GENOMIC INSTABILITY ORIGINATES FROM
LEUKEMIA STEM CELLS IN A
MOUSE MODEL OF CML-CP

A Dissertation
Submitted to
the Temple University Graduate Board

In Partial Fulfillment
of the Requirements for the Degree of
DOCTOR OF PHILOSOPHY

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May 2013

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ABSTRACT

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Doctor of Philosophy

Temple University 2013

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In chronic myelogenous leukemia (CML), activation of BCR-ABL, the product of the *bcr-abl* chimeric gene, leads to constitutive activation of pathways that increase genomic instability through endogenous production of reactive oxygen species (ROS) that cause oxidative DNA damage and inactivate the function of repair proteins leading to unfaithful DNA repair. If misrepaired, oxidative DNA damage, such as 8-oxoguanine (8-oxoG), may result in point mutations and/or DNA double-strand breaks (DSBs) leading to drug resistance to the BCR-ABL kinase inhibitor imatinib mesylate (IM) and accumulation of chromosomal aberrations associated with malignant CML progression from a benign chronic phase (CP) to a fatal blast phase (BP).

To determine which population of CML-CP cells, leukemia stem cells (LSCs) and/or leukemia progenitor cells (LPCs), displays elevated levels of ROS and oxidative DNA damage, and whether these elevated levels of ROS and oxidative DNA damage in CML-CP subpopulations result in the accumulation of genomic instability, we employed the tetracycline-inducible SCLtTA/BCR-ABL transgenic mouse model. We showed that LSCs, including the quiescent subpopulation, but not LPCs, displayed elevated levels of ROS and oxidative DNA damage, perhaps due to deregulated expression of genes involved in ROS metabolism, resulting in genomic instability manifested by both point

mutations and genetic alterations. We also examined the effect of IM on ROS, oxidative DNA damage and genomic instability displayed by CML-CP subpopulations, and determined that elevated ROS and oxidative DNA damage were not inhibited by IM in quiescent LSCs, nor was genomic instability and deregulated gene expression prevented.

To explore underlying mechanisms, i.e. BCR-ABL expression levels, by which CML-CP cells accumulate genomic instability, we examined the effect of low and high BCR-ABL expression on ROS and oxidative DNA damage in BCR-ABL-transduced human CD34⁺ cells. We detected elevated ROS and oxidative DNA damage in high BCR-ABL-expressing CD34⁺ cells compared to low BCR-ABL-expressing cells. Furthermore, BCR-ABL exerted a kinase-dependent effect on ROS-dependent DNA damage.

These data support the hypothesis that genomic instability may originate from LSCs, but do not exclude the potential role of LPCs, and may have important clinical implications for CML treatment since additional genetic aberrations that encode primary resistance may protect LSCs, including the quiescent subpopulation, from eradication by tyrosine kinase inhibitors (TKIs), and the continuous accumulation of genetic errors may trigger disease relapse and progression.

ACKNOWLEDGMENTS

First and foremost, I would like to thank my mentor Dr. Tomasz Skorski, who provided exceptional support and guidance in the pursuit of my Ph.D. From Dr. Skorski I learned the importance of experimental design and analysis, and to think independently. Additionally, he afforded numerous opportunities for me to present our research, as well as learn from the research of others, which significantly contributed to my growth as a scientist. His hard work and dedication in studying genomic instability in CML has been inspiring, and I am forever thankful for the opportunity to work under his instruction.

I would also like to thank both past and present members of Dr. Skorski's laboratory, including Shuyue Ren, Mateusz Koptyra, Artur Slupianek, Margaret Nieborowska-Skorska, Kimberly Morales, and Yashodhara Dasgupta, whose mentorship, knowledge, support and friendship have been invaluable to my growth both professionally and personally.

I would like to extend my gratitude to the faculty and staff from the Department of Microbiology and Immunology for providing an outstanding educational experience in the classroom, as well as their time and support throughout my years as a graduate student.

I am abundantly grateful to the distinguished scientists who served as members of my Doctoral Examining Committee: Tomasz Skorski, M.D., Ph.D., D.Sc., Alexander Tsygankov, Ph.D., Kenneth Soprano, Ph.D., Dan Liebermann, Ph.D., and Scott Shore,

Ph.D. Their time, energy, insight and encouragement have been instrumental in enhancing my analytical skills and it has been a privilege to learn from their expertise.

I would also like to thank all who are mentioned within this work for their collaboration and expertise.

Lastly, I am eternally grateful for the unconditional love, support and patience from my family throughout my graduate studies, including Michael, Mom, Dad, Stephan, Brooke, Olivia, Reilly, Barbara, Mark and Jude.

For my mother, Beth, whose continuous encouragement and support to pursue higher education enabled me to achieve this milestone.

For my husband, Michael, whose patience and love are boundless.

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CHAPTER 1

INTRODUCTION

Chronic Myelogenous Leukemia (CML)

Chronic myelogenous leukemia (CML) is a clonal myeloproliferative disorder initiated by the acquisition of the *bcr-abl* translocation in the hematopoietic stem cell (HSC) and propagated by the hematopoietic progenitor cell (HPC) that is characterized by three stages of disease progression: chronic phase (CP), accelerated phase (AP), and blast phase (BP). CML-CP is characterized by the expansion of myeloid granulocytes in the bone marrow, spleen, and peripheral blood [1]. More specifically, discordant maturation of leukemia stem cells (LSCs) with a slight delay in maturation within the myeloid compartment combined with a decrease in apoptosis of myeloid cells contributes to increased myeloid mass in the bone marrow where all stages of myeloid maturation are present; the result is a hypercellular bone marrow that is devoid of fat [2]. Additionally, defective adherence of immature myeloid cells to the bone marrow stroma results in their premature release into circulation leading to the expansion of myeloid cells into the spleen and peripheral blood [3]. Furthermore, plasma levels of growth factors are significantly increased to modulate angiogenesis and increase vascularity within the bone marrow [4]. Regardless of the biological effects of CML-CP, the majority of patients have fully competent immune systems, are asymptomatic, and often diagnosed during routine physical examination [5, 6]. However, when symptoms ensue, CML-CP patients may experience fatigue, weight loss, bleeding, sweats, and abdominal discomfort due to splenomegaly and hepatomegaly [7].

After 3-5 years, the majority of CML-CP patients will progress to a transitional CML-AP, which is characterized by an increasing arrest of myeloid maturation and associated with the detection of an increased number of cytogenetic abnormalities [8]. Within 1-2 years, CML-AP patients will progress to a terminal CML-BP, characterized by an uncontrollable, rapid expansion of chemotherapy-refractory blast cells associated with the emergence of additional genetic lesions, and symptoms such as fever and bone pain, increased risk of infection and bleeding, and massive splenomegaly [1, 8, 9]. CML-BP resembles acute leukemia and can be characterized as myeloid in two-thirds of patients or lymphoid in one-third of patients, but is rarely characterized as a mixed myeloid-lymphoid phenotype [9-11]. CML-BP persists for approximately 3-6 months ultimately ending in death [8].

In the United States, approximately 5,430 new cases of CML are estimated for 2012, of which more than 11% are estimated to end in death [12]. CML represents approximately 12% of all leukemias and 15-20% of adult leukemias, affecting 1.6 out of 100,000 adults every year with a slight male preponderance (1.4 male:1 female) [8, 12]. CML rarely affects children and incidence increases with age; the median age at diagnosis is 65 years [13]. Though CML has no known etiology there is increased incidence in those exposed to radiation [14]. The median survival of CML patients without treatment is approximately 5-6 years [10].

bcr-abl: t(9;22)

In 1960, Nowell and Hungerford detected a similar minute chromosome, termed the Philadelphia chromosome, in peripheral blood cells and/or bone marrow cells among

7 patients diagnosed with CML; however, no other chromosomal abnormality was detected in these patients suggesting a causal relationship [15]. More than a decade later, Janet Rowley deduced that the Philadelphia chromosome was the result of a translocation between the long arms of chromosomes 22 (22q) and 9 (9q) resulting in a shortened chromosome 22q⁻ and an elongated chromosome 9q⁺; results were consistent in 9 patients analyzed with quinacrine fluorescence and Giemsa staining [16]. In 1982, de Klein et al. discovered that the translocation between chromosomes 22q and 9q was reciprocal through detection of the *c-abl1* gene from 9q on 22q⁻ in CML patients, suggesting a role for the *abl* gene in CML [17], and just two years later, Groffen et al. detected a sequence of the chromosomal breakpoint cluster region (from the *bcr* gene) on 22q⁻ in seventeen of nineteen CML patients, suggesting a role for the *bcr* gene in CML [18]. Shortly thereafter, the juxtaposition of the *bcr* and *abl* genes on 22q⁻ was confirmed, as well as the determination that this genetic juxtaposition resulted in a fused transcript and subsequent expression of the 210 kilodalton BCR-ABL protein [19, 20]. Research since has broadly focused on deducing pathways regulated by p210 BCR-ABL.

p210 BCR-ABL

BCR-ABL is a cytoplasmic non-receptor tyrosine kinase (TK) that harbors deregulated kinase activity manifested by constitutive autophosphorylation leading to the recruitment of adaptor proteins, phosphorylation of signaling molecules and activation of downstream signaling events required for oncogenic transformation [21]. More specifically, BCR-ABL kinase upregulates cell proliferation through enhanced expression of phosphoproteins and diminished expression of tyrosine phosphatases [22], decreases

apoptosis through modulation of apoptotic pathways [23-29], increases cytokine-independent growth through enhanced expression of growth factors (GFs) and their receptors [30-36], decreases adhesion to bone marrow stroma and produces cytoskeletal abnormalities [37-41].

BCR promotes the dimerization/tetramerization of BCR-ABL thereby facilitating autophosphorylation and constitutively activating ABL tyrosine kinase resulting in an increase in phosphotyrosine residues on BCR-ABL creating binding sites for the SH2 domains of other proteins, which can then be phosphorylated on tyrosine residues and thus activated [42, 43]. There are a variety of cell signaling pathways and cytoskeletal proteins through which BCR-ABL chronically activates and mediates transformation, proliferation, survival, and adhesion: RAS-MAPK, JAK-STAT, and PI3K/AKT [22].

Treatment of CML

In the 1950s, CML patients were treated with busulfan, an oral alkylating agent that damages and disrupts DNA structure leading to cytotoxicity; however, busulfan was associated with severe and prolonged myelosuppression, and it took another 20 years for treatment with fewer adverse side effects to be employed [44]. Hydroxyurea was largely used as first-line treatment in the 1970s, which produced rapid hematologic responses and increased survival in CML patients over busulfan. Although both busulfan and hydroxyurea induced complete hematologic responses (CHR) in CML patients, neither prevented patients from progressing to terminal CML-BP [45].

Interferon-alpha (IFN- α) exerts a wide range of anti-leukemic effects on the immune system (anti-proliferative, anti-angiogenic) and greatly enhances survival of

CML patients over cytotoxic agents [46]. IFN- α induces complete cytogenetic responses (CCyR) in CML patients [47-49], which are enhanced further in combination treatment with cytarabine, a subcutaneously-administered form of hydroxyurea [50, 51]. However, IFN- α has moderate to severe side effects that may not be tolerated well by many.

While the only curative treatment for CML is bone marrow transplant, the discovery of BCR-ABL-targeted therapy with imatinib mesylate (IM; Gleevec[®]) has revolutionized the treatment of CML. IM is a potent and selective inhibitor for ABL tyrosine kinase, platelet-derived growth factor receptor (PDGFR) and c-Kit receptor [52-54]. In CML, IM binds to the ATP-binding site of BCR-ABL tyrosine kinase and stabilizes it in its inactive conformation thereby preventing its catalytic activity of auto-phosphorylation and tyrosine phosphorylation of downstream substrates [55]. Ultimately, IM induces apoptosis and/or exerts an anti-proliferative effect on dividing CML cells [56, 57]. IM treatment has achieved complete hematologic responses (CHRs) in the majority of CML-CP patients, cytogenetic responses (CyRs) in about half of CML-CP patients, and molecular responses (MR) in a small cohort of CML-CP patients [58]; additionally, long-term treatment with IM has prolonged survival and responses in CML-CP patients [59-61]. Furthermore, IM has induced CHRs and CyRs in patients who have progressed to CML-AP/BP [62, 63].

Despite successful clinical outcomes achieved in CML patients treated with IM, there are multiple mechanisms of both primary and secondary resistance that cause patients to relapse and progress to advanced disease phases. Kinase domain mutations have been detected in 90% of CML patients who relapse [64]. More specifically, varying levels of resistance to IM are conferred by more than 90 different point mutations of

more than 55 residues in the *bcr-abl* gene that disrupt binding of IM to the ABL tyrosine kinase (TK) domain, thereby preventing kinase inhibition [65-68]. Other mechanisms of IM resistance include gene amplification [69], overexpression of the BCR-ABL transcript [55], overexpression of Src family kinases [70, 71], overexpression of efflux proteins [72], under-expression influx proteins, and clonal cytogenetic evolution [73]. Upon developing resistance to IM, dose escalation with IM or treatment with second-generation tyrosine kinase inhibitors (TKIs) may be employed.

Dasatinib (Sprycel[®]) is a dual Src/Abl kinase inhibitor that is significantly more potent than IM by binding to both the inactive and active conformations of BCR-ABL [74-76]; in addition, dasatinib targets a more primitive population of progenitor cells than IM, but not the quiescent fraction [77]. Nilotinib (Tasigna[®]) is a derivative of IM that also inhibits the inactive conformation of BCR-ABL, however, displays a better fit in the ATP-binding pocket, and, therefore, exerts greater potency than IM [75, 78]. Both dasatinib and nilotinib induce hematologic, cytogenetic, and molecular responses in CML patients of all phases and in some who display various mechanisms of IM resistance, with the exception of the T315I kinase domain mutation [79-86].

Reactive Oxygen Species (ROS)

Reactive Oxygen Species (ROS) are molecules containing unpaired electrons within a molecular orbital that confer the reactivity of the molecule [87]. ROS play roles in biological functions and can act as second messengers to regulate activities of redox-sensitive enzymes and signaling molecules to promote cell growth and regulate gene expression [88, 89]. ROS are produced endogenously, perhaps in response to

hematopoietic growth factors, through oxidative metabolism and the electron transport reaction by which electrons are leaked to and accepted by molecular oxygen (O_2) [89-93]. Univalent, bivalent, and trivalent reduction of molecular oxygen (O_2) results in the production of superoxide anion radical ($\bullet O_2^-$), hydrogen peroxide (H_2O_2), and hydroxyl radical ($\bullet OH$), respectively [94].

