

Reactive oxygen species in BCR-ABL1-expressing cells – relevance to chronic myeloid leukemia

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Chronic myeloid leukemia (CML) results from the t(9;22) reciprocal chromosomal translocation producing the *BCR-ABL1* gene, conferring growth and proliferation advantages in the CML cells. CML progresses from chronic, often syndrome-free, to blast phase, fatal if not treated. Although the involvement of BCR-ABL1 in some signaling pathways is considered as the cause of CML, the mechanisms resulting in its progression are not completely known. However, BCR-ABL1 stimulates the production of reactive oxygen species (ROS), which levels increase with CML progression and induce BCR-ABL1 self-mutagenesis. Introducing imatinib and other tyrosine kinase inhibitors (TKIs) to CML therapy radically improved its outcome, but TKIs-resistance became an emerging problem. TKI resistance can be associated with even higher ROS production than in TKI-sensitive cells. Therefore, ROS-induced self-mutagenesis of BCR-ABL1 can be crucial for CML progression and TKI resistance and in this way should be taken into account in therapeutic strategies. As a continuous production of ROS by BCR-ABL1 would lead to its self-destruction and death of CML cells, there must be mechanisms controlling this phenomenon. These can be dependent on DNA repair, which is modulated by BCR-ABL1 and can be different in CML stem and progenitor cells. Altogether, the mechanisms of the involvement of BCR-ABL1 in ROS signaling can be engaged in CML progression and TKI-resistance and warrant further study.

Key words: chronic myeloid leukemia, reactive oxygen species, DNA damage, DNA repair, cancer stem cells, imatinib resistance

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Abbreviations: AKT, protein kinase B; AP site, apurinic, apyrimidinic or abasic site; ATM, ataxia telangiectasia mutated; ATR, ataxia telangiectasia and Rad3 related; *BCR*, the breakpoint cluster region gene; BCR-ABL1, fusion tyrosine kinase; BER, base excision repair; *c-ABL1*, the Abelson proto-oncogene; CAT, catalase; CCR7, C-C chemokine receptor type 7; CML, chronic myeloid leukemia; CSC, cancer stem cells; Cripto, a ligand for the GRP78 protein; DDR, DNA damage reaction; DNA-PK_{cs}, DNA-dependent protein kinase, catalytic subunit; DSB, double-strand break; FANCC, Fanconi anemia complementation group C; FOXO, forkhead box O transcription factor; G-CSF, granulocyte colony stimulating factor; GRP78, 78 kDa glucose-regulated protein; HDACi, histone deacetylase inhibitor; HIF-1/2, hypoxia inducible factor-1/2; Hox-A9, homeobox protein; HSC, hematopoietic stem cell; ICAM-1, intercellular adhesion molecule 1; LT-HSC, long-term hematopoietic stem cell; MAPK, mitogen-activated protein kinases; Meis1, myeloid ecotropic viral integration site 1 homolog; PBX1, Pre-B-cell leukemia homeobox 1; PPAR γ , peroxisome proliferator-activated receptor- γ ; Ph, Philadelphia chromosome; PI3K, phosphatidylinositol 3-OH kinase; PLK1, polo-like kinase 1; PTEN, phosphatase and tensin homolog; ROS, reactive oxygen species; SOD 1,2, superoxide dismutase 1,2; SSB, single-strand break; ST-HSC, short-term hematopoietic stem cell; TGF- β , transforming growth factor β ; TKI, tyrosine kinase inhibitor; TLS, translesion synthesis; TNF- α , tumor necrosis factor- α

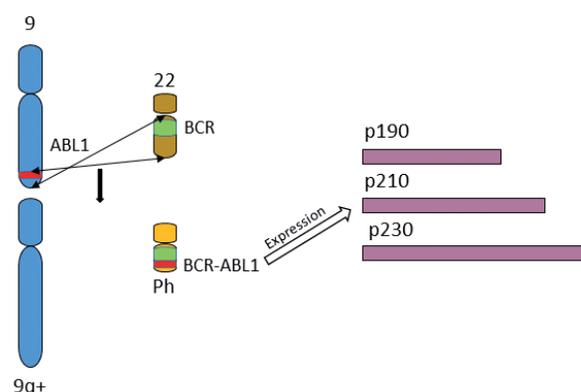


Figure 1. The t(9;22) chromosomal reciprocal translocation results in the Philadelphia (Ph) chromosome containing the BCR-ABL1 gene, which expression can give three protein products. The p210 protein displays the strongest oncogenic properties and can occur in two variants.

INTRODUCTION

Chronic myeloid leukemia (CML) is caused by the t(9;22) chromosomal reciprocal translocation in hematopoietic stem cells, leading to the shortening of chromosome 22, which is called the Philadelphia chromosome (Ph) and contains the *BCR-ABL1* fusion gene (reviewed in Druker, 2008) (Fig. 1).

The product of this gene, the BCR-ABL1 protein, is a tyrosine kinase, which phosphorylates many downstream targets, including those important in cancer (Fig. 2).

Initially, the role of BCR-ABL1 in the transformation of bone marrow cells was attributed to conferring increased growth and proliferation to the transformed cells, but at present, an increased resistance to apoptosis is considered as the main effect induced by BCR-ABL1 in transforming cells (Leber, 2011).

