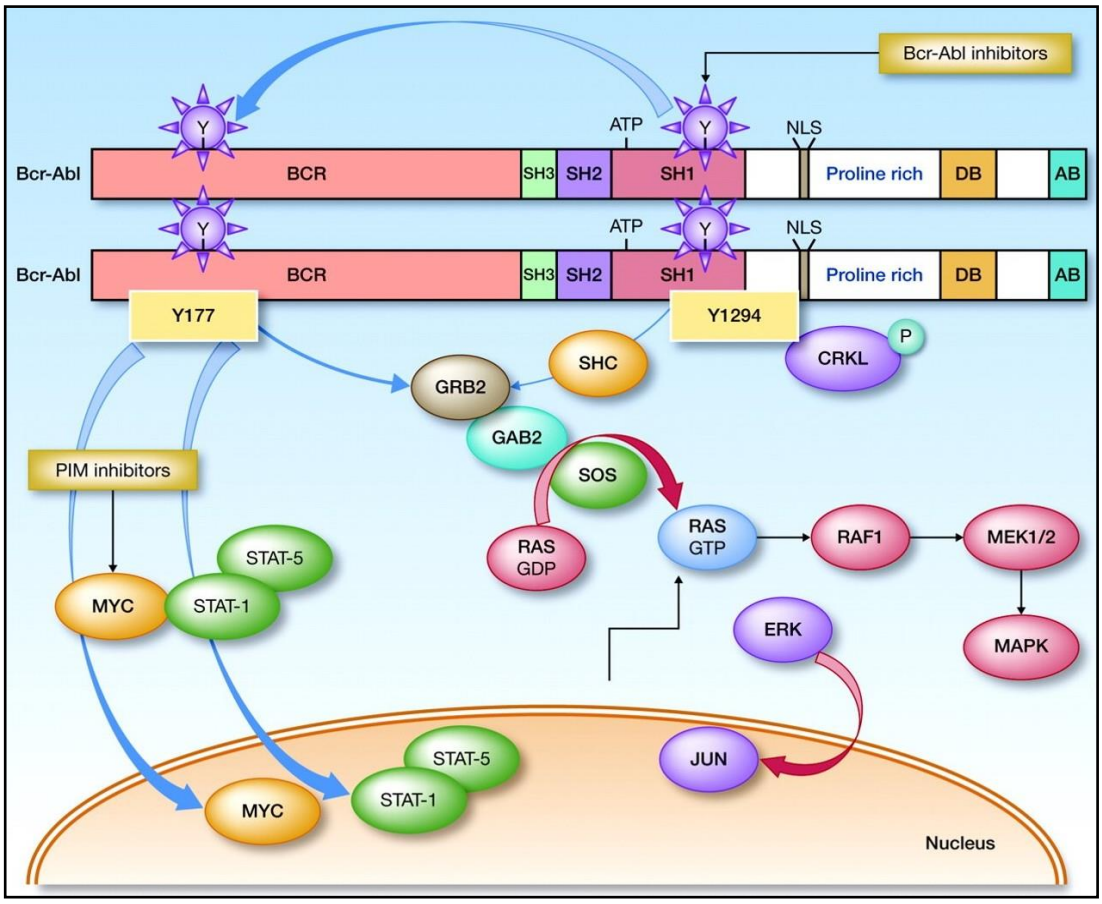


Figure X. Molecular pathways activated by BCR-Abl. The BCR-Abl proteins can form dimers or tetramers through their CC domains, and trans-autophosphorylate. Phosphorylation at the Y177 residue generates a high-affinity binding site for growth factor receptor-bound protein 2 (GRB2). GRB2 binds to BCR-Abl through its SH2 domain and binds to SOS and GRB2-associated binding protein 2 (GAB2) through its SH3 domains. SOS in turn activates Ras downstream pathway, thereby activating MEK1/2 and MAPK and resulting in abnormal cell proliferation. In addiction the complex activates the PI3K/AKT pathway (**Cilloni D. et al. 2012**).

3'UTR mediated gene regulation

Eukaryotic gene expression is controlled at the transcriptional and translational levels. After transcription, the messenger RNA (mRNA) is either translated into protein or degraded, depending upon its function in the cell. The stability of mRNA depends on different regions containing *cis*-acting elements and *trans*-regulating factors binding directly or indirectly to these *cis*-acting elements. The formation of mRNA-protein complexes followed by a series of re-modelling events, influence the translation and decay of mRNAs. These *cis*-regulatory elements may be located in the 5' untranslated region (UTR), the open reading frame (ORF) or the 3'UTR (**Figure XI**) (**Mignone F. et al. 2002**).

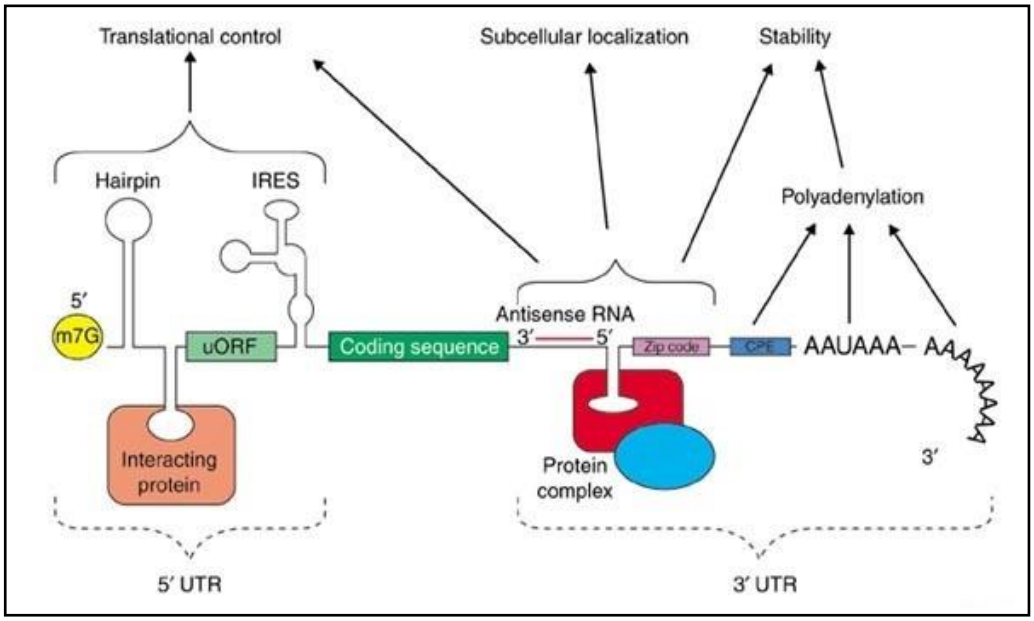


Figure XI. Structure of a eukaryotic mRNA. Some post-transcriptional regulatory elements that affect gene expression are illustrated. Abbreviations (from 5' to 3'): UTR, untranslated region; m7G, 7-methyl-guanosine cap; hairpin, hairpin-like secondary structures; uORF, upstream open reading frame; IRES, internal ribosome entry site; CPE, cytoplasmic polyadenylation element; AAUAAA, polyadenylation signal (**Mignone F. et al. 2002**).

Control at the 5' UTR level involves RNA secondary structure such as internal ribosome entry site, upstream ORF, 5'-terminal polypyrimidine-rich sequences, and the methylation state of the cap structure. In the ORF, a synthesized peptide may affect translation efficiency of its own mRNA, while the presence of rare codons may cause ribosome pausing during translation. Coding region determinants (CRDs) of mRNA instability can also be found in the ORF and target mRNAs for rapid degradation by endonucleolytic cleavage (*c-myc*) or rapid deadenylation and degradation (*c-fos*) (**Audic Y. et al. 2004**; **Hosoda N. et al. 2003**; **Lopez de Silanes I. et al. 2007**).

Although regulatory elements are found in all parts of the mRNA from a mechanistic point of view, the 3' UTR is a place of choice for such elements. Indeed, it is likely that the 3'UTR is not scanned by the ribosomes (**Poyry T.A. et al. 2004**). Therefore, any RNA/protein interaction taking place in this region persists through translation, enabling regulation to take place at any time. Indeed, many 3' UTR contain regulatory sequences that bind RBPs and sncRNA, resulting in the stabilization or destabilization of a given mRNA or its translational activation or repression (**Lopez de Silanes I. et al. 2007**).

It has been shown that alterations in the 3'UTR of many transiently-expressed genes leads to enhanced stability of their mRNAs, leading to gene overexpression and oncogenesis (**Mayr C. et al. 2007**), which points to the role of 3'UTR in gene expression.

There are several recognised motifs in 3'UTRs that could be crucial for the regulation of mRNAs: the iron response element (IRE), that stabilises transferrin receptor (TFR) mRNA when the intracellular iron concentration is low, resulting in iron uptake, and the most commonly found AU-rich element (ARE) (**Lopez de Silanes I. et al. 2007**). Moreover, in the last few years, there is mounting evidence that the 3'UTR of several mRNAs harbour binding sites for microRNA, a class of small noncoding RNAs that regulate gene expression.

AU-rich elements in the 3'UTR

Adenylate/uridylate rich elements or AREs are the highly conserved elements found in the 3'UTR of many mRNAs that code for proto-oncogenes, cytokines and nuclear transcription factors (**Chen C.Y. et al. 1995**) and function as elements that are important for mRNA stability. 10% of human mRNAs are reported to contain AREs in their 3'UTR.

The first direct evidence that the ARE can function as a potent mRNA destabilizing element came from a study by Shaw and Kamen in 1986. The stability of β-globin mRNA was reduced considerably by inserting a conserved region of 51 nucleotides containing AUUUA motifs from the 3'UTR of human granulocyte-macrophage colony-stimulating factor (GM-CSF) into its 3'UTR (**Shaw G. et al. 1986**). Later, the mRNA degradation of *c-fos*, *c-myc*, β-interferon and many more genes was also shown to be the effect of AREs in their respective 3'UTR (**Chen C.Y. et al. 1994**; **Chen C.Y. and Shyu A.B. 1994**; **Jones T.R. et al. 1987**; **Peppel K. et al. 1991**).

AREs are proposed to be of three types, class I and II AREs containing various repeats of AUUUA signature motifs and class III lacking this pentamer (**Xu N. et al. 1997**). There appears to be a sequence hierarchy by which the three classes of AREs are composed from several key sequence features. AREs classified as class I are found in early-response gene mRNAs that encode various transcription factors and mRNAs for some cytokines like IL-4, IL-6. They contain one to three copies of AUUUA motifs coupled with nearby U-rich sequences. Each of these sequence features plays a distinct role and together they determine the destabilizing potency of the class I AREs (**Chen C.Y. et al. 1994**). Class II AREs possess multiple repeats of the AUUUA pentamer clustered together and the class III AREs contain only a couple of U stretches and a U-rich domain. The class II AREs direct asynchronous cytoplasmic deadenylation which is consistent with a processive ribonucleolytic digestion of polyA tails whereas class I and AUUUA less class III AREs mediate synchronous polyA shortening followed by the decay of the mRNA. Existence of cross-talk between the AREs and the 3' end polyA tail dictates the kinetics of cytoplasmic deadenylation and thereby the fate of the corresponding mRNA (**Xu N.**

et al. 1997). The length of AREs, especially the number of overlapping pentamers may also contribute to mRNA half-life (**Akashi M. et al. 1994**).