The one-electron reduction of molecular oxygen (O_2) yields the production of superoxide anion radical ($\bullet O_2^-$). Though short-lived, $\bullet O_2^-$ can exert damage *in vivo*. $\bullet O_2^-$ can behave as a reducing agent, through a metal-catalyzed Haber-Weiss reaction, or an oxidizing agent. When $\bullet O_2^-$ behaves as a reducing agent, the catalytic activity of metal ions, i.e. Fe^{3+} , bound to biomolecules mediated by $\bullet O_2^-$ generates hydroxyl radical ($\bullet OH$), which then exerts a damaging effect on a target molecule, i.e. DNA. When $\bullet O_2^-$ behaves as an oxidizing agent, Cu^{2+} or Fe^{3+} catalyzes the decomposition of $\bullet O_2^-$ to O_2 , where $\bullet O_2^-$ is a precursor to Cu^{3+} and Fe^{4+} formation, which are strong oxidants involved in oxidative damage [95-98].

The one-electron reduction of superoxide anion radical ($\bullet O_2^-$) yields the production of hydrogen peroxide (H_2O_2), however, $\bullet O_2^-$ also can be dismutated to H_2O_2 via superoxide dismutases (SODs) [99]. H_2O_2 is relatively stable by comparison to $\bullet O_2^-$, and can both induce tyrosine phosphorylation and inhibit protein tyrosine phosphatase (PTPase) activity; in fact, oxidation of cysteine residues in the active site of PTPases leads to loss of enzymatic activity [100, 101].

The one-electron reduction of hydrogen peroxide (H_2O_2) yields the production of hydroxyl radical ($\bullet OH$), however, the metal-catalyzed reduction of H_2O_2 via the Fenton reaction also leads to the generation of $\bullet OH$ [102-106]. $\bullet OH$ reacts by one of three

mechanisms: hydrogen abstraction, electron transfer, and addition reactions; for example, $\bullet\text{OH}$ adds to the double bonds of DNA bases or abstracts a hydrogen atom from each of the 5 carbon atoms of 2' deoxyribose [107]. Most often, however, $\bullet\text{OH}$ abstracts carbon-bound hydrogen atoms non-selectively to produce another radical of lower reactivity [91]. $\bullet\text{OH}$ is highly reactive and therefore reacts close to its site of formation. $\bullet\text{OH}$ attack on membrane lipid can lead to severe damage of the membrane, whereas attack on DNA bases or the deoxyribosyl backbone of DNA can lead to the generation of oxidative products resulting in damaged bases or strand breaks, respectively [108].

In normal cells, ROS are counteracted by antioxidants that quench or reduce ROS to less reactive substrates to prevent damage of biomolecules, and reduce tyrosine phosphorylation thereby counteracting the signaling effects of ROS. Although there are multiple systems, both enzymatic and non-enzymatic, involved in ROS detoxification, ROS can interact with and damage cellular components, such as lipids, proteins, DNA bases and the deoxyribosyl backbone of DNA, leading to base modification and/or DNA double-strand breaks (DSBs), and inactivate the function of DNA repair proteins leading to unfaithful repair and genomic instability [92, 106, 109]. Altogether, oxidative stress can lead to DNA lesions and genomic mutations [110].

There are many sources of electron transport and ROS generation within the cell: mitochondria, membrane NADPH oxidases, cytochrome P450, xanthine oxidase, microsomes and peroxisomes [111-113]. The majority of superoxide anion radical ($\bullet\text{O}_2^-$) production occurs within the mitochondria, which is responsible for ATP production and cellular respiration through the electron transport chain (ETC) where electrons are passed between molecules to produce energy. Even under normal conditions, redox carriers

within the mitochondrial ETC can leak single electrons to molecular oxygen (O_2) and convert it into $\bullet O_2^-$ [114-116]. The primary site of damage from mitochondrial $\bullet O_2^-$ is mitochondrial DNA (mtDNA), which, if extensive, will eventually shut down the mitochondria. The major sites of ROS production within the mitochondrial ETC are Complexes I through V, however, there are many other sources of ROS present within the mitochondria with varying sub-mitochondrial localization [117-120].

NADPH oxidases are plasma membrane-associated enzymes that, when activated, take NAD(P)H from the hexose monophosphate shunt in the cytoplasm and pass electrons to molecular oxygen (O_2) to produce superoxide anion radical ($\bullet O_2^-$) within the plasma membrane or to its outer surface [121, 122]. NADPH oxidases generate significant amounts of ROS and play an important role in the respiratory burst of immune cells providing oxidizing agents for microbicidal action, as well as exerting potential damage to molecules and cellular components [123, 124]. Cytochrome P-450, whose primary role is to detoxify compounds into less toxic products through oxidation and hydroxylation, is the terminal component of the monooxygenase system found within the endoplasmic reticulum (ER); during these oxidation and hydroxylation reactions, electrons may be leaked onto O_2 leading to the formation of $\bullet O_2^-$ [125]. Xanthine oxidase is a molybdenum iron-sulfur flavin hydroxylase that catalyzes the hydroxylation of purines, for example, the reaction of hypoxanthine to xanthine, resulting in the reduction of O_2 to $\bullet O_2^-$, and xanthine to uric acid, resulting in the reduction of O_2 to hydrogen peroxide (H_2O_2) [126, 127]. Microsomes produce the majority of H_2O_2 at hyperoxia sites and peroxisomes produce H_2O_2 via oxidation of fatty acids [98, 128].

Oxidative DNA Damage and Repair

DNA damage can induce transcriptional changes, activation of cell cycle checkpoints, DNA repair and/or apoptosis [92]. There are multiple pathways that exist to repair DNA damage, yet each DNA repair mechanism targets a specific type(s) of DNA damage. In normal cells, DNA damage response pathways are tightly regulated, however, misrepair of DNA damage may result in mutation and disease [129, 130].

8-oxoguanine (8-oxoG) and Base Excision Repair (BER)

Due to an electron-rich purine structure and the lowest oxidation potential of all four nucleotides, guanine is preferentially attacked by free radicals [131, 132]. More specifically, hydroxyl radical ($\bullet\text{OH}$) can add to guanine at carbon 4, 5, and 8 positions; however, the one-electron oxidation of the hydroxylated C8 radical yields 7,8-dihydro-8-oxoguanine (8-oxoguanine; 8-oxoG) [133-135]. 8-oxoG is easily formed, pro-mutagenic, and has an even lower oxidation potential than guanine thereby permitting an even higher reactivity with free radicals [136-140]. 8-oxoG is produced abundantly *in vivo* and therefore is used as a biomarker of oxidative DNA damage [140-142].

Repair of DNA base damage occurs primarily by base excision repair (BER), however, misrepair could result in mutation, such as base substitution and deletion, thereby promoting carcinogenesis [143-145]. BER is stimulated upon alkylation, oxidation, or deamination of bases forming non-bulky lesions [130]. Glycosylase and endonuclease enzymes are responsible for the repair of human 8-oxoG lesions [146]. More specifically, oxidized guanine is recognized and cleaved by a DNA glycosylase (OGG1, OGG2) and released from the sugar-phosphate backbone; then the one-nucleotide gap is filled by Polymerase β and ligated [130]. Misrepair of 8-oxoG can

result in loss of base pairing specificity, misreading of adjacent pyrimidines, or insertion of adenine opposite the lesion resulting in GC→TA transversions [135, 147]. If misrepaired, 8-oxoG can even result in the formation of DNA double-strand breaks (DSBs) [106, 148].

DNA Double-Strand Breaks (DSBs) and Repair

DNA double-strand breaks (DSBs) are the most dangerous type of DNA damage, and may result from both exogenous sources, i.e. irradiation and chemotherapy, and endogenous sources, i.e. recombination and ROS [129]. In response to DSBs, ATM kinase induces cell cycle arrest by activation of cell cycle checkpoints through downstream effectors that mediate repair, arrest or apoptosis [149, 150]. There are two primary mechanisms of DSB repair: homologous recombination (HR) and non-homologous end joining (NHEJ). HR is a highly accurate pathway that uses homologous DNA as a template, whereas NHEJ directly ligates the broken ends of DNA without using any DNA template and may be accompanied by the loss of a few or long stretches of nucleotides [129, 130, 151-153]. In fact, Karanjawala et al. showed that both free radicals and aberrant NHEJ result in increased DNA DSBs [106]. Repair pathway selection depends, in part, on cell cycle stage; NHEJ is the predominant DSB repair pathway in G₀, G₁ and early S phase, whereas HR is most prevalent during S and G₂ phases [130, 154]. ATM/ATR-dependent phosphorylation of histone H2AX at Ser139 (γ-H2AX) rapidly occurs around DSBs and spreads into adjacent chromatin to attract downstream proteins for efficient DSB repair [155-157]. Therefore, DSB repair can be inferred from the formation of foci containing DNA repair-associated proteins and chromatin modifications, i.e. γ-H2AX, where the number of repair foci correlates with

the number of DSBs [150, 158]. Aberrations in pathways regulating the DNA damage response include delayed checkpoint activation, which may result in the misrepair of DSBs leading to chromosomal aberrations [130, 150].

BCR-ABL-induced ROS-dependent DNA Damage

BCR-ABL displays chronic activation of signaling pathways (RAS, PI3K, STAT) that lead to elevated levels of ROS and create an imbalance between ROS production and ROS scavenging. In fact, BCR-ABL mimics some signaling events activated by growth factors (GFs) and growth factor receptors (GFRs), and activation of BCR-ABL, GFs and GFRs coincides with an increase of intracellular ROS levels in hematopoietic cells [90, 159, 160]. Additionally, the tyrosine phosphorylation patterns of signaling proteins induced by BCR-ABL transformation and hydrogen peroxide (H_2O_2) stimulation overlap; in contrast, cellular PTPase activity is decreased by both BCR-ABL and H_2O_2 in hematopoietic cell lines [160]. Increase in ROS is believed to be due directly to BCR-ABL kinase activity since both ROS and BCR-ABL kinase activity are inhibited by IM [160], and it appears that increased ROS amplify BCR-ABL signaling through a positive feedback loop. However, ROS levels also may be regulated partially by the bone marrow microenvironment, which provide additional stimuli for survival and self-renewal.

Elevated ROS in BCR-ABL-transformed cells depend on mechanisms that regulate glucose metabolism and is linked to the mitochondrial electron transport chain (ETC) [160, 161]; furthermore, BCR-ABL-transformed cells exert a “Warburg effect”, which contributes to elevated ROS production through hyperactive glucose metabolism

thereby fueling the mitochondrial ETC [162]. Additionally, BCR-ABL-transformed cells enhance and regulate glucose uptake [163, 164].

BCR-ABL induces elevated levels of intracellular ROS through increased glucose metabolism in a PI3K/mTOR-dependent manner [161, 165]. Furthermore, BCR-ABL activates PI3K/AKT and RAS/ERK pathways through recruitment of GRB2/GAB2 complex to autophosphorylated residue Y177 in BCR, and GAB2 phosphorylation-dependent activation of PI3K is associated with superoxide formation [166]. BCR-ABL regulates expression of both MYC and HIF-1, which transcriptionally control many glycolytic enzymes, through RAS and AKT, and PI3K/mTOR activation, respectively [167-170]. Lastly, complex formation of BCR-ABL with VAV leads to activation of RAC, an important regulatory subunit of NADPH oxidase [171, 172].

BCR-ABL stimulates genomic instability through enhanced DNA repair, prolonged activation of the G₂/M checkpoint to extend time for DNA repair, and inhibition of pro-apoptotic pathways [173-181]. More specifically, BCR-ABL elevated ROS levels resulting in 8-oxoG and DSB formation, and promoted enhanced yet unfaithful DNA repair resulting in mutation [182-184]. Therefore, it seems that BCR-ABL acquires more mutations than non-transformed cells because there is greater DNA damage and more enhanced yet less faithful DNA repair. Altogether, BCR-ABL kinase chronically activates survival pathways (RAS, PI3K, and STAT) [92], stimulates ROS production that enhances signaling and causes DNA damage in CML cells [160, 182, 185, 186] and alters DNA repair mechanisms [160, 174-177, 187-191] resulting in genomic instability responsible for mutation associated with drug resistance and disease progression.

BCR-ABL Dosage

Expression of BCR-ABL is associated with a mutator phenotype; therefore, up-regulation of BCR-ABL may cause an increase in genetic instability and mutation capable of driving disease progression and IM resistance [175, 180, 184]. Expression of BCR-ABL mRNA [192-196] and protein [197] increases with disease progression and has been associated with the development of resistance to IM [69, 198-203]. Elevated BCR-ABL in cell lines is also sufficient to mediate drug resistance [204]. Furthermore, BCR-ABL transcripts and protein kinase are found at the highest levels in the most primitive cells, although consequences of BCR-ABL expression may lead to diverse effects in different cell types [205]. These findings suggest that high expression of BCR-ABL may underlie mechanisms of CML progression and drug resistance, of which ROS and oxidative DNA damage are the primary suspects.

The Tetracycline-inducible (tet-off) SCLtTA/BCR-ABL Transgenic Mouse Model

In 2005, Daniel Tenen's group developed conditional transgenic mice where p210 BCR-ABL expression was targeted in the stem and progenitor cells of murine bone marrow using the tet-off system to induce a CML-like disease phenotype [206]. First, they developed a construct consisting of the SV40 minimal promoter and the rabbit β -globin intron 2. Downstream, the tetracycline transactivator protein (tTA) with poly A sequence was placed under the control of the murine stem cell leukemia (*SCL*) gene 3' enhancer, which restricted expression within adult murine bone marrow to hematopoietic stem and progenitor cells, and megakaryocytes [207, 208]. This fragment was purified and injected into the pronucleus of BDF mice. Previously, they generated TRE-BCR-

ABL mice [209]. They developed a construct consisting of a tetracycline-responsive element (TRE) and the cytomegalovirus (CMV) promoter; downstream, p210 BCR-ABL was placed under the control of the TRE, followed by rabbit β -globin intron 8, and a poly A sequence. This fragment was purified and injected into the pronucleus of FVB/N mice. To generate the SCLtTA/BCR-ABL double-transgenic murine line, male SCLtTA BDF mice were crossbred with female TRE-BCR-ABL FVB/N mice [206]. To date, this is the only mouse model of CML that initiates BCR-ABL expression at the level of the hematopoietic stem cell (HSC).

