EFFECTS OF THE BCR-ABL ONCOGENE ON DNA DAMAGE AND REPAIR

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ABSTRACT

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Chronic myeloid leukemia (CML) is a two-stage disease caused by the p210 BCR-ABL protein. BCR-ABL is a constitutively activated tyrosine kinase that activates signaling pathways to enhance proliferation and inhibit apoptosis. BCR-ABL kinase activity directly causes CML chronic phase, the first stage of CML, but the role of BCR-ABL in transitioning to blast crisis (BC), the second stage of disease, remains under investigation. Given that CML BC is marked by additional chromosomal abnormalities, the role of BCR-ABL in contributing to genomic instability through effects on DNA damage and DNA repair processes has been studied. We investigated what impact BCR-ABL has on the development of DNA double strand breaks (DSBs) and mis-repair in a cell system that is unable to undergo apoptosis. We determined that the failure of cells to undergo apoptosis after DNA damage leads to genomic instability, which is not further increased by BCR-ABL expression. We expressed BCR-ABL in a mouse hematopoietic cell line null for the pro-apoptotic proteins Bax and Bak (DKO). Both DKO cells and the BCR-ABL-expressing cell line (DBA) fail to undergo apoptosis after γ-irradiation (IR). DKO cells are dependent on interleukin-3 (IL-3) for growth and proliferation, but expression of BCR-ABL renders them IL-3 independent. We compared the induction of DSBs after IR and determined that unlike apoptosis-competent cell lines, which demonstrate increased DSB formation in the presence of BCR-ABL, there was no difference in DSB formation comparing DKO to DBA cells. This suggests that the Bax/Bak-mediated induction of apoptosis may be important in the DNA damage response. We also evaluated the level of misrepair in DKO and DBA cells after IR using spectral karyotype (SKY) analysis. We determined that DKO and DBA cells showed a similar accumulation of new chromosomal abnormalities, including gains and losses of chromosomes as well as

translocations, after IR and repair. These results suggest that the BCR-ABL-mediated effects on DNA damage and repair pathways may occur through inhibition of apoptosis rather than or in addition to direct effects on DNA repair pathways. We postulate that BCR-ABL shifts the apoptotic threshold, allowing a group of cells with an increased amount of damage to survive.

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I. INTRODUCTION

A. Chronic myeloid leukemia

Chronic myeloid leukemia (CML) is a disease of the bone marrow that occurs in two or three stages in afflicted patients. The disease was first described in 1845 by a number of different investigators as a blood cell disease characterized by excessive white blood cell accumulation.[1-3] The first stage of disease, known as chronic phase (CP), is frequently asymptomatic, marked only by myeloid hyperplasia in the bone marrow and peripheral blood.[4] The second stage of CML, blast crisis (BC), occurs 3-5 years after diagnosis of untreated CML CP.[4] This phase progresses rapidly like an acute leukemia, and is marked by greater than 20% of the blood or bone marrow consisting of undifferentiated myeloblasts or lymphoblasts.[5] Importantly, over 80% of patients in BC possess cytogenetic abnormalities in addition to the original Ph^{I} translocation.[6] These abnormalities are diverse, ranging from trisomy 8 to i(17q)[6, 7] to mutations that alter function or expression of p53[8, 9], RB[10], or MYC[7]. There is an intermediate phase that is sometimes recognized as occurring between CP and BC, known as accelerated phase (AP). Criteria for diagnosing AP are somewhat variable, and it is not always recognized as a phase of CML. These criteria can include 10–19% myeloblasts or >20% basophils in the blood or bone marrow, cytogenic evolution, and increasing splenomegaly.[11-14]

1. Epidemiology

CML incidence is roughly 1 in 50,000 per year[15], and increases with increasing age.[16] It was estimated that 5,050 men and women would be diagnosed with CML in the US in 2009.[16] Mortality increases with increasing age. The trend in mortality from 1975 to the present has improved[16], that is, mortality has decreased due to advances in therapy (discussed below). It was estimated that 470 men and women would die of CML in 2009.[16]

B. The Molecular Basis of CML

1. The Philadelphia Chromosome

Peter Nowell and David Hungerford first reported the existence of a minute chromosome in metaphases from patients with CML (then called chronic granulocytic leukemia) in 1960.[17] This chromosome was named the Philadelphia chromosome (Ph^{I}) for the city in which it was discovered. Ph^{I} was the first example of a consistent chromosome change associated with a neoplasm (other leukemias had been shown to have abnormal but variable cytogenetics).[18] While it was initially thought that the Ph^{I} was chromosome 21 with partially deleted material[18, 19], it was later determined to be a deletion of part of chromosome 22.[20] In 1973 Janet Rowley determined that CML patients also possessed a common additional faint band at the end of chromosome 9, which appeared to be the same size as the deleted material from chromosome 22.[21] She suggested that this may be a balanced translocation between the two chromosomes.

2. ABL

Armed with this information as well as the knowledge that the *c-Abl* sequence was located on chromosome 9[22]), de Klein et al. determined that *c-Abl* was in fact translocated from chromosome 9 to chromosome 22[23] in CML patients. This demonstrated that the translocation proposed by Rowley was reciprocal, as it was now known that material from chromosome 22 was fused to chromosome 9 and vice versa.

Abl is a tyrosine kinase found mainly in the nucleus.[24, 25] c-Abl is the human cellular homolog of the retroviral oncogene v-Abl[26], both of which demonstrate elevated protein kinase activity.[27] The v-Abl protein was known to transform a variety of cell types, including NIH 3T3 fibroblasts *in vitro*[28] and pre-B-lymphoid cells both *in vitro* and *in vivo*[29] and was shown to be able to eliminate the growth-factor dependence of hematopoietic cells.[30-35] This transformation is dependent on kinase activity of Abl.

3. BCR

The region on chromosome 22 involved in the translocation was described in 1984 by Groffen et al.[36] Rather than being located in one specific site in all patients, the breakpoints were initially found to be contained in a 5.8 kb region. This region was then termed the “breakpoint cluster region” (*BCR*).[36] Three different *BCR* regions have now been described[36-40], with more than 90% of CML cases having breakpoints in intron 13 or 14 of *BCR*.[41] Reaction of the Ph^{I}-specific protein product with BCR-specific antiserum confirmed BCR involvement in the translocation.[42] BCR is a serine/threonine kinase[43-45] that can function as a GTPase-activating protein for members of the Rho family of guanine nucleotide exchange factors[46, 47] and can phosphorylate histones and casein.[43] No evidence exists to suggest that the serine/threonine kinase activity of BCR is important for cellular transformation in CML.

4. p210 BCR-ABL

The gene product of the Ph^{I} translocation was initially determined to be an 8.5 kb mRNA[48], termed BCR-ABL. Transcription of this gene in CML produces the 210 kDa protein known as the BCR-ABL fusion tyrosine kinase.[42, 49] The 210 kDa protein co-immunoprecipitated with ABL antiserum and was detectable by both ABL- and BCR-specific antibodies.[42] There can be slight differences in the exact splice sites of BCR and ABL. The vast majority of patients exhibit mRNA transcripts with b3a2 or b2a2 junctions, and some patients maintain both variants.[50] Other variants include e1a2 and e19a2. These variants do not appear to have a strong impact on tumorigenicity *in vivo*, as there has been no significant correlation between M-bcr breakpoint location and disease outcome.[51, 52]

5. p190 and p230 BCR-ABL

Two other isoforms of BCR-ABL exist, a p185/190 form and a p230 form. Neither of these proteins are expressed in most patients who fit the clinical criteria for CML. Rather p190 BCR-ABL can be seen in patients with acute lymphoblastic leukemia (ALL) [38, 53-55] and p230 BCR-ABL is expressed in leukemic cells of some patients with chronic neutrophilic leukemia.[40]

6. BCR-ABL possesses constitutive tyrosine kinase activity

Initial studies on the function of BCR-ABL focused on the ABL domain because of previous work on the viral homolog v-ABL.[29] A number of proteins were found to be tyrosine phosphorylated in the human CML cell line K562, including the 210 kDa BCR-ABL protein.[56] BCR-ABL can both autophosphorylate as well as phosphorylate other proteins[27, 49], with a tyrosine kinase activity greater than that of c-ABL.[57] This kinase activity was shown to be Abl-specific, as only anti-Abl antisera could neutralize BCR-ABL kinase activity.[58] This autophosphorylation ability of BCR-ABL is necessary for its transforming potential.[59] Deregulated tyrosine kinase activity is not sufficient to induce oncogenesis, as BCR-ABL mutations that do not affect its kinase activity can decrease its ability to transform cells.[59-61]

7. Domains of BCR-ABL

a. Oligomerization domain

The amino-terminal-most portion of BCR-ABL consists of a coiled-coil oligomerization domain from BCR.(51) This region is documented to lead to constitutive oligomerization of BCR-ABL and this oligomerization is necessary for kinase activation.[62-64] It also contributes to BCR-ABL binding to actin.[65] Based on these and other structural studies, it is proposed that BCR-ABL is a cytoplasmically localized and constitutively activated tyrosine kinase.

b. Y177-GRB2 site

C-terminal to the coiled-coil region is an additional functional domain of BCR. This region is required for the serine/threonine kinase activity, leading to its being studied in more detail in the context of BCR-ABL.[43] This region contains a tyrosine autphosphorylation site (Y177), which complexes with GRB2 adaptor protein linking BCR-ABL to the RAS-MAPK pathway.[66-68] Deletion of this domain or mutation of Y177 suppressed the ability of BCR-ABL to transform Rat1 fibroblasts[60, 64, 69], but this mutant still possessed tyrosine kinase activity and was sufficient to render hematopoietic cells cytokine-independent.[61, 69] Additionally, this region can bind to the SH2 domain of Abl, an interaction that is not dependent on tyrosine phosphorylation.[70] Further, BCR-ABL with mutated Y177 is unable to induce a CML-like disease in mice.[71] Overall, these studies concluded that Y177 of BCR is necessary for the transforming function of BCR-ABL in hematopoietic cells that are relevant to the human disease.

c. DBL and PH Domains

p210 BCR-ABL has a region homologous to the guanine nucleotide disassociation-stimulating domain of the differentiated B-cell lymphoma *(dbl)* oncogene.[72] This region can stimulate GTP binding to Rac1, Rac2, and RhoA *in vitro*.[46] Close to this domain is a region with homology to the pleckstrin homology (PH) domain, which has a highly conserved three-dimensional structure.[73] These two domains are not found in the p190 isoform of BCR-ABL, and are not required for BCR-ABL-induced oncogenesis. The fact that p190 BCR-ABL is five-fold more enzymatically active than p210 BCR-ABL[74] suggests that this region may play a negative-regulatory role in BCR-ABL transformation.

d. SH3

The Abl portion of BCR-ABL contains a number of domains including two non-catalytic src homology (SH)2 and SH3 domains that are involved in signal transduction and modulating protein-protein interaction.[75] The SH3 domain of BCR-ABL is a negative regulator of tyrosine

kinase activity. Removal of the SH3 domain from BCR-ABL enhances cellular transformation[76, 77], and deleting the SH3 domain restores tyrosine kinase activity and transforming ability of a transformation-defective BCR-ABL oligomerization domain-deleted clone.[77] Further, deletion of the SH3 domain has no effect on efficient induction of a CML-like disease in mice.[78]

e. SH2

The importance of the SH2 domain, which regulates intracellular signaling cascades though interactions with phosphotyrosine-containing target peptides[79] may be cell-type dependent. It was determined that mutations in the SH2 domain did not impair the ability of BCR-ABL to prevent apoptosis or to render hematopoietic cell lines growth factor-independent.[61] However, SH2 mutants were unable to transform Rat1 fibroblasts or pre-B cells.[25, 60, 63, 69] The SH2 domain was reported to activate such pathways as phosphoinositide 3-kinase (PI3K)[80], but subsequent studies have demonstrated no defect in PI3K signaling in SH2 mutants.[81] Importantly, the BCR-ABL SH2 domain contributes to the efficient induction of a CML-like disease in mice.[80, 81]

8. Downstream signaling pathways of BCR-ABL

The consequences of BCR-ABL expression are similar to that of cytokine stimulation, including promoting growth and inhibiting apoptosis. These occur via BCR-ABL's regulation of a variety of downstream signaling pathways.

a. STAT5

Signal transducer and activator of transcription 5 (STAT5) is constitutively activated by tyrosine phosphorylation and induction of DNA binding activity in BCR-ABL-transformed mouse hematopoietic cells.[82] STAT1 and STAT3 are also activated to a lesser extent. This activation was found to be a direct activation by BCR-ABL, as dominant-negative mutants of Janus kinase

2(JAK2), which is upstream of STATs, did not block STAT5 activation. Further, BCR-ABL only moderately phosphorylated JAKs 1, 2 and 3, and did not complex with these proteins.[82] STAT5 signaling was shown to be required for efficient induction and maintenance of CML in mice[83], however it was found to not be absolutely required for BCR-ABL leukemogenesis.[84] However, the STAT5 mutations used in these studies were later determined to be hypermorphic rather than true null alleles,[85] and overall studies suggest that STAT5 signaling contributes to BCR-ABL-mediated leukemogenesis. This signaling pathway is important for efficient cellular transformation as phosphorylation of STAT5 by BCR-ABL leads to its translocation to the nucleus and binding to promoter elements to regulate gene expression.

b. Ras

Ras signaling is important for transmitting signals from receptor tyrosine kinases, and is required for transformation by oncogenic tyrosine kinases. BCR-ABL expression leads to an increase in the amount of GTP loaded onto Ras.[61, 80, 86-88] Grb2 forms a stable complex with BCR-ABL through the SH2 domain of Grb2 and Y177 on BCR-ABL.[67] This association can recruit son of sevenless (SOS, the Ras-activating nucleotide exchange factor) to the complex, which activates Ras GDP/GTP exchange.[67] Mutating Y177 in BCR-ABL eliminates Grb2 binding ability and compromises Ras activation.[89] Importantly, mutated forms of Grb2 have a dominant-negative effect on BCR-ABL-induced transformation[66], underscoring the importance of the BCR-ABL-Grb2 interaction. Inhibition of Ras signaling suppresses BCR-ABL-induced oncogenesis in terms of cell growth and focus formation[90, 91], suggesting that signaling through RAS is essential for the transforming ability of BCR-ABL.

c. PI3K

BCR-ABL is associated with the activation of phosphatidylinositol 3-kinase (PI3K).[92, 93] BCR-ABL activates PI3K through complex formation between the p85 subunit of PI3K and the SH2

domain of BCR-ABL.[80] The activation of the PI3K pathway is essential for the growth of primary CML cells,[93] and BCR-ABL-mediated leukemogenesis, as a kinase-deficient Akt mutant with dominant-negative activity suppressed BCR-ABL-dependent leukemia development in SCID mice.[80] Activation of PI3K can transduce tyrosine kinase signals[94, 95] and affect several downstream targets including RAS[96], Akt[97] and S6 kinase.[98, 99]

9. BCR-ABL effects on cellular properties

a. Proliferation

BCR-ABL has been shown to transform a number of different cell types including murine bone marrow cells[100, 101] and endothelial cells.[102] Interestingly, though BCR-ABL can transform Rat1 fibroblasts, it is unable to transform NIH3T3 fibroblasts, which may be due to species-specific or other cellular differences in BCR-ABL expression, compartmentalization or expression of additional factors that interact with BCR-ABL[74, 103, 104] This phenomenon is reflective of a frequently seen cell type-dependent difference in BCR-ABL-dependent properties. Transformation of cells by BCR-ABL renders them growth factor-independent and able to undergo dysregulated cell proliferation.[101]

b. Adhesion

BCR-ABL augments cellular adhesion to fibronectin in a tyrosine kinase-dependent manner.[105] BCR-ABL may also affect cellular adhesion in a tyrosine-independent manner.[106, 107] However, this function is likely dispensable for BCR-ABL-induced oncogenesis because deletion of cytoskeletal-interacting regions does not diminish cytokine-independent growth of hematopoietic cell lines or fully diminish foci formation.[63, 106, 108]

c. Apoptosis

Apoptosis[109], or programmed cell death, occurs through extrinsic signals activating death receptors, or intrinsically when a cell encounters DNA damage or ER stress.[110] In the case of cell-intrinsic apoptosis, stress signals lead to the activation of members of the anti- and pro-apoptotic Bcl-2 family that are responsible for mitochondrial outer membrane permeabilization. A pro-apoptotic death decision leads to the activation of caspases and chromatin degradation. Bcl-2 family anti-apoptotic members include Bcl-2, Bcl-xL and Mcl-1, and the proapoptotic members include Bax and Bak among others. Bax and Bak are required for apoptosis,[111, 112] as they oligomerize to form a pore in the outer mitochondrial membrane to release cytochrome C. The Bcl-2 family of proteins is important in controlling the apoptotic threshold.[113]

BCR-ABL has been shown to inhibit apoptosis. McGahon et al. determined that the resistance of K562 cells to apoptosis[114] was a consequence of BCR-ABL expression, as antisense oligonucleotide treatment against BCR-ABL rendered the cells susceptible to apoptosis.[115] Leukemic HL-60 cells expressing p185 BCR-ABL were also found to be more resistant to apoptosis after cytotoxic agents than wild type HL-60 cells. Expression of BCR-ABL in these cells led to the down-regulation of Bcl-2 and increase in Bcl-xL levels, though Bax levels did not change. The authors determined that Bcl-xL was important in BCR-ABL-mediated resistance to apoptosis in HL-60 cells, as knockdown of Bcl-xL increased susceptibility of HL-60.BCR-ABL cells to staurosporine, an ATP-competitive kinase inhibitor used to induce apoptosis.[116] BCR-ABL also inhibits apoptosis in hematopoietic progenitors[117, 118], where the pro-apoptotic molecule p38MAPK was suppressed and the anti-apoptotic Bcl-xL was upregulated.

Despite confirming that BCR-ABL-expressing cells were resistant to apoptosis induced by drug treatment, Keeshan et al. determined that BCR-ABL cells actually had a pro-apoptotic expression profile that is determined by the level of expression of the protein. Normal 32D cells and transformed cells expressing a small amount (low level) of BCR-ABL protein were found to

upregulate p53, p21 and Bax, and downregulate Bcl-2 in response to cytotoxic insult. In contrast, BCR-ABL-transformed cells expressing a large amount (high level) of BCR-ABL expressed constitutively high levels of p53, p21 and Bax, and low levels of Bcl-2, and cytotoxic insult did not alter these levels.[119] An explanation for this expression profile was elucidated in a later paper, where it was determined that the translocation of pro-apoptotic proteins Bax and Bad to the mitochondria was blocked in cells with high BCR-ABL expression. Additionally, BCR-ABL was found to prevent late mitochondrial depolarization and prevent caspase 9 and caspase 3 processing.[120] Therefore, despite the unexpected pro-apoptotic protein expression in cells expressing high levels of BCR-ABL, these proteins do not function normally to carry out apoptosis after cytotoxic insult.

10. BCR-ABL causes CML CP but the transition to CML BC is still in question

The incidence of CML CP is the direct result of the constitutive kinase activity of BCR-ABL and activation of downstream signal transduction pathways.[121, 122] However, the exact role BCR-ABL plays in the transition to CML BC is still not completely known. Activation of cytoplasmic signaling proteins does not appear to explain the progression to CML BC by itself. One of the hallmarks of CML BC is the acquisition of additional chromosomal aberrations beyond the initial t(9;22) chromosomal rearrangement. While a few abnormalities are more common than others, such as trisomy 8, isochromosome 17q, or the presence of an additional Philadelphia chromosome, secondary mutations vary greatly and reflect an overall genomic instability rather than a direct effect on any particular locus.[123] Whether this genomic instability is a direct consequence of BCR-ABL, making this disease a rare example of a one hit malignancy, or whether there are other, unrelated events driving CML BC is the focus of our studies described below.

C. Treatment of CML

For many years, therapies for CML were limited. Treatment with hydroxyurea would decrease cell proliferation and alleviate symptoms, but did not lead to cytogenetic remissions or longer life expectancy.[124] The only curative treatment was ablative chemotherapy to destroy proliferating cells, followed by allogenic bone marrow transplant. This treatment is not always an option for patients due to comorbidities or lack of a suitable donor.[125, 126] Interferon-α (IFN-α) therapy was commonly used and has been extremely effective in achieving remission in a number of CML patients and increasing disease-free survival, though it is not tolerated by all patients due to toxicity problems.[126-128]

1. Imatinib and second-generation BCR-ABL tyrosine kinase inhibitors

In 1996 treatment of CML was revolutionized by the Abl-specific tyrosine kinase inhibitor (TKI) imatinib[129] (also known as Gleevec, CGP 57148, or STI571). Imatinib functions as a competitive inhibitor of ATP binding to the BCR-ABL active site by binding to BCR-ABL in the inactive state. Imatinib was found to selectively inhibit proliferation, tumor formation and colony-forming ability specifically in BCR-ABL-expressing cells but not in normal cells.[129] This drug quickly moved into clinical trials in patients, which demonstrated both a high level of safety and efficacy in achieving cytogenic responses in CP CML after failed IFN-α therapy.[130] At 5 and 6-year follow-ups, responses to imatinib were found to be durable and well-tolerated. Overall the survival rate has been estimated at greater than 85%.[131]

However, despite this large success rate, approximately 20% of patients fail to achieve a complete cytogenetic response (the lack of any detectable Ph^{I}-positive metaphases in patient bone marrow), and other patients may be intolerant or develop resistance over time.[131] Further, CML AP and CML BC patients do not respond as favorably to imatinib as CML CP patients. This

has led to the development of second generation TKIs that are approved for use in imatinib-resistant or -intolerant patients.

Dasatinib, a dual BCR-ABL and Src kinase inhibitor that is effective in treating imatinib-resistant and -intolerant patients, inhibits BCR-ABL at lower doses than imatinib. Dasatinib has also been shown to inhibit platelet-derived growth factor receptor, c-kit, ephrin receptor kinases[132] and Src-family kinases.[133] Dasatinib triggers an irreversible commitment of cells to apoptosis[134] and is effective in imatinib-resistant and -intolerant patients, and has shown promise in the frontline treatment of CML.[135, 136]

Nilotinib is another second generation TKI that was rationally designed to better fit the inactive conformation of the BCR-ABL kinase domain. It is 30-fold more potent than imatinib,[137, 138] and more selective. While its chemical structure is similar to that of imatinib, it has a different intracellular transport mechanism[139] and has differing sensitivity to BCR-ABL mutations.[140, 141] It has been found to be effective in both imatinib-resistant and -intolerant CML[142], and has shown early success as a first-line BCR-ABL inhibitor.[143, 144]

Some patients are resistant to imatinib due to mutations in the BCR-ABL kinase domain[145] that do not allow proper binding of the drug in the BCR-ABL active site.[146] In most cases, these second generation TKIs have better success toward BCR-ABL kinase domain mutations, though they are still unable to inhibit BCR-ABL with mutations T315I or E255K/V. Additional drugs are under development that can inhibit these resistant BCR-ABL mutant forms, such as the third-generation BCR-ABL kinase inhibitors dasusertib[147] and AP25434,[148] and AP24163,[149] which have demonstrated activity against T315I BCR-ABL. Further, vaccine approaches are being tested in mice[150] and human clinical trials,[151] including vaccines directed at the T315I BCR-ABL mutant.[152] Early results in humans suggest that these vaccines may help to reduce

BCR-ABL transcript level, but further testing is required to determine their overall efficacy and safety.

Importantly, these TKIs can also be used *in vitro* to inhibit BCR-ABL in cell lines.