Regulation of ARE-gene expression: RNA binding proteins

The mechanisms through which AU-rich RNA binding proteins (AUBP) mediate post-transcriptional regulation, particularly in the context of immune regulation and the inflammatory response, have been reviewed extensively (**Stumpo D.J. et al. 2010**, **Gruber A.R. et al. 2011**, **Baou M. et al. 2009**, **Khabar K.S. 2009**, **Anderson P 2010**, **von Roretz et al. 2008**). Most AUBP function as accessory proteins to recruit mRNAs and to regulate their fate in various sub-cellular compartments, such as the exosome mediating 3'-5' decay, processing bodies for 5'-3' decay and stress granules for translational arrest. Some AUBP function cooperatively or antagonistically with each other or with argonaut (AGO) endoribonucleases within RNA-induced silencing complexes (RISC) mediating miRNA-dependent decay (**Anderson P. 2010**, **von Roretz C. et al. 2008**).

Three distinct types of AUBP (ARE poly-U-binding degradation factor-1/AUF1, Hu antigen/HuR/HuA/ELAVL1 and the tristetraprolin/ZFP36 family of proteins) are essential for normal hematopoiesis. Together with two further AU-rich RNA binding proteins, nucleolin and KHSRP/KSRP, the functions of these proteins are intimately associated with pathways that are dysregulated in various hematopoietic malignancies (**Baou M. et al. 2011**).

Among these, HuR play a wide role in leukemogenesis. Indeed, it is over-expressed in M4 Acute Myeloid Leukemia (AML) and correlated with high levels of elF4E (**Topisirovic I. et al. 2009**). It is also overexpressed in acute phase and blast crisis in CML as compared to chronic phase disease, with its expression increasing progressively during transit from chronic phase to blast crisis (**Radich J.P. et al. 2006**). In B-cell Chronic Lymphocytic Leukemia (BCLL), HuR mRNA is differentially expressed between cases with high and low levels of miR-16/miR-15 (**Calin G.A. et al. 2008**) consistent with its mRNA being targeted by miR-16 (**Xu F. et al. 2010**). Thus more emphasis on this protein is given throughout this work.

Results

Hypoxia reduces CML cell population expansion

The K562 and KCL22 stabilized cell lines were used, the first one isolated from BM and the others from pleural effusion of patients with CML in blast crisis. Thereby, both cell lines exhibit a highly undifferentiated, lymphoblast-like, phenotype.

Figure 1 shows the kinetics of the total number of viable cells in K562 and KCL22 cultures incubated for 7 days in severe hypoxia (0.1% O_2). Cell number increased over the first 2 or 3 days of incubation, respectively, in K562 and KCL22 cell cultures, and then decreased to reach, at day 7, about one tenth for K562 cells and one third for KCL22 cells with respect to the time-zero value. Thus, KCL22 cells resulted less sensitive to the effect of incubation in hypoxia.

These results led us to hypothesize that the different behavior of the two cell lines, although both derived from patients in blast crisis, may reflect differences in the mechanisms of regulation of BCR-Abl.

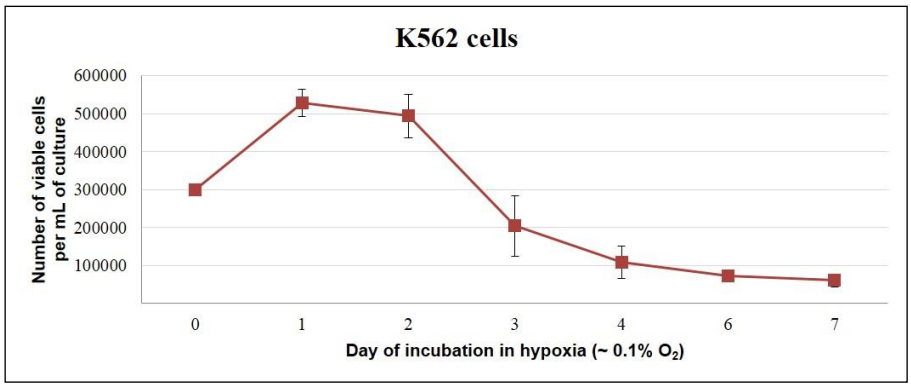
Hypoxia suppresses BCR-Abl protein expression

The expression levels of BCR-Abl protein and its phosphorylated form were determined by Western blotting in K562 and KCL22 cells during incubation in hypoxia.

Figure 2 shows that BCR-Abl protein remained more or less unchanged over the first 2 o 3 days of incubation, respectively, in K562 or KCL22 cells, while prolonged hypoxia led to the complete suppression of protein in both cell lines.

The complete removal of BCR-Abl protein in hypoxia suggests that its expression may be detrimental to the cell viability and growth in hypoxia.

A



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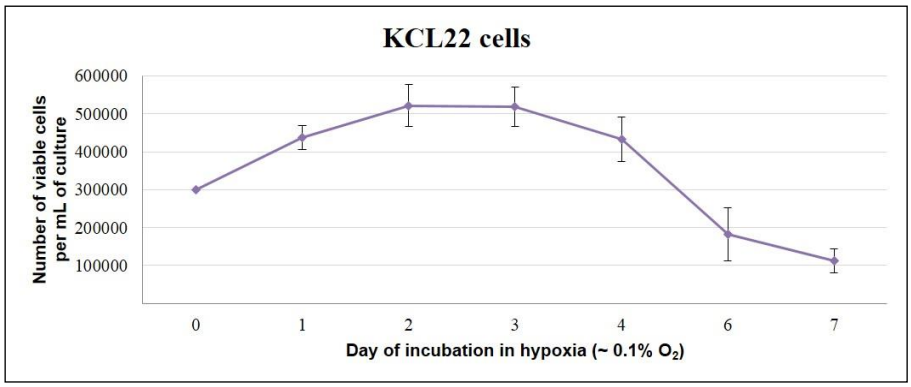

Figure 1. Effects of severe hypoxia on CML cell number and viability. Exponentially-growing K562 (A) and KCL22 (B) cells were plated at $3x10^5$/mL and incubated in hypoxia (0.1% O_2) until day 7; viable cells were counted by trypan blue exclusion at the indicated times. Data represent the Mean ± SD of four indipendent experiments.

Hypoxia reduces BCR-abl mRNA

To verify whether BCR-Abl protein suppression was associated with a reduction of BCR-abl mRNA, its levels were monitored daily during the incubation of K562 and KCL22 cells in hypoxia (**Figure 3**). Like in the case of BCR-Abl protein, BCR-abl mRNA was reduced in both cell lines. This reduction was more marked in K562 cells, where already after 24h of hypoxia mRNA levels were close to 50% of the time-zero value, to continue to decrease significantly during following incubation. In KCL22 cells, the reduction was of about 40% after 24h of hypoxia and this value was maintained more or less unchanged for the rest of time-course.

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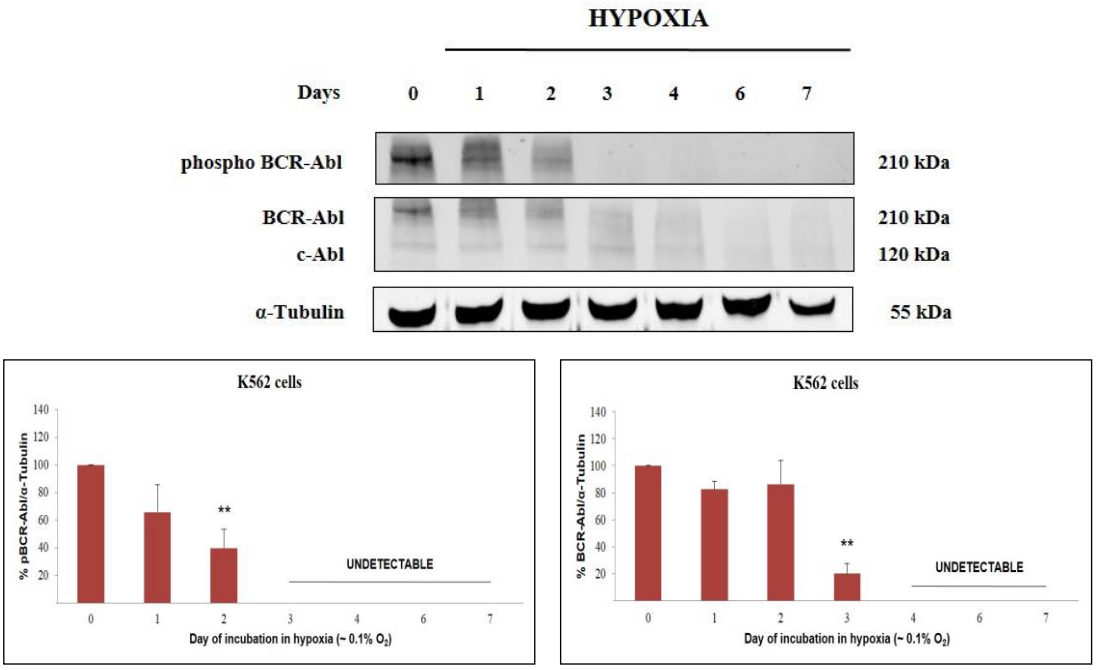

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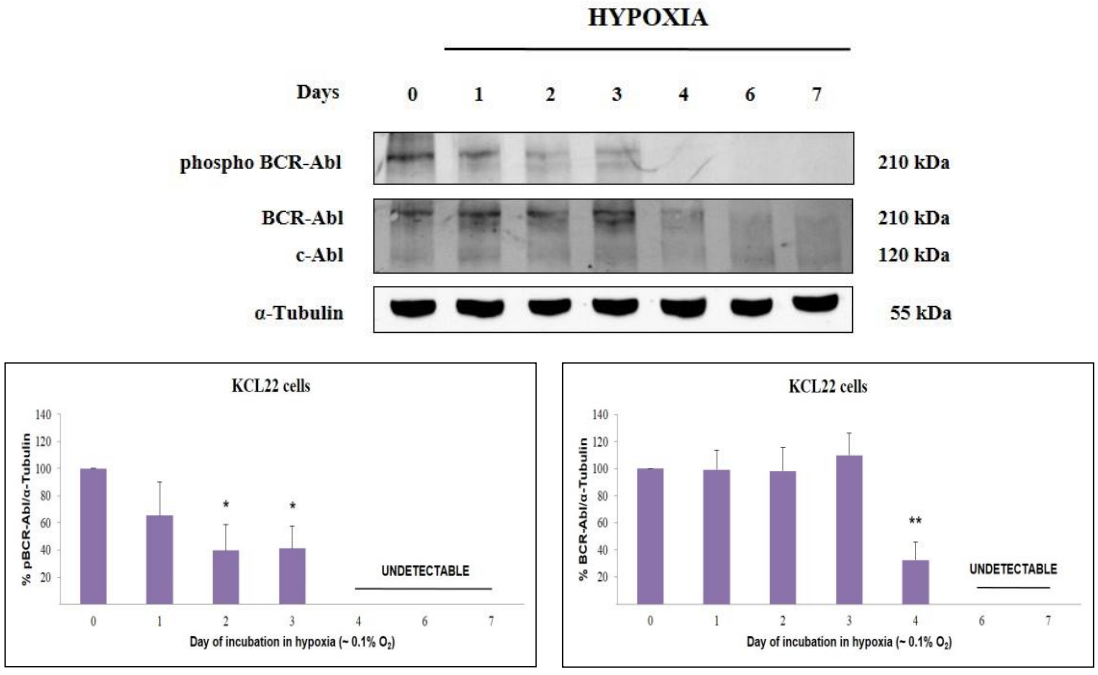