Rationale and Project Aims

There are two major obstacles in the treatment of CML: disease progression and drug resistance, both of which are a consequence of genomic instability. CML-CP is initiated by the leukemia stem cell (LSC) but is propagated by the leukemia progenitor cell (LPC), yet either population may exhibit drug resistance. Furthermore, disease progression to CML-BP is inevitable if CML-CP is either untreated or drug resistance is acquired through point mutation and/or additional chromosomal aberrations.

To determine which population of CML-CP cells, LSCs and/or LPCs, displays elevated levels of ROS and oxidative DNA damage, and whether these elevated levels of ROS and oxidative DNA damage in CML-CP subpopulations result in the accumulation of genomic instability, we employed the tetracycline-inducible SCLtTA/BCR-ABL transgenic mouse model. We also examined the effect of the BCR-ABL kinase inhibitor imatinib mesylate (IM) on ROS, oxidative DNA damage and genomic instability displayed by CML-CP subpopulations. Additionally, to explore underlying mechanisms,

i.e. BCR-ABL expression levels, by which CML-CP cells accumulate genomic instability, we examined the effect of low and high BCR-ABL expression on ROS and oxidative DNA damage in BCR-ABL-transduced human CD34⁺ cells.

Based on the accumulation of evidence, we suspect that both LSCs and LPCs display elevated levels of ROS and oxidative DNA damage that, when combined with unfaithful DNA repair mechanisms, results in genomic instability responsible for drug resistance associated with point mutations in the BCR-ABL kinase domain and CML progression associated with additional cytogenetic abnormalities. Furthermore, we suspect that levels of BCR-ABL expression and kinase activity may play a role in determining levels of ROS and oxidative DNA damage.

CHAPTER 2

MATERIALS AND METHODS

SCLtTA/BCR-ABL transgenic mice

Genotyping

Transgenic mice were generously provided by Steffen Koschmieder (University of Aachen, Germany). Mice were provided with drinking water supplemented with tetracycline hydrochloride (0.5g/L) (Sigma-Aldrich, Saint Louis, MO). Transgenic mice were identified by polymerase chain reaction (PCR) of tail snip DNA. DNA isolation and purification from mice tails were performed using the REDExtract-N-Amp Tissue PCR Kit (Sigma, Saint Louis, Missouri), and genotyping for the SCLtTA and p210 *BCR/ABL* transgenes was performed using transgene-specific primers (Eurofins MWG Operon, Huntsville, AL). 2X GoTaq polymerase Master Mix (Promega, Madison, WI) was used to amplify both SCLtTA and p210 *BCR/ABL* transgenes. *BCR/ABL*-specific primers (forward: 5'-GAGCGTGCAGAGTGGAGGGAGAACA-3'; reverse: 5'-GGTACCAGGAGTGTTTCTCCAGACTG-3') amplified a 500 basepair-long fragment using amplification conditions of 40 cycles at 94°C for 45 seconds, 55°C for 1 minute, and 72°C for 1 minute. SCLtTA-specific primers (*tTA*: 5'-TTTCGATCTGGACATGTTGG-3'; *SCL*: 5'-AGAACAGAATTCAGGGTCTTCCTT-3') yielded a 750 basepair product using amplification conditions consisting of 40 cycles at 94°C for 40 seconds, 60.5°C for 1 minute, and 72°C for 1 minute. PCR products were run in a 1.5% agarose gel containing ethidium bromide, and visualized using the Gel DocTM XR+ Molecular Imager[®] System (Bio-Rad, Hercules, CA).

Disease phenotype

Transgenic mice were euthanized by CO₂ inhalation followed by cervical dislocation. Peripheral blood (PB) was collected via cardiac puncture using a heparinized needle and syringe. Spleens (SPL) were removed, photographed (Sony Cyber-shot® Digital Camera W220), weighed (Sartorius BP211D, Goettingen, Germany), and then mashed to obtain cells in suspension. Bone marrow cells (BMCs) were flushed from tibias and femurs with culture medium and subjected to density separation by Lympholyte M (Cedarlane, Hornby, Canada) to remove erythrocytes. Enucleated red blood cells (RBCs) were lysed from total PBCs with ACK lysis buffer (0.15M NH₄Cl, 0.01M KHCO₃, 0.1mM EDTA·Na₂·2H₂O, pH 7.2-7.4). All cells were washed and resuspended in cold 1X phosphate-buffered saline (PBS) containing 1.0% BSA and purified rat anti-mouse CD16/CD32 (FcR γ III/II) antibody and incubated on ice for 10 minutes, followed by incubation with rat anti-mouse fluorescein isothiocyanate (FITC)-conjugated Ly-6G/C (Gr-1), phycoerythrin (PE)-conjugated CD11b (Mac-1), allophycocyanin (APC)-conjugated CD45R/B220, and PE-cyanin7 (PE-CyTM7)-conjugated CD3, or APC-conjugated Ter-119 and PE-conjugated CD41 (BD PharMingen, San Diego, CA) for 30 minutes in 4°C, and fixed with 1% paraformaldehyde (PFA) in 1X PBS. Cells were analyzed with FACS within 24-48 hours.

Real-time quantitative reverse transcription polymerase chain reaction (qRT-PCR)

qRT-PCR was conducted by Mirle Schemionek (University of Aachen, Germany), as described previously [210]. Isolation of DNase-treated RNA was performed using RNeasy® Mini Kit or RNeasy® Micro Kit for BM and SPL, respectively (Quiagen, Hilden, Germany). RNA isolation was followed by cDNA synthesis using the

Moloney murine leukemia virus (M-MLV) reverse transcriptase from Promega (Promega, Mannheim, Germany) following the manufacturer's protocol. For detection of p210 BCR-ABL transcripts, a real-time taqman assay (Eurogentec, Cologne, Germany) and Taqman Universal PCR Master Mix (Applied Biosystems, Darmstadt, Germany) were used in combination with the ABI 7500 Fast Real-Time PCR system (Applied Biosystems, Darmstadt, Germany). As an internal standard, the expression level of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used.

Detection of BM-derived stem and progenitor cells

BMCs from transgenic mice were flushed from tibias and femurs with Iscove's modified Dulbecco's medium (IMDM) (Mediatech, Manassas, VA) containing 10% FBS (Gibco, Grand Island, NY), 1X antibiotic-antimycotic solution (Mediatech, Manassas, VA), and pretested minimal concentrations of interleukin-3 (IL-3) and stem cell factor (SCF) required to maintain proliferation and viability, and subjected to density separation to remove erythrocytes by using Lympholyte M (Cedarlane, Hornby, Canada). Cells were resuspended in culture medium and placed in culture flasks to be incubated in 5% CO₂ and 37°C. 24-48 hours later cells were washed and resuspended in warm 1X PBS plus 1% BSA and stained with rat anti-mouse APC-conjugated lineage antibody cocktail, PE-conjugated CD117 (c-Kit), and PE-CyTM7-conjugated Ly-6A/E (Sca-1) for 30 minutes in 37°C (BD PharMingen, San Diego, CA). 1 X 10⁶ cells were assayed for ROS production; remaining cells were sorted for stem (Lin⁻c-Kit⁺Sca-1⁺) and progenitor (Lin⁻c-Kit⁺Sca-1⁻) populations, and subsequently used for cytospin preparation for immunofluorescence to assay for oxidative DNA damage.

ROS assay

Levels of intracellular reactive oxygen species (ROS) were analyzed in cells cultured in the presence of pretested minimal concentrations of IL-3 and SCF required to maintain viability and proliferation using the redox-sensitive fluorochromes dihydroethidium (DHE) to detect cellular $\bullet\text{O}_2^-$ (Sigma-Aldrich, Saint Louis, MO), Redox SensorTM Red CC-1 to detect $\bullet\text{O}_2^-/\text{H}_2\text{O}_2$ (Molecular Probes, Eugene, OR), and 2',7'-dichlorodihydrofluorescein-diacetate to detect $\text{H}_2\text{O}_2/\bullet\text{OH}$ (DCFDA). Briefly, cells were washed and resuspended in warm 1X PBS, then incubated with 5-10 μM of fluorochrome for 10-15 minutes in 37°C. Next, samples were washed and resuspended in cold 1X PBS, and analyzed using the BDTM FACSCanto or LSR II flow cytometry systems (BD Biosciences, San Jose, CA).

Immunofluorescence: oxidative DNA damage assay

Cytospins were prepared from cell suspensions containing 1% bovine serum albumin (BSA) (Sigma-Aldrich, Saint Louis, MO) or 2% FBS using the Thermo Shandon Cytospin 3 (Harlow Scientific, Arlington, MA). Cellspots were fixed with 4% formaldehyde for 20 minutes, then washed and permeabilized with 0.02% Triton X-100 and blocked with 2% BSA for 45 minutes. Cellspots were then incubated with 1:100-1:200 monoclonal anti-8-oxoguanine (8-oxoG) (Chemicon, Temecula, CA) or anti-phospho-histone H2A.X (Ser139) (Upstate, Temecula, CA) primary antibodies for 1 hour, followed by washing and blocking for 15 minutes. 1:4000-1:2000 Alexa Fluor[®] 594 or 488 goat anti-mouse IgG secondary antibody (Molecular Probes, Eugene, OR) was applied for 45 minutes to detect nuclear localization of 8-oxoG lesions and DNA DSBs; simultaneously, DNA was counterstained with 1:2000 blue-fluorescent nucleic

acid stain 4',6'-diamidino-2-phenylindole (DAPI), dihydrochloride (Molecular Probes, Eugene, OR). Cellspots were washed and fixed again before being treated with SlowFade® Gold antifade reagent (Molecular Probes, Eugene, OR) and mounted with a coverslip. Fixation took place at room temperature (RT), whereas all other steps were carried out at 37°C. Specific staining was visualized using an inverted Olympus IX70 fluorescence microscope (Olympus America, Melville, NY) equipped with a 100 X/1.35 numeric aperture UPlan Apo objective and Cooke Sensicam QE camera (Cooke, Auburn Hills, MI). A series of 3-dimensional images of each cell was stored in SlideBook software version 3.0.1 (Intelligent Imaging Innovations, Denver, CO). Deconvolution was applied to increase the resolution and contrast of the images, as described [174]. A collection of 3-dimensional images describing individual cells was converted to a one 2-dimensional photomicrograph. 2-dimensional photomicrographs of 8-oxoG lesions underwent further analysis using Adobe Photoshop (Adobe Systems, San Jose, CA) before final quantification of nuclear lesions in Slidebook.

Detection of BM-derived quiescent stem cells

BMCs from transgenic mice were flushed from tibias and femurs with IMDM supplemented with 10% FBS, 1% antibiotic, and pretested minimal concentrations of IL-3 and SCF, and then subjected to density separation by Lympholyte M (Cedarlane, Hornby, Canada) to remove erythrocytes. Cells were incubated overnight (12-16 hours) in serum-free medium (SFM) consisting of IMDM, 0.1% bovine serum albumin (BSA), and 1X antibiotic/antimycotic solution. Cells then were stained with rat anti-mouse APC- or PerCP-CyTM5.5-conjugated lineage antibody cocktail (BD PharMingen, San Diego, CA) and sorted for lineage-negative (Lin⁻) cells. Sorted cells were washed and

resuspended in 1X PBS and stained with 2 μ M carboxyfluorescein diacetate, succinimidyl ester (CFSE) (Molecular Probes, Eugene, OR) or Cell Proliferation Dye (CPD) eFluor[®] 670 (eBioscience, San Diego, CA) for 15 minutes at 37°C. Cells were washed with cold 1X PBS containing 10% FBS, and then cultured in IMDM containing 10% FBS, pre-tested minimal concentrations of IL-3 and SCF, and 1X antibiotic/antimycotic solution. A portion of cells stained with either CFSE or CPD eFluor[®] 670 were cultured with demecolcine solution (Sigma, St. Louis, MO) to serve as a positive control for quiescence. Cells were recovered, washed, resuspended in warm 1X PBS plus 1% BSA and stained with fluorochrome-conjugated cell surface receptors: rat anti-mouse APC- or PerCP-CyTM5.5-conjugated lineage antibody cocktail, PerCP-CyTM5.5- or PE-conjugated CD117 (c-Kit), and PE-CyTM7-conjugated Ly-6A/E (Sca-1) for 30 minutes in 37°C (BD PharMingen, San Diego, CA). Following cell surface receptor staining, assays for detecting phosphotyrosine levels, ROS (described above), and 8-oxoG in CFSE^{max} or CPD eFluor[®] 670^{max} Lin⁻c-Kit⁺Sca-1⁺ cells were conducted and analyzed by FACS.

Phosphotyrosine

Cells were washed with warm 1X PBS, then resuspended and fixed in BD Cytofix/Cytoperm[™] solution for 15 minutes at RT. Cells were washed once with BD Perm/Wash[™] buffer and then resuspended and permeabilized in fresh BD Perm/Wash[™] for 15 minutes at RT. Cells were washed once with BD Perm/Wash[™] then resuspended in fresh BD Perm/Wash[™] containing phosphotyrosine antibody for 30 minutes at RT. Cells were washed once with, then resuspended in fresh, cold 1X PBS for FACS analysis.

8-oxoG

Cells were washed with warm 1X PBS, then resuspended and fixed in 4% PFA in 1X PBS for 10 minutes at RT. Cells were washed once with 1X wash concentrate and then resuspended and permeabilized in 0.2% Tween in 1X PBS for 10 minutes at RT. Cells were washed once with 1X wash concentrate then resuspended in 1X wash concentrate containing FITC-conjugated 8-oxoguanine antibody (Biotrin, Dublin, Ireland) for 1 hour at RT. Cells were washed once with, then resuspended in fresh, cold 1X PBS for FACS analysis.