Chronic myeloid leukemia is usually characterized by three consecutive phases. The chronic phase is usually symptom-free or is associated with mild symptoms, frequently disappearing upon the start of treatment, and can last for several years. In accelerated phase, an increase in the number of immature white blood cells in blood and bone marrow is observed. Some other changes in DNA, beside Ph, can occur in this phase. Blast phase or blast crisis is fatal, if not treated, and patients in this phase display an uncontrolled increase in white blood cells and blasts population and additional genetic changes in blast cells. However, the disease can occur in only two phases as the accelerate phase may not follow. From the clinical point of view, it is obvious that CML can be diagnosed in blast phase, without any clinical evidence of preced-

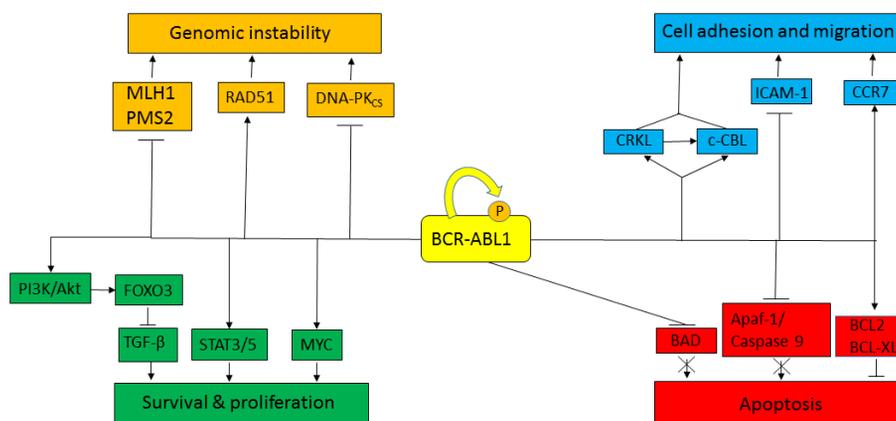


Figure 2. BCR-ABL1 is involved in cellular signaling pathways implicated in genomic instability, cell adhesion and migration, apoptosis, proliferation and survival, which all are important for cancer transformation supported by BCR-ABL1.

Presented are some pathways, covering only a small fraction of all possible interactions of BCR-ABL1. Moreover, whether the interaction leads to up- or downregulation of a particular protein can depend on the kind of cell or/and its environment. MLH1 and PMS2 are proteins of DNA mismatch repair, reducing errors of replication, RAD51 is a key protein for homologous recombination repair and DNA-PK_{cs} (DNA-dependent protein kinase, catalytic subunit) is involved in the repair of DNA double-strand breaks in non-homologous end joining pathway, which strongly influences genomic stability. CRKL, an adaptor protein, can mediate interaction between BCR-ABL1 and c-CBL, another adaptor, leading to defects in adhesion properties of the BCR-ABL1-positive cells. ICAM-1 (intercellular adhesion molecule 1) and CCR7 (C-C chemokine receptor type 7) are involved in cell migration and adhesion, which are defective in chronic myeloid leukemia cells. BCR-ABL1 can target the protein kinase Akt and FOXO3 (forkhead box 3), a transcription factor to hinder growth inhibition in response to TGF- β (transforming growth factor β). Increased survival and proliferation can result also from the interaction of BCR-ABL1 with STATs (signal transducer and activator of transcription) and the MYC oncoprotein. BCR-ABL1 can interact with proteins directly involved in apoptosis, including BCL family proteins and caspases, making BCR-ABL1-positive cells resistant to apoptosis.

ing phases. Time needed for moving of CML from the chronic phase to the accelerated phase and then to the blast crisis, may be different for different patients and the mechanisms underlying this variability are not completely known. This can be due to the fact, that although mechanisms of CML induction can be attributed to the anti-apoptotic action of BCR-ABL1, molecular processes resulting in progression of the disease are largely unknown. They can however, be associated with genetic changes accumulated during the course of the disease (Deininger *et al.*, 2000). They, in turn, induce and increase genomic instability, which is typical for almost all cancer cells. Reactive oxygen species (ROS) play an important role in the integrity of the genome, as they can damage DNA either directly or via their intermediates and increased ROS production is associated with BCR-ABL1 activity (Sattler *et al.*, 2000).

The efficacy of CML therapy used to be low and a real breakthrough was the introduction of imatinib (imatinib mesylate, Gleevec, STI573), a tyrosine kinase inhibitor (TKI), which changed CML from a fatal disorder into a chronic disease. However, imatinib resistance soon became a budding problem, which was not definitely solved by the next generation of TKIs. TKI-resistance is observed in many cases of blast crisis (Perrotti *et al.*, 2010). We and others showed that ROS can be important for imatinib resistance, which along with the accumulation of genetic changes in blast phase, suggest that ROS can play an important role in CML progression (Nieborowska-Skorska *et al.*, 2011; Nieborowska-Skorska *et al.*, 2014; Slupianek *et al.*, 2013; Koptyra *et al.*, 2006). This role should be considered in a general involvement of ROS in cancer transformation.

ROLE OF REACTIVE OXYGEN SPECIES IN GENOMIC STABILITY OF CANCER CELLS

Reactive oxygen species are ubiquitous and play an important role in cellular signaling (Ray *et al.*, 2012). How-

ever, their excess, typical for oxidative stress, can have a devastating impact on cell functioning. An increased level of ROS was observed in many cancer cases, but in the majority of them the question whether they are the reason or a consequence of cancer is still open. As the solution of this problem can be crucial for cancer prevention, diagnosis and therapy, studies on the role of ROS in cancer are justified. Many aspects of ROS signaling can contribute to cancer induction and progression, but one of the most direct associations is through genomic instability observed in almost all cancer cells.

Genomic instability can be expressed as the extent of DNA mutations and chromosomal rearrangements in the genome. These changes are derived from DNA damages, but the source of them in cancer is not completely known. In certain cases, there is a causal relationship between the exposure to DNA-damaging environmental factors and cancer with the involvement of genomic instability, but in many circumstances such relationship is unknown. The extent of pre-mutagenic DNA lesions is regulated by the cellular DNA damage reaction (DDR) and DNA repair in particular (Zhou & Elledge, 2000). Cancer-associated ROS can damage not only DNA, but also DNA repair proteins and other biomolecules important for DDR, leading to increased genomic instability. They can also damage enzymes of antioxidant defense, resulting in ROS accumulation.