Figure 2. Effects of hypoxia on BCR-Abl expression and phosphorylation. Exponentially-growing K562 (**A**) and KCL22 (**B**) cells were incubated in hypoxia for the indicated times. Cell lysates in Laemmli buffer were subjected to SDS-PAGE and blotted with antibodies to BCR-Abl and its phosphorylated form pBCR-Abl, and with anti-α-Tubulin as loading control. The histograms represent the data obtained from the densitometry of bands in blots of three indipendent experiments (Mean + SD) and are expressed as the ratio of phosphoBCR-Abl (*left*) or BCR-Abl to α-Tubulin (*right*). Statistical analysis was performed by paired Student's t-test. *$**p \leq 0.01$ vs T0*, *$*p \leq 0.05$ vs T0*

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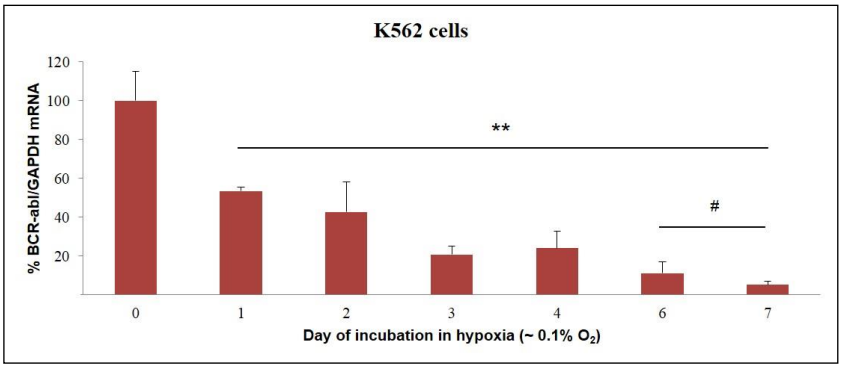

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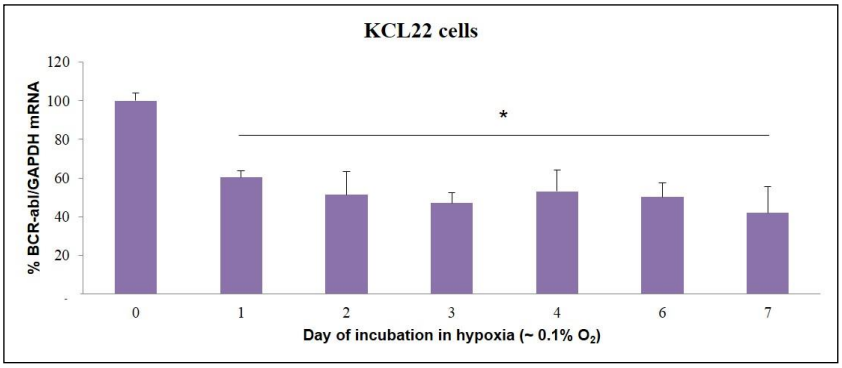

Figure 3. Effects of hypoxia on BCR-abl mRNA. Exponentially-growing K562 (**A**) and KCL22 (**B**) cells were incubated in hypoxia for the indicated times. Total RNA was isolated using TRIzol and retro-transcribed. BCR-abl mRNA levels were normalized with respect to GAPDH mRNA or to 18S rRNA (*data not shown*) and determined by quantitative Real Time-PCR. Data represent Mean + SD of three indipendent experiments: ***p ≤ 0.01 vs T0*, **p ≤ 0.05 vs T0*, *# p ≤ 0.01 vs day1, day2,* and *day4*

Different effects of hypoxia on BCR-abl mRNA stability

To investigate on whether the decrease of BCR-abl mRNA in hypoxia (**Figure 3**) could be attributed to reduced transcriptional activity or to regulation of mRNA stability, we incubated K562 and KCL22 cells under normoxia or for 24 hours in hypoxia before blocking transcription by adding of actinomycin D (5 μg/mL) for the indicated times.

K562 cells exhibited comparable BCR-abl mRNA stability ($t_{1/2}$ = 3.8 hours, R^2 = 0.7971) in normoxia and in hypoxia. In contrast, in KCL22 cells, the half-life of BCR-abl mRNA was significantly reduced in hypoxia compared to normoxia to 4.5 hours, R^2 = 0.805, vs 6.3 hours, R^2 = 0.9665, respectively (**Figure 4**).

These results led us to hypothesize that BCR-abl mRNA levels are subjected to transcriptional control in K562 cells, to post-transcriptional control in KCL22 cells (although we cannot exclude also the involvment of a transcriptional control).

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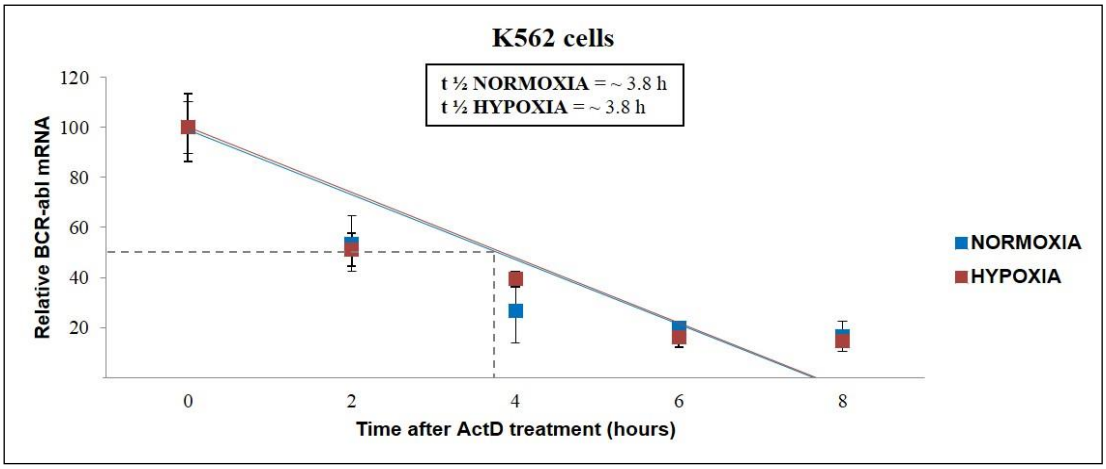

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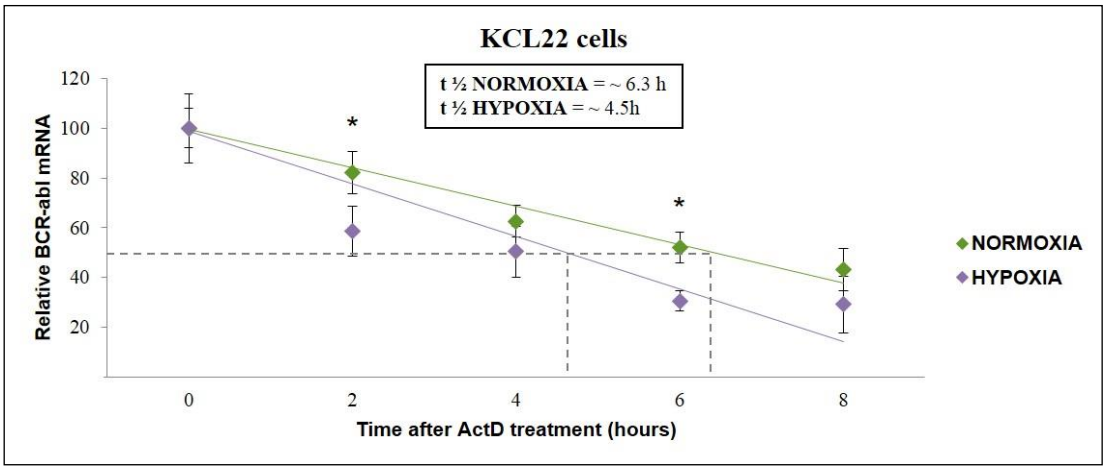

Figure 4. BCR-abl mRNA stability after the treatment with the transcription inhibitor Actinomycin D. K562 (**A**) and KCL22 (**B**) cells were exposed to normoxia or for 24 h to hypoxia and then transcription was blocked by the addition of actinomycin D (5 µg/mL) for the indicated times. BCR-abl and GAPDH mRNA were determined by quantitative Real Time-PCR. The ratios of BCR-abl to GAPDH mRNA at time-zero in normoxia or after 24 hours of hypoxia were set to 100. The transcript levels are reported as percentages of total transcript present at time-zero. The half-life of the BCR-abl mRNA was calculated by regression analyses. Data represent Mean ± SD of three indipendent experiments: **p ≤ 0.05 HYPOXIA vs NORMOXIA*

BCR promoter activity

To assess the potential involvement of the transcriptional machinery in the regulation of BCR-abl mRNA levels in hypoxia, we transiently transfected K562 cells with a luciferase reporter vector carrying the BCR promoter (**Marega M. et al. 2010**), involved in the activation of both BCR and BCR-abl transcription and we performed a gene report assay to compare hypoxic and normoxic cultures (**Figure 5**).

Differently from what expected from the clear and progressive suppression of the BCR-Abl protein, we did not observe a significant decrease of the transcriptional activity of BCR promoter in hypoxia.

These data indicated that probably BCR-abl transcription in CML cells subjected to prolonged hypoxia is not regulated at the promoter level, but rather by other mechanisms (for instance, by transcriptional repressors) acting on signal sequences that are located far from the BCR promoter.

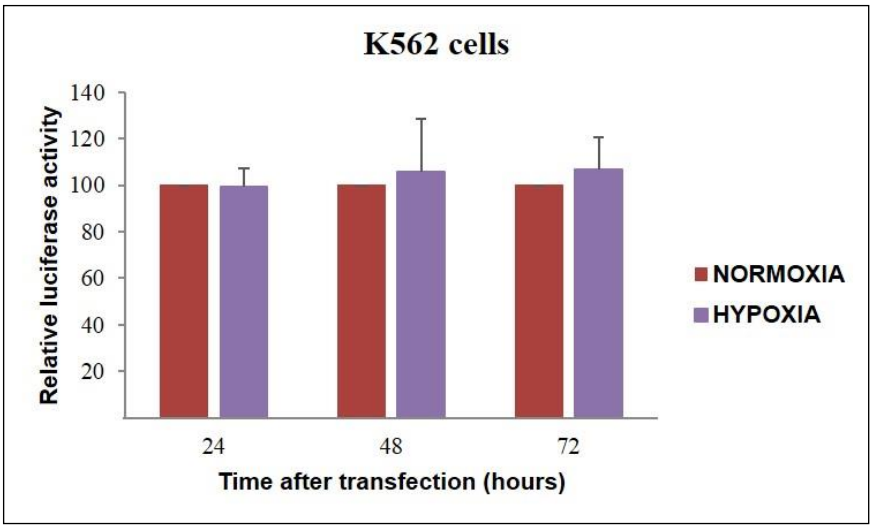

Figure 5. BCR promoter activity. A Firefly luciferase reporter construct containing either the BCR promoter or the pGL3 basic empty vector was transiently transfected into K562 cells. Cells were incubated in normoxia or hypoxia and luciferase activity was measured after 24, 48, and 72h from transfection. The ratio of pGL3-luc/BCR to pGL3-luc/empty luciferase activity under normoxia was set as 100 for each time point. Data represent Mean + SD of three indipendent experiments.