In vivo imatinib mesylate (IM) treatment

In vivo IM treatment of transplanted recipient mice was conducted by Mirle Schemionek (University of Aachen, Germany), as described previously [210]. IM treatment was performed starting 4 weeks after transplantation, over a 4-week period. IM (LC Laboratories) was dissolved in water and applied by oral gavage, using 100 mg/kg body weight twice daily.

Detection of BCR-ABL kinase domain mutations

Total BM or SPL cells were extracted from non-induced and induced SCLtTA/BCR-ABL mice, and subjected to density separation to remove erythrocytes by using Lympholyte M (Cedarlane, Hornby, Canada). Cells were stained with rat anti-mouse APC-conjugated lineage antibody cocktail (BD PharMingen, San Diego, CA) and sorted for Lin⁻ cells within 24 hours. For D-HPLC and ASO-PCR, Lin⁻ BMCs were washed once with 1XPBS then resuspended/shredded in 1mL TRIzol[®] reagent (Invitrogen) by repetitive pipetting with syringe and 20G needle to extract total RNA, which was then frozen at -20°C.

Denaturing high performance liquid chromatography (D-HPLC)

D-HPLC was conducted by Martin Mueller (Medical Faculty of Mannheim, Heidelberg University, Mannheim, Germany), as described previously [211]. Briefly, cDNA was synthesized from total RNA with random hexamer primers and reverse transcriptase, from which three overlapping fragments spanning the entire BCR-ABL KD were amplified by nested PCR and analyzed by D-HPLC followed by bidirectional sequencing to characterize BCR-ABL KD mutations.

Allele-specific oligonucleotide ligation-polymerase chain reaction (ASOL-PCR)

ASOL-PCR was conducted by Thoralf Lange and Jacqueline Maier (University of Leipzig, Germany), as described previously [212-214]. cDNA was synthesized from total RNA with random hexamer primers and reverse transcriptase, from which BCR-ABL sequences were amplified by PCR, and then followed by stringent high temperature hybridization to the PCR product of 2 probes which span one mutation region of interest. Each probe consists of an ABL-hybridizing sequence flanked by a PCR primer-binding site, whereas only the first probe carries the point mutation at its 3' end while the 5' end of the second probe corresponds to the neighboring base. A high temperature ligation reaction joins the 2 probes only when there is no mismatch at the mutation site. The yield of the ligated probe is determined by real-time PCR using primers complementary to the primer binding sites at the non-ligated ends of the two probes. PCR amplifications for 5 of the most common BCR-ABL KD mutations (G250E, Y253H, E255K, T315I, F359V) were performed using individual allele-specific 3' primers complementary to the respective mutations on *ABL*.

Selection and sequencing of IM-resistant colonies

Lin⁻ BM or SPL cells were cultured in IMDM supplemented with 10% FBS and 1% antibiotic/antimycotic solution containing 1μM IM for 7 days. Viable cells were then plated in semisolid methylcellulose medium in the absence of growth factors and in the presence of 1μM IM for another 7 days to allow colony formation of individual IM-resistant clones. IM-resistant single colonies were selected then expanded *in vitro* in IMDM supplemented with 10% FBS, 1X antibiotic/antimycotic solution and ideal concentrations of IL-3 and SCF required to stimulate proliferation. As described previously [184], total RNA was extracted and reverse transcribed into cDNA using random hexamers and MMLV transcriptase. cDNA was amplified using primers specific for the human c-ABL KD (forward: 5'-CGCAACAAGCCCACTGTC-3'; reverse: 5'-TCCACTTCGTCTGAGATACTGGATT-3'). Amplified fragments were sequenced at University of Pennsylvania using the c-ABL KD reverse primer.

High density single nucleotide polymorphism (SNP) array

BMCs from transgenic mice were flushed from tibias, femurs, and humeri with culture medium and subjected to density separation by Lympholyte M (Cedarlane, Hornby, Canada) to remove erythrocytes. High molecular weight (HMW) genomic DNA (gDNA) was extracted from FACS-sorted Lin⁻ BMCs or total BMCs and/or SPL using DNeasy Tissue Kit (Qiagen, Maryland) according to manufacturer's protocol, and resuspended in 75μLs 2mM Tris·Cl, 0.1mM EDTA buffer. 250ngs of HMW gDNA was run against a lambda DNA marker on a 0.5% agarose gel containing ethidium bromide and visualized using the Gel DocTM XR+ Molecular Imager[®] System (Bio-Rad, Hercules, CA). The quantity and quality of HMW gDNA was assessed using the ND-1000

spectrophotometer (NanoDrop®, Wilmington, DE). 3-5 µgs of HMW gDNA for application to Affymetrix GeneChips was processed at the Jackson Laboratory (Bar Harbor, Maine) to compare mouse DNA samples, as described [215]. The specific GeneChips used are capable of detecting polymorphisms in 623,124 well-characterized mouse SNPs. SNP calls were based on the C57BL/6J background and generated using the BRLMM-P algorithm. Annotated data were analyzed by Hans-Ulrich Klein (University of Muenster, Germany).

Microarray Analysis of hematopoietic stem cells (HSCs)

Microarray was conducted by Mirle Schemionek (University of Aachen, Germany), as described previously [210], and analyzed by Hans-Ulrich Klein (University of Muenster, Germany). BM-derived cells were isolated from 3 induced SCLtTA/BCR-ABL mice and 3 non-induced mice for controls. 5000 Lin⁻c-Kit⁺Sca-1⁺ stem cells were FACS-sorted directly into RNA extraction buffer. After addition of carrier RNA, RNA was DNase-treated, extracted, and frozen at -80°C. Linear amplification of less than 20 ng of total RNA was conducted [216]. The yield of biotinylated cRNA from purified stem cells of each mouse ranged between 15 and 25 µg. A total of 15 µg of the biotinylated cRNA was hybridized to Affymetrix Mouse Genome 430 2.0 GeneChips covering approximately 45,000 transcripts. After hybridization at 45°C for 16 hours, the GeneChips were analyzed using an Affymetrix GeneChip® Scanner 3000. Individual genes were analyzed using the MicroArraySuite 5.0 software for calculation of the presence or absence of calls and the D-chip Software. 361 genes were changed at least 1.5-fold ($P < 0.05$ according to Mann-Whitney test) in expression when induced mice were compared to non-induced mice. For evaluating the functional significance of altered

gene expression, a pathway analysis was performed, using Ingenuity Pathway Analysis software 7.0 (IPA, Ingenuity Systems).

Human CD34⁺ cells

CD34⁺ fractions of human bone marrow cells (BMCs) obtained from healthy donors were infected with pMIG-GFP retroviral construct containing empty vector (MIG R1), p210*BCR-ABL* (MIG 210), or p210*BCR-ABL*(K1172R) (MIG KD) cDNA by Hardik Modi (City of Hope National Medical Center, Duarte, CA), as described previously [217], and were generously provided by Ravi Bhatia (City of Hope National Medical Center, Duarte, CA). Cells were maintained in IMDM (Mediatech, Manassas, VA) containing 10% FBS (Gibco, Grand Island, NY), 1X antibiotic-antimycotic solution (Mediatech, Manassas, VA), and pretested minimal concentrations of SCF and granulocyte macrophage-colony stimulating factor (GM-CSF) required to maintain viability and proliferation, and incubated in 5% CO₂ and 37°C. 48 hours later the 15% lowest and highest GFP-expressing cells, or total GFP-positive cells were either unsorted and analyzed for ROS levels by FACS (described above), or FACS-sorted into the 20% lowest and highest GFP-expressing cells, or total GFP-positive cells, then analyzed by Western blot and for oxidative DNA damage by immunofluorescence (described above).

Western blotting

Cells were washed with cold 1X PBS and resuspended in 1X sodium dodecyl sulfate (SDS) buffer with β -mercaptoethanol. Samples were boiled for 2-3 minutes and then sonicated for 10-15 seconds. Proteins were separated by electrophoresis on a 9.0% SDS-polyacrylamide gel, transferred to a nitrocellulose membrane, and probed with

monoclonal 1:200 anti-c-Abl (Ab-3) (Calbiochem, Gibbstown, NJ), 1:1000 anti-phosphotyrosine, clone 4G10 (Upstate, Temecula, CA), 1:2000 anti- β -Actin (Abcam, Cambridge, MA), and polyclonal 1:500 anti-GFP (FL) (Santa Cruz, Santa Cruz, CA) primary antibodies. Monoclonal antibodies were countered with IRDye[®] 800CW goat anti-mouse IgG secondary antibody, whereas polyclonal antibodies were countered with IRDye[®] 680 donkey anti-rabbit IgG secondary antibody (1:10,000). Blots were analyzed with the Odyssey Infrared Imaging System (Li-Cor, Lincoln, NE).

Statistical analysis

Data are expressed as mean $\pm$ standard deviation (SD). Statistical analyses were performed using the unpaired Student *t* test or Mann-Whitney U test; *P* values equal to or less than 0.05 were considered significant.

CHAPTER 3

RESULTS

CML-CP Lin⁻c-Kit⁺Sca-1⁺ LSCs, including the quiescent population, display elevated levels of ROS and oxidative DNA damage that persist even during IM-inhibition of BCR-ABL kinase

Koschmieder et al. previously showed that up to 31% of induced SCLtTA/BCR-ABL mice exhibited a mixed myeloid/lymphoid disease phenotype suggestive of progression to lymphoid blast crisis [206]. Since we were interested in examining how hematopoietic cell subpopulations display levels of ROS and oxidative DNA damage during CML-CP, and how these levels translate into genomic instability, we utilized mice expressing CML-CP disease phenotype only.

qRT-PCR from the BM (6.296 ± 1.647) and SPL (4.602 ± 1.512) of induced SCLtTA/BCR-ABL mice displayed BCR-ABL mRNA expression, whereas BCR-ABL mRNA expression from the BM (0.000260 ± 0.000230 , $p=0.003$) and SPL (0 ± 0 , $p=0.006$) of non-induced mice was either nearly undetectable or not detectable (N.D.); results are representative of 2 experiments from 3 mice per group (Figure 1A, *left panel*). qRT-PCR from Lin⁻c-Kit⁺Sca-1⁺ stem (0.146 ± 0.0365) and Lin⁻c-Kit⁺Sca-1⁻ progenitor (0.0203 ± 0.00402) cells of induced SCLtTA/BCR-ABL mice displayed BCR-ABL mRNA expression, whereas BCR-ABL mRNA expression from Lin⁻c-Kit⁺Sca-1⁺ stem (0 ± 0 , $p=0.002$) and Lin⁻c-Kit⁺Sca-1⁻ progenitor (0.000453 ± 0.000785 , $p=0.001$) cells of non-induced mice was either N.D. or nearly undetectable (Figure 1A, *right panel*). Furthermore, Lin⁻c-Kit⁺Sca-1⁺ leukemia stem cells (LSCs) displayed significantly higher levels of BCR-ABL mRNA expression compared to Lin⁻c-Kit⁺Sca-1⁻ leukemia progenitor cells (LPCs) from induced SCLtTA/BCR-ABL mice ($p=0.004$), thereby

mimicking a feature of human CML-CP which is characterized by elevated BCR-ABL mRNA expression in Lin⁻CD34⁺CD38⁻ LSCs compared to Lin⁻CD34⁺CD38⁺ LPCs [218]. Additionally, elevated BCR-ABL transcripts in human CML-CP stem cells are independent of *bcr-abl* gene amplification, a phenomenon often associated with disease progression and prolonged exposure to IM [77, 200, 201, 219, 220]. However, we did not determine *bcr-abl* gene copy number in induced SCLtTA/BCR-ABL mice, so we cannot exclude the possibility that elevated transcript levels may be due to multiple copies of the BCR-ABL transgenes.

Induced SCLtTA/BCR-ABL mice displayed splenomegaly (264.864 mgs $\pm$ 101.633) compared to non-induced mice (124.551 mgs $\pm$ 23.918, $p < 0.001$) (Figure 1B). Induced SCLtTA/BCR-ABL mice displayed expansion of mature Gr1⁺/CD11b⁺ (53.190 $\pm$ 8.111) and immature Gr1^{low}/CD11b⁺ (27.253 $\pm$ 4.491) granulocytes, and a decrease of B220⁺ B cells (1.957 $\pm$ 0.232) and Ter119⁺ erythrocytes (9.873 $\pm$ 3.048) in the BM, compared to non-induced mice (Gr1⁺/CD11b⁺: 30.230 $\pm$ 0.721, $p = 0.008$; Gr1^{low}/CD11b⁺: 8.443 $\pm$ 0.595, $p = 0.002$; B220⁺: 18.190 $\pm$ 2.183, $p < 0.001$; Ter119⁺: 34.883 $\pm$ 0.889, $p < 0.001$) (Figure 1C, *left panel*). Induced SCLtTA/BCR-ABL mice displayed expansion of mature Gr1⁺/CD11b⁺ (13.510 $\pm$ 2.701) and immature Gr1^{low}/CD11b⁺ (7.537 $\pm$ 2.189) granulocytes, Ter119⁺ erythrocytes (34.307 $\pm$ 8.774), and CD41⁺ megakaryocytes (9.777 $\pm$ 2.673), and a decrease of B220⁺ B cells (23.040 $\pm$ 10.718) in the SPL, compared to non-induced mice (Gr1⁺/CD11b⁺: 2.2 $\pm$ 0.111, $p = 0.002$; Gr1^{low}/CD11b⁺: 1.207 $\pm$ 0.384, $p = 0.008$; Ter119⁺: 8.260 $\pm$ 2.1, $p = 0.007$; CD41⁺: 2.410 $\pm$ 0.145, $p = 0.009$; B220⁺: 44.267 $\pm$ 6.305, $p = 0.042$) (Figure 1C, *center panel*). Induced SCLtTA/BCR-ABL mice displayed expansion of mature Gr1⁺/CD11b⁺ (29.533 $\pm$ 4.141) granulocytes and CD41⁺

Figure 1. Induced SCLtTA/BCR-ABL transgenic mice exhibit a CML-CP-like disease phenotype. (A) BCR-ABL mRNA expression relative to the housekeeping gene GAPDH in non-induced (black bars) and induced (gray bars) mice by qRT-PCR ; n=3 mice per group, N.D.=not detected. *left panel*: BCR-ABL mRNA expression from bone marrow (BM) and spleen (SPL) cells; *right panel*: BCR-ABL mRNA expression in BM-derived Lin^c-Kit⁺Sca-1⁺ stem and Lin^c-Kit⁺Sca-1⁻ progenitor cells. (B) Spleen weight in non-induced (n=58) and induced (n=55) mice; *inset*: representative spleens. (C) Percentage of mature (Gr1⁺/Mac-1⁺) and immature (Gr1^{low}/Mac-1⁺) granulocytes, B cells (B220⁺), T cells (CD3⁺), erythrocytes (Ter119⁺) and megakaryocytes (CD41⁺) in BM (*left panel*), SPL (*center panel*) and peripheral blood (PB) (*right panel*) from non-induced (black bars) and induced mice (gray bars), as measured by FACS analysis; n=3 mice per group. Data are expressed as mean $\pm$ standard deviation (SD). *p $\leq$ 0.001, **p $\leq$ 0.005, ***p $\leq$ 0.01, ****p $\leq$ 0.05 in comparison to non-induced counterparts.

megakaryocytes (55.367 ± 6.646), and a decrease of CD3⁺ T cells (60.867 ± 3.710) in the PB, compared to non-induced mice (Gr1⁺/CD11b⁺: 5.3 ± 1.442 , $p < 0.001$; CD41⁺: 39.267 ± 7.222 , $p = 0.047$; CD3⁺: 81.8 ± 6.083 , $p = 0.007$) (Figure 1C, *right panel*). As expected, differences in Ter119⁺ erythrocytes from PB were not detected among non-induced and induced mice due to the experimental application of ACK lysis buffer, which also may have resulted in overall increased percentages of the other cell types detected in both groups.