Hydroxyl radical ($\cdot\text{OH}$) is one of the most, if not the most, reactive ROS, which easily reacts with DNA by the addition to the C5-C6 double bond or abstraction of a proton from the methyl residue of thymine and C-H bonds of the deoxyribose sugar (Cooke *et al.*, 2003). These effects can lead to the production of a variety of purine and pyrimidine adduct radicals, which are reduced or oxidized in dependence on the environment (Steenken, 1997). The final products can show different pairing properties from the originals and contribute to DNA mutations during next cellular divisions. Transferring the electrons released by certain endo- and exogenous com-

pounds to molecular oxygen, can lead to the formation of superoxide ($O_2^{\bullet-}$), which can support $\bullet OH$ production in the presence of iron ions (Nemmar *et al.*, 2016). Oxidatively modified DNA bases can constitute a significant part of all the bases present in the cell (Cooke *et al.*, 2003). Besides *in situ* formation of oxidatively modified DNA bases, they can be created by incorporation of a deoxynucleotide precursor, which was attacked by ROS and in fact, the majority of base modifications present in DNA can be produced in this way (Dumont *et al.*, 2016).

The action of ROS can bring several other consequences for cellular DNA than point mutations resulted from non-canonical base-pair forming by oxidatively-modified bases. Such bases can constitute a block of the replication fork as replicative DNA polymerase cannot recognize a nucleotide precursor with a modified base and is not able to fit it into its catalytic pocket. Stalled polymerase can be replaced by a translesion synthesis (TLS) polymerase, which can be highly error-prone and induce mutations (Livneh *et al.*, 2016). Otherwise, the replication fork can generate various DNA arrangements upon the encounter of certain modified bases, including chicken foot structures, which, when resolved, can produce DNA double-strand breaks (DSBs), which can result in chromosome breaks (Zhang *et al.*, 2016). The formation of DNA single-strand breaks (SSBs) resulted from the restart of stalled replication fork is also possible.

ROS-induced the oxidatively modified DNA bases are primarily repaired by DNA base excision repair (BER). BER starts from recognizing a modified base by a DNA glycosylase, which removes the base by cleavage of the N-glycosylic bond between the base and 2'-deoxyribose. Such a base-free site (apurinic, apirimidinic or abasic site, AP site) is then cleaved either by a lyase activity of the glycosylase or a specialized AP endonuclease, which leads to a SSB (reviewed in Bellacosa & Drohat, 2015). The termini of DNA at such SSB are then processed to become a substrate for DNA polymerase and DNA ligase. However, if the process of DNA repair synthesis is incomplete, which can be a consequence of several events, including ROS attack, a SSB will persist. Accumulation of SSBs formed in such way can result in DSBs and eventually chromosome breaks, if not repaired.

Therefore, ROS can induce a plethora of DNA damages, which in certain cases can be processed by overlapping DNA repair systems. If not repaired or misrepaired, they can result in DNA mutations or chromosomal breaks, which can induce genomic instability, a

prerequisite to cancer transformation. A ROS-induced mutation can be casual for the transformation if occurs in a proto-oncogene or a gene encoding tumor suppressor. If such a mutation occurs in genes involved in DDR (DNA maintenance genes), it can result in further increase and accumulation of DNA lesions, increasing genomic instability and the chance that the DNA damage brings a mutation occurring in a gene important for malignant transformation.

ROS SIGNALING IN HEMATOPOIETIC STEM CELLS

As CML is a cancer stem cell-derived disease, ROS regulation in hematopoietic stem cells (HSCs) can play a role in its pathogenesis. Several signaling pathways are involved in ROS functioning in HSCs, including ATM (ataxia telangiectasia mutated), PI3K (phosphatidylinositol-3-OH kinase)/AKT (protein kinase B), PTEN (phosphatase and tensin homolog), MAPK (mitogen-activated protein kinases) and others (reviewed in Shi *et al.*, 2012). In general, these pathways act to support the residence of HSCs in specific niches in hypoxia and a low ROS level (Fig. 3).

This issue is addressed in more detail in the next chapter.

Cytokines can play an important role in the interaction between HSCs and their niche, which was firstly demonstrated in association with telomere dysfunctionality (Schultz *et al.*, 2016). Granulocyte colony-stimulating factor (G-CSF) was identified as involved in this interaction. However, G-CSF can stimulate ROS production in the PI3K/AKT pathway and these ROS can affect the function of HSCs, leading to their transformation (Zhu *et al.*, 2006).

Reactive oxygen species are associated with inflammation, so it is justified to consider the influence of inflammation-related ROS on HSCs. The proinflammatory cytokine tumor necrosis factor- α (TNF- α) was reported to inhibit self-renewal of HSCs in Fanconi anemia complementation group C (FANCC) gene-deficient mice (Zhang *et al.*, 2007). Fanconi anemia (FA) is a human disease characterized by genomic instability and excessive ROS production (Li *et al.*, 2012). That TNF- α -induced inhibition of mouse HSCs self-renewal was attributed to ROS accumulation as pretreatment with N-acetyl-L-cysteine (NAC), an antioxidant, reduced this effect.

The forkhead box O (FOXO) is a subfamily of mammalian transcription factors, which target many proteins in the PI3K-AKT/PKB signaling pathway (Ma and

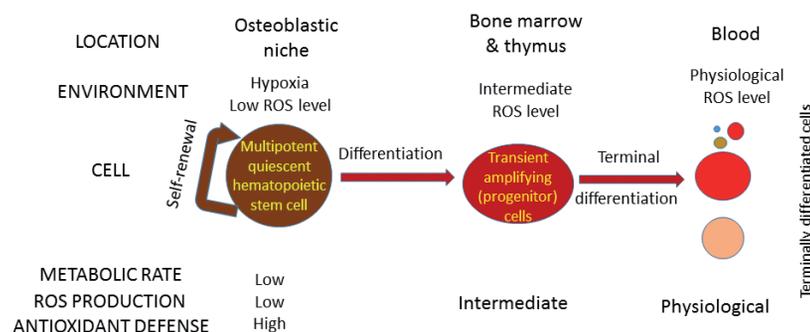


Figure 3. ROS affect hematopoiesis.