2. CML stem cells are resistant to imatinib

The cancer stem cell hypothesis suggests that only a subset of cells have the capacity to drive tumorigenesis. For example, cells with potential to reconstitute human AML exist in the CD34+CD38- population of hematopoietic cells.[153] Fialkow first demonstrated that CML was a clonal disease that originated from a pluripotent hematopoietic cell[154], or even a hematopoietic stem cell (HSC).[155, 156]

Some evidence supporting the existence of CML stem cells comes from the experience of patients treated with imatinib. Despite the huge success of this BCR-ABL kinase inhibitor in prolonging CML patient survival, BCR-ABL positive cells are not completely eradicated. Even patients who achieve a complete molecular response, or undetectable BCR-ABL transcripts, will relapse if treatment is discontinued, suggesting that there remains a fraction of leukemic stem cells (LSCs). In fact HSCs have been shown to be less sensitve to imatinib[157, 158], and residual BCR-ABL transcripts were detected almost exclusively in the HSC compartment of imatinib-treated patients.[159]

The differences in CML CP and BC have recently been described in terms of the difference in leukemic stem cells, that a new population of LSCs exists in BC. This is thought to occur though increasing genetic instability within the tumor population, which has been well-documented in CML BC([160], discussed below). Evidence suggests that the granulocyte macrophage progenitors (GMPs) of CML BC patients acquire stem-like self-renewal properties.[161, 162] It is not known whether BCR-ABL plays a role in inhibiting CML stem cell death. This does not explain

the development of the less common lymphoid blast crisis, which likely develops from a lymphoid progenitor cell acquiring stem-like properties.

D. Systems for studying BCR-ABL and its effects

1. Mouse Models of CML

A number of different attempts have been made to recapitulate the human CML disease in mice. These have included xenotransplantation into immunodeficient mice, transgenic mice, and transplantation of BCR-ABL retroviral-transduced bone marrow.[163] The latter model was developed by Drs. Daley, van Etten and Baltimore at the Whitehead Institute[121] and refined by Dr. Pear at the University of Pennsylvania[164] and has been widely utilized.

Xenotransplantation consists of injecting cells into severe combined immunodeficient (SCID) or non-obese diabetic/SCID (NOD/SCID) mice. CML cell lines derived from BC patients engraft efficiently in SCID mice.[165, 166] The ability to work directly with primary patient CML cells is a large advantage with this model and there has been some success when transplanting cells from patients in CML BC into these mice.[165, 167] However, there has still been limited success in establishing consistent or high-level engraftment with patients with CP CML.[165, 168] NOD/SCID animals were found to be superior to SCID for engraftment with CP CML, but a very large cell dose was still required.[169-171] Importantly, xenotransplanted mice frequently did not develop any clinical illness, limiting the usefulness of this model in recapitulating human disease.[163, 172]

A number of different transgenic mice expressing BCR-ABL under the control of various promoters have been established.[173-178] Interestingly, rather than developing a CML-like disease, most of these mice instead developed T- or B-ALL.[173-177] Transgenic mice expressing BCR-ABL under the control of the hMRP8 promoter did develop a myeloproliferative

disease in roughly 25% of founders, with one progressing to CML BC.[178] This low rate of myeloproliferative disease development limits the usefulness of this model in studying human CML.

BCR-ABL has also been expressed in mice in conditional transgenic systems. BCR-ABL under the control of a tetracycline response element resulted in the development of fatal B-lymphoid leukemia.[179] Importantly, turning off BCR-ABL in this system resulted in a rapid disappearance of disease[179], suggesting that continuous expression of BCR-ABL is required to maintain disease. Transgenic mice have been useful in demonstrating that BCR-ABL induces leukemia, but have not led to the development of a reliable CML mouse model.

The best mouse models of human CML are produced from an *ex vivo* retroviral bone marrow transduction followed by transplantation into syngenic or immunodeficient mice. Initial studies with this system led to a myeloproliferative disease arising in mice 4-12 weeks after transplantation,[121, 122] characterized by peripheral blood leukocytosis and infiltration of spleen liver and lungs with myeloid cells, reminiscent of CML CP. However, these mice died of respiratory failure very quickly after transplant, before the onset of BC. In fact, bone marrow in this model is typically comprised of less than 10% blasts. Importantly, disease in these animals is oligoclonal, demonstrating that BCR-ABL, as a single genetic abnormality, can drive myeloproliferation. The CML-like disease could be transferred to secondary recipients through bone marrow transplantation[180, 181], and in some cases this resulted in the development of an acute leukemia from the same clone that initiated disease in the original animal[180], suggesting a CML BC-like disease could develop if the animals did not succumb to respiratory failure. Importantly, these studies demonstrated that BCR-ABL is the direct cause of CML.

The use of transient retroviral packaging systems improved viral titer and has led to a more efficient induction of a CML-like disease in mice.[164, 182, 183] Use of these vectors has led to 100% induction of a polyclonal CML-like disease fatal within 4 weeks of transplantation. Importantly, treatment of donors with 5-fluorouracil, which enriches the donor population in stem cells, is required for the CML-like disease phenotype as opposed to a mixture of leukemic diseases.[182] This model has been useful in identifying the domains of BCR-ABL and signaling pathways required for leukemogenesis.[71, 78, 80, 84]

There are also mouse models that develop a CML-like myeloproliferative disease without the expression of BCR-ABL. *Alox15*-null mice are deficient in 12/15-lipoxygenase and develop a myeloproliferative disease reminiscent of CML.[184] Features of disease in this model include increased levels of circulating myeloid cells, splenomegaly, and splenic infiltration by immature myeloid progenitors. Interestingly, approximately 15% of *Alox15*-null mice developed a condition similar to CML BC, with increased immature myeloid cells, though whether the marrow was comprised of > 20% myeloblasts is unclear. In these mice, protein levels of the interferon consensus sequence binding protein (ICSBP) are reduced, which correlate with increased Akt activation. Importantly, ICSBP-null mice also develop a myeloproliferative disease and can undergo transition to a CML BC-like phase but lack transplantablility during the myeloproliferative phase.[152] Further study of these pathways may provide insights to the mechanism of transition to CML BC.

In mice, inactivation of JunB, a transcriptional regulator of myelopoesis, leads to embryonic lethality. However, reintroducing junB expression into all but the myeloid lineage leads to development of a progressive, transplantable myeloproliferative disorder resembling human CML.[185] These mice can also progress to a second stage resembling CML BC. While these mice do not express BCR-ABL and a low percentage of mice progress to CML BC, the disease is

transplantable to secondary recipients. This model could also be useful for studying the genes and signaling pathways associated with the transition to CML BC.

Lastly, another mouse model displays a CML BC-like disease, though lacks the transition from CML CP. This model was established by expressing BCR-ABL in a long-term culture of murine hematopoietic cells deficient in B-cell development.[186] The leukemia-initiating cells in this model were found to reside in the GMP compartment (similar to what has been reported in CML BC patients[161, 162]). This model again does not allow the study of what causes the transition of CML CP to CML BC as the mice only develop acute disease. Future studies will be required to develop a model that undergoes this transition in the presence of BCR-ABL.

2. Cells used to study BCR-ABL

A number of cell lines are commonly used to study BCR-ABL and its effects. A few of them are outlined below.

a. Ba/F3

Ba/F3 is a murine bone marrow-derived cell line dependent on IL-3.[187] Expression of BCR-ABL renders these cells IL-3-independent and tumorigenic in nude mice.[101] Ba/F3 pTET-ON 210 (TonB210.1) cells express BCR-ABL under the control of a tetracycline-inducible promoter.[188] Addition of doxycycline to the media leads to the expression of BCR-ABL, providing the cells with the same IL-3-independent proliferation properties as the non-conditional line. Ba/F3 pTET-ON p210 cells allow for study of BCR-ABL-dependent effects in an isogenic setting.

b. 32D

32D is an immortalized myeloblast-like cell line derived from C3H/HeJ murine bone marrow that is dependent on IL-3 for growth and proliferation.[189] 32D BCR-ABL cells were derived from this

line through infection with a replication-defective murine retroviral vector expressing p210 BCR-ABL. Both the parental and BCR-ABL-expressing 32D cells can be induced to differentiate along either the granulocytic or monocytic lineage. Expression of BCR-ABL renders these cells IL-3-independent.[190]

c. K562

K562 cells are a human erythroleukemic cell line derived from a female patient with CML BC.[191, 192] These cells are positive for BCR-ABL and also exhibit a second reciprocal translocation between the long arms of chromosomes 15 and 17. They do not express wild type p53.[193, 194]

d. Primary patient samples

Cell lines have limitations, such as cellular effects of the immortalizing mutation, and cell line-specific responses. Thus, most laboratories confirm observations in primary cells from patients with CML. Such cells can be stored as frozen, viable cells. Like normal bone marrow, cells can be thawed and incubated for limited periods of time (up to 2 weeks). Thus, limited confirmatory work can be done in primary samples.

E. BCR-ABL and DNA damage

1. BCR-ABL-expressing cells have increased DNA damage and genetic aberrations

As discussed above, CML patients gain cytogenetic aberrations as they transition to BC. As BCR-ABL is the cause of CP CML and the only genetic abnormality associated with disease, the consequences of BCR-ABL expression on DNA damage and genetic aberrations have been studied for many years. The theme of a growing body of literature is that BCR-ABL expression is associated with a consistent small increase in both DNA damage and genetic aberrations in cells.

Pierre Laneuville first suggested that interleukin 3 (IL-3)-dependent cell lines transformed to cytokine independence by expression of BCR-ABL demonstrated features of genomic instability.[195] Laneuville later used the Big Blue mouse, in which point mutations in the transgenic lac Z gene lead to loss of lac Z reactivity in tissues, and reported that expression of BCR-ABL led to a small but significant increase in point mutations in BCR-ABL-expressing cells *in vivo*.[196] However, it is still unresolved whether point mutations are a major mechanism of genetic instability associated with CML BC (see below).

Several groups have pursued more specifically the role of BCR-ABL in the generation of chromosomal instability, one phenotype clearly associated with CML BC. Deutsch and colleagues analyzed sister chromatid exchange (SCE) and chromosome-specific fluorescence *in situ* hybridization (FISH) to demonstrate that BCR-ABL-expressing cell lines have an increased incidence of SCE and an increase in chromosomal translocations after DNA damage.[197] This result was confirmed on a more genome-wide basis using spectral karyotyping (SKY) by the Skorski laboratory[198], and we have recently extended these observations to primary CML cells compared to normal cells.[199]

Several common themes emerge from these studies. Genetic and chromosomal abnormalities are consistently increased in BCR-ABL-expressing cells, although the increase is modest. Increases are seen both spontaneously after long periods of expression of BCR-ABL, and with increased frequency after induction of DNA damage by genotoxic agents. The alterations appear to be random (the cited experiments were done under conditions that did not allow for selection of mutations which provided a growth advantage) consistent with a general “mutator phenotype” rather than induction of a specific genetic lesion. Importantly, most of these assays are laborious and expensive and, as a consequence, few of these papers have in-depth structure-function

studies to identify which domain(s) of BCR-ABL is necessary for the alteration in DNA repair that leads to the accumulation of genetic abnormalities.

2. Potential causes of initial DNA damage in BCR-ABL-expressing cells

DNA damage may arise in BCR-ABL-expressing cells in a number of ways. BCR-ABL has been shown to induce the production of reactive oxygen species (ROS), which cause oxidative damage and mutations.[200-202] Recently, it was also shown that the B cell-specific mutator enzyme activation-induced cytidine deaminase (AID) is expressed in CML lymphoid BC cells and contributes to mutations within BCR-ABL itself.[203] In adults, lymphoid BC represents a minority of CML BC patients[160], however, and it is not clear that AID contributes to the more common myeloid BC. Alternatively, damage could develop due to the uncontrolled proliferation of cells expressing BCR-ABL. As polymerases themselves cause errors during DNA replication[204], an increase in the number of cells produced could simply lead to an increased chance of aberrancy. Alternatively, it is speculated that activated tyrosine kinase oncogenes decrease the fidelity of the G1/S cell cycle checkpoint[191] although this has not been confirmed. Overall, there are several proposed effects of BCR-ABL that may lead to an increased rate of DNA damage in CML CP cells. None of these are conclusively demonstrated to be necessary for progression to CML BC, and it is rather likely that there are several mechanisms that lead to a modest increase in the rate of DNA damage in BCR-ABL-expressing cells.

3. Overview of DNA damage response and repair mechanisms

The DNA damage response is quite complex but can be shortly summarized: DNA damage may occur either as single nucleotide alterations, single strand breaks (SSBs), or double strand breaks (DSBs). SSBs are prone to degrade to DSBs. Single nucleotide alterations are repaired by mismatch repair (MMR) or by nucleotide excision repair (NER). Strand breaks are repaired by either high-fidelity homologous recombination (HR) when a sister chromatid is available as a

template (during the S or G2 phase of the cell cycle), or by non-homologous end joining (NHEJ), which may lead to short deletions in the repaired strands.

4. Aberrant DNA repair

DNA mutations can occur as the result of several situations; when there are mutations in single nucleotide repair pathways, mutations in proteins necessary for the complex process of DNA DSB recognition and repair or, alternatively, when there is a failure of cell cycle checkpoints that allows subsequent replication of damaged DNA. The latter may occur because of defects in sensing DNA damage or defects in proteins necessary to execute the cell cycle arrest.

5. Effects of BCR-ABL on DNA repair

The effect BCR-ABL has on DNA repair pathways has been extensively studied (Figure 1) and the bulk of these results are outlined below.

a. Single nucleotide repair

Although point mutations in the kinase domain of BCR-ABL have been detected and are important for imatinib resistance[205, 206], it is unclear at this time just how prominent or deleterious point mutations in the rest of the genome are in patients transitioning to CML BC. It is notable that recent work in AML with normal cytogenetics has revealed such mutations.[207, 208] Given the rapid advances in DNA sequencing approaches, it is likely that more information point mutations in CML BC will be available in the next few years. The effects of BCR-ABL on single nucleotide repair have been studied in the context of mismatch repair and nucleotide excision repair.

i. Mismatch repair

Mismatch repair (MMR) corrects DNA mismatches when a base or a few bases are incorrectly

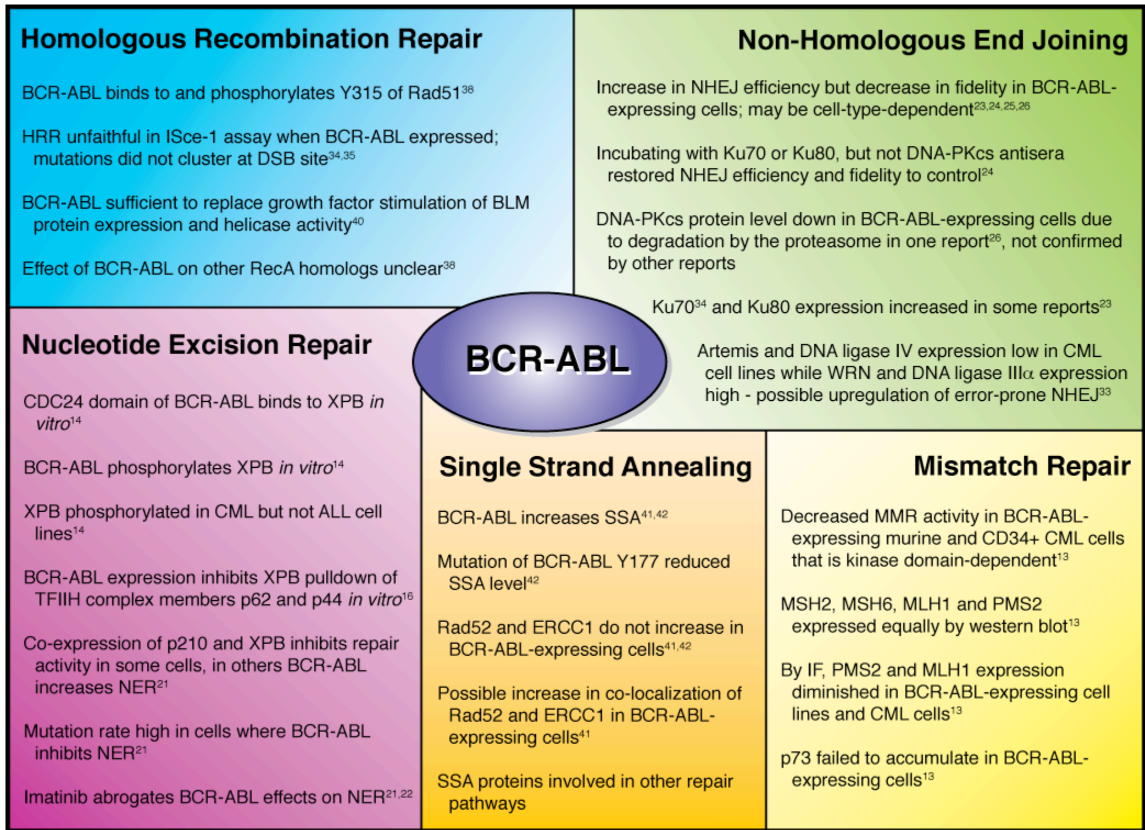


Figure 1: Reported BCR-ABL-mediated effects on DNA repair proteins

incorporated or mutated. In eukaryotes, mismatches in DNA are bound by MutSa (MSH2-MSH6 heterodimer) or MutSb (MSH2-MSH3 heterodimer), which initiates the repair process along with MutL1 (MLH1-PMS1 heterodimer).[209] A nick is made in one DNA strand, which is then degraded past the mismatch, followed by filling in of the proper bases by DNA polymerase δ.

Only one paper has examined the effect of BCR-ABL on MMR.[210] By incorporating an EGFP gene with a point mutation in the start codon into BCR-ABL-positive or -negative cells, the authors were able to detect MMR products as GFP+ cells. They determined that in both murine hematopoietic cells expressing BCR-ABL and primary CD34+ CML cells there is a decrease in MMR activity compared to controls. This was due to BCR-ABL kinase function, as imatinib treatment returned MMR activity to control level.[210]

The authors explored the expression of the four major MMR proteins, MSH2, MSH6, MLH1 and PMS2 after MMR induction. Western blot revealed no effect on expression of any of these proteins in BCR-ABL-expressing cells compared to parental cells.[210] However, in immunofluorescence (IF) studies, it appears that PMS2 and MLH1 expression is significantly diminished in BCR-ABL-expressing cell lines and CML cells compared to controls. These western and IF experiments are not functional assays, and also do not explore if MSH2-MSH6 or MLH1-PMS2 heterodimer formation, or MSH2-MSH6/MLH1-PMS2 complex formation is disrupted. However, the IF data suggest it may be valuable to further explore these interactions, as well as the function of other proteins that cooperate in the MMR response, such as PCNA, RPA, RFC, or EXO1.

ii. Nucleotide excision repair

Nucleotide excision repair (NER) is the main DNA repair pathway for ultraviolet (UV)-induced damage, and also works to correct other bulky helix distortions.[211] UV lesions are detected by

the damaged DNA binding protein (DDB) heterodimer, made up of DDB1 and DDB2, and helix distortions are recognized by the xeroderma pigmentosum group C (XPC)-Rad23B complex. These complexes then recruit other NER proteins to carry out repair. The transcription factor TFIIH, consisting of XPB, XPD, p62, p52, p44 and p34, is the NER helicase, and XPG and XPF create incisions in the DNA for PCNA and RPA binding and subsequent DNA polymerase δ or ε repair. Several groups have studied the interactions of BCR-ABL with this complex of proteins with conflicting results.[212-215]

Initial reports showed that the CDC24 homology domain of BCR-ABL[215] as well as BCR[214] interacted with the XBP protein in yeast cells. *In vitro* assays demonstrated that p210 BCR-ABL (but not p185 BCR-ABL) was pulled down by GST-XPB, an interaction that was not dependent on the kinase activity of BCR-ABL.[215] However, a complex of BCR-ABL and XPB was not convincingly demonstrated in human BCR-ABL-expressing cell lines, suggesting that the interaction may not occur at physiologic levels of BCR-ABL expression. It was later suggested that the mechanism of this NER defect may be a result of BCR-ABL interfering with overall formation of the TFIIH complex formation.[216] It was found that the presence of BCR-ABL prevented the pull-down of p62 and p44 with XPB *in vitro*[216], which may be the result of steric hindrance induced by BCR-ABL binding to XPB. This negative interaction may not be physiologically relevant, however. Though BCR-ABL has been shown to translocate to the nucleus[217, 218], subsequent attempts[219] have been unsuccessful in replicating this data, and we believe BCR-ABL to be primarily a cytoplasmic protein in the absence of substantial DNA damage. BCR-ABL and TFIIH therefore have different subcellular localizations and limited interaction *in vivo*, as suggested by the very weak ability to pull-down a BCR-ABL/XPB complex[215], and the absence of p210 from purified TFIIH complexes.[216]

The functional effects of BCR-ABL expression on NER are also controversial. Several groups have demonstrated that expression of BCR-ABL makes cells sensitive to UV- and cisplatin-induced (NER-type) damage.[213-215] The cell line 27-1 has a defect in NER that is corrected by expression of XPB. However, the presence of BCR-ABL leads to the inability of XPB to correct this defect.[216] An interesting interpretation comes from Canitrot et al., who determined that the effects of BCR-ABL on NER activity are cell-type dependent.[212] In BaF3 cells, BCR-ABL decreased NER activity and sensitized the cells to UV, in concordance with the above studies. However, in all other cell lines tested, the presence of BCR-ABL increased NER and decreased UV sensitivity.[212] In all cases, imatinib abrogated the effects, suggesting the necessity of the kinase domain of BCR-ABL.[212, 220] The results of Laurent et al. corroborate the data that suggest that BCR-ABL decreases sensitivity of MO7E and 4A2+ cells to UV.[213] Importantly, their data make clear the large number of variables at play in assessing the rapid and integrated series of events that occur after DNA damage. They demonstrate that after UV-induced DNA damage, BCR-ABL-expressing cells displayed less DNA damage than parental cells as measured by the number of cyclobutane pyrimidine dimers. However, BCR-ABL cells showed an increase in DNA breaks as assessed by the Comet assay. NER works through induction of DNA strand “nicks” (really SSBs) that will be converted to DSBs in the alkaline comet assay. Thus, the authors suggest that BCR-ABL effects in the comet assay indicate an effect on the DNA repair process leading to faster DNA repair.[213] Their studies leave open the question of the fidelity of this repair. On top of the conflicting results from different cell lines[212-215], the role of BCR-ABL in affecting NER is of questionable importance, as UV-induced damage is not likely to be seen in the hematopoietic system, which is not exposed to UV. At this point, although the effects of BCR-ABL on NER in cell lines is documented, its physiologic signficance in the progression of CML CP to CML BC remains unclear.

b. Double strand break repair

It is well documented that partial deletions, duplications and translocations are commonly seen in patients with CML BC.[123] Additionally, Dierov et al. demonstrated that BCR-ABL-expressing cell lines and CML cells acquire more translocations and aneuploid events than control cells after exogenous DNA damage (as assessed by SKY).[199] As translocations arise as a result of the mis-repair of DNA DSBs, many labs have investigated how BCR-ABL may play a role in altering the efficiency or fidelity of DSB repair pathways. The investigated repair pathways include non-homologous end joining, homologous recombination repair, and single strand annealing.

i. Non-homologous end joining

Non-homologous end joining (NHEJ) repair of DSBs occurs rapidly after breakage and does not require a homologous template, and thus is the preferred response to DSBs when cells are in G0 or G1 phase. The canonical NHEJ pathway involves the binding to a DSB by the Ku70/Ku80 heterodimer and recruitment of the DNA-PKcs-Artemis nuclease.[221] Ligation steps are carried out by DNA ligase IV along with XRCC4. NHEJ is unfaithful in that most breaks repaired in this manner are marked by short deletions and insertions of 1-4 nucleotides, and often translocations are formed when chromosomes are incorrectly juxtaposed.