Glucose availability influences CML cell survival and downregulates BCR-Abl protein

To test whether hypoxia *per se* is capable of driving the effects shown above or additional environmental/metabolic factors are required, such as glucose availability, K562 and KCL22 cells were incubated in normoxia or hypoxia and in the absence or the presence of glucose for 14 days.

As shown in **Figure 6**, cells maintained in hypoxia without glucose underwent a sharp increase of mortality, while cells grown in hypoxia with glucose, behaved as expected and described above. In cells incubated in normoxia without glucose, compared to cells maintained under standard conditions (normoxia with glucose), it is worth noting that under mild restriction of energy supply (normoxia without glucose or hypoxia with glucose) the time-course is identical until day 2 (K562 cells) or day 3 (KCL22 cells), the days of incubation which precede BCR-Abl protein suppression (see **Figure 2A** and **B**). Beyond these incubation times, the number of viable cells decreases more rapidly in hypoxia w/ glucose than in normoxia w/o glucose, likely due to the Pasteur effect.

To deepen the above issue, trying to understand whether there is a correlation between cell survival in the absence of glucose and the expression of the BCR-Abl protein, we determined this expression by Western blotting in K562 and KCL22 cells incubated in normoxia w/o glucose (**Figure 7**). As can be seen, cells cultured under normoxia in the absence of glucose, as well as cells maintained in hypoxia with glucose, showed that the time of BCR-Abl protein down-regulation coincides with that at which cell survival also starts to decrease.

These results led to conclude that survival of bulk of leukemia cell population depends on the expression of BCR-Abl.

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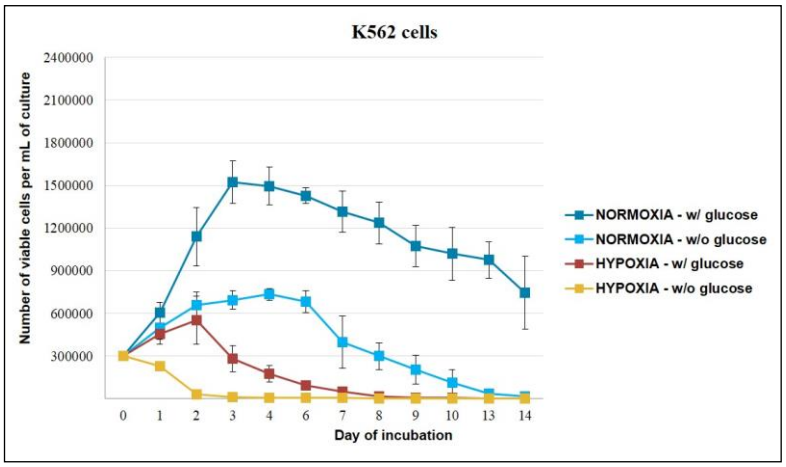

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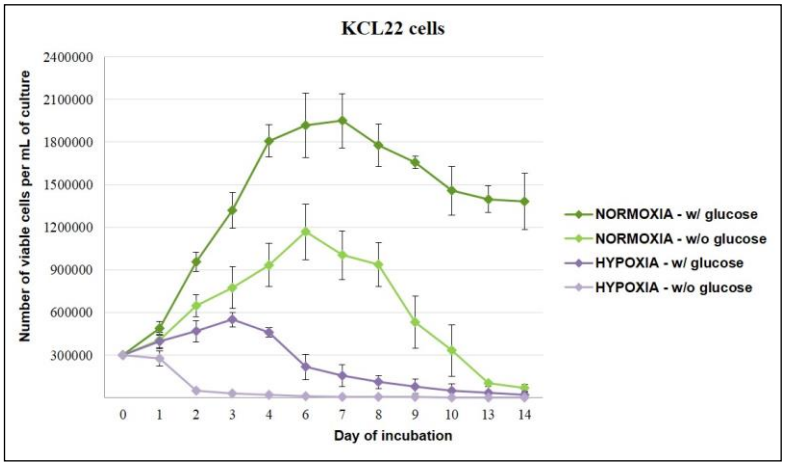

Figure 6. Glucose starvation influences cell survival. Exponentially-growing K562 (**A**) and KCL22 (**B**) cells were incubated in normoxia w/ or w/o glucose and in hypoxia w/ or w/o glucose and, at the indicated times, viable cells were counted by trypan blue exclusion. Data represent Mean ± SD of four indipendent experiments.

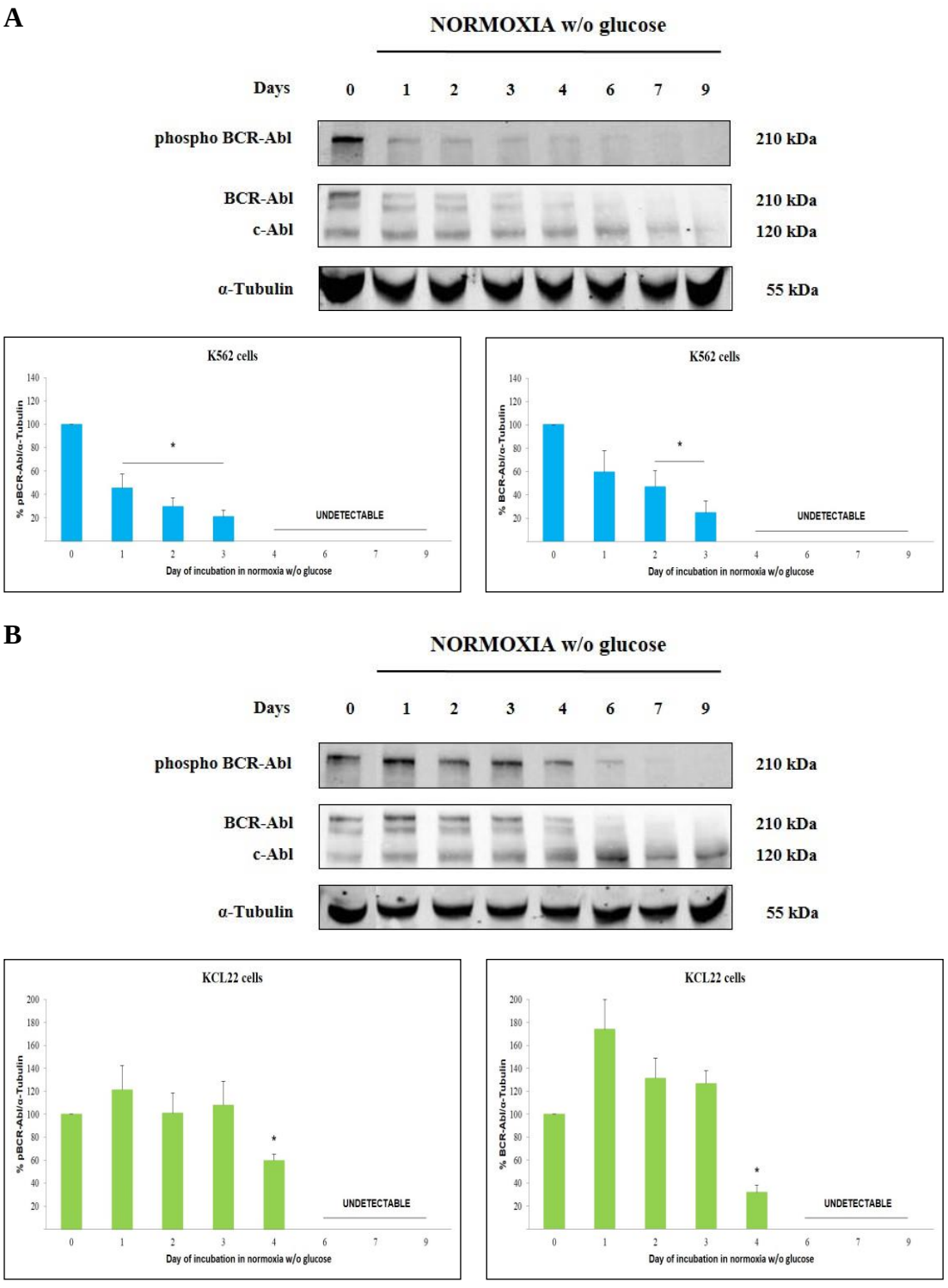

Figure 7. Glucose starvation inhibits BCR-Abl protein activity by inducing its down-regulation. Exponentially-growing K562 (**A**) and KCL22 (**B**) cells ($3x10^5$ cells/mL each) were incubated under normoxia w/o glucose for 9 days. Each day cells were harvested, and the expression of BCR-Abl and pBCR-Abl were determined by Western blot. α-Tubulin was blotted as a protein-loading control. The histograms represent data obtained by densitometry from three indipendent experiments (Mean + SD) and are expressed as the ratio between phosphoBCR-Abl (*left*) or BCR-Abl (*right*) to α-Tubulin. Statistical analysis was performed by paired Student's t-test. *$p \leq 0.05$ *vs T0*

Glucose deprivation does not affect BCR-abl mRNA levels

To verify whether, under glucose starvation, suppression of BCR-Abl protein was associated with that of BCR-abl mRNA, mRNA levels were determined during incubation in normoxia w/o glucose in K562 and KCL22 cells (**Figure 8**).

BCR-abl mRNA levels were unchanged across incubation in both cell lines, leading us to hypothesize a possible post-transcriptional control, translational and/or post-translational for cells incubated in normoxia, despite the energy shortage.

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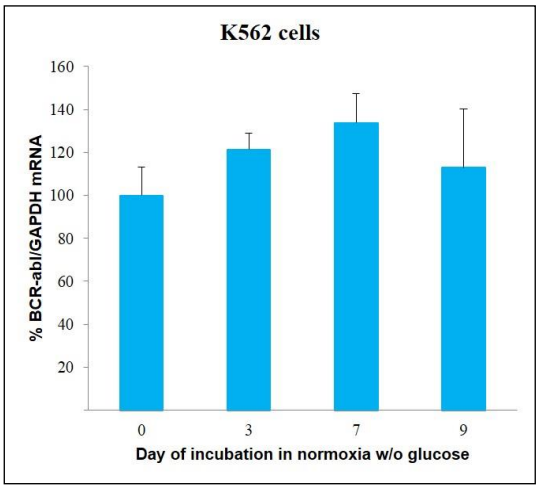

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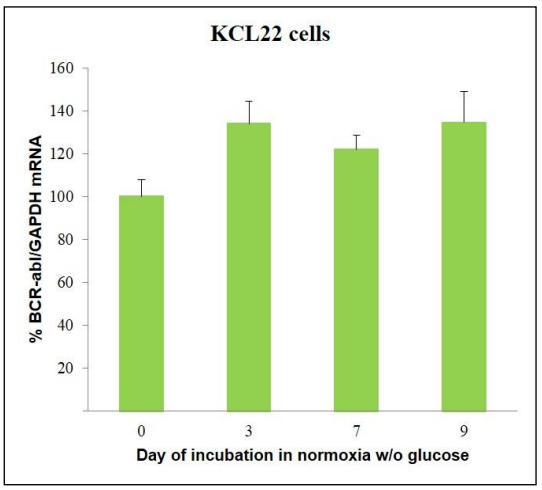

Figure 8. Effects of glucose starvation on BCR-abl mRNA. Exponentially-growing K562 (**A**) and KCL22 (**B**) cells were incubated in normoxia w/o glucose for the indicated times. Total RNA was isolated using TRIzol and reverse transcribed. BCR-abl mRNA levels were normalized to GAPDH mRNA or to 18S rRNA (*data not shown*) as determined by quantitative Real Time-PCR. Data represent Mean + SD of three indipendent experiments.