The formation of cellular components of blood is initiated by a multipotent hematopoietic stem cell (HSC) residing in an osteoblastic niche with low oxygen content. HSCs keep their metabolism at low level and produce low level of ROS, mostly neutralized by an effective antioxidant defense. HSCs replenish their population by self-renewal and occasionally differentiate to transit amplifying cells with various degree of potency and specifying different cell lineages. ROS can be involved in HSCs differentiation as well as terminal differentiation of transit amplifying cells. Oxidative stress associated with a high ROS level can induce HSCs senescence or death.

Wang, 2012). These proteins include antioxidant enzymes, crucial for ROS neutralization, such as superoxide dismutases (SOD1 and 2) and catalase (CAT). The *Foxo* genes can play an important role in HSCs functioning and this role includes ROS regulation (Miyamoto *et al.*, 2007; Tothova *et al.*, 2007; Yamazaki *et al.*, 2006).

ATM along with AKT and ataxia telangiectasia and Rad3 related (ATR), which are essential for maintaining genomic stability, can be involved in HSCs homeostasis, primarily in their self-renewal (Ito *et al.*, 2006; Maryanovich *et al.*, 2012). An upregulation of cyclin-dependent kinase inhibitors, p16^{Ink4a} and p19^{Arf}, essential for the cell cycle regulation, was observed in HSCs of ATM-deficient mice when the level of ROS was increased. Similar effect was observed in HSCs of the *Bmi-1*-deficient mice, which suggests the involvement of epigenetic factors in ROS regulation of HSCs, as *Bmi-1* is important for chromatin remodeling (Iwama *et al.*, 2004; Lessard *et al.*, 2003; Park *et al.*, 2003).

The p38 MAPK is a protein encoded by gene responsive to stress stimuli, including UV radiation, osmotic and heat shocks (Alam *et al.*, 2014). In fact it represents a class of proteins involved in important cellular functions, including differentiation, apoptosis and autophagy. It was shown that HSCs self-renewal strongly depended on normal control of ROS and functioning (Jang and Sharkis, 2007). The authors showed in that work two subpopulations of HSCs depending on their ROS intracellular content – ROS^{low} and ROS^{high}, different in their long-term self-renewal ability. Moreover, a different level of expression of phosphorylated p38 MAPK was shown in ROS^{high} and ROS^{low} HSCs.

As genomic instability is a feature of almost all cancer cells it is reasonable to consider mechanisms of this effect in HSCs. However, DDR, which is crucial for genomic stability, in stem cells is not so well recognized as in their non-stem counterparts. Moreover, it is not easy to identify the most primitive CML stem cells, so the term “stem” would be rather used in operational sense, and not necessarily always as “the very first”.

In general HSCs, as other stem cells, reside in a cellular compartment, called “niche”, where they are protected from external insults. Therefore, the niche is not only a spatial structure, but also has a specific microenvironment. A majority of HSCs are in G0 phase of the cell cycle, allowing them to decrease the risk of mutations following DNA replication, but they are able to re-enter the cycle to self-renew or differentiate to produce progenitor and terminally differentiated cells. This state is referred to as quiescence. Self-renewal occurs in the niche, whereas progenitor cells are produced outside of it.

Conditions in the HSCs niche should be precisely regulated to ensure proper functioning of the cells. Osteoblasts were identified as an important component of the HSCs niche in mice (osteoblastic niche) and it was shown that these cells were located in close proximity of vascular cells (vascular niche) (Kiel *et al.*, 2005; He *et al.*, 2016). Therefore, supporting HSCs by their various niches is crucial for maintaining their functions and it was shown that ROS could be involved in this effect (Jang & Sharkis, 2007). However, this is not really surprising, because the niche should have a decreased level of ROS to avoid damage to HSC biomolecules. As ROS are produced from oxygen, decreasing the level of oxygen in the niche seems to be the most direct way to diminish ROS extent (Simsek *et al.*, 2010). Other scenarios include increasing the activity of antioxidant enzymes, increasing the concentration of small antioxidants. The consequenc-

es of ROS action on DNA of HSCs can be reversed, at least in part, by effective DNA repair system or in general, by increased DDR. These and other research suggest that keeping ROS at low level in HSCs depends on the interaction of the cells with their niche(s) (reviewed in Naka *et al.*, 2008; Kiel *et al.*, 2008).

As mentioned, DDR in stem cells can differ from that functioning in non-stem cells. Because HSCs are probably the best explored and known adult stem cells, DDR in these cells is relatively well known, but our knowledge on it is still incomplete and may not reflect general features of DDR in all kinds of stem cells. Moreover, DDR mechanisms can be different in different species. However, general DDR also can be species-specific, which is supported by repairadox – cells of rodents repair some DNA damage with lower efficacy than human cells, but both have similar ratio of survival (Hanawalt, 2001). The most important features of DDR in HSCs are underlined by the difference in DDR in quiescent and differentiating HSCs, which can be attributed to different proteins with overlapping functions in DNA repair and self-renewal or differentiation. This can be exemplified by the involvement of p53 in DDR and self-renewal in human HSCs (Milyavsky *et al.*, 2010). The p53 protein is also considered as a mediator of HSCs “competition” for proper niche, which can be interpreted as a protective function of this protein in HSCs, independent of its role in DDR (Bondar & Medzhitov, 2010). In general DDR in HSCs can be less effective and more error-prone than in non-stem counterparts, especially in quiescent cells (Rossi *et al.*, 2007; Nijnik *et al.*, 2007; Mohrin *et al.*, 2010).