The effects of BCR-ABL on NHEJ repair may be cell type dependent. Slupianek et al. used a fluorescent detection assay to look at NHEJ repair products after restriction digest.[222] They found an approximately 2-fold increase in NHEJ activity in blunt-end repair in BCR-ABL-expressing 32Dcl3 cells compared to parental, and a 4-fold increase in the case of 5' overhang repair. The fidelity of the repair was compromised, with more small additions and larger deletions in the presence of BCR-ABL.[222] Importantly, these results have been recently confirmed.[223] Gaymes et al. present data which agrees with Slupianek, where BCR-ABL-expressing CML patient samples and K562 cells displayed a 3-5 fold increase in end-ligation efficiency compared to normal CD34+ cells.[223] This was accompanied by an increased frequency of misrepair,

which included large (30-400 bp) deletions. Mechanistically, they found that Ku70 and Ku80 were involved, as incubation with antisera against these proteins restored the frequency and size of deletions to control levels. The same was not true of DNA-PKcs, as the use of anti-sera to that protein did not alter deletion size.[223] In contrast to these results, Pastwa et al. found that K562 myeloid leukemia cells (which express BCR-ABL but not wild type p53) showed fewer repair products from DNA with 5' overhangs than normal human lymphocytes, but no difference in blunt-end repair between the two cell types.[224] When imatinib was included in the assay, the same result was obtained, leading the authors to question which genetic component may actually be responsible for the difference, the expression of BCR-ABL or the lack of wild type p53 in the K562 cells.[224] Overall, these data support the concept that BCR-ABL expression increases repair of DNA DSB through the error-prone NHEJ mechanism.

Deutsch et al. also explored the mechanism by which BCR-ABL may be affecting NHEJ.[225] They found that while there was no difference in the protein expression of Ku70 and Ku80, cells expressing BCR-ABL expressed less DNA-PKcs than their wild type counterparts.[225] This protein expression was not due to a difference in the amount of mRNA in the cells, but rather degradation of DNA-PKcs, as inhibition of the proteasome restored protein levels. This was also the case in CD34+ cells from CML patients compared to normal controls.[225] However, as with other results, this has not been confirmed. Preliminary work in our laboratory (Dierov, Carroll, unpublished) showed a decrease in expression of a full size DNA-PKcs enzyme but appearance of a smaller protein fragment. Gaymes et al. determined that DNA-PKcs was not involved in the large deletions in NHEJ products in CML cells[223], and Slupianek et al. detected similar expression levels of DNA-PKcs in normal and BCR-ABL-expressing cells.[222] They also determined that there was an increase in Ku70 and Ku80 protein expression in BCR-ABL cells after IR, though no difference in protein level was seen prior to IR.[222] This response could actually be a response to the increase in DNA DSBs, rather than a BCR-ABL-mediated protein

upregulation, but corroborates the importance of Ku70 and Ku80 in leading to large deletions in NHEJ products.[223, 226] Evidence that the response could be due to an increase in native breaks comes from Brady et al. who examined normal PBLCs and CD34+ cells after 6Gy IR and determined that they in fact have a similar pattern (though lower overall level) of NHEJ activity and misrepair compared to untreated myeloid leukemia cells.[226] The comparisons are not completely isogenic, but it is interesting to speculate that it is the excessive amount of DNA damage that is driving constitutive expression of NHEJ proteins in untreated myeloid leukemia cells, including the BCR-ABL-expressing K562 line. Certainly, there is increasing evidence that the effects of BCR-ABL may be mediated through alterations in the level of expression or effects on the function of Ku70 and Ku80 although the exact cause-effect relationships remain to be determined.

A second, more error-prone pathway of NHEJ exists, though it is not well-defined.[227] DNA ligase IIIa (which usually participates in SSB repair and base excision repair[228]) is thought to function as a backup for DNA ligase IV when it is unavailable or inefficiently recruited.[229] Additionally, poly ADP-ribose polymerase (PARP) was shown to play a role in NHEJ in cells deficient in Ku70/Ku80.[230] WRN protein, which may actually play a role in canonical NHEJ, is also important in preventing genomic instability, as cells lacking WRN generate extensive deletions after NHEJ repair.[231] These alternative NHEJ proteins lead to an increased frequency of errors in repair products as a result of microhomology-mediated ligation (low-fidelity repair). The error-prone repair of NHEJ products often seen led Sallmyr et al. to explore whether alternative NHEJ proteins were involved in repair of DSBs in CML.[232] CML cell lines expressed reduced steady state levels of Artemis and DNA Ligase IV as compared to normal hematopoietic cells, as well as increased levels of WRN and DNA Ligase IIIa as assessed by western blot. Though most comparisons are between non-isogenic cell lines, the same overexpression of WRN and DNA Ligase IIIa is seen in P210MO7e cells over parental MO7e, and a CML patient sample

with high BCR-ABL expression also has correspondingly high WRN and DNA Ligase IIIa compared to a CML patient sample with lower BCR-ABL expression. No changes were seen in the expression of Ku80, DNA-PKcs (in contrast with a previous report[225]), XRCC4, XRCC1 or PARP1.[232] Interestingly, siRNA knockdown of either WRN or DNA ligase IIIa resulted in a greater increase in both endogenous and IR-induced DSBs in K562 cells than NC10 cells, as well as a decrease in end joining efficiency. Additionally, Artemis overexpression in K562 cells led to a decrease in the size of DNA deletions at misrepaired DSBs, suggesting the down regulation of Artemis in CML cells may be important mechanistically in abnormal DSB repair.[232] This will be a compelling mechanism if it is confirmed in isogenic cell lines or CML patient samples, or with the use of imatinib to inhibit BCR-ABL expression.

ii. Homologous recombination repair

Homologous recombination repair (HRR) involves the use of a sister chromatid to repair a DSB with greater fidelity than NHEJ. DNA around the DSB is resected toward the 5’ end on both strands, followed by invasion of the sister chromatid using RAD51 and its accessory proteins.[233] RecQ family helicases (BLM, WRN, RTS, RecQL1 and RecQL5) unwind DNA during HRR both at initiation and resolution of intermediates.

Cells expressing BCR-ABL showed enhanced HRR efficiency as assessed by *ISce-I* expression assay[222, 234, 235], but the repair was unfaithful.[222, 234] While the number of repair events was similar to that of parental cells, the mutation frequency was 100-fold higher in terms of single base substitutions. Interestingly these mutations did not cluster near the DSB site.

A few proteins have been explored for their role in BCR-ABL-mediated HRR efficiency and fidelity. RAD51 expression increased in BCR-ABL-expressing cells after IR[222] and in one report under steady-state conditions.[235] Indeed, the addition of a RAD51 antisense construct to the HRR efficiency assay decreased the amount of RAD51 protein as well as the number of HRR

events that occurred.[235] Additionally, RAD51 was found to co-localize with phosphorylated H2AX in IF studies[222, 234], but there is some concern that this represents an artifact of increased expression of Rad51 after the induction of DNA damage. In addition, BCR-ABL was found to directly associate with RAD51 through IP and to phosphorylate RAD51 on the Y315 site. This phosphorylation could enhance RAD51/RAD52 complex[236] formation, stimulating HRR.[237] What role BCR-ABL may play in stimulating other RecA homologs is unclear, as it appears the expression level of some of these proteins may have changed, but more work is needed to clarify the overlapping roles of this family of proteins.[238]

BLM has been shown to be necessary for normal DSB repair.[239] Slupianek et al. examined the expression of BLM in BCR-ABL-expressing and control cells, and determined that BCR-ABL was sufficient to replace the growth factor stimulation of BLM protein expression and helicase activity.[240] There may also be an effect on the colocalization of BLM and RAD51 in BCR-ABL expressing cells, which could have an impact on HRR.

iii. Single-strand annealing

Single-strand annealing (SSA) is a non-conservative HRR process that repairs DSBs that occur between identical repeats. This process is very unfaithful, as it results in the deletion of sequences between repeats, as well as one of the repeats. SSA is not completely independent from other DSB repair mechanisms, as many of the proteins involved overlap between different repair pathways. Specifically, a number of HRR proteins and processes are common to both pathways, with the exception of Rad51, which does not participate in SSA.[241]

Cramer et al. investigated the role of BCR-ABL in promoting SSA, and determined there was an increase in SSA activity with increasing BCR-ABL expression.[242] Fernandes, et al. also determined that there was an increase in SSA with BCR-ABL expression, and that imatinib

abrogated the effect.[243] Control cells were not supplied with growth factors that would match the effect of BCR-ABL, and thus the ability of the cells to grow and proliferate may impact the results. In fact, adding stromal cell conditioned media at the same time as imatinib inhibited the abrogation. Like HRR, SSA is regulated by Rad proteins (particularly Rad52) and it is not clear that the effects described are isolated to SSA. Interestingly, no effects were seen on the protein levels of ERCC1 or Rad52 in BCR-ABL-expressing cells. At this point, further work needs to be done to clarify the effects of BCR-ABL on SSA.

c. Checkpoint regulation

An alternative hypothesis to a direct effect of BCR-ABL on DNA repair is that BCR-ABL expression alters the cellular ability to efficiently recognize and respond to DNA damage. Ataxia telangiectasia mutated protein (ATM) and its homolog ataxia telangiectasia and Rad3 related protein (ATR) are PI3K family members that sense and respond to DSBs. Signaling commences through the checkpoint kinases, as ATM phosphorylates Chk2 and ATR phosphorylates Chk1. ATM can also phosphorylate BRCA1, which plays a role in DSB repair. ATM has been shown to interact with and phosphorylate c-Abl after IR.[244]

When CML BC cell lines and patient samples were analyzed for mutations in ATM in the Abl binding region and the region implicated in sporadic hematological malignancy, no nucleotide changes were detected.[245] Additionally, ATM has been shown to associate with BCR-ABL under immunoprecipitation conditions, however no difference was seen in the kinase activity of ATM in terms of its ability to phosphorylate either p53 or Chk2.[217] It is therefore unlikely that ATM plays a significant role in the transition to CML BC. As an ATM homolog that regulates chromosome stability, ATR was also investigated as a potential instigator in the transition to CML BC. As with ATM, ATR was found to associate with BCR-ABL after DNA damage and Dierov et al. initially reported that BCR-ABL expression appeared to inhibit ATR function.[217] This was not

confirmed however in recent studies by Niebrowska-Skorska et al.[219] Although the concept remains intriguing, there is not conclusive evidence at this point that BCR-ABL alters the function of the ATR/Chk1 axis.

Both ATM and ATR have been found to phosphorylate histone H2AX[246, 247] and this action is intact in BCR-ABL-expressing cells as it has been used as a marker for DNA damage.[219, 222] It is not clear whether the increase in phosphorylation of H2AX in BCR-ABL expressing cells is solely a result of an increase in the number of DSBs present, or if H2AX itself is regulated by the expression or kinase activity of BCR-ABL.

The effect of BCR-ABL on BRCA1 was also investigated, and it was determined that an increase in BCR-ABL expression led to a marked decreased in BRCA1 protein. This was not correlated with any change in mRNA levels, which suggested degradation through the proteasome. Proteasome inhibition led to the recovery of BRCA1 protein, however, long incubations with the proteasome inhibitor also led to a decrease in the p210 protein, and thus a direct correlation could not be made. It was demonstrated that BCR-ABL kinase activity was necessary for the decrease in BRCA1 protein, as the use of either a kinase defective BCR-ABL or imatinib abrogated the decrease.[197]

Nbs1 has also been shown to be phosphorylated by ATM leading to checkpoint activation.[248] Rink et al. determined that BCR-ABL stimulated the expression of Nbs1 in various cell lines.[249] However, this may be an artifact of the system, as the increase in Nbs1 expression over wild type is only seen when the parental cells are grown for 12 hours without required growth factors. These cells are undergoing apoptosis as evidenced by the expression of active caspase 3 and PARP cleavage. There is no difference seen in Nbs1 expression when comparing parental and BCR-ABL expressing cells maintained in the necessary growth factors, suggesting that this

difference may not be relevant *in vivo*. After drug treatment, there is enhanced phosphorylation of Nbs1 in BCR-ABL cells over parental cells even in the presence of growth factors, though this may reflect the known increase in DNA damage in cells expressing BCR-ABL.

d. Centrosomal hypertrophy

Centrosomes are vital members of a cell's mitotic machinery as they organize the mitotic spindle to ensure bipolar separation of chromosomes. Consequently, centrosome aberrations are a possible cause of aneuploidy, which is a feature of CML BC. Additionally, centrosomes may play a role not only structurally, but also as regulators of mitotic entry through their association with p53.[250] The role of centrosome defects on causing genetic instability is under examination[251, 252], and one group has examined these defects in the context of CML.[253, 254]

Giehl et al. determined that centrosome abnormalities correlated with CML disease stage and preceded chromosomal aberrations.[254] Importantly, in the first study, primary patient samples were utilized, giving added weight to the data. The authors suggest that the acquisition of centrosome defects may be one of the driving forces of progression to CML BC and contribute to the chromosomal instability.[254] A causative relationship with BCR-ABL was later elucidated by this same group[253], where they utilized U937 cells with a tetracycline-inducible BCR-ABL in long-term culture experiments. They determined that constant expression of BCR-ABL led to increasing centrosome aberrations over time. Further, turning BCR-ABL off halfway through the experiment via doxycycline withdrawl led to a reversion toward baseline.[253] Overall these data suggest that BCR-ABL introduces centrosome abnormalities, which may contribute to the chromosomal aberrancies found in CML BC.

e. Apoptosis inhibition

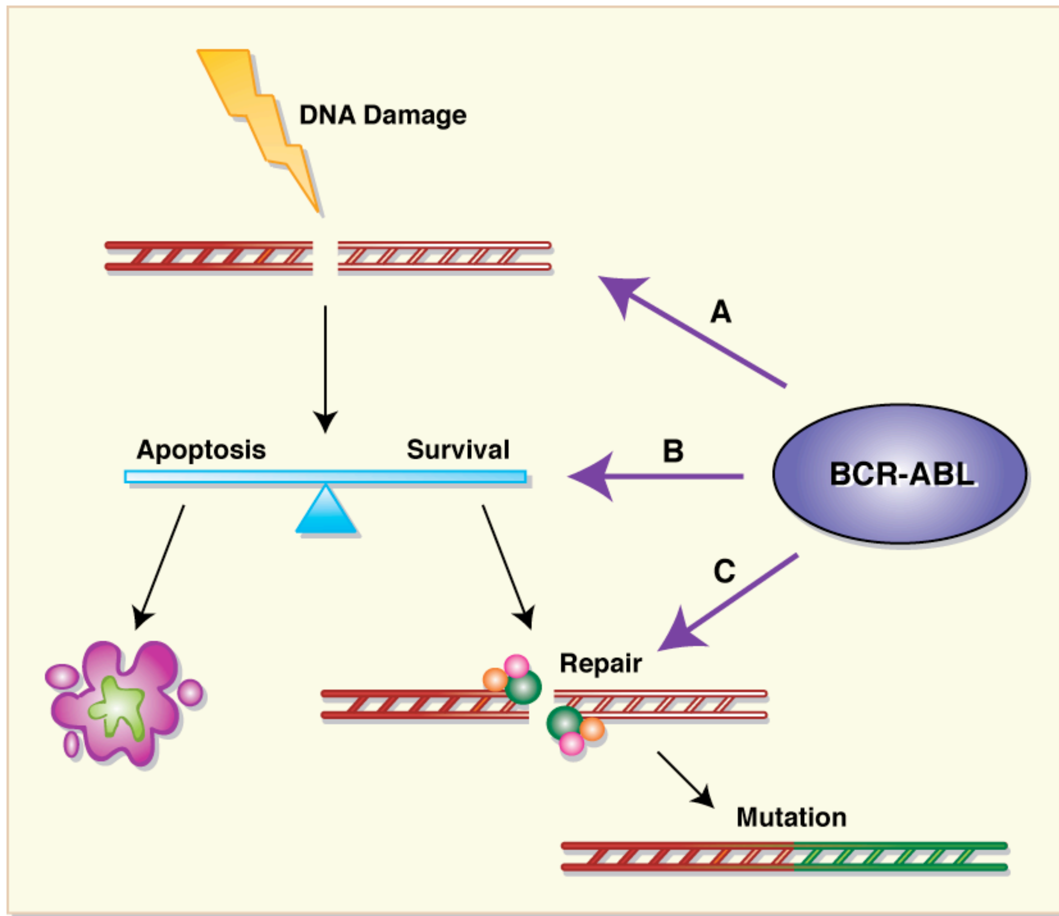


Figure 2: BCR-ABL affects DNA damage through various mechanisms. Arrows A, B and C demonstrate the three main ways BCR-ABL can affect DNA damage and repair. (**A)** represents DNA damage caused by BCR-ABL, which may occur through creation of reactive oxygen species. Once damage occurs, BCR-ABL may inhibit apoptosis, thus allowing cells to survive with more damage than can be effectively repaired **(B)**. BCR-ABL also interacts directly and indirectly with repair proteins and can contribute to raid but low-fidelity repair **(C)**.

When cells acquire DNA damage, the damage-sensing proteins recognize the error and stimulate repair, but when the damage is too great the cell may instead signal to undergo apoptosis. This prevents the replication of potentially harmful mutations, and evading apoptosis is one of the known hallmarks of cancer. Another of the known cellular roles of BCR-ABL is the inhibition of apoptosis which was described above.

A critical question is whether BCR-ABL expression allows cells with damaged DNA to survive long enough to recruit error-prone DNA repair pathways leading to survival of CML cells with mis-repaired DNA. Such a mechanism (c.f. Figure 2) may provide a model to integrate the multiple DNA repair abnormalities described. After DNA damage, cells either repair DNA rapidly and continue to grow (if damage is minimal); undergo apoptosis (after large amounts of damage); or arrest, repair DNA and then resume growth (after intermediate damage).

F. Hypothesis

The bulk of data convincingly demonstrates that BCR-ABL-expressing cells are subject to the accumulation of mutated DNA (Figure 2). It has been suggested that BCR-ABL expression leads to the development of DNA damage through the induction of ROS or the natural error rate of DNA synthesis and the hyperproliferation of cells. Further, the effect of BCR-ABL on various repair mechanisms has been explored, but a consistent mechanism has not been determined. We hypothesize that the known effects of BCR-ABL on inhibiting apoptosis play a role in the development of the DNA damage seen in CML BC. We speculate that BCR-ABL alters the apoptotic threshold of cells, increasing the pool of cells undergoing active DNA repair rather than programmed cell death.

II. <u>MATERIALS AND METHODS</u>

A. Cell Culture

Bax-/-Bak-/- (DKO) cells (a kind gift of Dr. Craig Thompson, University of Pennsylvania) were previously described.[255] Cells were maintained in RPMI supplemented with 10% FBS, 3 ng/ml r-mIL-3 (R&D Systems, Minneapolis, MN), Pen/Strep, HEPES, and L-glutamine. DKO BCR-ABL (DBA) cells were generated using Mig210 (a kind gift of Dr. Warren Pear, University of Pennsylvania). Control cells were infected with MigR1 empty vector (Dr. Warren Pear). Retrovirus was generated as previously described.[164] Cells infected with Mig210 or MigR1 were selected by flow sorting for expression of green fluorescent protein (GFP). Cells were maintained as above. For some experiments, DBA cells were washed three times in PBS and plated in the same media as above without IL-3 present. Subclones of DBA and DKO cells were generated by limiting dilution, plated at 1/2 cell per well in a 96 well plate. Less than 1/3 of wells resulted in cell outgrowth.

BaF3 pTET-ON p210 cells were maintained in RPMI supplemented with 10% FBS, 1-3 ng/ml r-mIL-3, Pen/Strep, HEPES, and L-glutamine.[74] For the induction of BCR-ABL, cells were washed three times in 1X PBS to remove IL-3 and re-plated in 1-2 μg/ml doxycycline (dox) a minimum of 24 hours before experiments. Cells were grown in both 1 ng/ml IL-3 and 1μg/ml dox where indicated. Dasatinib was obtained from Bristol Meyers Squibb. Imatinib (Gleevec) was obtained from Novartis Pharmaceuticals.

Our laboratory maintains a tissue bank that collects and stores human leukemia samples from the Hospital of the University of Pennsylvania after obtaining informed consent. These samples include a number of frozen CML cells from patients in both CML BC and CP. These cells can be

thawed and compared to normal patient bone marrow or sorted CD34+ cells, the cell fraction that contains the hematopoietic progenitor population.

B. Western Blot

Cells were lysed in lysis buffer (150mM NaCl, 50mM Tris, 1% Triton-X 100 (Sigma) plus EDTA (Gibco), sodium pyrophosphate, protease inhibitor cocktail (Sigma), and phosphatase inhibitor cocktails I and II (Sigma)) or RIPA buffer (50mM Tris-HCl, pH 7.4, 1% NP-40 (IGEPAL CA-630, Sigma), 0.25% Na-deoxycholate, 150mM NaCl, 1mM EDTA, 1mM PMSF, 1mM Na3VO4, 1mM NaF and protease inhibitor cocktail P8340 (Sigma) followed by gentle sonication for 5 minutes at 4 degrees. Lysate was centrifuged to remove insoluble components. Equal amounts of protein (quantified by Bradford Assay (Bio-Rad, Hercules, CA)) were loaded onto polyacrylamide gels and run by standard SDS-PAGE. Proteins were transferred onto nitrocellulose membrane (Bio-Rad) and blocked with Odyssey Blocking Buffer (LI-COR, Lincoln, NE) or 5% Baileys Irish Cream in 1X PBS. Primary antibodies, as follows, were used at 1:1000 concentrations: Phospho-c-Abl (Tyr245) (Cell Signaling Technology, Danvers, MA); Abl (BD Pharmingen, San Jose, CA); Bax (Santa Cruz Biotechnology, Santa Cruz, CA); Bak (Upstate/Millipore, Billerica, MA), Chk1 (Cell Signaling Technology), p-Chk1 rabbit mAb 133D3 (Cell Signaling Technology). α-tubulin (Sigma) was used at 1:5,000. This was followed by four washes in 1X TBST and subsequent incubation with secondary antibodies at a concentration of 1:10,000: IRDye 800 Conjugated Affinity Purified anti-Mouse IgG (H+L) (Rockland, Gilbertsville, PA); Alexa Fluor 680 goat anti-rabbit IgG (H+L) (Molecular Probes/Invitrogen, Eugene, OR). Membranes were then washed with 1X TBST three times and 1X PBS twice. Signal was detected with the Odyssey scanner (LI-COR) and quantified when indicated using the Odyssey software.

C. Cell Proliferation Assays

Cells were plated at densities ranging from 1-4 x 10^5 to ensure cells were in the exponential growth phase. Trypan blue exclusion assays were performed using a 1:1 dilution with cells cultured for the amount of time stated. Cells were counted manually under the microscope on a hemacytometer or using the Cellometer Auto T4 (Nexcelom Bioscience, Lawrence, MA).

D. Apoptosis Analysis

Cells were treated and harvested as described in the text. Cells were then washed in 1X phosphate buffered saline (PBS) prior to resuspension in 100μl Annexin V binding buffer (Invitrogen). 1μl of 20X PI (Molecular Probes) solution (20μl of 1mg/ml PI in 480 μl 1X PBS) and 10μl Annexin V-APC (Invitrogen) solution (1μl Annexin V-APC in 1ml binding buffer) were added to the suspended cells. After a 15-minute incubation at 4 degrees in the dark, an additional 200μl binding buffer was added for analysis. Cells were analyzed on FACSCalibur (BD) and live/dead analysis was performed using FlowJo software (Tree Star, Ashland, OR).