RNA-binding proteins involvment in BCR-Abl post-transcriptional control

Recent data demonstrate the involvement of different classes of RNA-binding protein in normal hematopoiesis and leukemogenesis (**Baou M. et al. 2011**) In particular, the RNA binding protein HuR appears to be overexpressed in blast crisis Chronic Myeloid Leukemia (CML) compared with chronic-phase disease with the level of expression increasing progressively during transit from the chronic phase to the blast crisis (**Radich J.P. et al. 2006**). HuR is known to play an important role in both the stabilization of many mRNAs both in the modulation of translational efficiency of different mRNAs.

We determined the HuR protein levels in K562 and KCL22 cells subjected to 7 days of hypoxia with glucose or 9 days of normoxia under glucose starvation, to evaluate whether HuR could be involved in BCR-Abl translational control (**Figure 9**).

HuR protein levels remained unchanged both in hypoxia and under glucose starvation. So, probably, HuR is not responsible for the decrease of BCR-Abl protein in these conditions.

Energy shortage does not affect BCR-Abl protein half-life

As reported by **Bartholomeusz G.A. et al. 2007**, BCR-Abl protein half-life in CML cells is approximately 40 hours. We wanted to determine whether hypoxia or glucose starvation could interfere with protein decay. K562 and KCL22 cells were incubated in normoxia or for 24 hours in normoxia without glucose or hypoxia with glucose before blocking translation by the addition of cycloheximide (50 μM) for the indicated times (**Figure 10**).

No significant differences in the BCR-Abl protein half-life were observed between mild energy shortage conditions (hypoxia w/ glucose or normoxia w/o glucose) and standard cultures conditions (normoxia w/ glucose).

These results strenghten the hypothesis of the involvment of translational control mechanisms in the BCR-Abl protein expression control.

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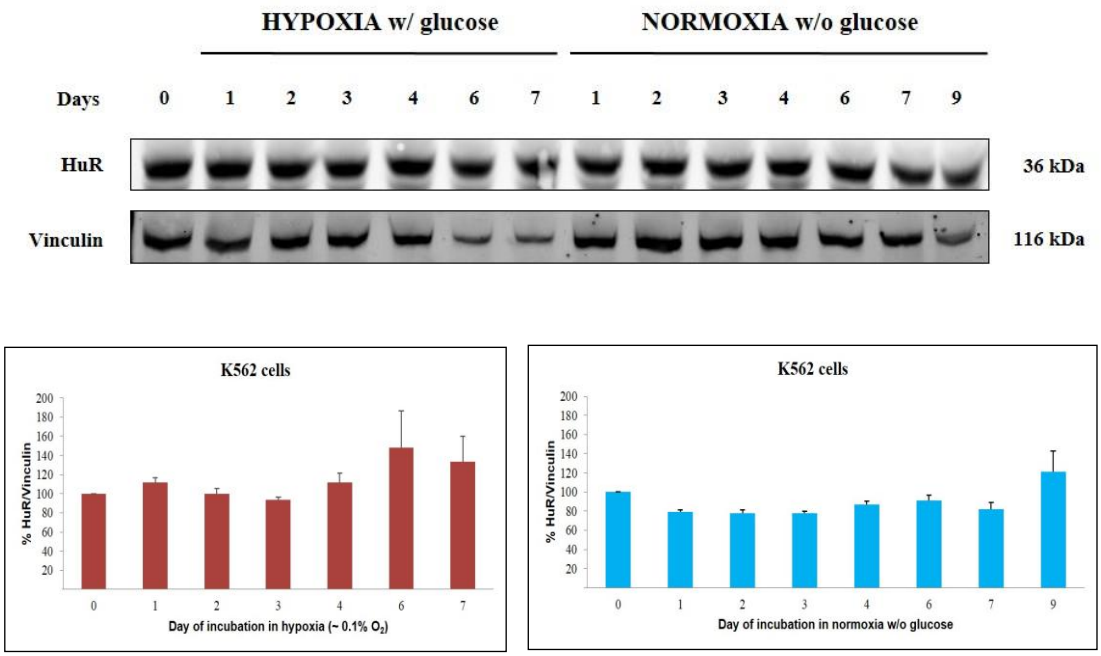

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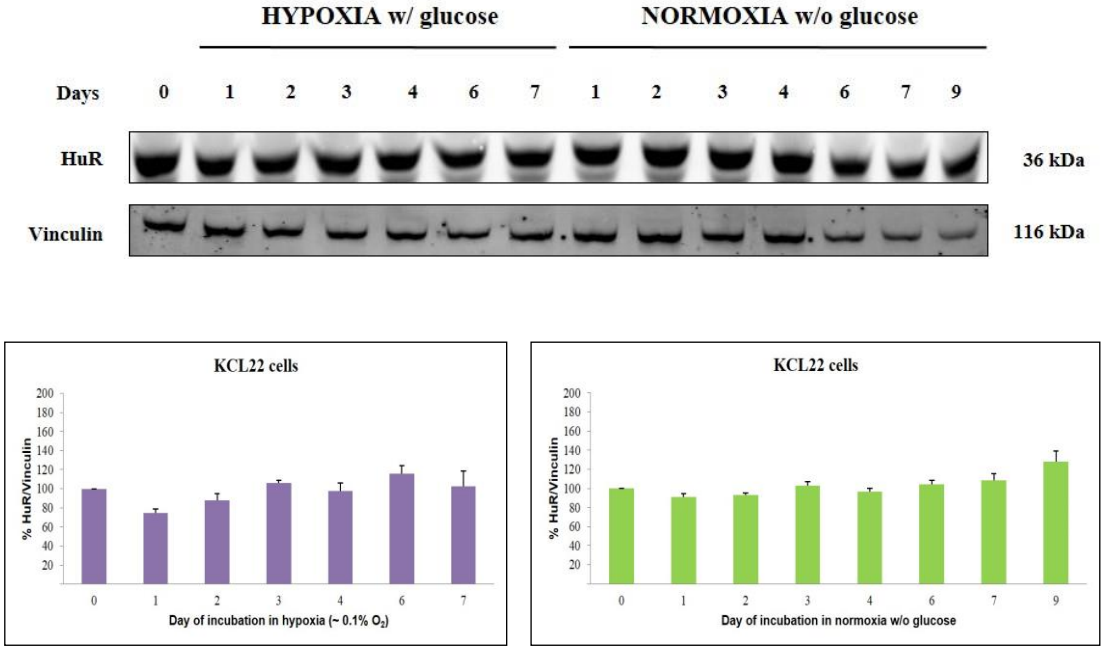

Figure 9. Levels of HuR protein under oxygen or glucose deprivation. Exponentially-growing K562 (**A**) and KCL22 (**B**) cells were incubated for the indicated times in hypoxia w/ glucose (*left*) or in normoxia w/o glucose (*right*). Cell lysates in Laemmli buffer were subjected to SDS-PAGE and blotting with antibodies raised against HuR, or Vinculin as loading control. The histograms represent densitometry data and are expressed as the ratio between HuR and Vinculin in three indipendent experiments (Mean + SD). Statistical analysis was performed by paired Student's t-test.

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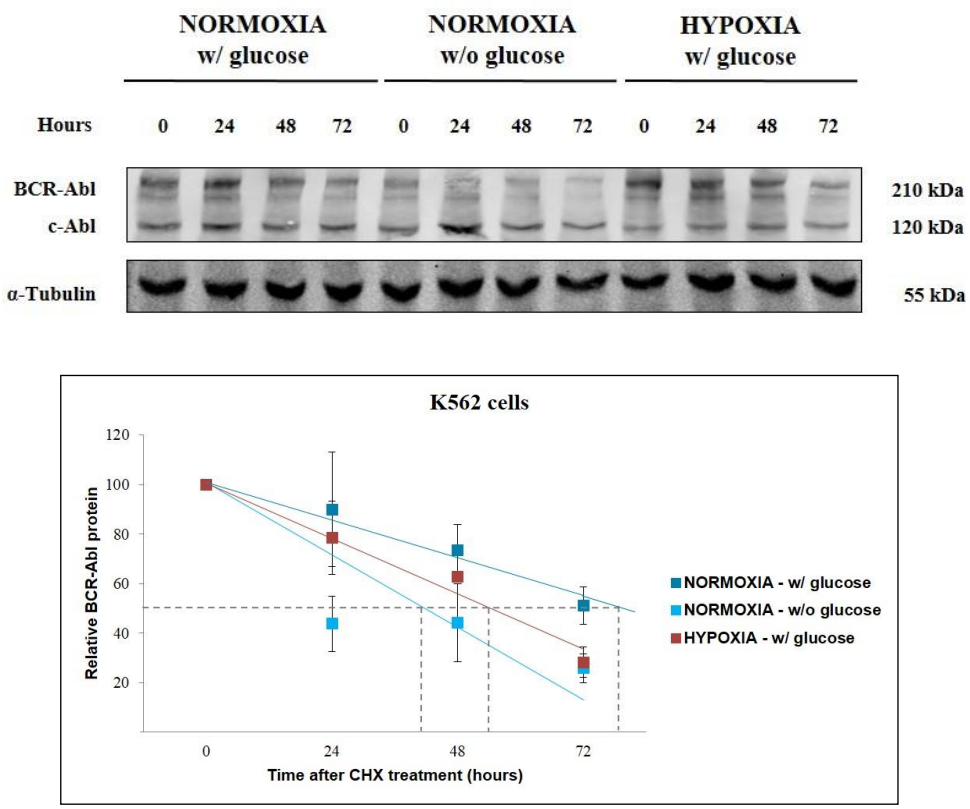
NORMOXIA
w/ glucose
NORMOXIA
w/o glucose
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Hours
0 24 48 72 0 24 48 72 0 24 48 72
BCR-Abl
c-Abl
210 kDa
120 kDa
α-Tubulin
55 kDa
K562 cells
Relative BCR-Abl protein
120
100
80
60
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24
48
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Time after CHX treatment (hours)
NORMOXIA - w/ glucose
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HYPOXIA - w/ glucose