ROS-DEPENDENT METABOLIC REGULATION OF HEMATOPOIETIC STEM CELLS IN THE HYPOXIC NICHE AND ITS ROLE IN LEUKEMOGENESIS

The regulation of the metabolism of stem cells is essential for their stemness. It is not only due to the fundamental functions of these cells, but also due to their need of a proper environment. Metabolic processes are the source of many chemicals and physical particles, which can exert a detrimental effect on the cell. That is why stem cells keep their metabolism at a minimal required rate and are located in an environment with a low level of harmful insults. This functional-structural object is called stem-cell niche. In the case of HSCs, the niche is located in the endosteal regions of the bone marrow. The majority of HSCs in the niche is in a quiescent state limiting their activity to self-renewal. They are usually called long term HSCs (LT-HSCs) as they have a long-term self-renewal capacity after transplantation in a mouse foreign organ. However, the need for generating of billions and billions functional blood cells during the human lifetime, causes these LT-HSCs to increase their activity to produce progenitors, including short-term HSCs (ST-HSCs), lineage-restricted progenitors and terminally differentiated cells (Fig. 4).

Although the location of the stem cell niche implies some physical protection of the cells against physical and chemical detrimental agents, LT-HSCs acquires some unique properties, including distinctive DNA damage response (DDR), decreasing the chance of changes to their genome (Blanpain *et al.*, 2011). However, DNA repair can be considered as a two-edged sword in HSCs – for example DNA double-strand breaks in the niche are paired by non-homologous end joining, a DNA repair system, which is fast but error-prone and may lead to a leukemic transformation (Rossi *et al.*, 2008; Pawlows-

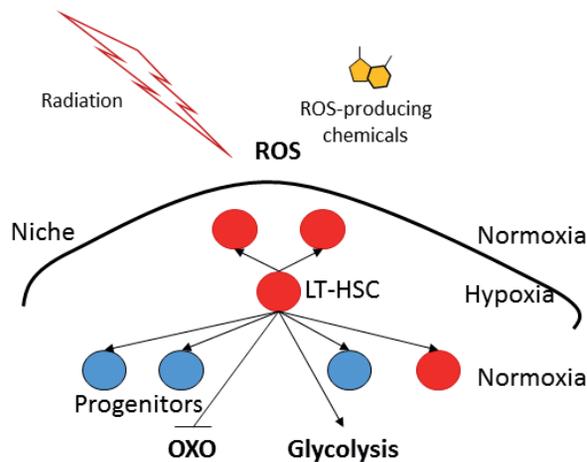


Figure 4. Long-term hematopoietic stem cells (LT-HSCs, red circles) reside in the bone marrow niche, a structure which shields the cells from detrimental external insults, including radiations and chemicals.

The metabolic activity of LT-HSCs is kept at minimum in the niche to avoid production of ROS and protect their DNA against detrimental factors. LT-HSCs essentially limit cellular division to self-renewal, but when needed they produce committed progenitor cells (blue circles), which can further differentiate. Hypoxia is typical for the niche to keep the ROS level low. HSCs in the niche preferentially use glycolysis as the source of energy than mitochondrial oxidative phosphorylation (OXO).

ka & Blasiak, 2015). Detailed study of the HSCs niche revealed that the cells reside in a hypoxic environment, which is in agreement with general protective role of the niche, as the low partial pressure of oxygen results in a low level of ROS (Harrison *et al.*, 2002). The consequences of hypoxic environment of the HSCs are not limited to their protection against detrimental action of ROS as hypoxia induces quiescence of these cells (Shima *et al.*, 2010).

Although hypoxia can contribute to better protection of HSCs against environmental insults, it creates a serious problem for the metabolism of these cells. A reduced availability of O₂ induces cellular and systemic response in many cells, including HSCs, and hypoxia inducible factor-1, HIF-1, plays a key role in this response (Semenza, 2010). This factor is responsible for quiescence of HSCs in hypoxia by forming heterodimers consisting of its two subunits, HIF-1 α and β , and by interaction with the 78 kDa glucose-regulated protein, GRP78 and its ligand, Cripto (Miharada *et al.*, 2012). As mitochondria are a rich source of ROS, HSCs display a low mitochondrial activity, which is associated with increased glycolysis (Wanet *et al.*, 2015). Active switch from oxidative to glycolytic metabolism is mediated by HIF-1, through the conversion of pyruvate into lactate by lactate dehydrogenase A (LDHA) or the process of pyruvate decarboxylation, in which pyruvate is converted into acetyl-CoA by the mitochondrial enzyme pyruvate dehydrogenase. As the switch between oxidative phosphorylation and glycolysis in ATP production is typical for cancer cells, HSCs have prerequisites required for functioning as leukemic cells.

Recently, it was shown that HIF-1 α , an oxygen-sensitive component of HIF-1, is transcriptionally activated by the homeobox protein Meis1 in human HPSCs (Kocabas *et al.*, 2015). This interaction was mediated by the cofactors Pbx1 and HoxA9. It seems important in the

context of HSPCs cancer transformation, as Meis1 was shown to be upregulated in acute leukemias (Kawagoe *et al.*, 1999; Eppert *et al.*, 2011; Becker & Jordan, 2011). Moreover, both Pbx1 and HoxA9 also can be implicated in leukemogenesis (Nakamura *et al.*, 1999; Aspland *et al.*, 2001). Therefore, hypoxic environment in the HPSCs niche can contribute to leukemic transformation of these cells and mechanisms of HIF-1 regulation can be important for leukemogenesis and detailed studies are needed to assess the potential of interaction between HIF-1 and BCR-ABL1 as well as its components, BCR and ABL1. In general, there is a need to establish the relationship between metabolic characteristics of HSCs in hypoxic microenvironment and their association with fundamental properties of these cells including self-renewal, quiescence, apoptosis and cancer transformation (Zhang & Sadek, 2014; Ryu *et al.*, 2015; Testa *et al.*, 2016).

To proliferate, HSCs activate the phosphatidylinositol 3-OH kinase (PI3K)/Akt/mTOR pathway with the involvement of growth factors and nutrients, including glucose (Yuan & Cantley, 2008). This activation is associated with ROS production or decreased ROS scavenging. As ROS are required for normal cellular homeostasis, their activity should be strictly controlled. Several molecules including antioxidant enzymes, DDR proteins, polycomb proteins and others are involved in this control (Suda *et al.*, 2011).