BCR-ABL tyrosine kinase is associated with elevated levels of ROS and oxidative DNA damage in both murine and human BCR-ABL-transformed cell lines in comparison to non-transformed cell lines [160, 182, 184]; similarly, both ROS and oxidative DNA damage are elevated in primary CML-CP and CML-BP cells in comparison to counterparts from healthy donors [184]. Most recently, Nieborowska-Skorska et al. published that ROS and oxidative DNA damage are elevated in human CD34⁺ progenitor cell-enriched and CD34⁺CD38⁻ stem cell-enriched populations of CML-CP patient cells in comparison to counterparts from healthy donors [221].

To determine the effects of BCR-ABL expression on ROS in murine BM-derived Lin⁻c-Kit⁺Sca-1⁺ stem and Lin⁻c-Kit⁺Sca-1⁻ progenitor subpopulations from the SCLtTA/BCR-ABL mouse model of CML-CP we applied the redox-sensitive fluorochrome dichlorofluorescein diacetate (DCFDA) to detect H₂O₂/•OH and analyzed cells by FACS (Figure 2A). Lin⁻c-Kit⁺Sca-1⁺ LSCs from induced SCLtTA/BCR-ABL mice displayed elevated levels of H₂O₂/•OH ($59,550 \pm 7,030$) compared to non-induced Lin⁻c-Kit⁺Sca-1⁺ stem cells ($47,150 \pm 1,640$; $p = 0.001$). Furthermore, Lin⁻c-Kit⁺Sca-1⁺ LSCs from induced mice displayed elevated levels of H₂O₂/•OH compared to induced

Lin⁻c-Kit⁺Sca-1⁻ LPCs ($35,590 \pm 14,640$; $p=0.026$). Non-induced Lin⁻c-Kit⁺Sca-1⁺ stem cells also displayed elevated levels of H₂O₂/•OH compared to non-induced Lin⁻c-Kit⁺Sca-1⁻ progenitor cells ($32,240 \pm 4,120$; $p<0.001$), which suggests that non-induced stem cells inherently harbor greater ROS levels than non-induced progenitor cells. Unlike the aforementioned recent finding that ROS are elevated in the human CD34⁺ progenitor cell-enriched population of CML-CP patient cells in comparison to counterparts from healthy donors [221], induced Lin⁻c-Kit⁺Sca-1⁻ LPCs failed to significantly elevate ROS levels above non-induced Lin⁻c-Kit⁺Sca-1⁻ progenitor cells, perhaps due to sub-threshold levels of BCR-ABL expression; therefore, further experiments on this subpopulation were deemed unnecessary.

To determine the effects of BCR-ABL expression on oxidative DNA damage in murine hematopoietic Lin⁻c-Kit⁺Sca-1⁺ stem cells from the SCLtTA/BCR-ABL mouse model of CML-CP we quantified nuclear staining of antibodies against 8-oxoguanine (8-oxoG) to detect oxidative lesions and histone γ -H2AX to detect DSBs (Figure 2B). Lin⁻c-Kit⁺Sca-1⁺ LSCs from induced SCLtTA/BCR-ABL mice displayed elevated levels of 8-oxoG (2.222 ± 2.064) compared to non-induced Lin⁻c-Kit⁺Sca-1⁺ stem cells (1.693 ± 1.354 , $p=0.011$) (Figure 2B, *left panel*). Similarly, Lin⁻c-Kit⁺Sca-1⁺ LSCs from induced mice displayed elevated levels of γ -H2AX foci (14.355 ± 8.974) compared to non-induced Lin⁻c-Kit⁺Sca-1⁺ stem cells (10.611 ± 6.466 ; $p<0.001$) (Figure 2B, *right panel*). A wide range of 8-oxoG lesions and DSBs were detected in both non-induced and induced Lin⁻c-Kit⁺Sca-1⁺ stem cells, leading to overlapping standard deviations (S.D.) among these two groups; however, the number of DSBs depend on cell cycle stage with the lowest number detected in G₀/G₁ and the highest number detected S and G₂/M [182].

Regardless, due to the large number of cells analyzed per group and the consistently higher levels of oxidative DNA damage detected in induced $\text{Lin}^- \text{c-Kit}^+ \text{Sca-1}^+$ LSCs a significant trend was detected.

Figure 2. $\text{Lin}^- \text{c-Kit}^+ \text{Sca-1}^+$ LSCs from induced SCLtTA/BCR-ABL transgenic mice display elevated levels of ROS and oxidative DNA damage. (A) $\text{H}_2\text{O}_2/\bullet\text{OH}$ were detected in BM-derived $\text{Lin}^- \text{c-Kit}^+ \text{Sca-1}^+$ stem and $\text{Lin}^- \text{c-Kit}^+ \text{Sca-1}^-$ progenitor cells from non-induced (black bars, n=7) and induced (gray bars, n=4) mice with DCFDA and analyzed by FACS. (B) *left panel*: 8-oxoG (n=163 cells from 5 non-induced mice, n=110 cells from 4 induced mice) and *right panel*: γ -H2AX (n=162 non-induced and n=279 induced cells possessing ≥ 5 foci each; n=4 mice per group) were detected by immunofluorescence in DAPI-counterstained nuclei of BM-derived $\text{Lin}^- \text{c-Kit}^+ \text{Sca-1}^+$ stem cells. Data are expressed as mean $\pm$ standard deviation (SD). * $p \leq 0.001$, ** $p \leq 0.05$ in comparison to non-induced counterparts.

Nieborowska-Skorska et al. recently published that ROS and oxidative DNA damage are elevated in the quiescent CD34^+ progenitor cell-enriched population of CML-CP patient cells in comparison to counterparts from healthy donors [221]. To determine the effects of BCR-ABL expression on ROS in murine BM-derived quiescent $\text{Lin}^- \text{c-Kit}^+ \text{Sca-1}^+$ stem cells from the SCLtTA/BCR-ABL mouse model of CML-CP we applied the redox-sensitive fluorochromes dihydroethidium (DHE) and Redox Sensor CC-1 (CC-1) to detect cellular $\bullet\text{O}_2^-$ and $\bullet\text{O}_2^-/\text{H}_2\text{O}_2$, respectively, in CFSE^{max} cells and analyzed cells by FACS (Figure 3A). CFSE^{max} $\text{Lin}^- \text{c-Kit}^+ \text{Sca-1}^+$ LSCs from induced SCLtTA/BCR-ABL mice displayed elevated levels of cellular $\bullet\text{O}_2^-$ ($7,636 \pm 2,187$) compared to

CFSE^{max} Lin⁻c-Kit⁺Sca-1⁺ stem cells from non-induced mice ($3,079 \pm 520$; $p < 0.001$) (Figure 3A, *left panel*). Similarly, CFSE^{max} Lin⁻c-Kit⁺Sca-1⁺ LSCs from induced mice displayed elevated levels of $\bullet\text{O}_2^-/\text{H}_2\text{O}_2$ ($7,269 \pm 1,563$) compared to CFSE^{max} Lin⁻c-Kit⁺Sca-1⁺ stem cells from non-induced mice ($4,409 \pm 868$; $p = 0.026$) (Figure 3A, *right panel*).

Figure 3. Quiescent Lin⁻c-Kit⁺Sca-1⁺ LSCs from induced SCLtTA/BCR-ABL transgenic mice display elevated levels of ROS and oxidative DNA damage. (A) Cellular $\bullet\text{O}_2^-$ and $\bullet\text{O}_2^-/\text{H}_2\text{O}_2$ were detected in BM-derived CFSE^{max} Lin⁻c-Kit⁺Sca-1⁺ stem cells from non-induced (black bars, $n=7$ and $n=4$ mice, respectively) and induced (gray bars, $n=3$ mice per group) mice with DHE (*left panel*) and Redox Sensor Red CC-1 (*right panel*), respectively, and analyzed by FACS. (B) 8-oxoG (*left panel*) and γ -H2AX (*right panel*) were detected in BM-derived CPD eFluor 670^{max} Lin⁻c-Kit⁺Sca-1⁺ stem cells from non-induced ($n=5$ mice per group) and induced ($n=5$ and $n=6$ mice, respectively) mice, and analyzed by FACS. Data are expressed as mean $\pm$ standard deviation (SD). * $p \leq 0.001$, ** $p \leq 0.05$ in comparison to non-induced counterparts.

To determine the effects of BCR-ABL expression on oxidative DNA damage in murine BM-derived quiescent Lin⁻c-Kit⁺Sca-1⁺ stem cells from the SCLtTA/BCR-ABL mouse model of CML-CP we detected fluorochrome-conjugated anti-8-oxoG and anti-histone γ -H2AX antibodies, respectively, in CPD eFluor 670^{max} cells and analyzed cells by FACS (Figure 3B). CPD eFluor 670^{max} Lin⁻c-Kit⁺Sca-1⁺ LSCs from induced

SCLtTA/BCR-ABL mice displayed elevated levels of 8-oxoG ($9,036 \pm 2,219$) compared to CPD eFluor 670^{max} Lin⁻c-Kit⁺Sca-1⁺ stem cells from non-induced mice ($3,490 \pm 1,133$; $p=0.001$) (Figure 3B, *left panel*). Similarly, CPD eFluor 670^{max} Lin⁻c-Kit⁺Sca-1⁺ LSCs from induced mice displayed elevated levels of γ -H2AX foci ($3,052 \pm 666$) compared to CPD eFluor 670^{max} Lin⁻c-Kit⁺Sca-1⁺ stem cells from non-induced mice ($1,168 \pm 574$; $p<0.001$) (Figure 3B, *right panel*).

Although IM eradicates most dividing CML cells, quiescent CD34⁺ cells from CML-CP patients are insensitive to IM [56]. To test whether elevated ROS and oxidative DNA damage were underlying insensitivity to IM in BM-derived quiescent Lin⁻c-Kit⁺Sca-1⁺ stem cells, we detected ROS and oxidative DNA damage in untreated (-IM) and treated (+IM) cells from both non-induced and induced mice. First, we applied a fluorochrome-conjugated antibody detecting cellular protein tyrosine phosphorylation in CPD eFluor 670^{max} cells to confirm that BCR-ABL kinase activity was inhibited by IM in murine BM-derived quiescent Lin⁻c-Kit⁺Sca-1⁺ stem cells from the SCLtTA/BCR-ABL mouse model of CML-CP (Figure 4A). As expected, CPD eFluor 670^{max} Lin⁻c-Kit⁺Sca-1⁺ LSCs from induced SCLtTA/BCR-ABL mice displayed elevated levels of tyrosine phosphorylation ($5,026 \pm 2,074$) compared to non-induced CPD eFluor 670^{max} Lin⁻c-Kit⁺Sca-1⁺ stem cells ($1,179 \pm 712$; $p=0.006$). Furthermore, IM inhibited tyrosine phosphorylation in CPD eFluor 670^{max} Lin⁻c-Kit⁺Sca-1⁺ LSCs from induced mice ($2,004 \pm 473$; $p=0.003$ compared to induced/untreated CPD eFluor 670^{max} Lin⁻c-Kit⁺Sca-1⁺ LSCs) to levels of non-induced CPD eFluor 670^{max} Lin⁻c-Kit⁺Sca-1⁺ stem cells treated with IM ($1,222 \pm 744$).

To determine the effects of IM-inhibition on ROS in murine BM-derived quiescent Lin⁻c-Kit⁺Sca-1⁺ LSCs from the SCLtTA/BCR-ABL mouse model of CML-CP we applied the redox-sensitive fluorochrome DCFDA to detect H₂O₂/•OH in CPD eFluor 670^{max} cells (Figure 4B). CPD eFluor 670^{max} Lin⁻c-Kit⁺Sca-1⁺ LSCs from induced SCLtTA/BCR-ABL mice displayed elevated levels of H₂O₂/•OH (12,019 ± 2,581) compared to non-induced CPD eFluor 670^{max} Lin⁻c-Kit⁺Sca-1⁺ stem cells (3,465 ± 533; p<0.001). IM significantly inhibited levels of H₂O₂/•OH in CPD eFluor 670^{max} Lin⁻c-Kit⁺Sca-1⁺ LSCs from induced mice (5,703 ± 1,320; p=0.02 compared to induced/untreated CPD eFluor 670^{max} Lin⁻c-Kit⁺Sca-1⁺ LSCs), however, not to levels of

Figure 4. Despite complete inhibition of BCR-ABL kinase, imatinib mesylate (IM) fails to completely inhibit ROS and oxidative DNA damage in quiescent Lin⁻c-Kit⁺Sca-1⁺ LSCs from induced SCLtTA/BCR-ABL transgenic mice. Lineage-negative BMCs from non-induced (black bars) and induced (gray bars) mice were untreated (-IM) or treated (+IM) with 2 consecutive doses of 1μM IM. **(A)** Phosphotyrosine (P-Tyr) was measured in CPD eFluor 670^{max} Lin⁻c-Kit⁺Sca-1⁺ cells from non-induced (n=4) and induced (n=7) mice, and analyzed by FACS. **(B)** H₂O₂/•OH were detected in CPD eFluor 670^{max} Lin⁻c-Kit⁺Sca-1⁺ cells from non-induced (n=5) and induced (n=3) mice with DCFDA, and analyzed by FACS. **(C)** 8-oxoG was measured in CPD eFluor 670^{max} Lin⁻c-Kit⁺Sca-1⁺ cells from non-induced (n=5) and induced (n=4) mice, and analyzed by FACS. Data are expressed as mean ± standard deviation (SD). *p ≤0.001, **p ≤0.005, ***p ≤0.01, ****p ≤0.05 in comparison to non-induced counterparts.

non-induced CPD eFluor 670^{max} Lin⁻c-Kit⁺Sca-1⁺ cells treated with IM ($3,027 \pm 572$; $p=0.006$).