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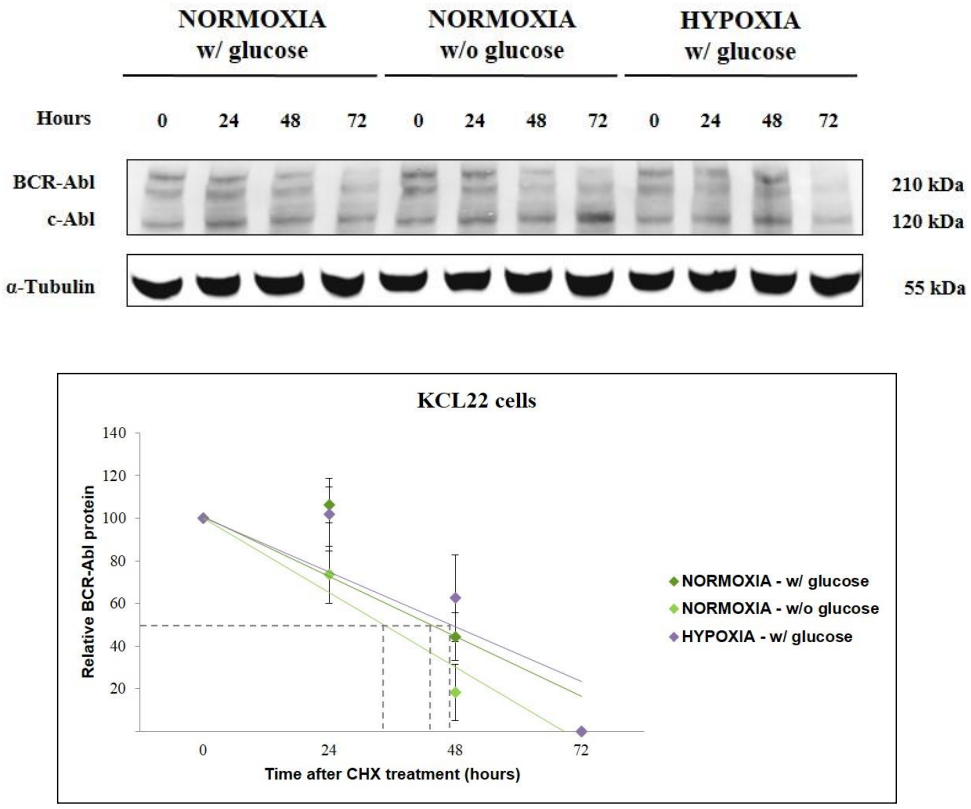
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α-Tubulin
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KCL22 cells
Relative BCR-Abl protein
140
120
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72
Time after CHX treatment (hours)
NORMOXIA - w/ glucose
NORMOXIA - w/o glucose
HYPOXIA - w/ glucose

Figure 10. Effects of hypoxia and glucose starvation on BCR-Abl protein half-life. K562 (**A**) and KCL22 (**B**) cells were exposed to normoxia or for 24 h to hypoxia w/ glucose or normoxia w/o glucose. Subsequently, translation was blocked by addition of cycloheximide (50 μM) for the times indicated. BCR-Abl protein levels were determined by Western blotting. The ratios of BCR-Abl to α-Tubulin at T0 in normoxia or after 24 hours of hypoxia w/ glucose or normoxia w/o glucose were set to 100. The protein levels are reported as percentages of total protein present at time-zero. The slope of the decay line was calculated by standard linear regression, and the BCR-Abl protein half-life was determined accordingly. For K562 cells BCR-Abl $t_{1/2}$ = 78 hours, R^2 = 0.9678 (NX - w/ glucose), $t_{1/2}$ = 54 hours, R^2 = 0.9727 (HX - w/ glucose), $t_{1/2}$ = 40 hours, R^2 = 0.6984 (NX - w/o glucose); for KCL22 cells, BCR-Abl $t_{1/2}$ = 44 hours, R^2 = 0.8152 (NX - w/ glucose), $t_{1/2}$ = 48 hours, R^2 = 0.7859 (HX - w/ glucose), $t_{1/2}$ = 34 hours, R^2 = 0.964 (NX - w/o glucose). Data represent Mean ± SD of three indipendent experiments.

Effects of hypoxia and glucose starvation on the maintenance of Leukemic Stem Cells

To identify the CSCs, we developed in our laboratory the Culture Repopulating Ability (CRA) assay. As mentioned before, this method estimates the culture-repopulating power of normal or leukemic cells undergoing a selection treatment in a primary liquid culture (LC1) by means of their wash and transfer in fresh medium to non-selective conditions in a secondary culture (LC2).

Stabilized leukemia cell lines, ensuring genetically homogeneous cells and enhancing repeatability of results, were found nevertheless phenotypically heterogeneous, comprising cell subsets exhibiting functional phenotypes of stem or progenitor cells. These subsets can be assayed separately, provided an experimental system capable to select one from another. On this basis, K562 and KCL22 cells were incubated in hypoxia for 7-10 days or in normoxia without glucose for 10-14 days (selective LC1) depending on the cell line, and after transferred to a normoxic non-selective LC2 (with glucose) culture for the indicated times.

As can be observed in **Figure 11**, both K562 cells that KCL22 showed a similar peak of LC2 repopulation to that we have seen previously (**Tanturli M. et al. 2011**) for Leukemic Stem Cells (LSCs) phenotype (at about 21-24 days of LC2).

These results confirm the hypothesis that is therefore energy shortage (hypoxia as well as glucose deprivation) the driving force to ‘select’ and allow the mainteinance of LSCs.

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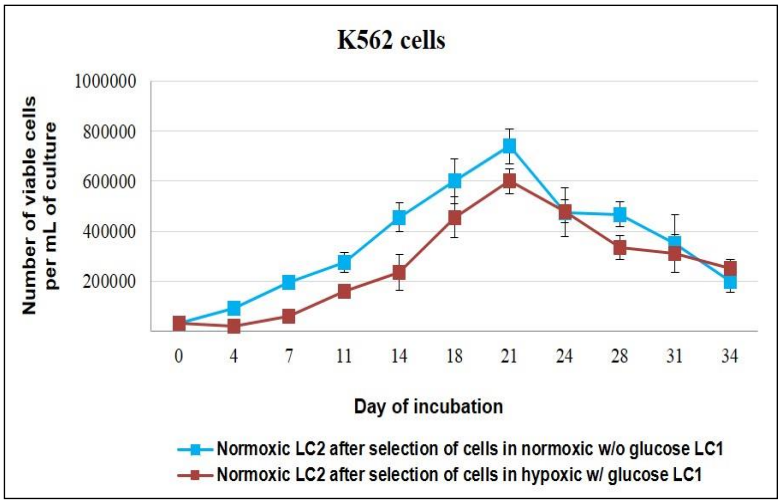

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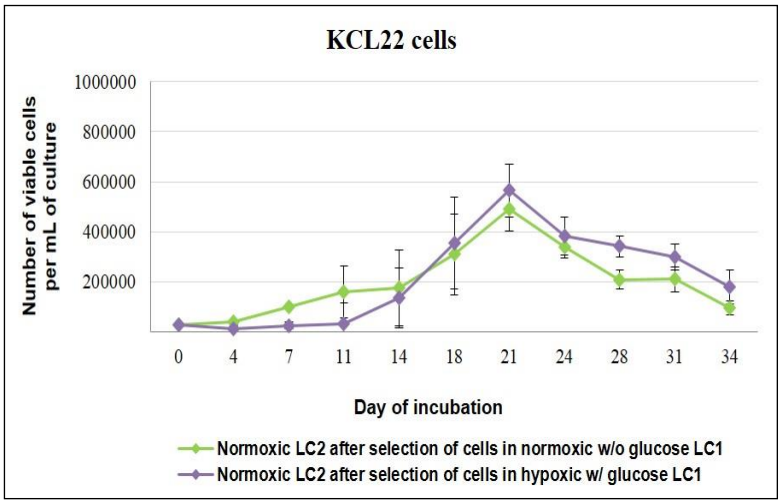


Figure 11. Kinetics of LC2 repopulation of hypoxia- or glucose starvation-selected CML cells. Exponentially-growing K562 (**A**) and KCL22 (**B**) cells were incubated for the indicated times in hypoxia w/ glucose or normoxia w/o glucose and then transferred at $3x10^4$ cells/mL to LC2. LC2 were incubated in air and viable cells were counted by trypan blue exclusion. Data represent Mean ± SD of three indipendent experiments.

Discussion

The results of the study presented here suggest that the suppression of BCR-Abl protein is a distinctive feature of CML cells adapted to energy shortage. This suppression heavily affects the natural history of disease, as it represents the crucial condition for the selection of BCR-Abl protein-negative LSC of CML.

The characterization of molecular mechanisms regulating BCR-Abl expression was undertaken using the blast crisis CML cell lines K562 and KCL22. We initially showed that incubation of CML cells in hypoxia, results in complete suppression of BCR-Abl protein, thereby inhibiting theirs growth. We then demonstrated that also BCR-abl mRNA is down-regulated during incubation in hypoxia.

To investigate if these findings could be due to differences in mRNA stability, BCR-abl mRNA half-life was determined. No significant differences in BCR-abl mRNA stability could be detected in K562 cells subjected to hypoxia compared to those incubated in normoxia. By contrast, in KCL22 cells the half-life of BCR-abl mRNA was significantly reduced in hypoxia compared to normoxia. These results led us to hypothesize that BCR-Abl suppression in hypoxia follows different pathways depending on the cell population considered, being subjected to transcriptional control in K562 cells and likely to post-transcriptional control in KCL22 cells.

To further characterize the possible transcriptional control mechanism, we focused our attention on the BCR promoter, which regulates the expression of both BCR and BCR-abl.

To test whether there were differences as for BCR-abl transcript in hypoxia compared to normoxia, we transiently transfected K562 cells with a luciferase reporter vector carrying the BCR promoter. Unexpectedly, the results of luciferase assay did not show a significant decrease in the transcriptional activity of BCR promoter in hypoxia. Thus, these results suggest that BCR-abl transcription in CML cells incubated in hypoxia is not regulated at the promoter level, but rather by other mechanisms (for instance, by transcriptional repressors) acting on signal sequences that are located far from the BCR promoter.

BCR-abl activates glucose metabolism as part of its transforming activity. Activation of glycolysis in Ph+ cells is associated with an increase of GLUT-1 glucose transporter at cell surface and suppression of p53 (**Barger J.F. et al. 2013**).

We therefore investigated on the metabolic effects of glucose starvation on BCR-Abl expression. To test whether hypoxia *per se* is capable of driving the effects shown above or additional environmental/metabolic factors such as glucose availability are required, K562 and KCL22 cells were incubated in normoxia or hypoxia and in the absence or the presence of glucose. Results showed that cells incubated in normoxia in the absence of glucose, as well as those maintained in hypoxia in the presence of glucose, undergo a significant decrease of viability and suppression of BCR-Abl protein, but not mRNA. This allows to hypothesize the involvment of post-transcriptional control, translational and/or post-translational.

Accumulating data demonstrated the involvement of different classes of RNA-binding protein in normal hematopoiesis and leukemogenesis (**Baou M. et al. 2011**) In particular, the RNA binding protein HuR appears to be overexpressed in blast crisis CML with the level of expression increasing progressively during transit from chronic phase to blast crisis (**Radich J.P. et al. 2006**). HuR is known to play an important role in both the stabilization and the modulation of translational efficiency of different mRNA.

We, therefore, evaluated by western blotting the expression levels of HuR in our experimental settings. In contrast with what expected, we did not observe significant changes of HuR protein level either in hypoxia with glucose or in normoxia without glucose. This means that HuR is unlikely to be one of the AUBP responsible for BCR-Abl protein suppression under energy shortage conditions.

The relationship of the level of protein synthesized to that of the corresponding mRNA is regulated by translation efficiency and the stability of the synthesized protein. In general, it is expected that increases of mRNA levels would result in concomitant increases of the corresponding protein. Deviations from this simple relationship during changes in gene expression may be due to translational control mechanisms, or could result from variations of translation efficiency of alternative mRNA isoforms (**Stevens G. et al. 2013**).