E. Cell Cycle Analysis

Cells were mock treated or treated with etoposide (Sigma), a topoisomerase II inhibitor, or γ-irradiation (Gammacell Cs-137 source) to induce DNA DSBs. Propidium Iodide (Molecular Probes) staining was carried out according to protocol after fixation in 70% ethanol. Briefly, cells were washed in 1X PBS and resuspended dropwise in 1ml 70% ethanol and stored at 4 degrees overnight or longer. Fixed cells were then washed twice with 1X PBS and resuspended in PBS containing 2% Propidium Iodide (Sigma, St. Louis, MO), 2% DNAse-free RNAse and 0.1% Triton-X 100. Cells were analyzed on a FACSCalibur (BD) and cell cycle analysis was performed using ModFit LT (Verity Software House).

F. Pulsed Field Gel Electrophoresis

2 x 10^5 cells were collected at specified times before or after γ-irradiation on ice, washed twice in ice cold 1X PBS and suspended in 50μl L Buffer (100mM EDTA pH 8.0, 10mM Tris pH 7.5, 20mM NaCl) before encapsulation in 1.5% SeaPlaque GTG agarose (Cambrex) plugs at a 1:1 ratio. Plugs were incubated in Lysis Buffer (L Buffer with 1% SDS and 1 mg/ml Proteinase K) at 50 degrees for 20-24 hours, then washed in 1X TE 8.0 (10mM Tris-Cl pH 8.0, 10mM EDTA). Plugs were then incubated in 1X TE pH 8.0 for 3+ hours at 4 degrees, followed by 1X TAE (40 mM Tris-acetate, 20 mM sodium acetate, 1 mM EDTA, pH 8.0) incubation at 4ºC overnight. Pulsed field electrophoresis was performed using a CHEF-DR II Pulsed Field Electrophoresis System (Bio-Rad) in 0.8% PFGE-certified agarose (Bio-Rad) in 1X TAE at 2.8V/cm with a 400-1800s switch time for 60 h at 4ºC. DNA was stained using SYBR Green I (Invitrogen). Imaging was performed on a Storm 860 scanner (Molecular Dynamics) and quantification was performed using ImageQuant (Molecular Dynamics). *S. cerevisiae* and *S. pombe* molecular weight standards (Bio-Rad) were used for fragment size estimation.

G. Spectral Karyotype Analysis

3 x 10^6 cells in 5 ml of media were irradiated (5 Gy) or not and allowed to repair for 48 h before the addition of 5ml fresh media and 50μl colcemid (Roche, Nutley, NJ). In the case of cells treated with dasatinib, dasatinib was washed out 48 h after IR and cells were replated in IL-3 media for an additional 24 h before colcemid addition in order to stimulate proliferation to acquire sufficient metaphases. After 6 h, cells were harvested and washed twice with 1X PBS. After final discard of supernatant, cells were gently flicked into suspension in remaining PBS. 1ml of 75mM KCl was slowly added with gentle mixing. After inversion of the tube, cells were incubated for 20 min at room temperature before centrifugation. After supernatant was discarded, cells were gently suspended in remaining buffer before addition of 1 ml ice cold 3:1 methanol:acetic acid (fixative). Cells were incubated on ice for 10 min then spun down and resuspended in fixative 2 more times.

After final resuspension, 15μl of cells was dropped onto wet slides in a glass dish in a 37-degree water bath.

Slides were equilibrated in 2X saline-sodium citrate (SSC) then treated with pepsin (Sigma) in 0.01 M HCl at 37 degrees for 5 minutes. After washing in PBS and PBS/$MgCl_2$, slides were fixed in 1% formaldehyde in PBS/$MgCl_2$. Slides were again washed in PBS, then serially dehydrated in room temperature ethanol, 70%, 90%, 100%. After air-drying, slides were denatured in 70% deionized formamide/2x SSC, followed by serial ethanol dehydration (70% at 0 degrees, 90% at room temperature, 100% at room temperature). After air-drying, slides were hybridized with mouse DNA SKY Paint Kit (Spectral Imaging, Israel) at 37 degrees for 72 hours. Slides were then washed in 50% formamide (Fluka BioChemika)/2X SSC, followed by 1X SSC. Slides were dipped in 4X SSC/0.1% Tween 20 (Sigma) and blocked with 3% BSA/4X SSC/0.1% Tween 20 at 37 degrees for 30 minutes. Slides were then stained with mouse anti-digoxigenin (Sigma) and Avidin-Cy5 (Jackson Immuno Research Lab) in 1%BSA/4X SSC/0.1% Tween 20 for 45 minutes at 37 degrees. After washing in 4X SSC/0.1% Tween 20, slides were incubated with sheep anti-mouse Cy5.5 (Amersham) in 1% BSA/4X SSC/0.1% Tween 20 at 37 degrees for 45 minutes. Slides were washed and stained for 5 minutes in DAPI (Sigma)/2X SSC. After a final wash, slides were serially dehydrated in ethanol (70%, 90%, 100%), air dried, and covered with anti-fade solution and a cover slip. Images were acquired with an Olympus BX51 equipped with a spectracube/camera SD301 controlled by the full acquisition and analysis software Case Data Manager 5.5 (Applied Spectral Imaging, Vista, CA).

H. Southern Blot

Cells were spun down and washed in PBS before DNA extraction using DNeasy (Qiagen) according to protocol. 10ug of whole genomic DNA was digested using restriction endonucleases *Hind* III (10 U/ul) or *Eco* RI (10 U/ul) (Roche, Manheim, Germany) overnight at 37 degrees C

according to manufacturer's instructions. Digested products were run on a 0.9% agarose gel in 1X TAE for 14 hours at 30V. Ethidium bromide was used to detect DNA in the gel before transfer. The gel was washed in ddH2O, then incubated with 0.025M HCl for 30 minutes. After washing with ddH2O, denaturation was carried out in 1.5M NaCl/0.5 M NaOH twice for 20 minutes with shaking. After washing with ddH2O, neturalization was carried out using 1.5M NaCl/0.5M Tris*Cl pH 7.0. The gel was transfered to a Nytran SuPerCharge Nylon membrane (Whatman, Sanford, ME) using the capillary transfer method overnight.[256] The membrane was crosslinked twice at 1200 x 100 $\mu J/cm^2$ in a UVP CL-1000 ultraviolet crosslinker before probing.

A radioactive probe was created by digesting empty MIGR1 vector with *Eco* RI and Nco I to cut out the IRES (592 bp) portion. The digested DNA was run on a 1% agarose gel containing ethidium bromide and run at 100V for 2h. The 592 bp band was cut out and extracted from the gel using the QIAquick Gel Extraction Kit (Qiagen). The probe was labeled using Amersham Ready-To-Go DNA Labelling Beads (-dCTP) (GE) and 50uCi P-32 a dCTP (EHRS, UPenn) according to manufacturer's protocol. Excess labeled nucleotides were removed using illustra MicroSpin G-50 Columns (GE) according to manufactuer's instructions. Probe was denatured and hybridized to membrane according to QuikHyb (Stratagene) protocol for double-stranded probes. After washing according to QuikHyb protocol, membrane was exposed to Hyperfilm (Amersham) at -80 degrees with an intensifying screen.

I. Immunofluorescence

CML BC patient #623 cells were thawed and maintained for 1 day in RPMI + 10% FBS and 1 ng/ml IL-3. Normal peripheral blood mononuclear cells from 4 donors were combined and maintained for 1 day in RPMI + 10% FBS and 1 ng/ml IL-3. Ba/F3 p-TET-On p210 cells were maintained in IL-3 or doxycyline media. Cells were harvested and washed with PBS, then resuspended at 10^6 cells/ml. Serially diluted aliquots were cytospun onto slides at 1,000 rpm for

10 minutes. Slides were fixed for 30 minutes at -20 degrees C in methanol then washed with PBS + 0.05% Tween (PBST). Blocking was carried out for 1 h at 37 degrees C in 5% milk in PBST, followed by 3 washes with PBST. Slides were then incubated in primary antibody (polyclonal rabbit pericentrin #PRB-432C (Covance, Berkeley, CA) or monoclonal mouse GTU-88 #T6557 (Sigma)) in 3% BSA in TBST for 2 h at 4 degrees. This step was followed by 3 washes with PBST at RT then incubation with secondary antibody (Alexa Fluor 488 goat anti-rabbit # A11034 (Molecular Probes) or Alexa Fluor 594 donkey anti-mouse A21203 (Molecular Probes)) at RT in the dark for 30 min. Slides were washed 5 times with PBST then dried at RT for 10 min before the addition of 15uL Prolong Gold + DAPI and a coverslip. Slides were imaged on a Nikon Eclipse E800 microscope using IPlab v.3.0 software.

J. Fragile Site Analysis

BaF3 PTET-ON p210 cells were treated for 24-72 h with IL-3 or doxycyline or both. Cells were split to 2.5×10^5/ml and 0.2μM aphidicolin (Sigma) or DMSO was added to the culture, and in some cases 10uM imatinib. After 24 h, cells were treated with colcemid in 5ml additional media for 45 minutes. Cells were harvested and treated with 12 ml of 0.075M KCl at 37° for 18 minutes, before the addition of 1 ml 3:1 MeOH:Acetic Acid and inversion. Cells were then washed with fresh ice cold 3:1 MeOH:Acetic Acid 3 times and dropped onto slides pre-washed with 3:1 MeOH:Acetic Acid. 50 Giemsa-stained metaphases were counted per treatment.

K. Statistical Analysis

A one-tailed, unpaired student's t-test was used to determine whether mean levels of damage in BCR-ABL-expressing cells exceeded control cells in PFGE experiments. For SKY experiments, we carried out two two-way analyses of variance with the goal of determining whether differences in the mean number of IR-induced chromosomal aberrations differed between treatment groups. The first analysis compared the DKO and DBA cell-lines and tested the null hypothesis that the

mean number of irradiation-induced chromosomal abnormalities in the DBA line was no greater than in the DKO line. The second compared DBA cells treated with dasatinib and IL-3 to those with IL-3 alone, and DBA with IL-3 to DBA without IL-3. Here we tested the null hypotheses that the mean number of irradiation-induced chromosomal abnormalities was (1) no greater in IL-3-alone treated cells than cells with IL-3 and dasatinb, and (2) no greater in cells untreated with IL-3 compared to lines treated with IL-3 alone. Type I error rates were set to 0.05. One-sided 95% confidence intervals (CIs) were constructed to assess upper or lower boundaries for differences in IR-induced abnormalities; two-sided confidence intervals were used to estimate individual abnormality levels.

III. BCR-ABL MEDIATED EFFECTS ON GENOMIC INSTABILITY ARE SECONDARY TO INHIBITION OF APOPTOSIS

Note: Parts of this chapter have been submitted to Leukemia *for evaluation for publication.*

A. Introduction

CML is a two-stage disease of the bone marrow caused by the BCR-ABL fusion tyrosine kinase.[21, 36, 257] The first stage (CML CP) is marked by a hyper-proliferation of white blood cells that retain the capacity to differentiate, and is a direct result of BCR-ABL activation. This activation leads to increased proliferation and decreased apoptosis in BCR-ABL-expressing cells.[61, 74, 114, 115] The second stage of disease (CML BC) is marked by additional DNA damage, maturation arrest, and resistance to chemotherapy.[258, 259] How exactly BCR-ABL is involved in promoting DNA damage remains under debate.

The inhibition of apoptosis contributes to the development of cancer[260] as well as genomic instability.[261] BCR-ABL is able to inhibit apoptosis, including apoptosis induced by DNA damage stimuli.[262, 263] Cytochrome C release from the mitochondria (an early step in the apoptotic pathway) is blocked in BCR-ABL-expressing cell lines.[264, 265] Further, BCR-ABL has been found to modulate expression of pro- and anti-apoptotic Bcl-2 family members [116, 266-268] through different signaling pathways. The translocation of the pro-apoptotic proteins Bax and Bad to the mitochondria was blocked in cells with high BCR-ABL expression.[119] Additionally, BCR-ABL was found to prevent late mitochondrial depolarization and caspase 9 and caspase 3 processing.[120] Thus, clearly, BCR-ABL inhibits apoptosis but the role of this function of the protein in CML pathogenesis is incompletely defined.

We have recently demonstrated that BCR-ABL-expressing cell lines and primary CML cells accumulate chromosomal translocations after induction of DNA DSBs.[199] Here we address

whether this is a consequence of the inhibition of apoptosis by BCR-ABL. We introduced BCR-ABL into IL-3-dependent cells that lack expression of Bax and Bak and treated the parent cell line and the BCR-ABL-expressing cells with γ-irradiation (IR). In contrast to apoptosis-competent cells, the two cells lines demonstrated equivalent amounts of DNA double strand breaks (DSBs) and chromosomal translocations after repair. These results support the hypothesis that inhibition of apoptosis is critical in the BCR-ABL-mediated alteration of the cellular response to DNA damage.

B. Results

DBA cells express BCR-ABL but not pro-apoptotic proteins Bax and Bak

As noted above, previous reports have demonstrated that BCR-ABL-expressing cells are subject to error-prone DNA repair after DNA DSBs. However, as BCR-ABL can inhibit apoptosis, and the inhibition of apoptosis leads to genomic instability, it is unclear whether the error-prone repair is a direct effect of BCR-ABL, or secondary to its inhibition of apoptosis. In order to address what effect BCR-ABL has on DNA damage independent from its effects on apoptosis, we have generated a cell line that expresses BCR-ABL but does not express the pro-apoptotic proteins Bax and Bak.[255] DKO cells (Double Knock Out) are a mouse hematopoietic line null for pro-apoptotic genes *Bax* and *Bak*. These cells are dependent on IL-3 for growth and proliferation, but fail to undergo cell intrinsic apoptosis.[255] We infected these cells with Mig210[164], a retrovirus expressing BCR-ABL under the control of the MSCV promoter and containing GFP under the control of an internal ribosomal entry site (IRES). Cells were then selected for expression of GFP. The resulting cell line is designated DBA, for DKO BCR-ABL. As shown in Figure 3a, we determined that BCR-ABL was both expressed and activated in DBA cells (lane 3), while it was not expressed in the DKO parental cells (lane 2). A BCR-ABL-expressing cell line (BaF3 pTET-ON p210, grown in the presence of doxycycline) was used as a positive control for BCR-ABL expression and phosphorylation (lane 1). We confirmed the absence of Bax and Bak expression

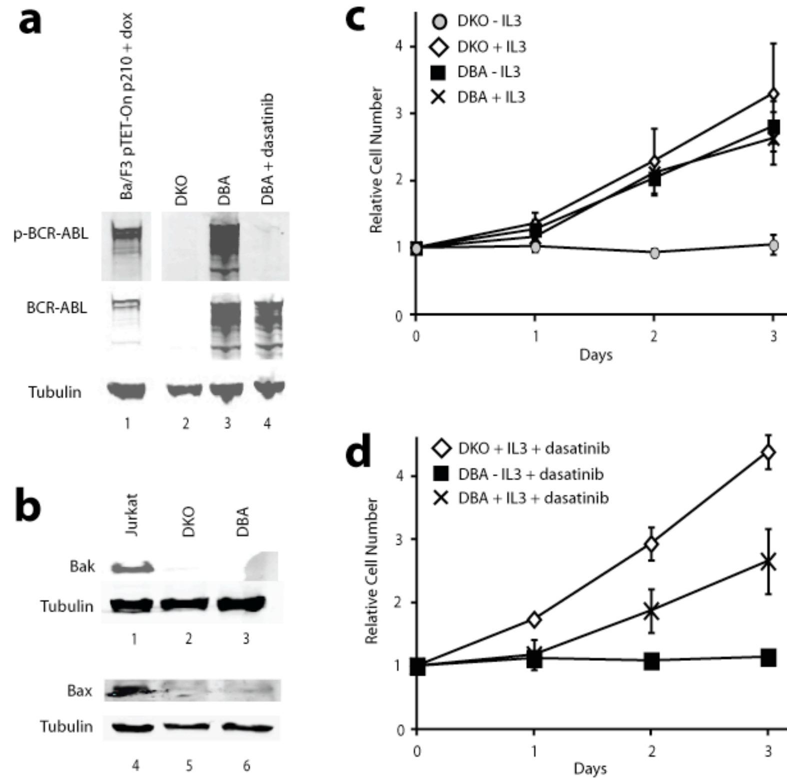


Figure 3: BCR-ABL is expressed in DBA cells and inhibited by dasatinib. a) Western blot was carried out using Abl and p-Abl antibodies, along with Tubulin as a loading control. 50μg of protein was loaded into each well. Ba/F3 PTET-ON p210 cells grown with doxycycline are used as positive control for BCR-ABL expression. **b)** DKO and DBA cells were counted in the presence or absence of IL-3 during their exponential growth phases. All points were calculated relative to the starting concentration of cells. Error bars are +/-SD for at least three independent experiments. **c)** Western blot was carried out using Bax and Bak antibodies, along with Tubulin as a loading control. 50μg of protein was loaded into each well. A small non-specific streak can be seen in the Bax blot that is the same size as Bax. Jurkat cells were used as a positive control for Bax and Bak expression. **d)** DKO and DBA cells were counted in the presence of 10nM dasatinib during their exponential growth phases. All points were calculated relative to the starting concentration of cells. Error bars are +/-SD for at least three independent experiments.

in the DBA cells (Figure 3b, lanes 3 and 6) like the parental DKO cells (lanes 2 and 5). Jurkat cells were used as a positive control for protein expression. We conclude that BCR-ABL is expressed in DBA cells, while Bax and Bak are not expressed.

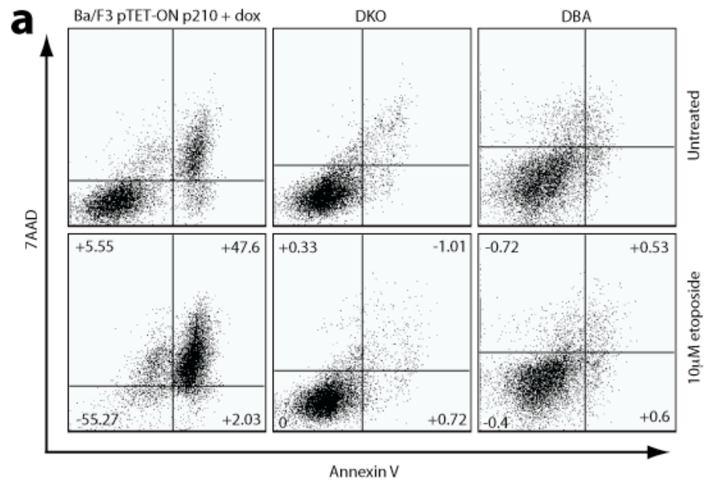
DBA cells are IL-3-independent

We next determined whether the expression and activation of BCR-ABL was sufficient to cause cytokine-independent growth and proliferation. The parental DKO cell line is dependent on IL-3 for proliferation [255] (Figure 3c, compare open diamonds to gray circles). We determined that the proliferation rate of DBA cells in either the presence (crossed lines) or absence (black squares) of IL-3 is similar to the growth of DKO cells. We therefore conclude that BCR-ABL is expressed and active in DBA cells, which confers IL-3-independent growth.

Previous results have shown that the effect of BCR-ABL on cell growth is dependent on its kinase activation. To demonstrate that this is true in DBA cells, we incubated cells with the Abl kinase inhibitor, dasatinib, to determine the effects on kinase activity and cell proliferation. We found that after 2 hours of dasatinib treatment, BCR-ABL was no longer tyrosine phosphorylated in DBA cells (Figure 3a, lane 4). This corresponded with a failure of DBA cells to proliferate in the presence of dasatinib but absence of IL-3 (Figure 3d, black squares). Parental cells still proliferated at a normal rate when treated with dasatinib (open diamonds), and DBA cells maintained in IL-3 were still able to proliferate, but at a slightly decreased rate. This confirms that BCR-ABL is functionally active in DBA cells and is required for the IL-3-independent growth properties in these cells.

DBA cells are resistant to cell death

We next wanted to confirm that DBA cells do not undergo apoptosis after DNA damage. Cells were treated with 10 μM etoposide for 24 h to induce apoptosis and then stained with Annexin V



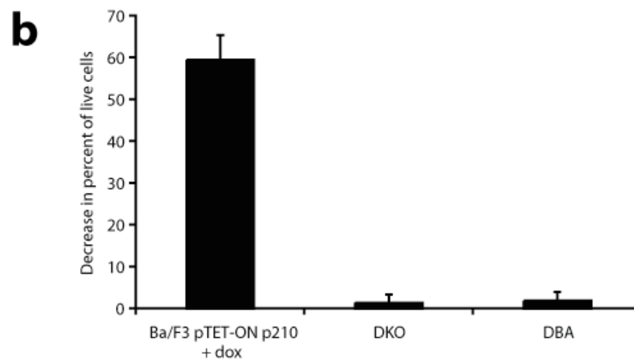


Figure 4: DNA damage does not induce cell death in DBA cells. a) Cells were treated with 10μM etoposide for 24 h. Cells were harvested and stained with Annexin V-PE and 7AAD and analyzed using FlowJo software. Ba/F3 pTET-ON p210 grown in the presence of doxycycline cells were used as a positive control for cell death in an apoptosis-competent BCR-ABL-expressing cell line. Numbers shown indicate the change in percentage of cells in each gate between untreated and etoposide treated. **b)** The decrease in percent of live cells for each condition when treated with etoposide compared to untreated is represented graphically. Error bars are +SD for three independent experiments. The same result was seen after IR (data not shown).

and 7AAD. After this incubation there is no decrease in the number of live cells in either DKO (Figures 4a and b) or DBA cells. Note that there is no change in the percent of cells in the lower left Annexin V-negative, 7AAD-negative quadrant. Ba/F3 pTET-ON p210 cells in the presence of doxycycline were used as a positive control for cell death in an apoptosis-competent BCR-ABL-expressing cell line. Similar results were seen after treatment with γ-irradiation (IR) and with higher concentrations of etoposide (data not shown). We conclude that even after a strong dose of DNA damaging agent, DKO and DBA cells remain viable.

DKO cells display a larger increase in G2/M than DBA cells after DNA damage

Previous data has suggested that BCR-ABL expression leads to an enhanced G2/M arrest after DNA damage.[262, 269] To determine if this is the case in DBA cells, and thus an effect independent of the anti-apoptotic effects of BCR-ABL, we treated cells with IR or etoposide and observed the fraction of cells in G2/M by propidium iodide staining. After treatment with IR, *bax-/- bak-/-* DKO control cells show an increase in the G2/M portion of the cell cycle (Figure 5a). This number peaks around 24 hours after treatment, and then decreases toward basal level after 48 hours. In DBA cells, the initial increase in G2/M peak is lower than in DKO, but the percent of cells in G2/M remains elevated after 48 hours rather than starting to decrease (Fig 5a). The same result was seen after a two hour 10 μM etoposide exposure (data not shown). Control cells not treated with IR maintain a constant lower percentage of cells in G2/M (Fig 5b). These results were confirmed using individual subclones of DKO and DBA cell lines (Figure 6). Interestingly, cells continue to proliferate at wild type rates after 5Gy irradiation (Fig 5c). This suggests that the G2/M delay is not an outright cell cycle arrest, but an increase in the relative duration of G2/M for several days after DNA damage.