To determine the effects of IM-inhibition on oxidative DNA damage in murine BM-derived quiescent Lin⁻c-Kit⁺Sca-1⁺ LSCs from the SCLtTA/BCR-ABL mouse model of CML-CP we applied a fluorochrome-conjugated antibody detecting 8-oxoG in CPD eFluor 670^{max} cells (Figure 4C). In accordance with Figure 3B (*left panel*), CPD eFluor 670^{max} Lin⁻c-Kit⁺Sca-1⁺ LSCs from induced SCLtTA/BCR-ABL mice displayed elevated levels of 8-oxoG ($42,870 \pm 10,055$) compared to non-induced CPD eFluor 670^{max} Lin⁻c-Kit⁺Sca-1⁺ stem cells ($17,066 \pm 5,233$; $p=0.002$). However, IM failed to significantly inhibit formation of 8-oxoG in CPD eFluor 670^{max} Lin⁻c-Kit⁺Sca-1⁺ LSCs from induced mice ($33,767 \pm 12,752$); in fact, post-treatment 8-oxoG levels remained significantly above those of non-induced CPD eFluor 670^{max} Lin⁻c-Kit⁺Sca-1⁺ stem cells treated with IM ($15,175 \pm 4,033$; $p=0.017$).

These data show that induced Lin⁻c-Kit⁺Sca-1⁺ leukemia stem cells (LSCs), including the quiescent fraction, from the tetracycline-inducible SCLtTA/BCR-ABL transgenic mouse model of CML-CP, display elevated levels of ROS, including superoxide anion radical ($\bullet\text{O}_2^-$), hydrogen peroxide (H_2O_2) and hydroxyl radical ($\bullet\text{OH}$), and oxidative DNA damage, including 8-oxoguanine and DNA double-strand breaks (DSBs), and that these precursors to genomic instability are not inhibited by treatment with the small molecule tyrosine kinase inhibitor imatinib mesylate (IM).

Genomic instability originates from CML-CP Lin⁻c-Kit⁺Sca-1⁺ LSCs and is not prevented by IM-inhibition of BCR-ABL kinase

Different mechanisms inducing both primary and secondary resistance against the BCR-ABL inhibitor IM have been identified, however, the majority of cases are due to point mutations within the ABL kinase domain (KD) leading to impaired ability to bind IM [55, 68, 69]. To examine whether or not elevated ROS and oxidative DNA damage in Lin⁻c-Kit⁺Sca-1⁺ LSCs from both untreated (-IM) and treated (+IM) induced SCLtTA/BCR-ABL mice resulted in genomic instability manifested by point mutations in the BCR-ABL KD, we employed three increasingly sensitive methods of detection on BM- and/or SPL-derived lineage-negative cells: D-HPLC (denaturing high performance liquid chromatography), ASOL-PCR (allele-specific oligonucleotide ligation-polymerase chain reaction), and sequencing of resistant colonies.

D-HPLC failed to detect KD mutations in both untreated and treated induced SCLtTA/BCR-ABL mice (0/7 and 0/5, respectively). However, ASOL-PCR detected two KD mutations in one out of seven untreated, induced mice: E255K and T315I. ASOL-PCR also detected one KD mutation (T315I) in one out of five treated induced mice. Two of the five untreated induced mice that harbored detectable mutations as determined by sequencing of IM-resistant colonies harbored two mutations each (E255K and T315I; T315I and H396P) though from different clones. Three of the five induced untreated mice that harbored detectable mutations as determined by sequencing of IM-resistant colonies harbored one mutation each: T315I or H396P. Two of the seven untreated induced mice failed to form resistant colonies though they conferred growth factor independence *in vitro*.

Total RNA from 9 IM-treated mice was submitted for mutation analysis by D-HPLC and ASOL-PCR. 3 out of 9 mice were IM-treated for 4 weeks before cells were isolated for RNA extraction, whereas 6 out of 9 mice were IM-treated for 4 weeks, followed by 3 weeks without treatment to permit leukemic expansion of cells. BCR-ABL amplification was not accomplished in 4 of the 9 mice, all from the second group of IM-treated mice; therefore, these 4 samples were not included in the total number of mice analyzed. The T315I mutation that was detected by ASOL-PCR was from 1 out of 2 mice from the second treatment group.

As expected, there were no detectable mutations in total mRNA from untreated non-induced mice using D-HPLC or ASOL-PCR (0/4 mice, not shown). Furthermore, Lin⁻ cells from untreated non-induced mice failed to confer growth factor independence *in vitro* prior to plating cells in methylcellulose, thereby suggesting the absence of resistant colonies. Also, we extracted BMCs from untreated non-induced SCLtTA/BCR-ABL mice that were maintained with tetracycline-supplemented drinking water *in vivo*, and then induced BCR-ABL expression *in vitro* by culturing Lin⁻ BMCs without tetracycline for one week. Although these cells exhibited growth factor independence they failed to exhibit resistance upon *in vitro* incubation with IM, thus confirming that point mutations did not arise as a result of *in vitro* culture.

Table 1. Imatinib-naïve and imatinib-treated induced SCLtTA/BCR-ABL mice harbor leukemia clones expressing imatinib-resistant BCR-ABL kinase domain (KD) mutations.

Assay: Sensitivity	Mice ^a	Detection ^b	KD mutations
D-HPLC: 10 ⁻² -10 ⁻³ (wt:mutant mRNA)	-IM	0/7	-
	+IM	0/5	-
ASOL-PCR: 10 ⁻³ (wt: mutant mRNA); 10 ⁻³ (cells)	-IM	1/7	E255K, T315I
	+IM	1/5	T315I
Sequencing of IM-resistant colonies: 10 ⁻⁶ (cells)	-IM	5/7	E255K, T315I, H396P

^aInduced mice were untreated (-) or treated (+) with imatinib mesylate (IM) as described in Materials and Methods

^bNumber of mice with mutations/total number of mice analyze

Copy number alterations (CNAs) have been detected in many CML patient samples by single nucleotide polymorphism (SNP) and cytogenetic hybridization (CGH) arrays and associated with resistance to tyrosine kinase inhibitors (TKIs) and/or progression from CML-CP to CML-AP/BP [222-226]. To examine whether or not elevated ROS and oxidative DNA damage in Lin⁻c-Kit⁺Sca-1⁺ LSCs from induced SCLtTA/BCR-ABL mice exhibiting CML-CP resulted in genomic instability manifested by CNAs in specific genes, we employed high density SNP array [215] of high molecular weight (HMW) genomic DNA (gDNA) extractions from the Lin⁻ BMCs of 8 untreated (-IM), induced and 4 untreated, non-induced mice, and 4 tail gDNA samples from untreated, induced mice. SNP results from induced Lin⁻ BM gDNA were compared to non-induced Lin⁻ BM and/or induced tail gDNA in order to deduce copy number losses (CNLs) and gains (CNGs) in candidate genes that either regulate ROS levels and DNA repair, or candidate genes previously reported to be associated with TKI-resistance (TKI-R) and/or CML progression.

In untreated, induced SCLtTA/BCR-ABL mice we detected CNAs in many genes that regulate ROS levels (Table 2). More specifically, we detected CNAs in many genes encoding proteins involved in the mitochondrial respiratory chain (MRC). NADH dehydrogenase-ubiquinone genes (*Ndufs2*, *Ndufa4l2*) encode proteins that are subunits of Complex I (NADH-ubiquinone oxidoreductase), an integral inner membrane multi-protein complex of the mitochondrial ETC that reversibly oxidizes NADH in a reaction coupled to a proton pump that generates transmembrane potential and results in •O₂⁻ generation into the mitochondrial matrix in the presence of NADH and tightly bound ubiquinone, which acts as a redox shuttle between the Fe-S centers and O₂ [116, 227];

therefore, CNGs in *Ndu* family genes may lead to enhanced $\bullet\text{O}_2^-$ production. The α -ketoglutarate dehydrogenase complex is tightly associated with the matrix side of the inner mitochondrial membrane and catalyzes the oxidation of α -ketoglutarate to succinyl-CoA using NAD^+ as an electron acceptor which may lead to the production of $\bullet\text{O}_2^-$ and H_2O_2 [228]. The α -ketoglutarate dehydrogenase complex is composed of three enzymes, one of which is α -ketoglutarate dehydrogenase, also known as oxoglutarate dehydrogenase (*Ogdh*); therefore, CNG of *Ogdh* may result in enhanced production of $\bullet\text{O}_2^-$ and H_2O_2 . Complex III (ubiquinone-cytochrome *c* reductase) is an enzyme complex oxidizing coenzyme Q during the “Q-cycle”, using cytochrome *c* as an electron acceptor, coupled to the translocation of protons to generate transmembrane potential [116, 229]. Complex III is a major producer of $\bullet\text{O}_2^-$, due to an unstable semiquinone formed in the Q_0 site, to both the mitochondrial matrix and cytosolic side, which can easily be dismutated to H_2O_2 [230-235]. Ubiquinol cytochrome c reductase 10 (*Uqcr10*) encodes a subunit of Complex III, therefore, CNG of *Uqcr10* may lead to enhanced $\bullet\text{O}_2^-$ production. Monoamine oxidases are located in the outer mitochondrial membrane and catalyze the oxidation of biogenic amines accompanied by robust H_2O_2 release [116]; CNGs in two genes encoding family members of monoamine oxidase (*Maoa*, *Maob*) may lead to enhanced production of H_2O_2 . Glutathione-S-transferase A3 (*Gsta3*) encodes an enzyme involved in ROS detoxification; it catalyzes the conjugation of ROS with the reduced form of glutathione (GSH) by acting as an electron acceptor. CNL of *Gsta3* may result in net gain of mitochondrial ROS due to loss of antioxidant activity, leading to mitochondrial lipid peroxidation [236].

In addition to CNAs detected in mitochondrial-associated genes that may regulate ROS levels, we detected CNGs in endoplasmic reticulum (ER)-associated genes that may regulate ROS levels. Cytochrome P-450 is the terminal component of the monooxygenase system within the ER that detoxifies compounds into less toxic products through oxidation and hydroxylation reactions, during which electrons may be leaked onto O₂ leading to the formation of •O₂⁻ [125]. Therefore, CNGs in multiple genes encoding different family members of cytochrome P450 (*Cyp4a12a*, *Cyp4f39*, *Cyp24a1*) may lead to enhanced production of •O₂⁻.

In untreated, induced SCLtTA/BCR-ABL mice we detected CNAs in many genes that regulate DNA repair (Table 2). RecQ helicases function in DNA replication, recombination and repair; more specifically, they have specific roles in DSB repair, mismatch repair (MMR) and base excision repair (BER) [237]. Therefore, CNLs of RecQ family members (*Recql*, *Recql5*) may result in the aberrant repair of DSBs leading to chromosomal translocations and/or megabase deletions of DNA, and/or mismatched basepairs and oxidative lesions leading to point mutations. Ring finger proteins act as tumor suppressors and recruit necessary repair proteins to DSB formation sites [238]; therefore, CNL of *Rnf40* may lead to aberrant DSB repair. The X-ray repair cross-complementing protein 1 (*Xrcc1*) interacts with various substrates involved in BER and single strand break (SSB) repair [239]; CNG in *Xrcc1* may result in enhanced, yet unfaithful, DNA repair—an effect associated with BCR-ABL activity.

Additionally, we submitted gDNA extracted from the total BM and SPL of 3 treated (+IM), induced SCLtTA/BCR-ABL mice and compared SNP array results to gDNA extracted from the total BM of 3 untreated, non-induced mice. Samples within

each group were pooled prior to the array to account for low cell number (due to *in vivo* IM-treatment) resulting in poor gDNA yield. In IM-treated, induced SCLtTA/BCR-ABL mice we detected CNAs in many genes that regulate both ROS levels and DNA repair. Complex IV (cytochrome c oxidase) is a terminal component of the mitochondrial ETC which adds four electrons onto O₂ in a series of reduction reactions, each of which may yield the formation of •O₂⁻ [123]. CNGs were detected in multiple genes encoding subunits of cytochrome c oxidase (*Cox6b2*, *Cox18*), which may result in the enhanced production of •O₂⁻. *Mcm9* encodes a DNA helicase crucial for effective HR [240]; therefore, CNL of *Mcm9* may be associated with defective DSB repair. Poly (ADP-ribose) polymerase proteins are involved in the repair of DNA strand breaks and maintain telomere length of chromosomes [241]. Consequently, CNG in *Parp4* may result in enhanced, yet unfaithful, DNA strand break repair and/or telomere lengthening resulting in enhanced survival of leukemia cells.