BCR-ABL1 AND ROS PRODUCTION

As we mentioned, elevated level of ROS can be associated with many cancers, but it is difficult to determine whether ROS belong to the reasons or consequences of the disease. It seems that both outcomes can occur in very many cases. If a cell is exposed to ROS, resulting in damage to a DNA repair gene, this will result in an increased chance of accumulating DNA damages upon the next cellular divisions, which can lead to genomic instability, increasing the probability of acquiring a mutation in cancer-related gene. DNA repair deficiency would result in a weaker control over intracellular ROS, leading to an elevated level of these species in cancer, despite the fact that the primary ROS, the reason of the cancer, are no longer present. This is a general scenario, but in certain cancers, the source of ROS can be attributed to some cancer-specific feature. This is the case in CML.

BCR-ABL1 was identified to stimulate ROS production in mouse-derived hematopoietic cells as this stimulation was blocked by imatinib (Sattler *et al.*, 2000). Therefore, BCR-ABL1, the cause of CML, induces ROS production leading to oxidative stress, typical for many cancers. When this stress was induced by hydrogen peroxide in non-transformed hematopoietic cells, several events typical for BCR-ABL1-expressing cells occurred. Therefore, that important work suggests at least two crucial facts. Firstly, CML can be initiated by anti-apoptotic and proliferative signaling of BCR-ABL1, but the development of the disease can be related to oxidative stress induced by BCR-ABL1. Secondly, the progression of CML can be influenced by chemicals modulating ROS levels, including small-molecular weight antioxidants.

Kim and coworkers showed that the Tyr177 residue in the BCR-ABL1 protein was a major regulatory site in the ROS production pathway in BCR-ABL1-expressing cells (Kim *et al.*, 2005). Two main mechanisms can be involved in enhanced ROS production by BCR-ABL1: increase in glucose metabolism and elevated activity of the electron transport chain in mitochondria (Kim *et al.*,

2005). The association between BCR-ABL1 and glucose pathway can be seen by the inhibition of glucose metabolism leading to a decreased level of protein phosphorylation. In general, the increase in ROS production in BCR-ABL1-positive cells can be dependent on the PI3K/mTOR signaling pathway and PI3K activation itself was reported to be sufficient for ROS overproduction in BCR-ABL1-expressing cells. As PI3K is involved in glucose uptake and metabolism, these results confirm that glucose metabolism is important for ROS production by BCR-ABL1. These important results obtained by Kim *et al.*, suggest possible mechanisms underlying mutual relationship between the activity of the BCR-ABL1 kinase and ROS production, which can be important for cancer biology and therapeutic targeting of the kinase in CML and other malignancies.

Increased ROS production stimulated by BCR-ABL1 can contribute to increased genomic instability in CML cells and this is sometimes interpreted as the consequence of the potential of BCR-ABL1 to induce genomic instability independently of its leukemogenic effect (reviewed in Quintas-Cardama & Cortes, 2009). However, it should be taken into account that such conclusion is based on the recognition of cancer phenotype onset, which depends on the detection threshold of facilities to be used for this purpose. Moreover, this statement should be rather limited to the leukemia-onset effect, as genomic instability and associated mutagenesis can be attributed to CML progression.

Ilaria *et al.* presented two mechanisms of increasing genomic instability by BCR-ABL1 (Ilaria *et al.*, 2002). One of them assumed the involvement of BCR-ABL1 in DNA repair, which is supported by the evidence of the participation of normal *c-ABL* in DNA repair (Ilaria *et al.*, 2002; Brain *et al.*, 2001). However, *c-ABL* can be involved in DNA repair, but is not indispensable for this process (Matt and Hofmann, 2016). This issue will be discussed in more details in the next chapter. In their other explanation of the involvement of BCR-ABL1 in genomic instability, Ilaria *et al.*, hypothesized that BCR-ABL1 can directly induce mutations at the level exceeding the repair capacity of the cell. In addition, an elevated expression of DNA polymerase β was observed in BCR-ABL1-expressing cells (Troung *et al.*, 2003; Canitrot *et al.*, 1999).

The association between BCR-ABL1-stimulated ROS production and mutagenesis is sometimes considered as a feed-forward loop: BCR-ABL1 increases the extent of ROS, which, in turn, attack its gene, inducing mutations. Mutated *BCR-ABL1* produces more ROS, leading to self-mutagenesis and an increase in genomic instability. Therefore, this feed-forward loop can be rather seen as a vicious cycle leading to apoptosis of BCR-ABL1-expressing cells. Because this is apparently not the case, there must be some control mechanisms of this self-mutagenesis and its influence on genomic stability. We and others showed that BCR-ABL1 could modulate DNA repair and this modulation was in many cases expressed as an increase in DNA repair efficacy, including efficacy of removing oxidative DNA damage (Slupianek *et al.*, 2013; Muvarak *et al.*, 2015; Nieborowska-Skorska *et al.*, 2006; Somsedikova *et al.*, 2014; Wang *et al.*, 2014; Takagi *et al.*, 2013; Brady *et al.*, 2003). Therefore, the modulation of DNA repair by BCR-ABL1 can be associated with the control of ROS-induced self-mutagenesis to adjust it to such level, which would stimulate cancer transformation of CML cells, but below the threshold, which cannot be tolerated by the cell, leading to its apoptosis. This picture is additionally complicated by the fact that

this extra DNA repair, stimulated by BCR-ABL1, can be unfaithful, leading to increased mutagenesis in BCR-ABL1-expressing cells, including the *BCR-ABL1* gene itself (Takagi *et al.*, 2013; Brady *et al.*, 2003). However, this represents rather a general conception and further studies are needed to shed light on this problem, which can be crucial for CML therapy in TKIs-resistant patients. Nevertheless, this seems to be an unsolved problem assuming that the CML cells population is homogenous. Therefore, a closer look should be taken at the intrinsic heterogeneity of CML cells.