Subclones of DBA and DKO cells behave like polyclonal cell population

The results in the previous figures were determined from a single cell line. In order to confirm that

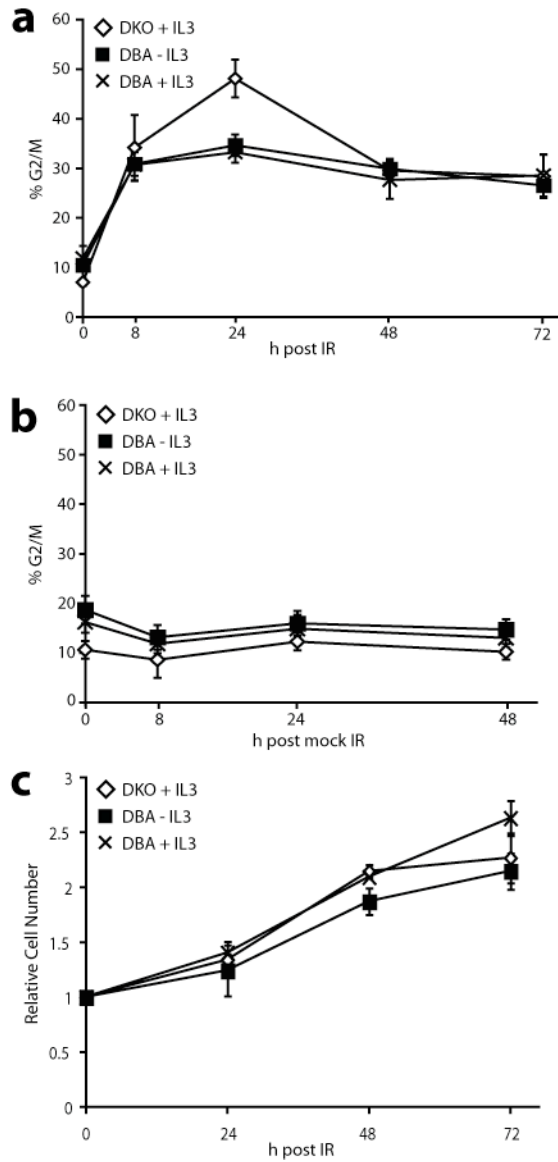


Figure 5: DBA cells remain in G2/M after DNA damage. a-b) Percent of cells in G2/M phase of the cell cycle as measured by PI staining. **a)** Cells were exposed to 5Gy IR and harvested at the indicated times. Error bars are +/-SD for at least three independent experiments. The same results were seen after 2 h treatment with 10μM etoposide (data not shown). **b)** Cells were mock treated and harvested at the indicated times. **c)** Cells were counted after IR. All points were calculated relative to the starting concentration of cells. Error bars are +/-SD for at least three independent experiments.

the experimental effects seen are a result of the BCR-ABL expression, we generated individual subclones of the cell lines. We confirmed that these subclones yielded similar results to the cell lines as a whole. We determined that the DBA subclones express BCR-ABL, which is constitutively phosphorylated (Figure 6a). Further, incubation with dasatinib abrogated BCR-ABL phosphorylation (Figure 6a). Additionally, subclones of the DBA line all proliferate in both the presence and absence of IL-3 (Figure 6b). However, DBA subclones fail to proliferate when treated with Imatinib in the absence of IL-3. These results confirm that BCR-ABL is expressed and phosphorylated in the DBA subclones, yields them independent of IL-3 for growth, and that the kinase activity of BCR-ABL is required for this growth factor independence. Experiments are ongoing to confirm by Southern blot that the various clones have integrated the BCR-ABL-expressing vector at different point in the genome and results seen are not an artifact based on the disruption of the locus at the point of insertion. Initial results have been suggestive of a difference but not conclusive.

DKO and DBA cells have similar numbers of DNA DSBs immediately after IR

Previous work by our lab and others[199, 222, 270] has demonstrated that expression of BCR-ABL alters the cellular response to DNA double strand breaks (DSBs). We showed previously using the comet assay that BCR-ABL expression in Ba/F3 pTET-ON p210 cells increased the olive tail moment over parental cells, meaning more DSBs were present when BCR-ABL was expressed.[217] Here we used pulsed field gel electrophoresis (PFGE) to examine DSBs in DKO and DBA cells. This method reveals DSBs in large fragments of DNA, as only those fragments are able to migrate into the gel while undamaged DNA remains in the well. As seen (Figure 7a), both DKO and DBA cells show an increase in DNA DSB after IR as expected (compare lane 2 to 1 and 8 to 7). The amount of DNA that ran into the gel compared to the total amount of DNA in the well was quantified for each lane, representing the total amount of damaged DNA. The ratio of damaged DNA immediately after IR (lane 2) to damaged DNA before IR (lane 1) was set equal

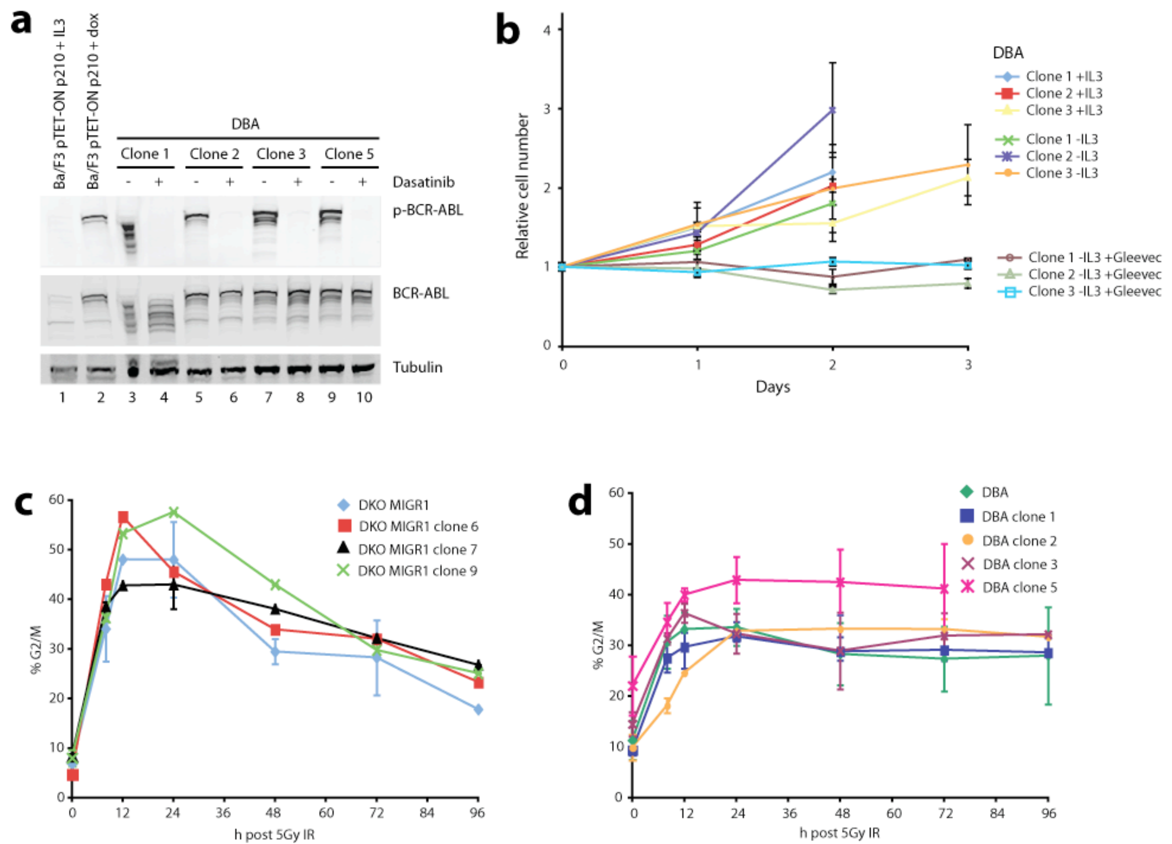


Figure 6: DKO and DBA subclones demonstrate properties similar to polyclonal cell line population. a) DBA clones express BCR-ABL and it is phosphorylated. 10nM dasatinib turns off the kinase activity of BCR-ABL as shown by the lack of p-BCR-ABL expression in the dasatinib treated lanes (4, 6, 8, 10). **b)** DBA clones proliferate in the absence of IL-3. They fail to proliferate in the presence of the BCR-ABL kinase inhibitor imatinib. **c)** Percent cells in G2/M after 5Gy IR for DKO MIGR1 clones. **d)** Percent cells in G2/M after 5Gy IR for DBA clones. These results are consistent with what was seen in the DBA and DKO lines.

to 1 for DKO cells. This allowed comparison of the amount of damage in DKO vs. DBA cells across multiple gels where absolute values determined by ImageQuant software were different. We determined that DBA and DKO cells contained similar amounts of DSBs after IR (Figure 7b, P = 0.32). In other words, expression of BCR-ABL in this apoptosis-incompetent cell line did not increase DNA DSBs in comparison to control after IR. This is in contrast to what was seen previously in Ba/F3 pTET-ON p210 cells[217] and suggests that the accumulation of a greater number of DNA DSBs in BCR-ABL-expressing cells is not observed in this Bax/Bak-null cell line. To confirm that the result seen in this experiment compared to previous work in apoptosis-competent cells (Ba/F3 pTET-ON p210) was not based on assay conditions, we also performed PFGE on Ba/F3 pTET-ON p210 cells grown with IL-3 or doxycycline to induce BCR-ABL expression (Figure 7c). We confirmed that in this cell line using this assay, BCR-ABL-expressing cells demonstrate more DSBs immediately after DNA damage than parental cells (Figure 7d). This suggests that these results from PFGE are similar to previously published results from the comet assay[217] and that using this assay, BCR-ABL-expressing, apoptosis-competent cells accumulate increased DNA DSBs after IR compared to isogenic control cells.

Cell intrinsic apoptosis is mediated by caspases. To explore whether an apoptosis-competent cell line treated with a caspase inhibitor would yield similar results to DKO and DBA cells, we again examined DNA DSB after IR using Ba/F3 pTET-ON p210 cells grown with the pan-caspase inhibitor quinolyl-valyl-O-methylaspartyl-[-2,6-difluorophenoxy]-methyl ketone (Q-VD-OPh)[271] (Figure 7e). Cells were either grown in IL-3 as a control or in doxycycline to induce BCR-ABL expression. Cells were then treated with Q-VD-OPh and irradiated, then assayed for DNA damage. BCR-ABL-expressing cells showed a small increase in the number of DNA DSBs immediately after 5Gy IR compared to parental cells growing in IL-3 after Q-VD-OPh treatment (Figure 7f). However, this difference is not statistically significant. This suggests that the addition of a caspase inhibitor abrogates the increase in DSBs seen in apoptosis competent BCR-ABL-

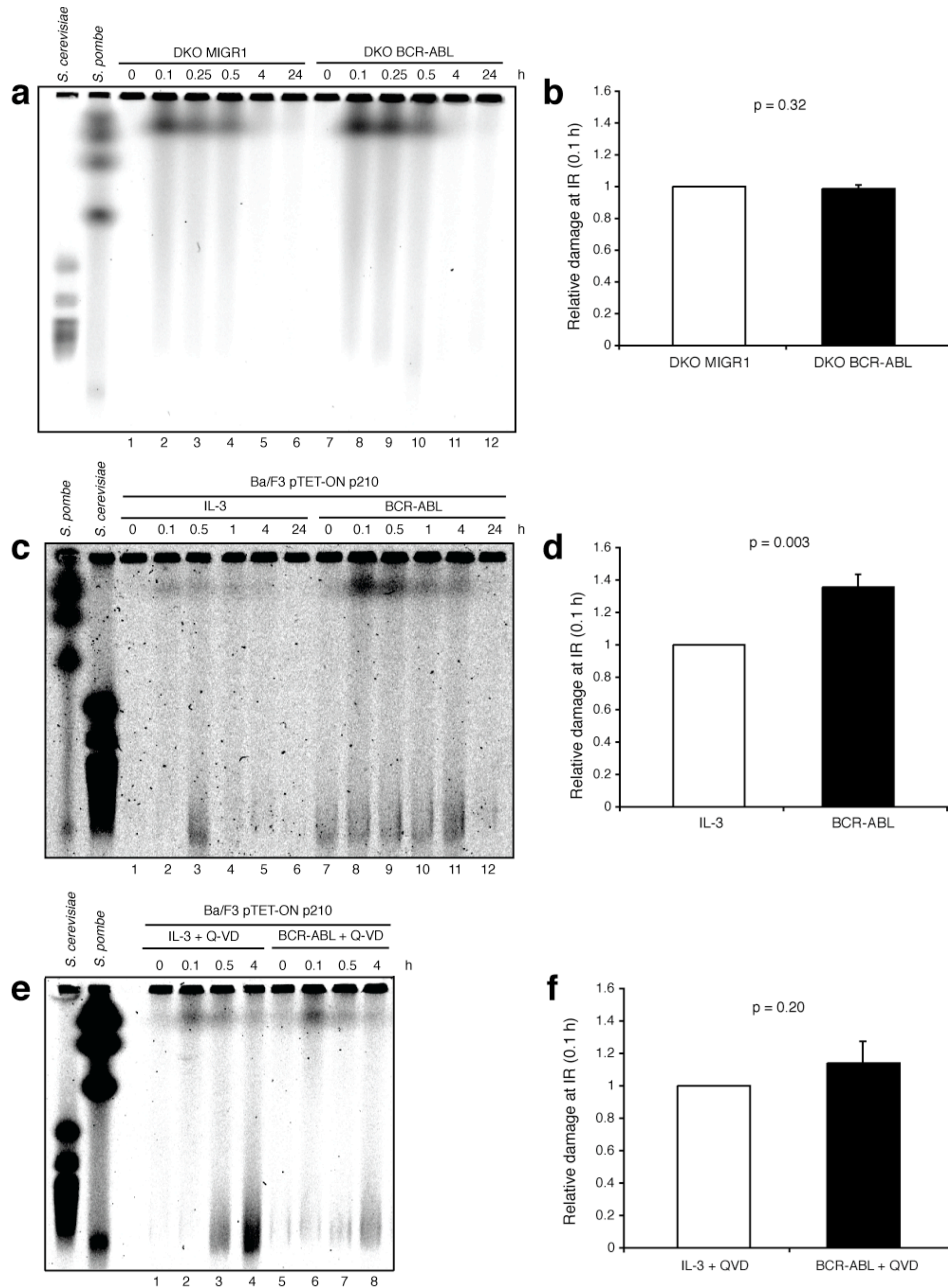


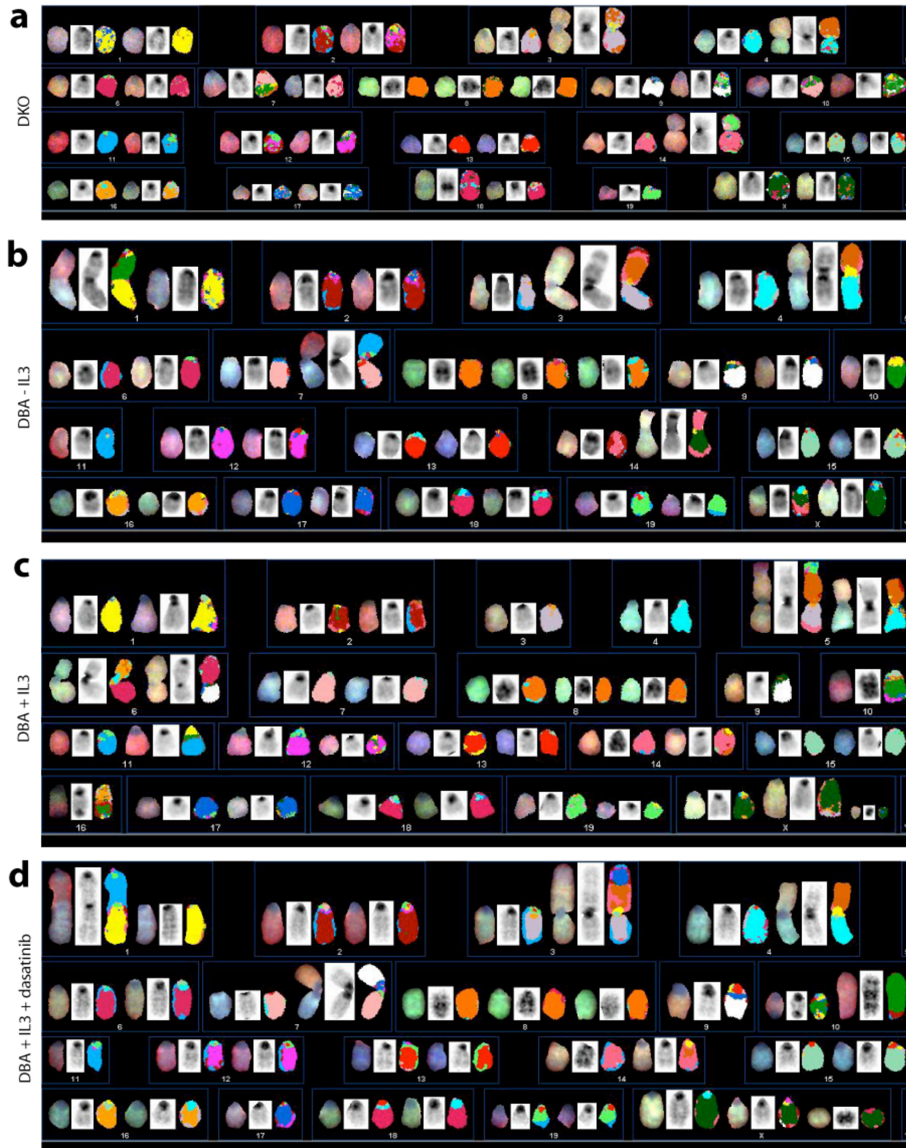
Figure 7: DBA and DKO cells have equivalent DSBs after IR. a, c, e) Scanned images of pulsed field gels stained with SYBR green. **a)** DKO and DBA cells run in a pulsed field gel with individual lanes representing various times before (0) or after IR. Gel shown is representative of at least 3 independent experiments. *S. cerevisiae* and *S. pombe* are run as size standards. **b)** Quantification of amount of DNA in smear (DSBs) divided by total amount of DNA in well for each condition immediately after IR relative to BCR-ABL-negative cell value. Error bars represent standard error over at least three independent experiments. **c)** Ba/F3 pTET-ON p210 cells grown with (BCR-ABL) or without (IL-3) doxycycline run in a pulsed field gel with individual lanes representing various times before (0) or after IR. Gel shown is representative of at least 3 independent experiments. **d)** Same as (**b**) but for smears represented by (**c**). **e)** Ba/F3 pTET-ON p210 cells grown with Q-VD-OPh to inhibit caspase activity, with (BCR-ABL) or without (IL-3) doxycycline run in a pulsed field gel with individual lanes representing various times before (0) or after IR. Gel shown is representative of at least 3 independent experiments. **f)** Same as **(b)** for smears represented by **(e)**.

expressing cells (compare Figure 7f to 7d). As discussed below, this result is unexpected since we are measuring DNA damage within minutes to hours after irradiation, prior to the onset of apoptosis. However, it demonstrates that the effect of BCR-ABL on DNA DSB repair may be associated not only with the lack of Bax and Bak expression but also with the ability of cells to undergo caspase-mediated apoptosis.

DKO and DBA cells have similar numbers of chromosomal alterations after IR and repair

We next wanted to determine if BCR-ABL expression would confer an increase in chromosomal abnormalities after IR and repair in a non-apoptotic background, as it did in an apoptosis-competent background.(22) Since BCR-ABL has been shown to affect the fidelity of repair[222], we wished to determine if this was the case when apoptosis was inhibited. We utilized SKY analysis to explore genetic alterations in individual DKO and DBA cells that were left untreated or exposed to 5 Gy IR and allowed to repair (Figure 8, a-d).

We first confirmed ($P < .0001$) that there were more chromosomal abnormalities (including gains or losses of whole chromosomes or centromeres and new translocations) in the irradiated cells than in the untreated cells (Figure 8e, compare black lines representing median number of abnormalities in irradiated (blue bars) to untreated (green bars)). The majority of cells demonstrated new chromosomal abnormalities, including simple unbalanced chromosomal translocations and gains and losses of whole chromosomes after IR and repair. When scoring aberrancies, we noted that both cell lines have a small number of clonal karyotypic abnormalities, which include a Robertsonian (Rb)(3;5) translocation, a Rb(4;5) translocation, and an additional chromosome 8 (trisomy 8). We did not count these clonal abnormalities toward the total number of new abnormalities. Interestingly, while untreated DKO cells displayed only clonal abnormalities, untreated DBA cells were found to have both clonal and additional non-clonal abnormalities.


a
DKO
b
DBA - IL3
c
DBA + IL3
d
DBA + IL3 + dasatinib

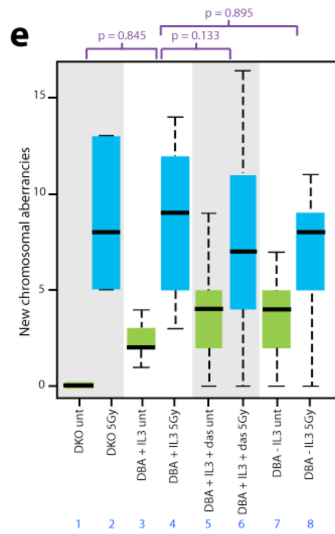

e
p = 0.895
p = 0.845
p = 0.133
New chromosomal aberrancies
15
10
5
0
DKO unt
DKO 5Gy
DBA + IL3 unt
DBA + IL3 5Gy
DBA + IL3 + das unt
DBA + IL3 + das 5Gy
DBA - IL3 unt
DBA - IL3 5Gy
1
2
3
4
5
6
7
8

Figure 8: DKO and DBA cells have equivalent amounts of DNA damage after IR and repair. a-d) SKY images, with each chromosome represented by its spectral color, DAPI, and pseudocolor images. **a)** DKO cells after 5Gy IR and 48 h repair. **b)** DBA cells grown without IL-3 after 5Gy IR and 48 h repair. **c)** DBA cells grown with IL-3 after 5Gy IR and 48 h repair. **d)** DBA cells grown with IL-3 and dasatinib after 5Gy IR and 48 h repair, followed by dasatinib washout and an additional 24 h in IL-3 alone to get a sufficient number of cells into metaphase. **e)** Boxplot of the number of new chromosomal aberrancies (median shown as thick black line) for DBA vs. DKO, and DBA with different treatment modifications. Colored boxes indicate the interquartile range (IQR) of the data and whiskers extend to the nearest datapoint within 2.5 IQR of the median.

However, the sample size of untreated DKO cells was small, and it is possible that variability would increase with a larger sample size, as in a non-apoptotic cell line one would expect to find some chromosomal abnormalities at baseline once the cells have gone through a few divisions.

We next compared the number of new chromosomal abnormalities after 5 Gy IR and repair between conditions to determine if the presence of BCR-ABL in the DBA cells impacted the repair of breaks. For the DKO cells, 5Gy IR was associated with an average increase of 8.4 (95% CI of 4.4, 12.8) more chromosomal abnormalities compared to control conditions. On average the 5Gy IR-associated increase in DBA cells was not significantly larger than for DKO cells ($P = 0.845$). In fact, compared to DKO, DBA cells averaged 2.4 fewer abnormalities in the IR versus untreated groups; a one-sided 95% CI (-2.43, 1.6) had an upper bound of 1.6 IR-induced abnormalities more for DBA than DKO, adding weight to the argument that the magnitude of any increase due to BCR-ABL was not significant in this comparison. Note that we did not statistically compare the difference between lanes 1 and 3, only the change between lanes 1 and 2 vs. lanes 3 and 4. We felt this was the relevant comparison in order to eliminate any baseline differences and compare the difference in BCR-ABL-induced chromosomal abnormalities after IR, not spontaneous BCR-ABL-induced abnormalities.