We detected CNAs in many genes previously associated with TKI-resistance and disease progression to CML-AP/BP in both untreated and IM-treated, induced SCLtTA/BCR-ABL mice (Table 2). CNGs in multiple genes encoding downstream effectors of BCR-ABL kinase signaling were detected: rac-γ serine/threonine protein kinase 3 (*Akt3*), rho/rac guanine nucleotide exchange factor 2 (*Arhgef2*), mitogen-activated protein kinase 1 (*Mapk1*), protein kinase c α (*Prkca*), and platelet-derived growth factor receptor β (*Pdgfrb*). Furthermore, CNL in dual specificity phosphatase 18 (*Dusp18*) may potentiate further BCR-ABL kinase signaling. WD repeat-containing protein 5 (*Wdr5*) may mediate self-renewal [242]; therefore, CNG in *Wdr5* may contribute to the enhanced self-renewal capacity associated with blastic transformation of

CML. Gain-of-function mutations in the isocitrate dehydrogenase 1 (*Idh1*) gene modify myeloid cell development and promote leukemogenesis [243], thus supporting the concept that CNG of *Idh1* could contribute to CML progression. Ikaros zinc finger family 1 (*Ikzf1*) is an important regulator of lymphoid development; loss of *Ikzf1* activity was associated previously with lymphoblastic transformation of CML, thus supporting the potential role of CNL in disease progression [244]. Overexpression of Zinc finger protein (*Zfp423*) is associated with blastic transformation of CML [245]; thus, CNG of *Zfp423* also may play a role in disease progression. Lastly, CNLs in multiple genes encoding known tumor suppressors were detected: dachshund homolog (*Dach1*), transformation related protein 53 (*Trp53*), and retinoblastoma (*Rb1*), thereby amplifying BCR-ABL-mediated transformation.

We detected CNAs in many genes regulating ROS levels and DNA repair, as well as genes previously associated with TKI-resistance and disease progression in human CML, in both untreated and IM-treated, induced SCLtTA/BCR-ABL leukemia cells. Although there appears to be biased distribution of CNAs detected in untreated, induced

Table 2. Imatinib-naïve and imatinib-treated induced SCLtTA/BCR-ABL mice accumulate aberrations in genes that regulate ROS levels and DNA repair resulting in resistance to TKIs and progression to CML-AP/BP

CNA	Mice ^a	Candidate genes	
		ROS and DNA repair	TKI-R and CML-AP/BP
CNLs	-IM	<i>Gsta3, Recql, Recql5, Rnf40</i>	<i>Cabin1, Ccdc91, Csf3r, Dach1, Ddx11, Dusp18, Eng, Ifi74, Ikzf1, Mapk8ip1, Pik3c2g, Recql, Rere, Rnf40, Sugt1, Tesk1, Trp53</i>
	+IM	<i>Mcm9</i>	<i>Asf1a, Map3k7, Mcm9, Rars2, Rb1</i>
CNGs	-IM	<i>Cyp4a12a, Cyp4f39, Cyp24a1, Maoa, Maob, Ndufs2, Ndufa412, Ogdh, Uqcr10, Xrcc1</i>	<i>Abcc3, Akt3, Arhgef2, Card14, Cep170, Gabpb2, Idh1, Mapk1, Nup85, Nup188, Pax6, Pi4ka, Prkca, Psmc1, Ptbp2, Spire1, Ush2a, Usp37, Wdr5, Zfp423</i>
	+IM	<i>Cox6b2, Cox18, Parp4, Recql5</i>	<i>Nup85, Pax6, Pdgfrb, Ptpn14, Wdr26</i>

^aInduced mice were untreated (-) or treated (+) with imatinib mesylate (IM) as described in Materials and Methods

mice, we analyzed nearly three times as many samples in this small cohort (n=8 untreated, induced mice versus n=3 IM-treated, induced mice) and we cannot exclude the possibility of detecting a greater number of different CNAs associated with ROS, DNA repair, TKI-R, and CML-AP/BP if we had analyzed more IM-treated, induced mice.

These data show that elevated levels of precursors to genomic instability, i.e. ROS and oxidative DNA damage, displayed by induced Lin⁻c-Kit⁺Sca-1⁺ leukemia stem cells (LSCs), including the quiescent fraction, from the tetracycline-inducible SCLtTA transgenic mouse model of CML-CP result in the accumulation of genetic mutations including, but not limited to, point mutations in the BCR-ABL kinase domain and alterations in genes previously associated with resistance to tyrosine kinase inhibitors (TKIs) and progression from a relatively benign CML-CP to CML-AP/BP human patients. Additionally, the accumulation of genomic instability is not inhibited by the small molecule tyrosine kinase inhibitor imatinib mesylate (IM). Furthermore, alterations in genes associated with ROS, i.e. gains in genes that contribute to ROS formation and losses in genes that inhibit ROS formation, and DNA repair may be underlying factors leading to elevated levels of ROS, oxidative DNA damage and genomic instability originating from these induced LSCs.

CML-CP Lin⁻c-Kit⁺Sca-1⁺ LSCs display deregulated expression of genes associated with ROS and DNA repair

To identify molecular mechanisms underlying enhanced levels of ROS and oxidative DNA damage resulting in genomic instability of Lin⁻c-Kit⁺Sca-1⁺ LSCs from induced SCLtTA/BCR-ABL mice, we performed gene expression profiling of Microarray data previously generated by Steffen Koschmieder's group [210]. A list of selected genes

with more than 1.5-fold change in expression levels associated with regulation of ROS levels and DNA repair is shown in Table 3. We detected enhanced expression of genes encoding polyamine oxidase (*Paox*) and a subunit of cytochrome c oxidase (*Cox6a2*) of Complex IV of the MRC in induced LSCs, which may result in enhanced levels of H₂O₂ and •O₂⁻, respectively [123, 246]. Furthermore, we detected decreased expression of genes encoding pyruvate kinase, liver and red blood cell (*Pklr*) and glutathione-s-transferase μ 2 (*Gstm2*); both pyruvate and glutathione are ROS scavengers, therefore, down-regulation of *Pklr* and *Gstm2* may result in net gain of mitochondrial ROS due to loss of antioxidant activity [236, 247]. TCDD-inducible poly (ADP-ribose) polymerase (*Tiparp*) gene expression is amplified and up-regulated in head-and-neck squamous cell carcinoma (HNSC) [248]; enhanced gene expression detected in induced LSCs may contribute to malignant transformation as a consequence of enhanced, yet unfaithful, DNA repair resulting in genomic instability. Unbiased pathway analysis using Ingenuity Software revealed highly significant changes in pathways related to mitochondrial dysfunction, oxidative phosphorylation, glycolysis/gluconeogenesis, pyruvate and glutathione metabolism, and DNA repair (data not shown).

Table 3. Microarray analysis of the expression of genes potentially involved in the enhanced levels of ROS and repair of oxidative DNA damage in Lin⁻c-Kit⁺Sca-1⁺ LSCs from induced SCLtTA/BCR-ABL mice in comparison to non-induced counterparts

Gene symbol	Probe set ID	Fold change	P value	Function
ROS				
<i>Paox</i>	1428859_at	1.55	0.023794	Catalyzes oxidation yielding H ₂ O ₂
<i>Cox6a2</i>	1417607_at	1.90	0.039070	Mitochondrial electron transport
<i>Pklr</i>	1421259_at	-1.65	0.004456	Pyruvate synthesis
<i>Gstm2</i>	1416411_at	-2.01	0.012738	Conjugates ROS with glutathione
DNA repair				
<i>Tiparp</i>	1426721_s_at	1.58	0.042406	DSB repair

These data show that deregulated expression of genes associated with ROS, i.e. elevated expression of genes that contribute to ROS formation and decreased expression of genes that inhibit ROS formation, and DNA repair may be underlying factors leading to elevated levels of ROS and oxidative DNA damage resulting in genomic instability originating from induced Lin⁻c-Kit⁺Sca-1⁺ LSCs of the tetracycline-inducible SCLtTA transgenic mouse model of CML-CP.

BCR-ABL exerts a dose- and kinase-dependent effect on ROS and oxidative DNA damage in BCR-ABL-transduced human CD34⁺ cells

Elevated mRNA and protein expression of BCR-ABL in CD34⁺ progenitor cells from CML patients has been associated with disease progression from CP to BP, the acquisition of additional genetic abnormalities and IM resistance [192-197, 203]. These findings suggest that high expression of BCR-ABL may underlie mechanisms of genomic instability associated with CML progression and drug resistance, of which ROS and oxidative DNA damage are the primary suspects.

To investigate the effect of increased levels of BCR-ABL expression on ROS and oxidative DNA damage we employed healthy human donor CD34⁺ cells transduced with infectious particles (MIG R1, MIG 210) expressing GFP generated by transient transfection of 293 cells [217]. Cell populations were selected based on low (L) and high (H) GFP expression levels determined by FACS. First, we assessed whether BCR-ABL expression levels corresponded to GFP expression levels selected by FACS. Western blot analysis using anti-c-Abl antibody confirmed increased BCR-ABL protein expression in MIG 210 H cells compared to the MIG 210 L cells (Figure 5A, *upper panel*). Additionally, MIG 210 H cells demonstrated increased tyrosine kinase activity and

altered patterns of tyrosine phosphorylation compared to MIG 210 L cells as assessed by Western blot analysis using anti-phosphotyrosine antibody (Figure 5A, *center panel*).

Both MIG 210 L and MIG 210 H cells exhibited increased phosphotyrosine levels compared to control MIG R1 L and MIG R1 H cells expressing GFP alone.

Next, we determined whether BCR-ABL expression levels affected ROS levels in transduced CD34⁺ cells (Figure 5B). As detected by DHE and analyzed by FACS, MIG 210 H cells displayed elevated levels of •O₂⁻ (14,646.333 ± 469.118) compared to MIG 210 L cells (7,542.472 ± 417.389, p<0.001) and MIG R1 H cells expressing GFP alone (5,562 ± 736.996, p<0.001) (Figure 5B, *left panel*). Additionally, MIG 210 L cells displayed elevated •O₂⁻ levels compared to MIG R1 L cells (5,104.667 ± 276.553, p=0.001). As detected by CC-1 and analyzed by FACS, MIG 210 H cells displayed elevated levels of •O₂⁻/H₂O₂ (2,229.056 ± 213.705) compared to MIG 210 L cells (1468.889 ± 376.708, p=0.038) and MIG R1 H cells (1,423.5 ± 157.622, p=0.006) (Figure 5B, *right panel*). However, MIG p210 L cells failed to elevate •O₂⁻/H₂O₂ levels significantly above that of MIG R1 L cells.

Lastly, we determined whether BCR-ABL expression levels affected levels of oxidative DNA damage in transduced CD34⁺ cells (Figure 5C). As detected by immunostaining of anti-8-oxoG antibody in DAPI-counterstained nuclei, MIG 210 H cells displayed enhanced nuclear staining of 8-oxoG (12.64 ± 7.67) compared to MIG 210 L cells (7.24 ± 4.303, p=0.016) and MIG R1 H cells (4.675 ± 2.75, p<0.001) (Figure 5C, *left panel*). Similarly, as detected by immunostaining with anti-γ-H2AX antibody in DAPI-counterstained nuclei, MIG 210 H cells displayed enhanced γ-H2AX foci (24.038

Figure 5. ROS and oxidative DNA damage are proportional to BCR-ABL protein expression and kinase activity in BCR-ABL-transduced human CD34⁺ cells. Human CD34⁺ cells were transduced with pMIG-IRES-GFP (MIG R1) or pMIG-p210BCR-ABL-IRES-GFP (MIG 210) retroviral constructs. Cells were maintained in IMDM containing 10% FBS, 1% antibiotic-antimycotic solution, and growth factors (SCF, GM-CSF) required to promote survival and proliferation. **(A)** Cells expressing the 15-20% lowest (L) and highest (H) GFP were sorted by FACS and analyzed by Western blot to detect p210BCR-ABL and c-ABL protein expression (*upper panel*), cellular protein tyrosine phosphorylation (p-Tyr; *center panel*), and β-Actin for loading control (*lower panel*). **(B)** Cellular $\bullet\text{O}_2^-$ and $\bullet\text{O}_2^-/\text{H}_2\text{O}_2$ were detected in the 15-20% lowest (light green bars) and highest (dark green bars) GFP-expressing cells with DHE (*left panel*) and Redox Sensor CC-1 (*right panel*), respectively, and analyzed by FACS; n=3 experiments per group. **(C)** Cells expressing the 15-20% lowest (light green bars) and highest (dark green bars) GFP were sorted by FACS. *left panel*: 8-oxoG (MIG R1 L: n=15 cells, MIG R1 H: n=31 cells, MIG 210 L: n=16 cells, MIG 210 H: n=21 cells) and *right panel*: γ-H2AX (MIG R1 L: n=31 cells, MIG R1 H: n=24 cells, MIG 210 L: n=45 cells, MIG 210 H: n=53 cells) were detected by immunofluorescence in DAPI-counterstained nuclei. Data are expressed as mean ± standard deviation (SD). *p ≤ 0.001, **p ≤ 0.005, ***p ≤ 0.01, ****p ≤ 0.05 in comparison to MIG R1 counterpart(s) and MIG 210 L.

± 15.111) compared to MIG 210 L cells (18.533 ± 11.567 , $p=0.049$) and MIG R1 H cells (13.5 ± 9.217 , $p=0.002$) (Figure 5C, *right panel*). However, MIG p210 L cells failed to enhance both 8-oxoG lesions and DSB formation significantly above that of MIG R1 L cells.

The transformation potential and mutator phenotype of BCR-ABL has largely been attributed to its kinase activity [175, 180, 184, 249]. To investigate the effect of BCR-ABL kinase activity on ROS and oxidative DNA damage we employed healthy human donor CD34⁺ cells transduced with infectious particles expressing GFP alone (MIG R1), co-expressing GFP and BCR-ABL (MIG 210), or co-expressing GFP and BCR-ABL kinase-deficient K1172R mutant (MIG KD) generated by transient transfection of 293 cells. Cell populations were selected based on total GFP expression determined by FACS. First, we determined whether BCR-ABL KD mutant affected $\bullet\text{O}_2^-$ levels in transduced CD34⁺ cells (Figure 6A). As detected by DHE and analyzed by FACS, MIG 210 cells displayed elevated levels of $\bullet\text{O}_2^-$ ($12,028 \pm 796$) compared to MIG R1 cells ($7,072 \pm 1,437$, $p<0.001$) and MIG KD cells ($8,303 \pm 1,929$, $p=0.004$). Then, we determined whether BCR-ABL KD mutant affected levels of oxidative DNA damage in transduced CD34⁺ cells (Figure 6B). As detected by immunostaining of anti-8-oxoG antibody in DAPI-counterstained nuclei, MIG 210 cells displayed enhanced nuclear staining of 8-oxoG (0.842 ± 0.714) compared to MIG R1 cells (0.342 ± 0.32 , $p<0.001$) and MIG KD cells (0.434 ± 0.279 , $p=0.034$). Similarly, as detected by immunostaining with anti- γ -H2AX antibody in DAPI-counterstained nuclei, MIG 210 cells displayed enhanced γ -H2AX foci (33.683 ± 27.017) compared to MIG R1 cells (21.475 ± 20.262 , $p=0.024$) and MIG KD cells (19.969 ± 20.150 , $p=0.019$). MIG KD cells failed to enhance

•O₂⁻ levels, 8-oxoG lesions and DSB formation significantly above that of MIG R1 cells, suggesting that BCR-ABL kinase activity is necessary for ROS-induced DNA damage to occur.