ROS IN CHRONIC MYELOID LEUKEMIA STEM CELLS

Due to the cancer stem cells theory, cancer depends on a small population of “cancer stem cells (CSCs)”, which are responsible for cancer maintenance, especially in unfavorable conditions for its development, e.g. when an anticancer drug is present in its immediate environment. It is commonly accepted that CSCs constitute the root of CML, but their precise identification and characterization have not been done yet (reviewed in: Zhou *et al.*, 2015). It is accepted that CML cancer stem cells are within the Lin⁻CD34⁺CD38⁻ population, whereas CML progenitor cells (CPCs) are characterized by the Lin⁻CD34⁺CD38⁺ phenotype. The former can be responsible for the initiation and maintenance of the disease, whereas the latter – for its progression. It is important that general therapeutic strategy in CML with TKIs targets only actively dividing cells and CSCs are often quiescent.

Skorski and his laboratory showed that both CSCs and CPCs in CML displayed high levels of ROS, independently of whether they were treated with TKIs or not (Nieborowska-Skorska *et al.*, 2012; Nieborowska-Skorska *et al.*, 2013; Nieborowska-Skorska *et al.*, 2014; Bolton-Gillespie *et al.*, 2013). As mentioned above, PI3K is involved in increased ROS production

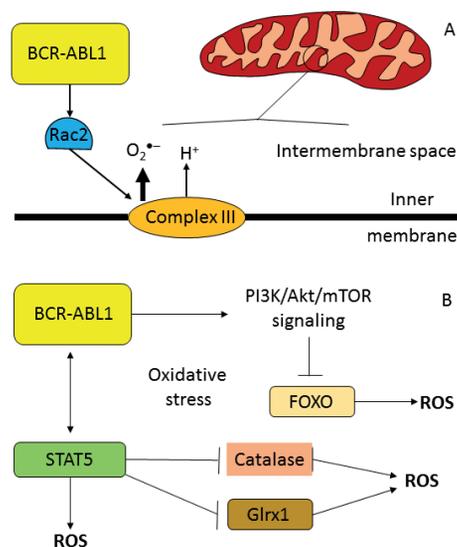


Figure 5. BCR-ABL1 interacts with the Rac2 GTPase and stimulates ROS production via the mitochondrial electron chain complex III (A). BCR-ABL1 can stimulate the Akt signaling pathway, which in turn inhibits forkhead O transcription factor (FOXO), essential for defense against oxidative stress (B).

In BCR-ABL1+ cells STAT5 transcriptionally regulates genes causing the enhanced ROS production. Prolonged stimulation of STAT5 by BCR-ABL1 downregulates two antioxidant enzymes: catalase and glutaredoxin-1 (Glx1), which results in an enhanced level of ROS.

in BCR-ABL1-expressing cells. Further research showed that RAC2, a PI3K effector, could be responsible for ROS increase in CSCs and CPCs by changing the electron flow through respiratory complex III (Nieborowska-Skorska, 2012) (Fig. 5).

Next, it was shown that the activation of the AKT protein, the key factor in DNA damage response signaling, could be inhibited in BCR-ABL1-expressing cells (Kim *et al.*, 2005). Inhibition of AKT activation was observed in imatinib-treated CSCs and CPCs as compared with their imatinib-naïve counterparts (Nieborowska-Skorska *et al.*, 2014). Searching for the mechanisms of these changes led to unexpected conclusion that AKT regulated cellular reaction to oxidative DNA damage in CPCs, but not in CSCs. It is somehow surprising, as the AKT kinase plays the major role in DNA damage signaling also in stem cells and targeting this kinase is considered as a therapeutic strategy to eradicate the most primitive CSCs (Shimura *et al.*, 2012).

ROS IN CHRONIC MYELOID LEUKEMIA THERAPY WITH TYROSINE KINASE INHIBITORS

Apart from the involvement of ROS in physiological cellular signaling, they can contribute to cancer transformation induced by oncogenic tyrosine kinases (Bourgeois *et al.*, 2013). As mentioned above and shown in many studies, imatinib and other TKIs, decreased ROS level in BCR-ABL1-positive cells and this effect was underlined rather by the interaction of TKIs with the kinase, than TKIs scavenging effect or their interaction with cellular antioxidative system. Therefore, CML therapy with TKIs can be strengthened by the use of ROS scavengers. It was demonstrated that N-acetyl cysteine, a ROS inhibitor, increased apoptosis induced by imatinib in BCR-ABL1-positive cells, both sensitive and resistant to this drug (Rakshit *et al.*, 2009). This effect was associated with the production of nitric oxide mediated by endothelial nitric oxide synthase. However, these studies showed that glutathione, which is a ROS scavenger, partially decreased this effect, suggesting that the augmentation of the effect of imatinib by ROS inhibitors can result from different mechanism(s) than direct ROS scavenging.

Histone deacetylase inhibitors (HDACi), which induce more open structure of chromatin, enabling easier access of proteins involved in cell differentiation and death, were showed to potentiate BCR-ABL1-positive cells killing by KW-2449, a TKI targeting BCR-ABL1 and Src kinases, in both imatinib-sensitive and resistant cells (Nguyen *et al.*, 2011). This effect was associated with an increased ROS production and enhanced extent of DNA double strand breaks, evaluated indirectly by H2AX phosphorylation. Similar results were obtained for a combined treatment of CML cells with another HDACi, vorinostat and BI2536, a PLK1 (polo-like kinase 1) (Dasmahapatra *et al.*, 2013). The effect observed in that study was independent of the imatinib-sensitivity/refractory status of the cells.

These and other results indicate that ROS can contribute to killing BCR-ABL1-positive cells, possibly via the mechanisms associated with the increase in genomic stability to the level, which cannot be tolerated by the cell and leads to its death. These mechanisms can include several processes, including ROS-induced damage to cellular structures, DNA damage, interference with BCR-ABL1 pro-survival signaling pathways and DNA repair, as well as the modulation of cell cycle checkpoints, which in fact is involved in all these effects.