We next wished to determine whether the presence of IL-3 had any effects on protecting cells from damage. Comparing DBA cells grown in IL-3 to those grown without IL-3, we found that cells without IL-3 had 2.7 fewer IR-induced abnormalities than cells with IL-3. There was no evidence that IL-3 treatment offered significant protection from IR-induced damage ($P = 0.895$, upper bound on the one-sided confidence interval of 0.8 more abnormalities in IL-3-treated versus untreated).

Lastly, we wished to determine if BCR-ABL kinase activity was important for chromosomal abnormalities after IR and repair. We determined that inhibiting BCR-ABL kinase activity with

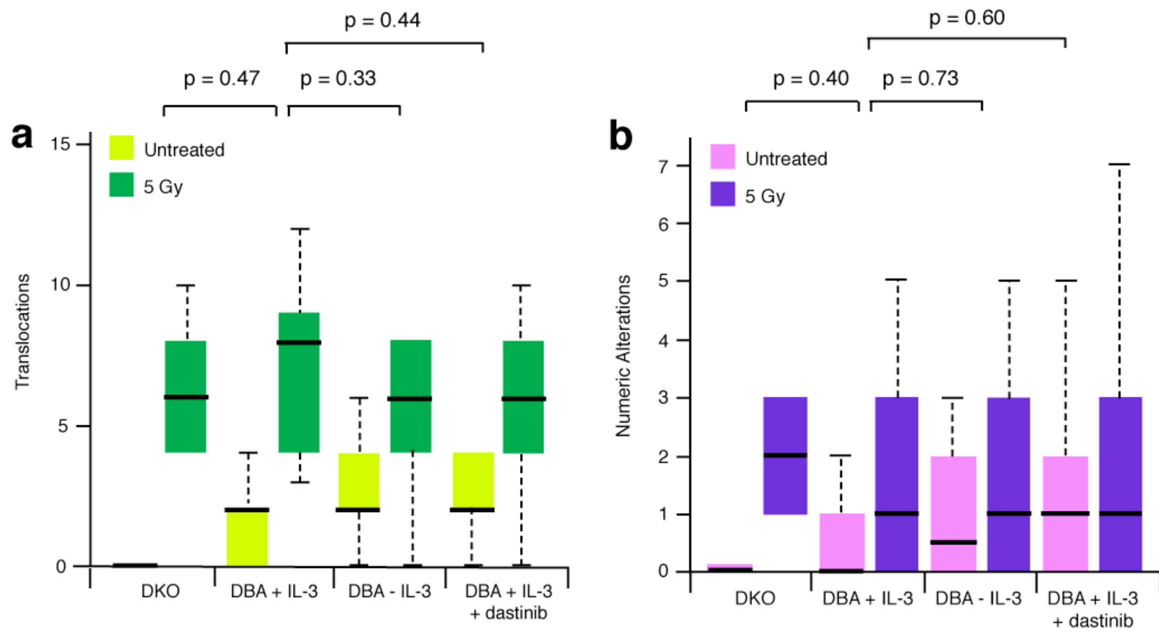


Figure 9: DKO and DBA cells show similar increases in translocations and numeric alterations after DNA damage and repair. Boxplots of the number of **(a)** new chromosomal translocations or **(b)** numeric alterations (median shown as thick black line) for DKO and DBA cells left untreated or treated with 5 Gy IR, followed by SKY analysis. Colored boxes indicate the interquartile range (IQR) of the data and whiskers extend to the nearest data point within 2.5 IQR of the median.

dasatinib was associated with a mean of 2.1 (lower bound on 95% CI of 5.9) fewer IR-associated increases in chromosomal abnormalities than DBA+IL-3 alone, a value which was not significantly different from zero (P = 0.133, Figure 8e). As a reference the mean number of IR-associated chromosomal abnormalities in the DBA+IL-3 group was 6.0 (95% CI of 3.0,8.9).

We also wished to determine whether the expression or function of BCR-ABL in DKO cells would affect numeric alterations or translocations differently, as these alterations occur in different stages of the cell cycle. We therefore examined the above data separately to investigate whether BCR-ABL led to an increase in either gains or losses of chromosomes or translocations in DKO cells. As with the combined data, we determined that there was an increase in both translocations and numeric alterations after IR compared to untreated. Overall the number of translocations increased more after IR than the number of numeric alterations (Figure 9), suggesting that in these cells IR may play a bigger role in inducing mis-repaired DSBs than in affecting chromosome segregation during mitosis.

No significant differences were observed when comparing the increase in translocations or numeric alterations before and after IR among the different treatment conditions. The mean difference in translocations before and after IR was slightly but not significantly greater than in DKO cells (P = 0.47), while the converse was true of numeric alterations (P = 0.40). The mean difference in IR-induced translocations was slightly higher in DBA cells treated with IL-3 than those without IL-3 (P = 0.33), while the mean difference in IR-induced numeric alterations was slightly higher in DBA cells without IL-3 (P = 0.73). The mean difference in IR-induced translocations was slightly higher in DBA cells treated with IL-3 than those treated with IL-3 and dasatinib (P = 0.44), while the mean difference in IR-induced numeric alterations was slightly higher in DBA cells treated with IL-3 and dasatinib (P = 0.60).

Overall, these data suggest that BCR-ABL does not increase the number of chromosomal abnormalities after IR and repair in a non-apoptotic background and supports the hypothesis that the BCR-ABL-mediated increase in DNA damage may be secondary to the inhibition of apoptosis.

C. Discussion

We and others have previously addressed the role of BCR-ABL in promoting the progression of CML chronic phase cells to CML blast crisis. It has been proposed that BCR-ABL expression causes an inherent increase in DNA damage or impairs DNA repair processes. Many of these effects are modest and there is not a consensus as to the critical DNA repair mechanisms affected by BCR-ABL expression. As an alternative, we hypothesized that many of these observations may reflect a difference in cell survival after DNA damage in BCR-ABL-expressing cell lines. In order to test this hypothesis, we have generated a hematopoietic cell line from Bax/Bak null cells. These cells are normally dependent on IL-3 for growth, but this requirement is replaced by expression of BCR-ABL (demonstrating that the mitogenic effects of BCR-ABL are retained in this cell line). Both DKO and DBA cells are resistant to apoptosis. In contrast to expression of BCR-ABL in apoptosis competent cells, DBA cells do not have an increase in DNA DSBs compared to control cells after IR. Importantly, both DKO and DBA cells show error-prone DNA repair after IR as demonstrated by spectral karyotyping. Taken together, these data suggest that the effect of BCR-ABL on the inhibition of apoptosis may play a role in the accumulation of secondary genetic events in oncogene-expressing cells.

Here we chose to utilize PFGE to detect DSBs, rather than the comet assay, which we and others have used previously.[217] The alkaline comet assay shows both SSBs and DSBs as a comet tail radiating from the cell, whereas PFGE only allows DNA with DSBs to be mobilized. We demonstrate that results are similar with the two assays, showing that either the damage

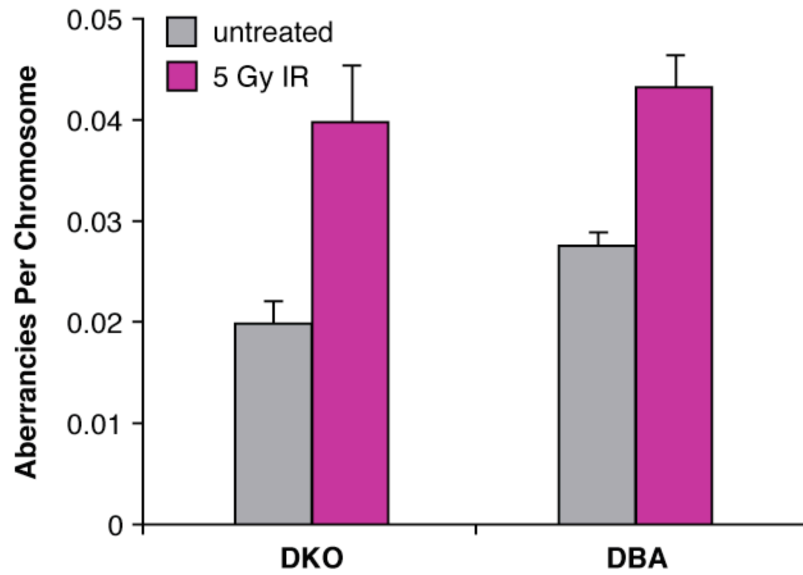


Figure 10: BCR-ABL expression does not increase chromosome breaks after 5 Gy IR. DKO and DBA cells were treated with 5 Gy IR followed by addition of colcemid 4 h later. Metaphases were harvested 48 h after colcemid addition, stained with Giemsa and scored for SSBs and DSBs. Note that this experiment is an n of 1. Error bars represent standard deviation in approximately 50 metaphases per condition.

experienced is primarily DNA DSBs, or that BCR-ABL impacts the generation of both SSBs and DSBs to similar extents in apoptosis-competent BaF3 pTET-ON p210. Of note, we also manually scored stained metaphases of DKO and DBA treated with colcemid a few hours after IR and did not see a difference in the overall number of aberrations, including SSBs, DSBs, tri-radials, quadriradials and rings (Figure 10), consistent with the data presented in Figure 7b. Thus, these several assays of DNA damage show similar results.

It was previously stated that a delay in the G2/M transition was necessary for resistance to DNA damage in BCR-ABL-positive cells.[269] It has been suggested that the delay in G2/M gives BCR-ABL-positive cells an opportunity to repair potentially lethal DNA damage rather than undergoing apoptosis.[262] Interestingly, we found that BCR-ABL-expressing cells that are unable to undergo apoptosis do not have a robust early G2/M arrest but have a trend towards a prolonged G2/M phase over several days after IR. This was not a true “G2/M arrest” as cells continued to proliferate and this was not due to a greater amount of DNA damage in the BCR-ABL+ cells. These data suggest that BCR-ABL may affect the G2/M checkpoint but the mechanism of such an effect is unclear.

We have analyzed both early DNA DSBs by PFGE, and the development of chromosomal abnormalities present 48 hours after DNA damage by SKY. As discussed further below, we hypothesize that either BCR-ABL expression or the absence of Bax and Bak, may allow cells with significant DNA damage to survive. DNA repair is error-prone in both DKO and DBA cells, perhaps because of the recruitment of low-fidelity repair pathways, or alternatively reflecting the normal but error-prone cellular response to DSBs that would lead to cell death in apoptosis-competent cells. The effects of BCR-ABL expression and the role of Bax and Bak on the early accumulation and repair of DNA DSBs is harder to explain. The apoptotic machinery is not known to be activated this quickly after DNA damage. The results here suggest a relationship between

the activation of the apoptotic machinery and the DNA damage response, but the mechanism of such a relationship is unclear. Overall these data suggest that the apoptotic regulatory proteins may play a role in the appearance of DSBs after damage.

As BCR-ABL normally interferes with apoptotic machinery, we believe that inhibiting apoptosis, as in the DKO cell line, “normalizes” the apoptosis background compared to BCR-ABL-expressing cells. Because the PFGE assay is only robust for comparing the levels of relative DNA damage and does not allow for a quantitative analysis of DNA damage, it is not clear if there is increased DNA damage in parental cells, or decreased damage in BCR-ABL-expressing cells.

Overall, we would propose that BCR-ABL expression allows a larger group of cells with significant DNA damage to survive long enough to undergo DNA repair. This DNA repair appears to be error-prone. It has been previously suggested that this is because of specific effects of BCR-ABL on DNA repair pathways (Figure 1). However, the results presented here suggest that low-fidelity DNA repair may be intrinsic in cells with a high apoptotic threshold, whether induced by BCR-ABL or by loss of Bax and Bak. As BCR-ABL-mediated effects can be cell type-dependent, future studies will be required to determine whether this is a generalizable phenomenon.

IV. OTHER EFFECTS OF BCR-ABL ON DNA DAMAGE REGULATION

As noted in the introduction above, BCR-ABL has been reported to have diverse effects on chromosomal structure and on cell cycle checkpoint. I have explored a number of these observations as described below.

A. BCR-ABL and ATR activation

1. Introduction

The DNA damage response proteins ataxia-telangiectasia mutated (ATM) protein, ataxia-telangiectasia and rad 3-related (ATR) protein and DNA-dependent protein kinase, catalytic subunit (DNA-PKcs) are members of the phosphatidylinositol 3-OH-kinase-related kinase (PIKK) superfamily. They are important for sensing and initiating DNA repair, checkpoints, and apoptosis.[272-274] ATM and ATR function by signaling through their major downstream effectors, Chk1 and Chk2 to regulate cell cycle progression after DNA damage.[275, 276] While ATM primarily phosphorylates and activates Chk2 and can regulate the G1 checkpoint[277], ATR primarily activates Chk1. There is some redundancy between the pathways, and both can induce S phase checkpoints.

c-ABL is known to be activated by DNA damage to bind to ATM.[244, 278] This led our lab to investigate whether BCR-ABL also could activate ATM or its homolog ATR after DNA damage. We determined that while etoposide induced BCR-ABL binding to ATM, it did not disrupt ATM function.[217] In contrast, BCR-ABL disrupted the function of ATR, as we observed that BCR-ABL-expressing cells demonstrate radioresistant DNA synthesis and decreased Chk1 phosphorylation after DNA damage (a readout for ATR kinase activity).[217]

In contrast to our published results, another group published data that ATR-mediated

phosphorylation of Chk1 was enhanced in BCR/ ABL-positive leukemia cells after DNA damage.[219] While this appeared convincing in cells treated with mitomycin C, cells treated with etoposide appeared to show neither an increase in Chk1 phosphorylation nor a decrease (as we had seen previously[217]).

As we were interested in further pursuing the importance of Chk1 activation or inactivation by BCR-ABL as a regulator of the DNA damage response, we revisited Chk1 phosphorylation after etoposide treatment.

2. Results

BCR-ABL does not consistently inhibit Chk1 phosphorylation

We wished to determine if in fact BCR-ABL affected ATR kinase activity. As was done initially, we have used phosphorylation of Chk1 on serine 345 as a surrogate assay for ATR kinase activity. We treated Ba/F3 cells with 10 uM etoposide for the indicated time and examined Chk1 S345 phosphorylation levels (Figure 11). We determined that in both IL-3 and BCR-ABL-expressing cells, Chk1 was phosphorylated after DNA damage (Figure 11a, compare lane 2 to lane 1 and lane 5 to lane 4). Additionally, there was no change in either Chk1 expression or phosphorylation up to 2 h after etoposide exposure (Figure 11a). Further, the same result was seen after a 1 h exposure with varying doses of etoposide. This suggests that BCR-ABL does not have an effect on ATR kinase activity in these cells.

DKO and DBA cells express less Chk1 protein than Ba/F3 pTET-ON p210 cells.

As different cell types have been shown to respond differently to DNA damage, we also wished to investigate how BCR-ABL expression would impact ATR activity in DKO cells. DKO and DBA cells were irradiated and Chk1 expression and phosphorylation levels were examined by western blot 1 h after treatment. We found that Chk1 protein was expressed at very low levels, which

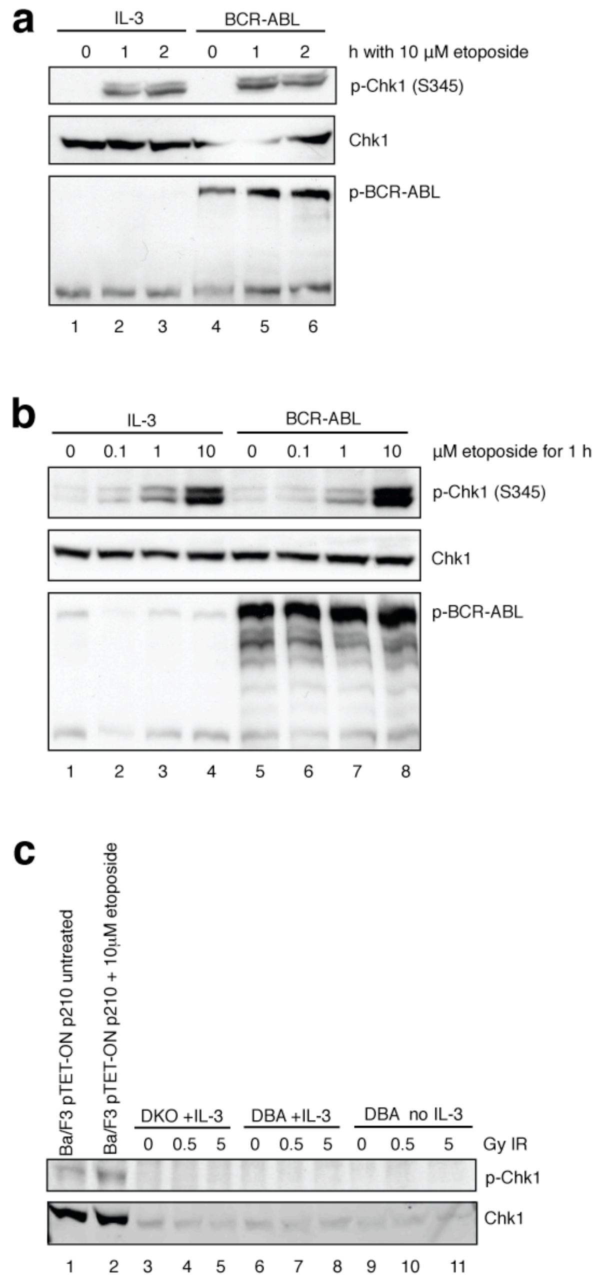


Figure 11: Chk1 phosphorylation after DNA damage. a) Ba/F3 pTET-ON p210 cells grown in IL-3 or doxycycline to induce BCR-ABL expression were treated with 10μM etoposide for the indicated times. 100μg of protein was run per lane on an SDS-PAGE gel and blotted with the indicated antibodies. **b)** Ba/F3 pTET-ON p210 cells grown in IL-3 or doxycycline to induce BCR-ABL expression were treated with the indicated concentrations of etoposide for 1 hour. 100μg of protein was run per lane on an SDS-PAGE gel and blotted with the indicated antibodies. **c)** Chk1 expression and phosphorylation in DKO and DBA cells relative to Ba/F3 pTET-ON p210 cells

made it difficult to see any obvious changes in Chk1 phosphorylation after DNA damage. These data suggest that Chk1 protein is expressed at lower levels in DKO and DBA cells than Ba/F3 pTET-ON p210 cells, and the importance of ATR in responding to DNA damage in these cells is unknown.

3. Discussion

While our lab had previously demonstrated that BCR-ABL expression led to decreased Chk1 phosphorylation by ATR, this was not repeated in Figure 11. There are a few possible explanations for this discrepancy. One is that the dose of etoposide used previously may have been cytotoxic, leading to the breakdown of proteins. An XTT assay showed that 7uM etoposide for 48 h killed all the cells, and 10uM was used in this assay, albeit for much shorter times.[217] However, this explanation is unlikely if Ba/F3 pTET-ON p210 cells were treated with a sufficient amount of doxycycline to induce BCR-ABL expression. In order to test this, an XTT assay was performed, which showed maximal and complete cell killing at 7 mM etoposide.[199] As the induction of Chk1 phosphorylation experiment was done with 10 mM etoposide, we hypothesize that there may have been artifacts induced by the induction of apoptosis during the experiment. The nature of these changes is unclear but may include induction of caspases or breakdown of the nuclear membrane. Breakdown of the nuclear membrane would also explain the variable results studying translocation of BCR-ABL to the nucleus after DNA damage (Sanchez and Carroll, unpublished).

Another possibility is that the difference in results could be because of the use of two different antibodies. Both antibodies recognize phosphorylated S345 of Chk1, but the one used in the 2004 paper[217] was polyclonal and the one used in Figure 11 was monoclonal. As the difference was more pronounced at later time points, it may be worth exploring whether the original

decrease in Chk1 phosphorylation in BCR-ABL-expressing cells is seen after 2 hours with the newer monoclonal antibody.

In order to look at Chk1 phosphorylation in DKO and DBA cells, which express low levels of Chk1 protein, one could try two possible methods. The first is to IP Chk1 followed by western blot to detect p-Chk1. This is problematic due to the size of Chk1 (54 kDa) and the size of the IgG heavy chain (55 kDa) used to pull down Chk1. This would make it very difficult to get enough separation on the gel to separate those bands. In this case the other possibility is to increase the amount of lysate loaded in the SDS-PAGE gel to greater than 100mg. The amount of total protein needed to see p-Chk1 in these cells is unknown. Overall, at this point, although other methods could be pursued to clarify the difference between these results and those in Dierov et al., we conclude that Chk1 is likely phosphorylated after DNA damage in BCR-ABL-expressing cells although whether other functions of ATR are affected by BCR-ABL expression remains unclear.

B. BCR-ABL and Centrosomes

1. Introduction

The centrosome is an organelle that is important in maintaining genomic stability. It serves as the microtubule organizing center of the cell; during mitosis it is important in regulating the mitotic spindle. The centrosome is composed of two centrioles surrounded by pericentriolar material (including the proteins γ-tubulin, pericentrin and ninein).[279, 280] The centrosome replicates once per cell cycle during S phase, and unsuccessful replication can lead to mis-segregation of chromosomes and aneuploidy. Aberrant numbers of centrosomes in a cell have been associated with cancer.[281-287]

Giehl et al examined primary CML samples to investigate the effect of CML stage on centrosome aberrancies.[254] They determined that compared to normal CD34+ cells, all CML samples

displayed centrosome alterations. Further, more abnormalities were detected in CML BC than CML CP. This contrasted with karyotypic abnormalities, which were seen almost exclusively in CML BC. This suggests that centrosome aberrancies precede karyotypic aberrancies, and may play a role in the genomic instability seen in CML BC.

They later investigated whether BCR-ABL expression could lead to the generation of centrosomal aberrancies.[253] Utilizing long term cultures of BCR-ABL-expressing human U937 cells, they determined that expression of BCR-ABL led to a time-dependent increase in centrosome aberrancies, which was partially abrogated by siRNA knockdown of BCR-ABL. Concomitantly, we were performing a similar experiment using long-term cultures of Ba/F3 pTET-ON p210 cells grown in doxycycline to induce BCR-ABL expression. We examined centrosomes at various times using immunofluorescence to look for aberrancies.