Thus, we determined BCR-Abl protein stability in K562 and KCL22 incubated in normoxia or hypoxia with or without glucose, to estimate if hypoxia or glucose starvation could interfere with protein decay. The results showed no significant differences in BCR-Abl protein half-life in any condition. This further confirms a key role of translational control in BCR-Abl expression.

It was previously shown in our laboratory that different types of leukemias contain hypoxia-resistant cells exhibiting stem cell properties, as well as less immature cells the numerical expansion of which is inhibited in hypoxia and glucose shortage. The existence of different, metabolically-characterized, functional cell subsets within leukemia cell populations reflects the organization of normal hematopoiesis, where the regulatory role of glycolysis and respiration within the stem/progenitor cell hierarchy has been long known. Such an organization is functional to the maintenance of stem cells within restricted, "metabolically-defined" bone marrow areas which we called the "hypoxic" or "ischemic" stem cell niches (**Giuntoli S. et al. 2011**, **Cipolleschi M.G. et al. 2013**, **Rovida E. et al. 2014)**. LSC selected under these conditions remain genotypically leukemic (BCR-abl-positive), but phenotypically BCR-Abl-negative (and indipendent of BCR-Abl signaling), a feature which makes of these LSC ideal candidates to represent the main CML cell subset resistant to IM and responsible for Minimal Residual Disease of CML.

To address the question wheter hypoxia or glucose starvation might select LSC, CML incubated in the absence of glucose for 14 days (LC1), were transferred at different times to non-selective secondary cultures (LC2) to estimate the maintenance of LSC/LPC potential in LC1 by CRA assay. The fact that LC2 established with cells rescued from LC1 incubated either in hypoxia or under glucose starvation showed a similar kinetics of repopulation, with a peak at days 21-24, confirmed our hypothesis that energy shortage (hypoxia or glucose deprivation) 'selected' and maintained LSC. Hypoxia, indeed, which is characterized by a high rate of glucose consumption, only accelerates cells selection.

Perspectives

The data reported in this thesis, addressing the regulation of BCR-Abl expression in blast crisis-CML cell lines, provide a useful background for the elucidation of the molecular mechanisms of BCR-Abl suppression and thereby induction of primary resistance (refractoriness) of CML cells to therapy. Possible avenues of future work in this field are discussed below.

Identification of possible regulatory elements involved in BCR-abl transcriptional control in hypoxia

One of the mechanisms through which protein levels in the cell are controlled is transcriptional regulation. Certain DNA regions, called *cis*-regulatory elements, are footprints for *trans*-acting proteins involved in transcription, either for the positioning of the basic transcriptional machinery or for the regulation. The basic transcriptional machinery is constituted of the DNA-dependent RNA polymerase (RNAP), which synthesizes various types of RNA. Core promoters on the DNA are used to position the RNAP. Other nearby regions regulate transcription: in eukaryotic organisms, in fact, proximal promoter regions, enhancers, silencers, and insulators are present (**Riethoven J.J. 2010**).

As showed above, BCR-abl gene seems to be subjected to transcriptional control in K562 cell line in hypoxia. However, the luminometric assay used in this thesis allows to investigate only what happens at the level of BCR promoter. Thus, many regulation and binding events may have remained undetected in these experiments. To facilitate the identification of all possible binding sites within or outside the BCR promoter regions, alternative methods should be employed. Tiling arrays covering a broader region of a particular gene locus of interest, or whole-genome tiling arrays could be used to study the binding outside the promoter region (**Horak C.E. et al. 2002**; **Lee T.I. et al. 2006**). With the development of next generation sequencing technologies, ChIP-seq could be performed for whole genome to map the binding

sites in an unbiased manner (**Barski A. et al., 2007**; **Johnson D.S. et al.,2007**; **Schones D.E. et al., 2008**).

Functional assays for the putative BCR-abl *silencer*

The identification of regions possibly exerting a negative control could give us the opportunity to further characterize the regulation of BCR-abl fusion gene. So, to determine whether the negative control region could act as a silencer, the next step could include a functional assay of these sequences, via their sub-cloning in a high expression plasmid, in different positions and orientations and fused, for example, to the bacterial CAT gene. These plasmids will be transfected into K562 cells, and CAT activity determined.

Investigations of histone modifications associated with BCR-abl gene regulation machinery

Another component of a transcription regulatory network is the chromatin domains and the modifications associated. Chromatin structure affects the binding of either sequence-specific or general transcription factors to regulatory elements, thus playing a crucial role in the transcriptional regulation of target genes. As a result, integrating the information for chromatin modifications and binding of transcription factors could facilitate a more complete understanding of transcription machinery and regulation.

Histone lysine acetylation has been shown to be associated with active transcription whereas deacetylation correlates with repression of transcription (**Tse C. et al. 1998**). Methylation of lysine 4 of histone H3 is linked to active genes, while methylation of H3/K is linked to a repressed state (**Lachner M. et al. 2002**). ChIP in combination with qPCR or microarray analysis could be used to study the modification status at the promoter and other regulatory elements of BCR-abl gene. This would allow to determine the role of histone modifications in the regulation of this gene, as well as to investigate on the activation or repression of the gene.

Further confirmation of the role of translational control in BCR-Abl expression

The translation of mRNAs into polypeptides is carried out in ribosomes (80S), that in eukaryotes are composed of two subunits. The small subunit has a sedimentation value of 40S, whereas the large subunit of 60S. The mechanism of protein synthesis can be described as having four distinct phases: (1) initiation: the small ribosomal subunit binds to the mRNA, and upon selection of the initiator AUG codon, the large ribosomal subunit is recruited to form the translation-competent 80S (70S in prokaryotes) ribosome; (2) elongation: decoding of mRNA sequence, delivery of amino acids by aminoacyl-tRNAs and incorporation into the growing polypeptide chain via the formation of peptide bonds (3) termination: release of the polypeptide upon recognition of a stop codon on mRNA and (4) recycling of the ribosomes into free subunits that continue translation of other mRNAs. An experimental approach which is widely used to study structure/function of ribosomes and also to monitor the efficiency of mRNA translation is the use of sucrose gradients to separate free ribosomal subunits from translating ribosomes by velocity sedimentation. Because ribosomal subunits are larger than free mRNA protein complexes, this methodology can also separate the two populations from cellular extracts. In addition, translating mRNAs, which are bound to several ribosomes, forming polyribosomes, migrate with much larger fractions in sucrose gradients. The sedimentation velocity of polyribosomes depends on their size, which relates to the efficiency of translation of the corresponding mRNA. The larger the corresponding mRNA and the number of bound ribosomes, the higher the sedimentation velocity. Sedimentation of particles is monitored by measuring the absorbance of the fractions at 254 nm, which provides the so-called 'polysome profile'. A typical polysome profile shows distinct peaks for the free ribosomal subunits (small 40S and large (60S), followed by the 80S ribosomes and heavier polyribosomes (**Krokowski D. et al. 2011**).

So, to better understand whether the energy shortage causes BCR-Abl translational initiation inhibition, polysome profile technique could allow us to determine whether CML cells we used are able to form ribosomal dimers,

translationally active. In fact, if translation initiation is inhibited, an increased accumulation of free ribosomal subunits and non-translating monosomes and a decrease in the polyribosome pool should be obtained. On the contrary, sustained association of mRNAs with polyribosomes would be associated with efficient translation of the corresponding mRNAs.

Determination of the possible role of other RNA binding proteins in response to energy shortage

Post-transcriptional mechanisms are key determinants in the modulation of the expression of final gene products. AU-rich elements are frequently found within the 3' UTRs of mRNAs coding for proteins with roles in the immune system, growth and survival. Within this context, fundamental players are RNA-binding proteins. Their dysregulation can lead to disease (**Pichon X. et al. 2012**).

As described above, RNA binding proteins are able to affect every aspect in the processing of transcripts, from alternative splicing, polyadenylation, and nuclear export to cytoplasmic localization, stability, and translation. Of interest is the fact that more than one RNA-binding protein can bind simultaneously the same mRNA; each protein is endowed with different properties, so that the balance of these interactions dictates the ultimate fate of the transcript, especially in terms of stability and rate of translation (**Pascale A. et al. 2012**).

Since recent data demonstrated the involvement of different classes of RNA-binding proteins in normal hematopoiesis and leukemogenesis (**Baou M. et al. 2011**), we could investigate whether other AUBP are involved in the post-transcriptional/translational control of BCR-Abl. If one or more AUBP were involved in BCR-Abl regulation, the next step would be silencing and overexpression of them, to confirm their role.

Potential miRNA involvment in BCR-Abl control during energy shortage conditions

Micro RNA (miRNA) are small non-coding RNA molecules (22-24 nucleotides) that regulate gene expression via post-transcriptional mechanisms. Following binding to target sites in mRNA, miRNA can both repress translation and destabilize specific mRNA, although recent studies have indicated that the predominant form of regulation may be mRNA destabilisation (**Stevens S.G. et al. 2013**).

A miRNA in its mature form can bind to the 3′-UTR of a target mRNA due to the partial sequence complementarity between the two (**Lewis B.P. et al. 2005**). Binding of a miRNA to its target triggers one or more of the following mechanisms of mRNA post-transcriptional inactivation: 1) mRNA cleavage, 2) inhibition of mRNA translation, or 3) mRNA deadenylation and rapid degradation. Of these mechanisms, miRNA-induced inhibition of translation primarily involves the prevention of targeted mRNA from undergoing translation initiation or elongation, without affecting the levels of mRNA expression (**Zeng Y. et al. 2003**).

Recently, it was demonstrated the role of different miRNAs, including MIR150, MIR203, MIR328, MIR10A, MIR181A, MIR130 in CML, many of them directly correlated with BCR-abl transcript levels (**Gordon J.E.A. et al. 2013**).

So, we could investigate the possible miRNA deregulation (up-regulation or down-regulation) during energy shortage (hypoxia or glucose starvation) and correlate these alterations to BCR-Abl down-regulation. We would use quantitative RT PCR-based methods to detect and quantify miRNA. These are also incorporated into higher throughput approaches including the widely used Taqman low density array system (**Chen C. et al. 2005**). Hybridization methods employ differing labelling strategies. One recently developed platform is the NanoString system where miRNA are counted directly, with minimal pre-amplification. This approach utilizes a barcoding system with sufficient diversity to allow discrimination between highly similar miRNA variants (**Wyman S.K. et al. 2011**).

Materials and Methods

Cell cultures

The human BCR-Abl positive K562 and KCL22 CML cell lines were obtained from ATCC (Rockville, MD, USA). Cells were cultured in RPMI 1640 medium (Gibco by Life Technologies, Carlsbad, CA, USA) supplemented with 10% heat-inactivated foetal bovine serum (FBS), 2 mM L-glutamine, 50 units/mL penicillin and 50 µg/ml streptomycin (all from EuroClone, Paington, UK) with high D-glucose (4.5 g/L), at 37°C. In some experiments, we used RPMI 1640 medium without D-glucose, supplemented as above.