Therefore, ROS can play a dual role in the fate of CML cells: they can promote their genomic instability and accelerate CML progression associated with acquiring TKI resistance and resistance to apoptosis, but on the other hand they can contribute to CML cell killing. Therefore, there seems to be a subtle balance between pro- and anti-malignant effects of ROS in CML cells. Consequently, using ROS scavengers in combination with TKIs and other agents mediating CML cells elimination should be carefully planned to avoid unwanted effects.

In the light of BCR-ABL1-associated ROS production, imatinib-based CML therapy can be supplemented with chemicals that can inhibit the activity of antioxidant enzymes. The expression of a key enzyme of this system, SOD1, was reported to increase in CD34+ subpopulation of BCR-ABL1-positive K562 cells as compared with the remaining, CD34-, subpopulation (Liu *et al.*, 2011). Therefore, increased expression of this antioxidant enzyme could be attributed to leukemic CSCs, which are considered to be responsible for the disease relapse and imatinib resistance. Eradication of CSCs is the primary goal of CML therapy. It was observed that the knockdown of the *SOD1* gene did not change the growth of the CD34+ BCR-ABL1-expressing cells, but sensitized them to imatinib (Liu *et al.*, 2011). Interestingly, it was observed that NAC, a ROS scavenger, inhibited the pro-apoptotic effect of SOD1 knockdown, which suggested that increased SOD1 activity could be really associated with CML stem cells. NAC reversed also the inhibition of proliferation of CML cells evoked by oxidative DNA damage induced by a high level of ROS (Wu *et al.*, 2015).

The balance between pro- and anti-carcinogenic effects of ROS in CML cells seems especially important in TKIs resistance, as imatinib-resistant cells displayed a higher level of ROS than their sensitive counterparts. We showed lastly that this result could be associated with mitochondria-mediated effects, including mitochondrial DNA damage, mitochondrial potential and expression of mitochondrial genes encoding proteins of the electron transport chain (Blasiak *et al.*, 2016; Synowiec *et al.*, 2015).

CONCLUSIONS AND PERSPECTIVES

BCR-ABL1 is the hallmark and cause of chronic myelogenous leukemia, conferring the advantage of growth and proliferation on CML cells. However, the progression of the disease may not necessarily be related directly to this fusion protein. Instead some effects associated with genomic instability, provoked by increased ROS production, can play an important role. That ROS overproduction can be related to BCR-ABL1 signaling. ROS produced by BCR-ABL1 can damage DNA, increasing genomic instability and promote BCR-ABL1 self-mutagenesis. However, there must be mechanisms preventing BCR-ABL1-directed cellular self-destruction. BCR-ABL1 can modulate DNA repair, but it can be an error-prone repair, contributing to BCR-ABL1 self-mutagenesis. Therefore, the balance between BCR-ABL1-induced ROS production and the extent of genomic instability is essential for survival of CML cells.

The problem of ROS-induced self-mutagenesis of BCR-ABL1 is directly associated with CML therapy. Imatinib and other TKIs, are effective in eliminating most of CML cells, but some of them survive TKIs treatment and cause CML relapse. They are CML stem cells, but their nature has not been known yet. The hierarchy of hematopoietic lineage is from hematopoietic

stem cells, through transient amplifying cells and terminally differentiated cells found in blood, bone marrow, thymus and nervous tissue. Similarly, CML cells can contain a small subpopulation of cancer initiating cells, characterized by a high stem potential and responsible for the induction and maintenance of the disease and cancer progenitor cells, responsible for CML phenotype. It was suggested that some phenomena related to BCR-ABL1 mutagenesis, including the repair of ROS-induced oxidative DNA damage, can have different effects in CSCs and CPCs and this can contribute to “sustained” mutagenesis provoked by BCR-ABL1, supporting cancer transformation, but not leading to cell destruction. It is therefore tempting to supplement CML therapy with ROS-scavengers to inhibit BCR-ABL1 self-mutagenesis and decrease the level of genomic instability. However, increasing ROS level in BCR-ABL1 positive cells by several mechanisms, including the inhibition of the activity of antioxidant enzymes, can be effective in eradicating CML cells. Therefore, understanding the mechanism of DNA damage reaction in CML stem cells and their progenitors is crucial for the success of CML therapy.

It was shown that agonists of peroxisome proliferator-activated receptor- γ (PPAR γ) represented by glitazones (antidiabetic drugs) decreased the population of LT CML stem cells of CML patients, whereas imatinib alone was not able to significantly reduce this population (Prost *et al.*, 2015). The combination of pioglitazone with imatinib improved the results of the treatment. In searching of mechanisms underlying observed effects, it was shown that the activation of PPAR γ was associated with downregulation of STAT5 and its two downstream targets, HIF-2 α and CITED2, which are essential for the stemness of LT-HSCs (Du & Jang, 2013; Wierenga *et al.*, 2014). Residual CML patients treated temporarily with pioglitazone displayed sustained complete molecular response up to about 5 years after withdrawal of the drug. This seminal work confirmed the relevance of cancer therapy aimed at cancer stem cells. Another supporting evidence came from the results of recent work showing that perturbation of the p53 and c-MYC protein resulted in selective killing and differentiation of LT CML stem cells, while normal HSCs were unaffected (Abraham *et al.*, 2016).

The identification of the most primitive CML stem cells is essential for the complete understanding of the pathogenesis of this disease and its therapy. However, as the most primitive cancer stem cell corresponding to a fertilized egg or embryonic stem cell could be rather a conception, than a real entity, current therapeutic strategies should be planned with taking into account intrinsic heterogeneity of CML cells population. This heterogeneity is also associated with different ROS production and their scavenging in CSCs and CPCs. This may create somehow puzzled situation, in which using particular antioxidant or ROS-inducer, can cause a reversed effects in this two kinds of cells. Therefore all clinical and molecular markers of CML should be analyzed at the same time to have a full picture of changes occurring after the treatment.

Conflicts of interest

The authors declare that there is no conflict of interest regarding this manuscript.

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