2. Results

CML BC cells demonstrate centrosome aberrancies

We first wished to confirm that we saw abnormal centrosomes in CML BC patient blasts. We stained cytospins from CD34+ cells from normal PBMCs and a CML BC patient with anti-g tubulin antibody to examine centrosomes. We determined that while normal CD34+ cells usually displayed 1 centrosome per cell (Figure 12a), CML BC cells frequently displayed a cluster of centrosomes (Figure 12b) consistent with what was shown previously.[254]

BCR-ABL expression in Ba/F3 pTET-ON p210 cells does not lead to obvious changes in centrosomes

We next wished to determine if long-term BCR-ABL expression in Ba/F3 pTET-ON p210 cells would lead to centrosome aberrancies. Cells were grown continuously in doxycycline to induce BCR-ABL expression, and cells were harvested after various times to examine centrosome

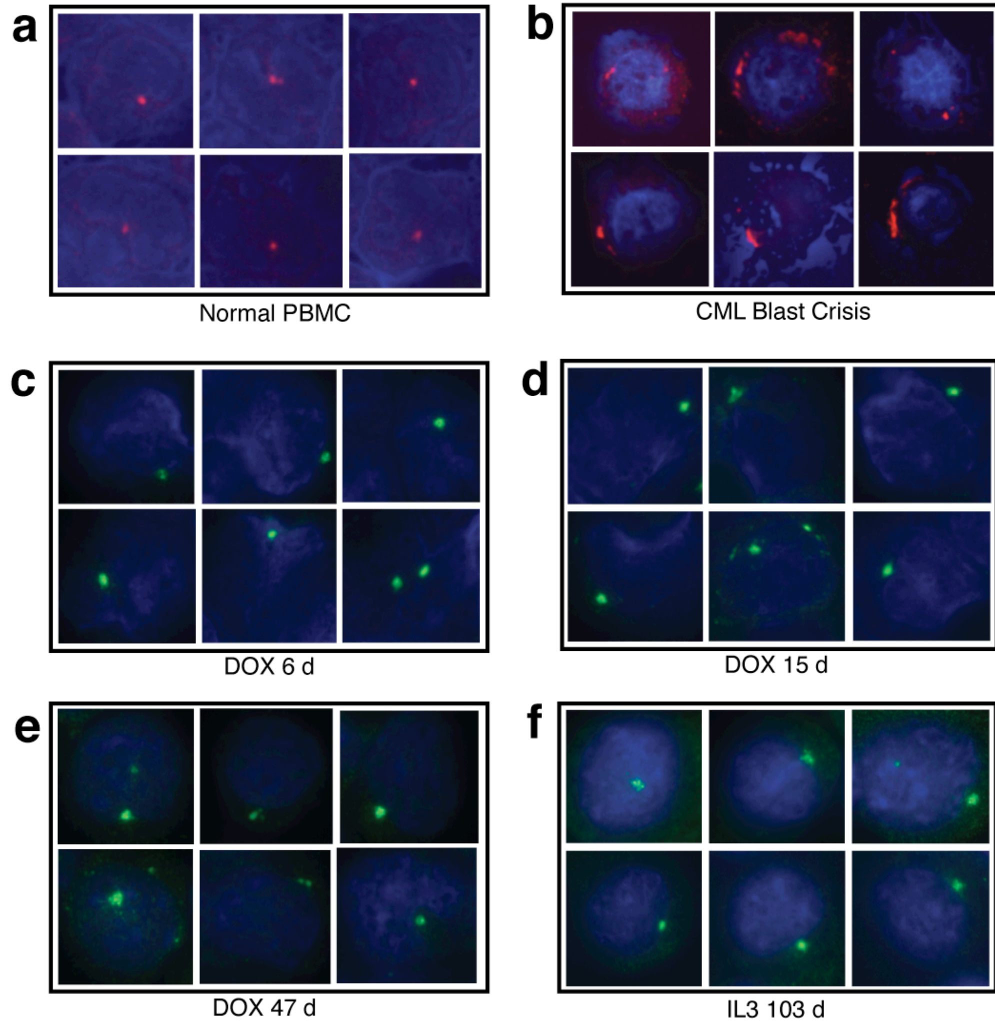


Figure 12: Centrosomes in CML and Ba/F3 pTET-On p210 cells. a) Normal PBMC stained with GTU-88 **b)** CML BC patient cells stained with GTU-88. **c-f)** Ba/F3 pTET-ON p210 cells stained with pericentrin grown with **c)** doxycyline for 6 days **d)** doxycyline for 15 days **e)** doxycyline for 47 days **f)** IL-3 for 103 days.

expression (Figure 12c-e). By looking at pericentrin staining, we observed that nearly all cells contained one or two centrosomes. We did not detect any increase in centrosomal aberrancies, which suggested to us that over the course of 47 days, BCR-ABL expression alone was not sufficient to induce centrosome aberrancies. As a control we confirmed that Ba/F3 pTET-ON p210 cells grown long-term in IL-3 did not have abnormal centrosomes (Figure 12f). This suggests that Ba/F3 pTET-ON p210 cells do not have unstable centrosomes under normal growth conditions.

3. Discussion

We began to investigate the effect of BCR-ABL on centrosome aberrancies in Ba/F3 pTET-ON p210 cells before the publication of the U937 data. We concluded that BCR-ABL was not sufficient to induce centrosome abnormalities after nearly 7 weeks in culture. However, U937 cells demonstrated centrosome abnormalities even earlier than this time. It is possible that this effect is cell-type dependent. Ba/F3 pTET-ON p210 cells are a mouse cell line while U937 cells are human, and there could be variability between species. Growing the cultures out even longer may yield a more obvious difference in centrosome expression in Ba/F3 pTET-ON p210 cells.

Another possibility is that the difference is very subtle and we did not take enough care to determine the exact quantity or extent of numeric alterations. It may be worth repeating this experiment with more controls, such as imatinib inhibition of BCR-ABL kinase activity. Additionally, concurrent metaphase spreads should be prepared to look at the development of aneuploidy over time. We would expect that if centrosome aberrancies cause genomic instability in the form of aneuploid cells, the centrosomal aberrancies would be detected prior to chromosomal changes. This was the case with CML cells in CP and BC.[254]

C. The Effect of BCR-ABL on Fragile Site Stability

1. Introduction

Fragile sites are chromosomal loci that exhibit breaks or gaps detectable in metaphase spreads due to replicative stress. Replicative stress can be induced by drugs such as aphidicolin[288], which is a selective inhibitor of DNA polymerase a[289] that blocks DNA synthesis.

Fragile sites play a role in genomic stability as they are hot spots for sister chromatid exchange, translocations, and deletions. Furthermore, ATR and BRCA1 are critical for maintenance of fragile site stability.[290, 291] We previously found that BCR-ABL associated with ATR and disrupted ATR signaling in Ba/F3 pTET-ON p210 cells and primary CML cells[217] and Deutsch et al. determined that BRCA1 was downregulated in BCR-ABL-expressing hematopoietic cells.[197] It is therefore possible that the inhibition of BRCA1 and ATR by BCR-ABL could lead to a decrease in fragile site stability. A significant increase in the frequency of aphidicolin-induced fragile sites was found in 4/4 CML patients compared to healthy matched controls.[292] We therefore hypothesized that BCR-ABL expression would lead to an increase in aphidicolin-induced fragile sites and utilized Ba/F3 pTET-ON p210 cells to test this hypothesis.

2. Results

The DNA fragile site assay is an assay for chromosomal breaks induced by aphidiocolin, which are scored manually by examination of stained chromosomes of mitotic cells. As shown in Figure 13a, aphidicolin induced an increase in chromosomal aberrations in Ba/F3 pTET-ON p210 cells (compare pink bars to gray bars) in all conditions. Importantly, BCR-ABL expression (DOX) increased the number of aphidicolin-induced aberrancies over control (IL-3). However, addition of IL-3 to the culture of BCRABL-expressing cells led to attenuation of increase in aphidicolin-induced breaks (IL-3+DOX pink bar). This suggests that BCR-ABL induces fragile site instability in Ba/F3 pTET-ON p210 cells, and IL-3 may have a protective effect.

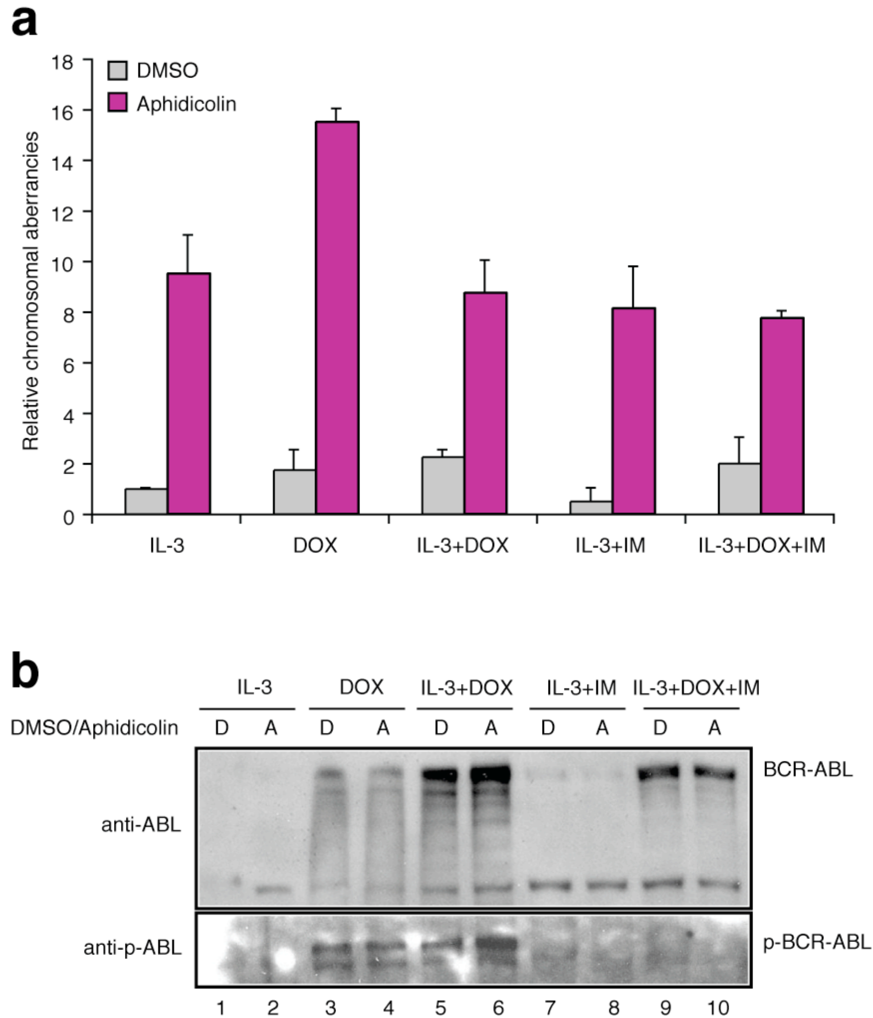


Figure 13: Chromosomal aberrancies after aphidicolin treatment. a) Ba/F3 pTET-ON p210 cells grown in the indicated media were scored for the number of chromosome and chromatid breaks, triradials, quadriradials and rings. In each case 50 metaphases were scored and compared to IL-3 treated with DMSO to normalize between experiments. n=2. **b)** Western blot for BCR-ABL expression and phosphorylation of cells scored in **(a)**. DOX: doxycycline; IM: imatinib

Addition of imatinib to the culture did not increase or decrease the number of aberrancies (compare pink bars for IL-3+IM to IL-3 and IL-3+DOX+IM to IL-3+DOX), suggesting that over a short time in these cells imatinib has no effect on fragile site stability.

Western blot control (Figure 13b) demonstrates that BCR-ABL is expressed in all cells grown with doxycycline (DOX). BCR-ABL is phosphorylated (kinase active) (lanes 3-6) but imatinib turns off BCR-ABL kinase activity (lanes 9, 10 – no p-BCR-ABL). Neither aphidicolin nor DMSO affected BCR-ABL expression or phosphorylation.

3. Discussion

Our results indicate that BCR-ABL expression leads to a decrease in fragile site stability. However, these results are called into question when one considers that the addition of IL-3 leads to the abrogation of the BCR-ABL-induced effect. Our doxycycline conditions actually alter two variables as there is addition of doxycycline to induce BCR-ABL expression and, usually, withdraw of IL-3. As noted above, BCR-ABL provides a mitogenic signal to cells and this switch does not alter the growth of the cells. There are a few possible explanations for our results. One is that the BCR-ABL-induced effect is real. This suggests that BCR-ABL may be interfering with the proteins responsible for maintaining fragile site stability. It was previously shown that ATR is critical in maintaining fragile-site stability.[290] WRN has also been shown to stabilize fragile sites by acting in a pathway with the ATR replication checkpoint.[293] Though WRN was found by one lab to be overexpressed in BCR-ABL-expressing cell lines and CML cells[232], we previously observed that BCR-ABL disrupts ATR activation and the ATR-dependent intra-S phase checkpoint[217], suggesting that disruption of this checkpoint may be a possible mechanism. Alternatively, BRCA1 was found to induce the G_2/M checkpoint after aphidicolin-induced replication stalling, and this checkpoint function is involved in fragile-site stability.[291] As BCR-

ABL expression was found to lead to a marked decrease in BRCA1 protein[197], this also may play a role in Ba/F3 pTET-ON p210 cells. Examination of these proteins side-by-side with examination of metaphase breaks and gaps should provide further insight.

What follows is that IL-3 is somehow providing a protective effect against breaks. In order to show that the BCR-ABL effect is real, the experiments would need to be repeated in a non-IL-3-dependent cell line. We initially tried to repeat this experiment in NIH 3T3 cells, but these cells are known to be aneuploid (data not shown, [294]), making them a poor choice for this assay. Another possibility is that there is a population of cells in the doxycycline-treated culture that are not expressing sufficient levels BCR-ABL to maintain normal growth in the face of genotoxic stress. The addition of IL-3 to the culture then allows these cells to grow out, skewing the results toward the non-BCR-ABL-expressing cells. This may be remedied by using a higher dose of doxycycline, which has been shown to induce increasing amounts of BCR-ABL.[199]

As the addition of IL-3 to the doxycycline culture itself abrogated fragile site instability, we could not determine whether the addition of imatinib had any effect on fragile site stability by inhibiting BCR-ABL kinase activity. Cells were treated with aphidicolin for 24 hours, which is too long to starve Ba/F3 cells of IL-3 or BCR-ABL. However, as Ba/F3 cells double approximately every 12 h, it may be possible to see the effects of aphidicolin addition over a shorter time. This would allow the addition of imatinib without IL-3, which would help determine whether BCR-ABL kinase activity is required for the effect on fragile sites. Controls would be needed to ensure that imatinib-treated cells are not dying. It is of note that imatinib itself did not decrease fragile site stability.

Overall, the results presented demonstrate that BCR-ABL may have an effect on fragile site stability, however experimental conditions can have a dramatic effect on results of these assays.

Thus, at this time, whether such an effect is physiologic or has a role in progression to CML BC is unclear.

V. CONCLUDING REMARKS

CML treatment is one of the most successful examples of molecular-based therapy translating into patient care. The knowledge that CML CP is caused by the BCR-ABL oncogene led to the development of drugs targeting this tyrosine kinase, and the use of imatinib and second-generation BCR-ABL inhibitors has drastically prolonged patient survival. However, some patients fail treatment and progress to CML BC, which is marked by an increase in chromosomal aberrancies. Additionally, patients frequently develop imatinib-resistant mutations in the BCR-ABL kinase domain. The potential cause of this genomic instability is the subject of much debate.

It remains unclear whether the transition from CML CP to CML BC and development of additional mutations is the direct result of expression of the BCR-ABL kinase, making this disease a rare example of a one-hit malignancy. It has been proposed that BCR-ABL expression causes an inherent increase in DNA damage or impairs DNA repair processes. Overall, effects of BCR-ABL on DNA damage appear to be modest, and there is a lack of consensus as to the mechanism(s) of DNA repair that may be affected by BCR-ABL. As an alternative, we hypothesized that the difference in DNA damage may reflect a difference in cell survival after DNA damage in BCR-ABL-expressing cell lines. To test this hypothesis, we generated a BCR-ABL-expressing hematopoietic cell line from Bax/Bak-null cells. These cells are normally dependent on IL-3 for growth, but this requirement is replaced by expression of BCR-ABL, demonstrating that the mitogenic effects of BCR-ABL are retained in this cell line. Both the parental DKO and BCR-ABL-expressing DBA cells are resistant to cell intrinsic apoptosis.

A. Distinguishing anti-apoptotic effects from DNA damage-response effects

We determined through PFGE that apoptosis-incompetent DBA cells do not have an increase in DNA DSBs compared to parental cells immediately after IR. This is in contrast to what was seen

in apoptosis-competent Ba/F3 pTET-ON p210 cells. The increase in DSBs in BCR-ABL-expressing apoptosis competent cells may simply represent the previously seen increase in DNA damage in BCR-ABL-expressing cells over controls. The addition of a pan-caspase inhibitor partially abrogated the BCR-ABL-induced effect in Ba/F3 pTET-ON p210 cells, but not to the extent of DKO compared to DBA. This begs the question of why a difference is seen between cell lines, and what impact the apoptotic machinery has on DNA damage. We hypothesize that BCR-ABL alters the apoptotic threshold after DNA damage, increasing the level of unrepaired or mis-repaired damage that can be present in proliferating cells. However, cells will not have initiated their apoptotic machinery immediately after IR, so it is interesting that the inability of cells to engage their apoptotic machinery could have an effect on DSB formation. This suggests that it is possible that apoptotic proteins could affect DNA damage proteins in a reverse direction (apoptosis is thought to only be triggered by DNA damage, not vice versa).

Cells will have initiated a NHEJ response very quickly after DNA damage, and the DNA damage response proteins are able to signal for the cell to initiate apoptosis. The NHEJ protein Ku70 has been shown to bind and sequester Bax in the cytosol, but release Bax following apoptotic stimuli.[295, 296] Further, ubiquitin-mediated Ku70 degradation induces Bax-dependent cell death.[297] BCR-ABL-expressing cells have been shown to have an increase in Ku70 protein expression after IR.[222] This increase in Ku70 could decrease Bax-dependent cell death in apoptosis BCR-ABL-expressing cells. It is interesting to speculate that Bax or Bak could play a dual role, not only initiating apoptosis after DNA damage, but also playing an unknown role in the appearance or repair of DSBs. It would be of interest to determine whether BCR-ABL affects Ku70 binding and sequestering of Bax, and whether Bax expression can have an impact on Ku70 or another DNA damage protein. NHEJ assays could be undertaken in the presence or absence of Bax, and the presence or absence of BCR-ABL.

Similarly, comparing Ba/F3 pTET-ON p210 cells with or without caspase inhibitor suggests that caspases may play a role in the BCR-ABL-induced development of DSBs. BCR-ABL is known to inhibit apoptosis, though this effect has been shown to be upstream of caspases.[264, 265] It may therefore be possible that the formation of DSBs after IR is either dependent on caspases, or can be blocked by caspase activation. The abrogation of DSBs when a pan-caspase inhibitor is added to Ba/F3 pTET-ON p210 cells may in fact be an *increase* in DSBs in IL-3-treated cells, rather than a decrease in DSBs in BCR-ABL-expressing cells. In order to determine this, Ba/F3 pTET-ON p210 cells grown in IL-3 without caspase inhibitor would need to be run on the same gel as Ba/F3 pTET-ON p210 cells grown in IL-3 and treated with caspase inhibitor.

The partial abrogation of BCR-ABL-induced breaks in Ba/F3 pTET-ON p210 cells treated with caspase inhibitor may be different from the total abrogation in BCR-ABL-induced increase in breaks seen in DKO vs. DBA cells simply due to the slight difference in pathway component affected. Bax and Bak are upstream of caspase activation, and are absolutely required for apoptosis. Alternatively, DKO and DBA cells are accustomed and acclimated to not expressing Bax and Bak, while Ba/F3 pTET-ON p210 cells are accustomed to being able to induce caspase activation and subsequent apoptosis. The difference seen in DSBs at IR may therefore reflect these cellular differences. While the data are suggestive, additional cell lines and conditions need to be used in these assays to adequately address the problem.

It is also possible that the caspase inhibitor may be working through other cellular mechanisms. Caspases are cysteine proteases that normally cleave substrates following aspartate residues. Caspase inhibitors are competitive inhibitors that prevent cleavage of caspase substrates. We used Q-VD-OPh, which is effective at inhibiting caspases at low concentrations, and is non-toxic to cells.[271] Further, it is not known to induce non-specific effects in the cell as do other caspase inhibitors. While it is possible that Q-VD-OPh may be inhibiting DNA damage through another

mechanism, no such effect is currently known.

B. Error prone repair is equivalent in DKO and DBA cells but needs greater statistical power

As noted above, our SKY analysis suggests that expression of BCR-ABL in Bax/Bak-null cells does not further increase error prone DNA repair. However, our SKY analysis was done on a modest number of metaphases and there was significant variation in results from cell to cell. The statistical analysis and possibility of over-interpretation of results are discussed below.

Importantly, both DKO and DBA cells show error prone DNA repair after IR as demonstrated by SKY. SKY analysis suggests that BCR-ABL does not cause a greater increase in chromosomal abnormalities after IR and repair than in non-apoptotic cells alone. Interestingly, it appears that the basal chromosomal abnormalities are higher in DBA cells than DKO cells (note that this difference was not statistically compared). Two possibilities exist here. One is that this is a real, fundamental difference. This agrees with what has been previously shown (though not always repeated), including our own SKY analysis of CML patient samples compared to normal CD34+ cells.[199] This is certainly an interesting observation, and not one to take lightly. The second possibility is that this difference is an artifact of the system. DKO cells are very difficult to thaw from frozen stock, while DBA grow up a bit more easily. This could lead to clonal selection in the DKO cells, while the DBA cells have a bit more variation in their basal damage states. In a non-apoptotic cell line, it is surprising that there were no non-clonal translocations or gains or loses or chromosomes at baseline. Based on the fact that these cells had undergone more than 5 divisions by the time they were analyzed with SKY, one would expect to find chromosomal abnormalities in some cells, and we expect to see that with a larger sample size. The difference between these two possibilities can be discerned through repetition in this cell population, in the subclones of the DKO and DBA lines, or in different cell systems.

Individual samples were not compared to each other, rather the increase in breaks after IR was compared between cell lines. We felt this was the relevant comparison in order to eliminate any baseline differences and compare the difference in BCR-ABL-induced chromosomal abnormalities after IR, not spontaneous BCR-ABL-induced abnormalities. The change in the baseline level of abnormalities can be examined by sampling parental cells and BCR-ABL-transformed cells growing in culture at various time points and analyzing using SKY or FISH.

The largest basal variations exist in the dasatinib-treated DBA cells and the DBA cells grown without IL-3. The high basal level of damage in these two groups may have impacted the comparison of these samples, by decreasing the mean increase in abnormalities after IR. This most strongly impacts the dasatinib-treated cells, which had the highest level of variation. While there was slightly less of an increase in the number of abnormalities in dasatinib-treated DBA cells over DBA+IL-3 alone, we are unsure of the significance of this finding. The high level of variability in abnormalities as well as the fact that DBA cells do not have a higher increase in abnormalities than DKO cells suggest to us that this dasatinib-induced difference may be an artifact.

It is of note that dasatinib treatment does not decrease aberrations in unirradiated cells, which could be the case if BCR-ABL caused the slight increase in baseline abnormalities. However, the cells are only incubated with dasatinib for a short period of time, which will not eliminate any aberrations that existed in the cells prior to dasatinib treatment. It is expected that long-term incubation with a BCR-ABL kinase inhibitor would decrease the BCR-ABL-mediated effects of the baseline cells. Here we were interested in eliminating BCR-ABL effects during a short time period, that is, only eliminating the BCR-ABL effect after a short period of damage and repair.

It is also possible that long-term incubation with dasatinib could have effects on damage acquisition. Patients treated with kinase inhibitors develop BCR-ABL point mutations, which can lead to drug resistance. It is unknown whether the development of these mutations is dependent on BCR-ABL expression or related to treatment with kinase inhibitors. One can surmise that as dasatinib has a number of additional effects, including inhibiting the Src family of kinases and PDGFR, it may itself impact DNA repair proteins and processes.

The variability seen in the SKY analysis is high, which gives us a low power to detect strong statistical differences. However, the likelihood that DBA cells possessed more chromosomal aberrancies than DKO cells was low, based on the 95% confidence interval (-2.1 to 7.8 more abnormalities in DKO cells; two-way analyses of variance with the goal of determining whether differences in the mean number of IR-induced chromosomal aberrations differed between treatment groups. Type I error rates were set to 0.05.) What the data actually lean towards, if there is a difference, is that there is a greater increase in abnormalities in DKO cells than DBA cells. This does not agree with previous data, but does suggest that DBA cells are unlikely to have more damage after repair than control cells. When we previously used SKY analysis in Ba/F3 and patient cells,[199] the effects of BCR-ABL were very pronounced, and we do not see that here. However, one must be careful to note that a Type II statistical error, or false negative, could be observed here. The small sample size (ranging from 4 to 26 metaphases), cell-to-cell variability, and lack of measuring precision suggest that it is possible that the hypothesis may have been falsely accepted. Larger sample sizes and greater precision are needed to rule out this type of error.