Experiments were performed with exponentially-growing cells, harvested from maintenance cultures subcultured in fresh medium 24 h before plating, and plated at $3x10^5$/mL. Incubation in normoxia (21% O_2) with or without glucose was carried out in a conventional cell culture incubator in a 5% CO_2, 95% air water-saturated atmosphere. Incubation in severe hypoxia (0.1% O_2) was carried out in a Ruskinn Concept 400 anaerobic incubator, flushed with a preformed gas mixture (0.1% O_2, 5% CO_2, 94.9% N_2) and water-saturated. This incubator allows easy entry and exit of materials and sample manipulations without compromising the hypoxic environment.

Viable cells were counted in a hemocytometer by trypan blue exclusion.

Protein extraction, separation and Western blotting

Cells were washed with ice-cold PBS containing 100 µM Na_3VO_4 and solubilized by incubating for 10 minutes at 95°C in Laemmli buffer (62.5 mM Tris/HCl, pH 6.8, 10% glycerol, 0.005% bromphenol blue, and 2% SDS). Lysates were clarified by centrifugation (14000*g*, 5 minutes, RT) and protein concentration in supernatants was determined by the BCA method.

Whole cell extracts (50 µg/lane) were boiled for 10 minutes in the presence of 100 mM 2-mercaptoethanol, separated by SDS-PAGE in 9% polyacrylamide minigels, and then transferred onto PVDF membranes (Merck Millipore, Billerica,

MA, USA) by electroblotting. Membranes were blocked in 1:1 Odyssey blocking buffer (OBB)/phosphate-buffered saline (PBS) for 1 hour at RT and incubated with primary antibody in 1:1 PBS-0,1% Tween (T-PBS)/OBB overnight at 4°C (LI-COR® Biosciences, Lincoln, NE, USA).

Primary antibodies used were: rabbit anti-phospho-c-Abl (Tyr245) (from Cell Signaling Technology, Danvers, MA, USA), mouse anti-α-Tubulin, mouse anti-Vinculin (from Sigma Aldrich, St. Louis, MO, USA), rabbit anti-c-Abl, mouse anti-HuR (from Santa Cruz Biotechnology, S. Cruz, CA, USA).

Secondary anti-IgG antibodies were IRDye®800CW- or IRDye®680-conjugated (LI-COR®). Antibody-coated protein bands were visualized by the Odyssey Infrared Imaging System Densitometry (LI-COR®) and images analyzed by Odyssey software to measure the mean fluorescence intensity (MFI) value of the area selected for each band. A background measurement was also taken.

RNA isolation and quantitative Real Time RT-PCR

For each experimental condition, the cells were washed once with ice-cold PBS and total RNA was extracted using TRIzol (Life Technologies, Carlsbad, CA, USA) according to the manufacturer's instructions. Residual DNA was removed by treatment with 5 units of DNase I (Roche Diagnostics, Lewes, UK) at RT for 15 minutes followed by inactivation at 65°C for 10 minutes. RNA concentration (µg/µL) was determined by spectrophotometric reading at 260 nm wavelength, and the purity of RNA was determined by calculating the ratio of absorbance at 260/280 nm. After, determination of quality and integrity of total RNA was assessed on 2% agarose gels.

Five hundred nanograms of total RNA were reverse-transcribed to cDNA using the ImProm-II™ Reverse Transcription System (Promega, Madison, WI, USA) according to the manufacturer's protocol.

Quantitative Real-Time PCR analysis of the BCR-ABL p210 transcript (b2a2 for the KCL22 cells, b3a2 for the K562 cells) was performed using the Applied Biosystems 7500 Fast Real-Time PCR System (Life Technologies) under the following conditions: Hot Start Activation at 95°C for 2 minutes (1 Cycle),

Denaturation at 95°C for 15 seconds, Annealing at 56°C for 20 seconds, and Extension at 60°C for 40 seconds (40 Cycles), Dissociation at 60-95°C (1 Cycle). The PCR mixture consisted of 2X GoTaq qPCR Master Mix (Promega, Madison, WI, USA), which includes GoTaq® Hot Start Polymerase polymerase, a new fluorescent DNA-binding dye that exhibits greater fluorescence enhancement upon binding to dsDNA than SYBR Green I, dNTPs, and PCR buffer. To this were added forward and reverse primers, and cDNA of samples in a total volume of 20µl. The amplification of a housekeeping gene, GAPDH or 18S rRNA (*data not showed*), was used to normalize the efficiency of cDNA synthesis and the amount of RNA applied.

Primers (from Integrated DNA Technologies, Coralville, IA, USA) were:
BCR-abl forward primer: 5'-GGAGCAGCAGAAGAAGTGTTT-3',
BCR-abl reverse primer: 5'-TGGGTCCAGCGAGAAGGTTTT-3',
GAPDH forward primer: 5'-AACAGCCTCAAGATCATCAGCAA-3',
GAPDH reverse primer: 5'-TCTGGGTGGCAGTGAT-3',
18S forward primer: 5'- CGGCTACCACATCCAAGGAA-3',
18S reverse primer: 5'- GCTGGAATTACCGCGGCT-3'.

Experiments were repeated with cDNA from at least 3 independent experiments, using a minimum of triplicates for each sample. Negative controls were performed with samples in which RNA templates in the reaction were replaced by nuclease-free water.

Based on the cycle threshold method, as recommended by the manufacturer, the observed measurements of the BCR-abl fusion transcripts and of the internal standard, GAPDH or 18S rRNA, in the tested samples were calculated against a standard curve previously obtained using serially diluted samples prepared from K562 and KCL22 leukemia cells. The rationale for the need of such a normalization was that PCR efficiencies varied from sample to sample. Therefore, the actual quantities of BCR-abl and GAPDH or BCR-abl and 18S rRNA transcripts in the tested samples were adjusted accordingly.

Analysis of mRNA stability following inhibition of transcription by actinomycin D

The half-life of BCR-abl mRNA stability was determined by treating K562 and KCL22 cells with Actinomycin D (Sigma-Aldrich). Cultures were incubated in normoxia or in hypoxia for 24 h, and then Actinomycin-D was added into the growth medium (5µg/mL) to block transcription. During the following 8 h, cells were harvested every two hours, and total RNA was extracted using TRIzol. The amounts of BCR-abl mRNA and GAPDH mRNA at each time point were determined by quantitative Real Time-PCR and mRNA stability among hypoxic and normoxic cultures compared.

Luciferase activity assay

The luciferase constructs, pGL3/BCR, containing the BCR promoter, and pGL3/empty vectors were kindly provided by professor Carlo Gambacorti Passerini (Unità di Ricerca Clinica-Ematologia, Azienda Ospedaliera San Gerardo/Università di Milano Bicocca, Monza, Italy).

K562 cells were transiently co-transfected with 1 µg pGL3-luc/BCR promoter plasmid or pGL3-luc/empty construct using Lipofetamine^{TM} 2000 (Life Technologies). The most efficient transfection was obtained using a Lipofectamine^{TM} 2000 to DNA ratio of 2:1 (µl:µg). pGL4 plasmid (0.1 µg) (Promega) expressing Renilla luciferase was used to normalize for transfection efficiency. After six hours from transfection, cells were diluited at $3x10^5$/mL and incubated in normoxia or hypoxia until the time of analysis in luminescence.

After 24 h, 48h, and 72h cells were washed, and a Dual-Glo™ Luciferase Assay System (Promega) was used to determine relative luciferase activity according to the manufacturer's protocol. Results were expressed as the ratio of Firefly luciferase/Renilla luciferase at equal amounts of protein. Each experiment represents the mean of three replicates and was repeated at least three times.

Analysis of protein stability by Cycloheximide assay

To test BCR-Abl protein stability, K562 and KCL22 cells were incubated in normoxia w/ glucose or incubated in normoxia w/o glucose or hypoxia w/ glucose for 24h. Cycloheximide (Sigma-Aldrich) was added at a final concentration of 50µM to block new protein synthesis and cells were harvested after 24, 48, or 72h. Cells were lysed and the expression of BCR-Abl protein was analyzed by Western blotting. Signals detected on blots were quantified by densitometry, and the data were plotted to determine protein half-life.

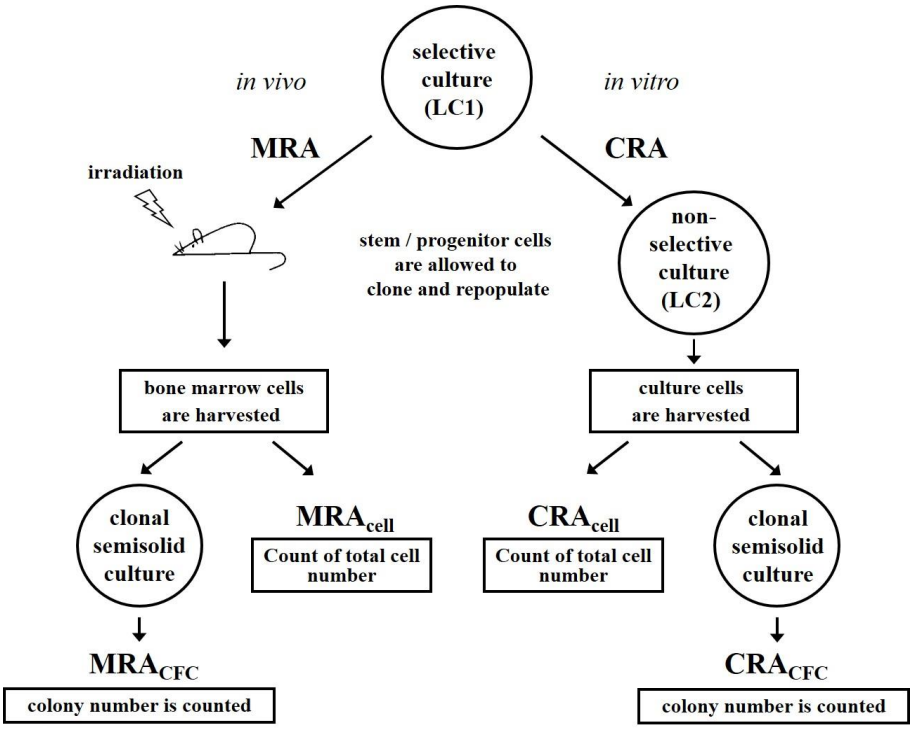
The Culture Repopulating Ability (CRA) assay

This assay estimates the culture repopulating potential of normal or leukemic hematopoietic cells undergone a selection treatment in a primary liquid culture (LC1) by means of their transfer in fresh medium to non-selective conditions in a growth-permissive secondary culture (LC2) and the measure of its repopulation following a further incubation therein. Before being replated into LC2, cells selected in LC1 were counted, centrifuged, and resuspended in fresh medium. Cell subsets rescued from LC1 at different times repopulate LC2 with different kinetics, the time necessary to reach the peak of LC2 repopulation reflecting the hierarchical level of stem/progenitor cells enriched in LC1.

The CRA assay is a non-clonogenic assay capable to reveal in vitro stem cells endowed with marrow-repopulating ability in vivo. The adaptation of CRA assay to leukemia cell populations enabled to detect different subsets of leukemia stem or progenitor cells.

Statistical analysis

All data were expressed as Mean ± standard deviation (SD) of three or more indipendent experiments. Differences were tested by the Student's *t*-test, considering p-values ≤ 0.05 and ≤ 0.01 statistically significant.



The CRA and MRA assays. Schematic representation of CRA (*in vitro*) and MRA (*in vivo*) assays and their relationship.

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