Figure 6. BCR-ABL kinase-deficient (KD) K1172R mutant does not induce ROS and oxidative DNA damage in transduced human CD34⁺ cells. Human CD34⁺ cells were transduced with pMIG-IRES-GFP (MIG R1), pMIG-p210BCR-ABL-IRES-GFP (MIG 210), or pMIG-p210BCR-ABL(K1172R)-IRES-GFP (MIG KD) retroviral constructs. Cells were maintained in IMDM containing 10% FBS, 1% antibiotic-antimycotic solution, and growth factors (SCF, GM-CSF) required to promote survival and proliferation. **(A)** Cellular •O₂⁻ was detected in GFP-positive cells with DHE and analyzed by FACS; n=5 experiments per group. **(B)** GFP-positive cells were sorted by FACS. *left panel*: 8-oxoG (MIG R1: n=83 cells, MIG 210: n=29 cells, MIG KD: n=16 cells) and *right panel*: γ-H2AX (MIG R1: n=40 cells, MIG 210: n=41 cells, MIG KD: n=32 cells) were detected by immunofluorescence in DAPI-counterstained nuclei. Data are expressed as mean ± standard deviation (SD). *p≤0.001, **p≤0.005, ***p≤0.05 in comparison to MIG R1 and MIG KD counterparts.

These data show that, in BCR-ABL-transformed human CD34⁺ cells, levels of BCR-ABL expression and/or kinase affect levels of ROS and oxidative DNA damage in a dose-dependent manner. Furthermore, these data suggest that there is a BCR-ABL kinase-dependent effect on levels of ROS and oxidative DNA damage. Therefore, elevated BCR-ABL expression levels and kinase activity may be underlying mechanisms contributing to elevated levels of ROS and oxidative DNA damage resulting in the accumulation of genomic instability.

CHAPTER 4

DISCUSSION

Primary and acquired genomic instability leading to drug resistance and disease progression poses as a major obstacle to achieving a cure for CML [68, 250]. To determine which subpopulation of leukemia cells, stem and/or progenitors, is responsible for the accumulation of genetic errors resulting in TKI-resistance and/or disease progression, we employed the tetracycline-inducible SCLtTA/BCR-ABL transgenic murine model of CML-CP which targets BCR-ABL expression to hematopoietic stem and progenitor cells. BCR-ABL mRNA expression in BM-derived Lin⁻c-Kit⁺Sca-1⁺ stem and Lin⁻c-Kit⁺Sca-1⁻ progenitor cells of induced SCLtTA/BCR-ABL mice lead to splenomegaly and granulo-monocytic myeloid expansion in the bone marrow, spleen and peripheral blood, thereby recapitulating the disease phenotype of human CML-CP.

We show here that induced Lin⁻c-Kit⁺Sca-1⁺ leukemia stem cells (LSCs), including the TKI-refractory quiescent population, but not induced Lin⁻c-Kit⁺Sca-1⁻ leukemia progenitor cells (LPCs), display elevated levels of ROS and oxidative DNA damage, an effect recently reported in human CML-CP CD34⁺CD38⁻ stem-enriched and CD34⁺ quiescent cells [221]. Also, we detected genetic aberrations and deregulated expression in genes that regulate ROS (*Gst*, *Cyp*, *Mao*, *Ndu*, *Ogdh*, *Uqcr*, *Cox*, *Paox*, *Pklr*) and DNA repair (*Recql*, *Rnf*, *Xrcc*, *Parp*), which may underlie the accumulation of ROS-induced oxidative DNA damage in induced Lin⁻c-Kit⁺Sca-1⁺ LSCs, including the quiescent population. Accumulation of ROS-induced DNA damage in induced Lin⁻c-Kit⁺Sca-1⁺ LSCs, including the quiescent population, resulted in the accumulation of clinically relevant point mutations in the BCR-ABL kinase domain (E255K, T315I,

H396P) and genetic aberrations associated with TKI-resistance and disease progression in human CML (*Ikzf1*, *Trp53*, *Idh1*, *Zfp423*, etc.). Altogether, these findings are in accordance with previous reports that BCR-ABL enhances unfaithful repair of ROS-induced DNA damage leading to genomic instability [182-184, 187], and suggests that there is regulation at both the genetic and transcriptional level. Furthermore, these results indicate that genomic instability may originate from the LSC, including the most primitive quiescent LSC, but does not exclude the possibility that genomic instability may originate from the LPC.

Primitive CML-CP cells are unresponsive to many tyrosine kinase inhibitors (TKIs) [56, 77, 218, 251, 252]. Despite inhibition of BCR-ABL kinase, CML stem cells may be insensitive to TKIs independently of quiescence [56, 252, 253], suggesting that there are other mechanisms underlying the insensitivity of quiescent CML stem cells to imatinib mesylate (IM). We show here that elevated levels of ROS and oxidative DNA damage in induced Lin⁻c-Kit⁺Sca-1⁺ quiescent LSCs persist despite IM inhibition of BCR-ABL kinase; similarly, accumulation of clinically relevant point mutations in the BCR-ABL kinase domain and genetic aberrations associated with TKI-resistance and disease progression in human CML is not prevented by IM. Thus, quiescent LSCs may serve as a reservoir for unfaithfully repaired ROS-induced DNA damage resulting in genetic aberrations that fuel both TKI-resistance and CML progression. Additionally, despite inhibition of BCR-ABL kinase, CML stem cells, including the quiescent (G₀) population, may be insensitive to IM independently of mutation [56, 77, 252, 253]. Interestingly, BCR-ABL transcripts are more abundant in G₀ CD34⁺ CML cells than in G₁/S/G₂/M CD34⁺ CML cells [254]. Even though we didn't detect BCR-ABL transcripts

in induced Lin⁻c-Kit⁺Sca-1⁺ quiescent LSCs, BCR-ABL expression levels, or “dosage”, may represent yet another mechanism underlying genomic instability in these cells.

In CML-CP patients, BCR-ABL transcripts and protein expression are more abundant in stem cells than in myeloid progenitors [77, 255], a characteristic that is mimicked in the induced Lin⁻c-Kit⁺Sca-1⁺ LSCs and Lin⁻c-Kit⁺Sca-1⁻ LPCs, respectively, of the SCLtTA/BCR-ABL mouse model; as a result, there appears to be a BCR-ABL dose-dependent effect on ROS levels in induced cells. However, non-induced Lin⁻c-Kit⁺Sca-1⁺ stem cells display elevated levels of ROS in comparison to non-induced Lin⁻c-Kit⁺Sca-1⁻ progenitors, suggesting that stem cells may be more prone to accumulate genomic instability due to intrinsic cellular properties, yet may also be better equipped to repair lesions [256, 257]. Induced Lin⁻c-Kit⁺Sca-1⁻ LPCs failed to recapitulate enhanced ROS levels previously reported in human CD34⁺ progenitor-enriched CML-CP cells [221], perhaps due to sub-threshold levels of BCR-ABL transcripts.

BCR-ABL transcripts and protein expression in myeloid progenitors are more abundant in CML-BC than in CML-CP [77, 217, 255, 258, 259]. Progression from CML-CP to CML-BC also is associated with the accumulation of additional genetic aberrations [260]. Furthermore, BCR-ABL expression exerts a dose-dependent effect on recruitment of unfaithful DNA repair mechanisms, an effect that may result in the accelerated acquisition of IM-resistant point mutations [187, 258, 261]. We report here that BCR-ABL expression exerts a dose-dependent effect on ROS and oxidative DNA damage in BCR-ABL-transduced human CD34⁺ cells, thereby strengthening the link between BCR-ABL “dosage” and unfaithfully repaired ROS-dependent DNA damage resulting in genomic instability associated with TKI-resistance and CML progression. Also, we report

that BCR-ABL exerts a kinase-dependent effect on ROS and oxidative DNA damage in transduced human CD34⁺ cells. However, IM inhibition of BCR-ABL kinase accompanied by persistently elevated levels of ROS and oxidative DNA damage in induced Lin⁻c-Kit⁺Sca-1⁺ quiescent LSCs from SCLtTA/BCR-ABL mice suggests that there are BCR-ABL kinase-independent mechanisms that maintain these elevated precursors to genomic instability; this notion is partially supported by a previous report suggesting that CML stem cells do not rely on BCR-ABL kinase activity for survival [262]. Accordingly, if BCR-ABL kinase-independent mechanisms of survival are either activated in response to the anti-proliferative effect of IM or already in place, both induced and innate quiescence may permit CML cells to accumulate genomic instability before re-entering the cell cycle as TKI-resistant and more aggressive CML cells leading to relapse.

Human CML CD34⁺CD38⁻ stem-enriched cells and CD34⁺ progenitor-enriched cells display elevated levels of ROS and oxidative DNA damage, and the detection of mutations in patients diagnosed with CML-CP supports the idea that genomic instability may originate from either or both compartments. However, if genomic instability originates from either compartment, the acquired mutation(s) must confer resistance in order to survive therapeutic intervention, such as imatinib mesylate (IM). Additionally, if genomic instability originates from the CML stem cell compartment then the acquired mutation(s) must also reactivate the cell cycle within the quiescent fraction in order to propagate the mutation(s) that confers resistance into the expanding pool of CML cells; if genomic instability originates from the CML progenitor cell compartment then the acquired mutation(s) must also equip these cells with the capacity to self-renew in order

to propagate the mutation(s) that confers resistance, which may otherwise be inconsequential upon terminal differentiation. Alternatively, CML cells may exhibit genomic instability that does not confer resistance to IM and/or induce progression to CML-AP/BP, thereby maintaining the susceptibility of CML cells to elimination by IM.

In the Phase III, open-label International Randomized Study of Interferon versus STI571 (IRIS), previously untreated CML-CP patients were randomized to receive treatment with imatinib mesylate (IM) or interferon- α (IFN- α) plus cytarabine [263]. Data from five- and six-year follow-ups of patients randomized to receive IM as first-line therapy for newly diagnosed CML-CP show that, despite spikes in the second and third year of treatment with IM, there is an overall decline in the annual rate of detectable events, characterized as loss of complete hematologic response (CHR), loss of major cytogenetic response (MCyR), progression to accelerated phase (AP) or blast phase (BP) CML, or death, with nearly undetectable events in the sixth year of treatment with IM [60, 264]. Furthermore, there is a steady decrease in the annual rate of detectable events over four years in patients who have achieved a complete cytogenetic response (CCyR) to treatment with IM, with nearly undetectable events in the fourth year.

The long-term success of IM in reducing the annual rate of detectable events may be due to the elimination of dividing CML cells perhaps displaying elevated ROS and oxidative DNA damage that results in irrelevant mutations that maintain the susceptibility of CML cells to IM rather than the inhibition of ROS and oxidative DNA damage and subsequent genomic instability resulting in resistance to IM and/or CML progression. Additionally, detectable events, of which genomic instability may be an underlying cause, are most likely manifested by the expanded CML progenitor population which is

successfully eliminated by IM, whereas the CML stem cell population may display genetic mutations that are present yet undetectable due to the limited size and/or quiescent nature of the compartment. IM may also exert anti-proliferative effects on a small fraction of dividing CML cells and fails to eliminate quiescent CML stem cells. More recent data suggests that there is an increase in the annual rate of detectable events during the seventh and eighth year of treatment with IM [60, 264, 265], which may be due to a resurgence of quiescent CML cells armed with mutations conferring IM-resistance and/or inducing CML progression that are re-entering the cell cycle to expand the resistant/malignant clone.

Some CML-CP patients display genetic mutations at diagnosis, whereas others display genetic mutations later throughout the course of disease, and some may never develop genetic mutations while under treatment. Because genomic instability is manifested heterogeneously among CML patients it is likely that precursors of genomic instability, i.e. ROS and oxidative DNA damage, are displayed heterogeneously among CML patients as well. Accordingly, it is possible that the tetracycline-inducible SCLtTA/BCR-ABL mouse model of CML-CP recapitulates genomic instability exhibited by CML-CP patients at higher risk for developing resistance and/or CML progression. Alternatively, the SCLtTA/BCR-ABL mouse model may serve as a better tool for detection of genetic mutations and may thus accurately mimic the genomic instability exhibited by the majority of CML-CP patients.

In conclusion, Lin⁻c-Kit⁺Sca-1⁺ LSCs, including the quiescent population, from induced SCLtTA/BCR-ABL mice exhibit ROS-induced DNA damage leading to genomic instability manifested by point mutations in the BCR-ABL kinase domain and

genetic aberrations resulting in TKI-resistance and CML progression, an outcome that is not inhibited by IM *in vivo* or *in vitro*. Elevated ROS and oxidative DNA damage may be due partially to elevated BCR-ABL protein expression and kinase activity, however, kinase-independent mechanisms may also pose as contributing factors. These results support previous findings that IM-resistant leukemic cells emerge from CML stem cells that acquire BCR-ABL gene mutations even before exposure to BCR-ABL-targeted agents [218], and suggest that primary CML stem cells display a genetically unstable phenotype that predisposes them to develop resistance prior to treatment. As a result, therapeutic efforts should be targeted toward both BCR-ABL kinase-dependent and -independent mechanisms of persistence and resistance in the CML stem cell compartment, including the quiescent fraction, in effort to eliminate every last CML stem cell. Although ROS, mechanisms that lead to ROS production, and redox-sensitive enzymes may be potential targets for therapeutic intervention in CML cells, these consist of signaling molecules that are also common to normal cells and therefore may not be ideal targets. Alternative DNA repair pathways and/or proteins that are enhanced by BCR-ABL-positive CML cells may serve as better therapeutic targets that would serve to eliminate CML cells while sparing normal cells.

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