We scored both alterations of chromosome number (gains or losses) as well as translocations Numeric alterations occur due to an unsuccessful mitosis and failure of the mitotic checkpoint to recognize this, while translocations can occur at any point in the cell cycle. They are common

during G0 and G1 phase when there is not a sister chromatid available for HR to take place, and are thus repaired by the less-faithful NHEJ mechanism. Interestingly, we did not find that DBA cells had a greater number of either aberrancy than DKO cells. This suggests that the presence of BCR-ABL in these cells is not causing an increase in aneuploidy or translocations. It does not rule out the possibility that there is no effect on other DNA damage, such as point mutations that occur during DNA synthesis in S phase. The number of IR-induced translocations was greater than the numeric alterations, which is in line with the expected result of IR. Full BCR-ABL-mediated effects on aneuploidy may require centrosomal hypertrophy seen in long-term cultures.[253, 254]

C. BCR-ABL affects the apoptotic threshold

Our results are suggestive of the possibility that BCR-ABL impacts the cell's apoptotic threshold, which we define as a level of DNA damage that the cell senses that it cannot repair and it therefore signals the intrinsic apoptotic machinery to undergo programmed cell death. This threshold is partially mediated by the Bcl-2 family of proteins, which includes Bax and Bak.[110] BCR-ABL inhibits apoptosis and prevents the translocation of Bax and Bad to the mitochondria.[120] We believe this causes a shift in the apoptotic threshold, allowing survival of cells with greater amounts of DNA damage than BCR-ABL-negative cells. We speculate that three groups of cells exist after DNA damage. One groups contains cells that are severely damaged and quickly committed to apoptosis. A second group experiences minimal DNA damage, repairs damage quickly, and remains genomically stable. The third group has a significant amount of damage but does not immediately commit to undergo apoptosis. Commitment of this group of cells to apoptosis is likely multi-factorial and dependent on the time required for DNA repair and the number of activated anti-apoptotic proteins and signaling cascades in cells. Along with directly affecting Bcl-2 family members, BCR-ABL is known to activate Akt, which can inhibit apoptosis as well as altering the ratio of anti-apoptotic to pro-

apoptotic Bcl-2 family members.[298] Through these or other mechanisms, we propose that BCR-ABL is allowing a group of cells with significant DNA damage to survive and undergo DNA repair, perhaps using low fidelity DNA repair pathways.

Further experiments are needed to rule out that these effects are cell-type dependent rather than a general BCR-ABL-mediated effect. As discussed below, alternate explanations exist for our results, and ruling them out is necessary to determine the true impact BCR-ABL has on genomic instability via the apoptotic threshold.

D. Alternate possibilities for PFGE results

The possibility exists that the amount of IR used was either too little or too much to detect quantitative differences in DNA damage using the PFGE assay. A brief experiment looking at DKO and DBA cells irradiated with 1-20Gy IR suggested that in this range there was a linear response to DNA damage, with no apparent difference between DKO and DBA cells (data not shown). However, it is possible that at very low levels or very high levels of IR there could be a difference in the DSBs between the cell lines. Looking at DSBs using PFGE on cells treated with less than 1Gy IR may be difficult due to the low level of damage and rapid repair. It is likely that most of the damage would be fixed by NHEJ in the time it took to get cells into plugs and into lysis buffer. The possibility exists that if cells were irradiated in plugs, they could be immediately transferred to lysis buffer to achieve a true zero time point. While there may be some effect on the efficiency of IR in cells irradiated in a plug versus cells irradiated in media, previous data suggest that this will not decrease the efficiency of IR. When live cells were irradiated in agarose, a linear dose response was observed up to 40 Gy and a significant signal as low as 2.5 Gy.[299] This suggests that while one may not be able to compare cells irradiated in media to those irradiated in agarose, there should be no issues with comparing cells irradiated in agarose to each other.

Irradiating cells produces reactive oxygen species (ROS). BCR-ABL is thought to promote the formation of ROS and thus lead to increased damage.[202, 234] There are numerous possibilities for how this may affect DNA damage after IR, including: BCR-ABL-expressing cells produce greater ROS than parental cells; the increase in ROS is greater in parental cells than those expressing BCR-ABL (due to the already existing level of ROS in BCR-ABL-expressing cells); or ROS production is equal in both. If one wanted to examine the effect of the production of ROS in these cells, the assays could be run in the presence of ROS scavengers.

It is also possible to use this assay to compare the rates of repair after IR. While we did not do an in-depth comparison, initial results suggested that DKO and DBA cells undergo repair at similar rates. This result is based on looking at the fraction of damaged DNA over time. We see in all cases that most repair is complete 4 h after IR, and repair is fully complete by 24 h. This suggests that there are no obvious gross changes in repair rate in BCR-ABL-expressing versus non-expressing cells, and if there are any subtle differences, the times examined after IR may need to be more focused. Since most repair is complete by 4 hours, it is likely that if there is any difference in repair rate it would be seen before that time. It would therefore be necessary to examine cells at smaller increments of time, which would be completed over multiple gels due to loading restrictions. These short time points also make it plausible to encapsulate cells in plugs prior to IR, which would also help make the time points far more accurate and precise. This provides the opportunity to precisely compare repair rates to distinguish subtle differences that may effect overall genomic instability.

E. Cell Cycle Effects

It was previously shown that BCR-ABL cells undergo a G2/M arrest after DNA damage, which is required for drug resistance.[269] It has been suggested that the delay in G2/M gives BCR-ABL-expressing cells an opportunity to repair potentially lethal DNA damage rather than undergoing

apoptosis.[262] It is unknown whether this G2/M arrest is a requirement for or a product of the BCR-ABL-mediated inhibition of apoptosis. Interestingly, we found that BCR-ABL-expressing cells that are unable to undergo apoptosis do not have a robust early G2/M arrest but have a trend towards a prolonged G2/M phase over the next several days. This was not a true “G2/M arrest” as cells continued to proliferate, and this was not due to a greater amount of DNA damage in the BCR-ABL-expressing cells compared to control cells, as both display a similar amount of damage after IR. This suggests that BCR-ABL may directly interfere with the G2/M checkpoint although the mechanism of such an effect is unclear. Also the effect is modest and this seems unlikely to explain the pattern of DNA damage repair and the chromosomal aberrations detected in these experiments.

The continued proliferation of DKO and DBA cells after DNA damage in the presence of an increased G2/M phase could reflect an escape of cell cycle checkpoints, or a relative decrease in the length of time the cell spends in the other phases of the cell cycle. Escape of cell cycle checkpoints has been associated with genomic instability and cancer (discussed below). The results of this experiment in DKO and DBA cells do not completely mirror what has been seen in the past in apoptosis-competent cells. Here we see that both DKO and DBA cells have an increase in G2/M 8 hours after IR, and this increases further in DKO cells by 24 hours. After 48 hours, both DKO and DBA have an equivalent G2/M portion. It is unclear if the further increase in DKO cells is meaningful, and if the expression of BCR-ABL prevents this increase. Apoptosis-competent cells showed a much more profound difference between parental and BCR-ABL-expressing cells, with the latter showing a much greater G2/M arrest. This difference could be explained by the difference in the amounts of DNA damage after IR in these different systems (where apoptosis-competent cells have demonstrated an increase in DNA damage in BCR-ABL-expressing cells while DKO and DBA show a similar level of damage). It could also be a cell type-related difference, or it could have a basis in the lack of Bax and Bak. Cells that cannot undergo

apoptosis that experience a level of damage that would normally initiate apoptotic signals may be likely to escape cell cycle checkpoints and survive with damage.

F. IL-3 as a variable in BCR-ABL-mediated effects on DNA damage and apoptosis

The PFGE experiments in BaF3 pTET-ON p210 cells were executed in the presence of IL-3 or doxycycline, but not both, while the DKO and DBA cells were run in the presence of IL-3 in both conditions. This leaves open the possibility that IL-3 could be a critical variable in determining the post-IR DNA damage levels.

While BCR-ABL can transform IL-3-dependent hematopoietic cells lines to cytokine independence[101, 190], and IL-3 is dispensable for BCR-ABL-induced myeloproliferative disease[300], an important question is whether IL-3 has an effects independent of those conferred by BCR-ABL expression.

Growth factors are necessary to provide proliferation signals and inhibit cell-intrinsic apoptosis.[301] Cytokine receptors associate with and activate cytoplasmic tyrosine kinases[302, 303] to turn on downstream signaling pathways. IL-3 activates a number of signaling pathways that stimulate growth and inhibit apoptosis, including the RAS and PI3K pathways.[304-306] Cytokine depletion not only turns off anti-apoptotic signaling, but also actively induces cell death by activating caspases.[306] In this way, IL-3 itself has a protective effect similar to that of BCR-ABL, and it is important to consider whether these effects completely overlap or maintain some independent processes.

Growth factors have also been shown to delay DNA damage-induced death, which could lead to increased clonogenic survival.[307, 308] It has previously been shown that IL-3 protects Ba/F3 cells from DNA damage-induced apoptosis.[309, 310] The PI3K/Akt pathway was found to be

sufficient to protect cells from growth factor starvation-induced apoptosis, but was not required for IL-3 inhibition of DNA damage-induced cell death.[311] Understanding the effects of IL-3 and BCR-ABL in this regard is crucial, as correct evaluation of experimental results depends on it.

The Carroll laboratory has published several observations on the effects of IL-3 in apoptosis-competent cells. We have shown that the combination of IL-3 and BCR-ABL expression has a modest combinatorial effect in protecting cells from apoptosis.[199] We have also studied the effect of IL-3 in combination with BCR-ABL expression on DNA damage after etoposide treatment. Using the comet assay, we determined that IL-3 did not provide further protection from (nor induction of) DNA damage over BCR-ABL expression alone.[217]

Evidence supporting that IL-3 has some independent protective effects compared to BCR-ABL comes from the above fragile site analysis using aphidicolin treatment (Figure 13). We found that after aphidicolin treatment there is an increase in DNA breaks in Ba/F3 pTET-On p210 cells treated with doxycycline compared to those treated with IL-3 alone. However, this effect was “rescued” by IL-3 treatment concomitant to doxycycline. We do not have an explanation for this discrepancy. The fragile site assay measures rare events that are generated during S phase and retained as the cell progresses through G2 and M. It is possible that the IL-3 rescue disproportionately enhances the late repair of fragile site breaks. Additional experiments using cells synchronized in S phase and followed in parallel with analysis by comet assay or PFGE (to assay DNA DSBs) and fragile site analysis would be required to address this discrepancy.

With the apoptosis incompetent DKO and DBA cells, we are unable to explore their response to drugs in an XTT assay. However, other experiments indicate that DBA cells behave the same regardless of the presence or absence of IL-3. This includes the G2/M profile after irradiation (Figure 5a) as well as cell proliferation in the absence of a DNA damaging agent (Figure 3c).

Further, we don't see a protective effect of IL-3 in the case of DBA and DKO cells in the SKY analysis. Though variability is high, results suggest that DBA cells grown with IL-3 have a slightly higher average increase in chromosomal aberrations after IR and repair than those grown without IL-3 (Figure 8). Further, IL-3 does not have a protective effect against the acquisition of baseline chromosomal abnormalities in the absence of IR. This suggests that IL-3, while certainly an important variable, may not be the critical variable in this system. Full analysis of this issue would require examination DBA cells grown both with and without IL-3 after DNA damage using PFGE analysis. This would also be compared to PFGE analysis of Ba/F3 pTET-ON p210 cells grown with IL-3, doxycycline, or both, with or without a pan-caspase inhibitor.

G. Technical Issues

1. PFGE vs. Comet

Here we chose to utilize PFGE to detect DSBs, rather than the comet assay, which we and others have used previously.[217] PFGE has been used to examine DNA DSBs in cells, and is thought to be the gold standard in the field of DNA damage. It provides better resolution of large DNA molecules than standard electrophoresis by applying an alternating voltage gradient to the gel.[312] The alkaline comet assay shows both SSBs and DSBs as a comet tail radiating from the cell after the application of a standard voltage gradient to single cells embedded in agarose plugs, whereas PFGE only allows DNA with DSBs to be mobilized. We wished to confirm that Ba/F3 pTET-ON p210 cells yielded consistent results in the PFGE assay and the comet assay. This was found to be the case, as in both assays cells expressing BCR-ABL had a higher incidence of DSBs after DNA damage, demonstrating that either the damage experienced is primarily DNA DSBs, or that BCR-ABL impacts the generation of both SSBs and DSBs to similar extents. Of note, we also manually scored stained metaphases of DKO and DBA treated with colcemid a few hours after IR and did not see a difference in the overall number of aberrations, including SSBs,

DSBs, tri-radials, quadriradials and rings (Figure 10) consistent with the data presented in Figure 7b. Thus, these several assays of DNA damage show similar results.

In the comet assay, typically only 50-200 cells are scored per treatment condition. PFGE shows the damage induced in the entire pool of cells, which can be 4- to 5-fold greater in number. While the larger sample size in PFGE decreases the chance that the cells selected for analysis in the comet assay are not representative of the entire population, PFGE is only a semi-quantitative assay and care must be taken to ensure accurate results.

In the comet assay one cannot consistently distinguish apoptosis from a “healthy” amount of DNA damage, as the fragmentation of DNA into kilobase-size fragments appears to be an early event in apoptosis, preceding the complete digestion of DNA into multiples of nucleosomal size fragments.[313] Any fragments of DNA are quantified in the comet tail as DNA damage. In PFGE, the low molecular weight band (which we see in Ba/F3 pTET-ON p210 cells after IR) is thought to represent cells undergoing apoptosis. The absence of this band in DKO and DBA cells supports this fact. However, no consistent changes were noted in this low molecular weight band in apoptosis-competent cells after DNA damage. Importantly, we calculated DNA damage both by including the full smear of DNA that migrated out of the well, or by only including the upper area of the smear – that which did not include this apoptotic portion. We found no difference in result when quantifying via either method. In both cases Ba/F3 pTET-ON p210 cells treated with doxycycline to induce BCR-ABL expression had a higher incidence of DSBs after IR than those treated with IL-3.

All PFGE quantifications have been done by comparing the IR lane to the pre-IR lane, which puts a lot of weight on the results of the pre-IR lane. This comparison is done to account for subtle differences in counting cells on the hemacytometer, which may lead to large differences when the

sample of cells is taken. While I am confident that the volume taken from the same flask 15 or 30 minutes apart contains a very similar number of cells, I am not confident that there is an equal number of cells in a sample from a different flask, due to the significant figures that can be achieved when scaling up from what was counted on the hemacytometer.

Alternatively, the ratio of mobile DNA to total DNA in the lane (smear plus well) could be used to normalize each sample so that comparisons can be made between flasks or between cell lines by normalizing each baseline to 1 and then comparing irradiated samples. This would allow comparison of the increase in breaks from baseline to irradiation between samples. However, since all baselines would then be normalized, the absolute value of the different samples cannot be compared, only the relative increases in damage.

This fact makes PFGE a semi-quantitative assay, which makes it difficult to compare between cell lines. However, some predictions can be made regarding what the state of DNA damage would be in the various cell lines compared to each other. These predictions are presented in Table 1. DKO and DBA cells are more resistant to DNA damage as they are unable to undergo apoptosis. It is difficult to determine if this will impact the overall level of DNA damage, and they may in fact have less damage at IR than Ba/F3 cells. The inhibition of apoptosis in Ba/F3 cells using a caspase inhibitor could lead to an increase in basal DNA damage, though the addition of the inhibitor just before irradiation should not create a large impact on the basal DNA damage condition. Ba/F3 cells expressing BCR-ABL in the absence of a caspase inhibitor may have a small increase in basal DNA damage over control, though data regarding this are mixed.

The irradiation for PFGE was conducted on ice, after a 10-15 minute incubation on ice prior to IR. In our gamma cell source, 5 Gy IR takes 10-11 minutes to complete. There is then approximately 5 minutes of travel time to return to the lab, at which point processing begins. Processing occurs

Cell line	Apoptosis	Basal DNA damage	DNA damage immediately after IR
DKO +IL-3	Inhibited	+	++
DBA +IL-3	Inhibited	+	++
DBA -IL-3	Inhibited	+	++
Ba/F3 pTET-ON p210 +IL-3	Competent	-	++
Ba/F3 pTET-ON p210 +Dox	Competent	+?	+++
Ba/F3 pTET-ON p210 +IL-3 +Dox	Competent	+?	+++
Ba/F3 pTET-ON p210 +IL-3 +Q-VD	Inhibited	-?	+++
Ba/F3 pTET-ON p210 +Dox +Q-VD	Inhibited	+?	+++
Ba/F3 pTET-ON p210 +IL-3 +Dox +Q-VD	Inhibited	+?	+++

Table 1: Predicted relative levels of DNA damage before (basal) and immediately after IR.

on ice and takes roughly 15 minutes to complete. While ice slows down the repair process, it is still likely that some repair takes place in this time frame. It is difficult to see a true zero time point, which may be of interest, due to the possibility that BCR-ABL could affect the amount of DNA damage produced, or the kinetics of DNA repair. This true zero would be useful in a number of regards. The best way to achieve this true zero may be to embed cells in agarose plugs prior to irradiation. It would then be possible to immediately lyse cells, eliminating the travel time factor. However, how much protection from IR the surrounding agarose would provide is unknown. All of these factors leave room for continued technical improvement of the detection of DNA DSBSs.

2. Limits of DKO and DBA cells

There are a number of limitations to the DKO and DBA cell lines that need to be considered when interpreting data and planning experiments for their use. First, the cells are infected with retrovirus at a very low rate. Infection of two million cells yielded 10^5 GFP+ cells. This disadvantage is even greater with the large MIG210 plasmid compared to MIGR1. This inability to infect a large number of cells limits what can be done rapidly without selecting for GFP+ cell and expanding the culture, forcing a need for stable cell lines to be produced for most experiments. Some assays, such as the comet assay, use a smaller number of cells that could perhaps be done in a transient setting, but experiments such as PFGE or SKY require a greater number of cells than one infection will provide. We generated subclones of DKO cells infected with MIGR1 and MIG210 by limiting dilution. Subclones were generated by limiting dilution, plating 1/2 cell per well in 96-well plates. Less than one in three wells grew out, as expected based on Poisson statistics, suggesting that wells showing growth are derived from a single cell. A difference in integration sites in the different subclones is presently being confirmed by Southern blot. This will ensure that the effects seen in the cells are due to the expression of BCR-ABL rather than the disruption of a common gene locus.

Despite the fact that DKO and DBA cells do not undergo apoptosis, they are very difficult to thaw from frozen stocks. DKO in particular are very difficult to thaw, though it is not surprising that these same cells expressing BCR-ABL have a slight growth advantage from frozen stock, as BCR-ABL expression leads to dysregulated growth. Often 5 million frozen cells will result in 100-1000 live cells at thaw, suggesting that the population of cells thawed may not be as polyclonal as the initial population that was frozen. This may have impacted the untreated SKY results (as discussed above) but the subcloned lines will be useful in confirming results obtained with the pooled cells.

Growth of healthy DKO and DBA cells is slow, with a doubling time greater than 24 hours. Further, some proteins do not seem to be expressed at high levels in these cells, such as Chk1 (Figure 11). Comparison of proteins between DKO and DBA cells is possible, but the variation from Ba/F3 pTET-ON p210 cells indicates that care must be taken to draw conclusions and all variables must be considered.

3. Limits of SKY Analysis

SKY is an expensive, time-consuming, and incomplete means of looking at genetic alterations. While SKY analysis can be utilized for determining numerical alterations and chromosomal translocations, it is insufficient to provide information about all genetic lesions. Point mutations and microdeletions are among the mutations not detected by SKY. As genomic sequencing becomes more cost and time effective, it will be possible to examine these types of lesions. Microdeletions can be detected by high-resolution chromosome banding, molecular chromosome analysis with FISH or DNA analysis. BCR-ABL point mutations are frequently detected in CML patients, suggesting that these lesions may be important in the transition to CML BC and require further examination.

The system we used in our SKY analysis was fairly static and not as manipulatable as is necessary to draw more solid conclusions (as discussed above regarding the limits of DKO cells). In many instances the effects of BCR-ABL on the repair of DNA damage have been found to be cell type-dependent (as discussed above). Therefore, the use of only one cell line in these experiments does not allow us to make any definitive statements about the lack of a BCR-ABL-mediated impact on genomic instability above that of apoptosis incompetence alone. To be able to say that BCR-ABL's ability to inhibit apoptosis is a major factor in the accumulation of chromosomal aberrancies, a number of additional experiments are necessary.

First, a cell system where pro- and anti-apoptotic factors can be manipulated is required to gain more information. The ability to knock out and then re-express Bax and/or Bak would would create a system whereby we could rule out that other factors in these cells are responsible for the lack of a difference. Alternatively, a system like Ba/F3 pTET-ON p210 cells treated with a pan-caspase inhibitor or not would give another cell line to add credence. The cost of SKY currently limits how many of these experiments can be undertaken but a more robust system may be of use in the future.

H. Additional possibilities for the effects of BCR-ABL on genomic instability

It is still possible that BCR-ABL causes genomic instability through other uninvestigated means, either directly or in concert with the inhibition of apoptosis. For example, BCR-ABL may usurp or disrupt the normal function of c-Abl, though this has not been documented. c-Abl can phosphorylate p73[314], which suppresses tumorigenesis and promotes apoptosis after DNA damage through transcriptional control of DNA repair genes.[315] By disrupting c-Abl function or cellular location, BCR-ABL could inhibit these p73-mediated effects.

The effects of BCR-ABL on the cell cycle have also been investigated.[86, 217, 235, 262] It is interesting to speculate that BCR-ABL could have a direct effect on cell cycle regulators, which could lead to genomic instability. Cyclin D1 forms complexes with its binding partners cyclin-dependent kinase 4 and 6 (CDK4 and CDK6) to promote G1 cell cycle progression. Overexpression of Cyclin D1 has been associated with a number of cancers[316-318], and degradation of Cyclin D1 was found to be necessary to prevent genomic instability following genotoxic insult.[319, 320] While cyclin D1 is not typically found in hematopoietic cells, it was found to be overexpressed in CML AP patient samples.[321] It is possible that BCR-ABL could affect the degradation of Cyclin D1 as has been demonstrated for DNA-PKcs.[225] The inhibition of apoptosis may present a background where cells are able to escape cell cycle checkpoints with DNA damage.

I. Clinical Relevance

Clinically, it is important to consider whether BCR-ABL may have an effect on the inhibition of apoptosis in CML stem cells. Even patients who have undetectable levels of BCR-ABL transcript after treatment with imatinib will relapse if taken off the drug. Therefore, not all BCR-ABL-expressing cells are being eliminated and some are experiencing clonal evolution, as evidenced by patients who lose major molecular response to imatinib due to BCR-ABL mutations. If BCR-ABL is shifting the apoptotic threshold in CML stem cells, that would allow the survival of cells with additional DNA damage.

Targeting CML stem cells with apoptosis-inducing agents in combination with imatinib or a second-generation BCR-ABL inhibitor may be the only means of fully-eradicating BCR-ABL cells, and such combination therapies are already under investigation.[322] This type of therapy is particularly necessary for patients who fail imatinib treatment and progress to blast crisis. Inducing the apoptosis of BCR-ABL-expressing CML stem cells is difficult, but necessary for

elimination of disease.

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