Role of c-Jun NH_2-Terminal Kinase in Bcr/Abl Induced Cell Transformation

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Abstract

The c-Jun NH_2-terminal kinase (JNK) group of kinases include ten members that are created by alternative splicing of transcripts derived from *Jnk1*, *Jnk2* and *Jnk3* genes. The JNK1 and JNK2 protein kinases are ubiquitously expressed while JNK3 is expressed in a limited number of tissues. The JNK signaling pathway is implicated in multiple physiological processes including cell transformation. There is growing evidence that JNK signaling is involved in oncogenesis. Nevertheless, the role that JNK plays in malignant transformation is still unclear. The aim of this thesis is to examine the role of JNK in malignant transformation. For this purpose, I used the Bcr/Abl oncogene as a transforming agent. Bcr/Abl is a leukemogenic oncogene that is created by reciprocal translocation between chromosome 9 and 22. The translocation breakpoint is variable and several different Bcr/Abl isoforms have been identified such as Bcr/Abl^{p185} and Bcr/Abl^{p210}, whose expression is associated with different types of leukemia. Bcr/Abl activates the JNK signaling pathway in hematopoietic cells and increases AP-1 transcription activity. Furthermore, dominant negative approaches demonstrate that inhibition of c-Jun or JNK prevents Bcr/ Abl-induced cell transformation *in vitro*. These data implicate the JNK signaling pathway in Bcr/Abl transformation although the role that JNK might have in this process is unclear. Thus, I examined the importance of JNK signaling in Bcr/Abl-

induced lymphoid or myeloid transformation. For this purpose I compared Bcr/Abl^{p185}- and Bcr/Abl^{p210}- induced transformation of wild-type and JNK1-deficient cells using three approaches: *in vitro*, *in vivo* and *ex vivo*. The results obtained with the *in vitro* approach suggest that both Bcr/Abl^{p185} and Bcr/Abl^{p210} require JNK activity to induce lymphoid transformation. While JNK1-deficiency inhibits Bcr/Abl^{p210} oncogenic potential in lymphoid cells both *in vitro* and *in vivo*, pharmacological inhibition of JNK activity (JNK1 and/or JNK2) blocked Bcr/Abl^{p185} induced malignant proliferation *in vitro*. The differential requirement for JNK observed in the two Bcr/Abl isoforms can be ascribed to the presence in Bcr/Abl^{p210} of the Dbl domain which can activate the JNK pathway *in vitro*. In the case of Bcr/Abl^{p210}, JNK1 is critical for the survival of the *ex vivo* derived transformed lymphoblasts upon growth factor removal. This result correlates with the fact that mice reconstituted with Bcr/Abl^{p210} transformed *Jnk1-/-* bone marrow showed normal malignant lymphoid expansion in the bone marrow yet they had reduced numbers of lymphoblast in the bloodstream and lacked peripheral organ infiltration. Thus JNK1 is essential for the survival of the transformed lymphoblast outside the bone marrow microenvironment in Bcr/Abl^{p210} induced lymphoid leukemia. Interestingly, while JNK1 is essential for lymphoid transformation, it is dispensable for the proliferation of transformed myeloblasts.

Taken together these results indicate that the JNK signaling pathway plays an essential role in the survival of Bcr/Abl^{p210} lymphoblasts and that JNK-deficiency decreases the leukomogenic potential of Bcr/Abl^{p210} *in vivo*. Thus, cell survival mediated by JNK may contribute to the pathogenesis of proliferative diseases.

Table of Contents

CHAPTER IV

List of Figures

List of Abbreviations

Abl	Abelson Leukemia viral oncogene homolog
ALL	Acute lymphoid leukemia
AP-1	Activator protein 1
Arg	Abl-related gene
ATM	Ataxia telangectasia-mutated kinase
Bad	BCL2 antagonist of cell death
Bak	Bcl2 antagonist killer
Bap-1	Bcr associated protein
Bax	Bcl2 associated X protein
Bcl2	B-cell lymphoma 2
$BclX_L$	Bcl2-related protein, Long isoform
BclW	Bcl2 homolog W
Bcr	Breakpoint cluster region
BH	Bcl2 homology domain
Bim	Bcl2 interacting mediator
Bmf	Bcl2 modifying factor
c-IAP2	Inhibitor of apoptosis 2

CML	Chronic myelogenous leukemia
CNL	Chronic neutrophilic leukemia
CrkL	Crk Likeprotein
DNA-PK	DNA-dependent protein kinase
Erk	Extracellular signal regulated kinase
FKHR-L1	Forkhead in rhabdomyosarcoma transcription factor
Gab2	Grb2-associated binding protein 2
GAP	GTP-ase activating proteins
GCKR	Germinal center kinase-related
GEF	Guanine-nucleotide exchange factor
GF	Growth factor
GM-CSF	Granulocyte-macrophage colony-stimulating factor,
Grb2	Growth factor receptor-bound protein 2
GTP	Guanosine Triphosphate
Hck	Hemopoietic cell kinase
IFN-α	Interferon alfa
IL-3	Interleukin 3
IR	Ionizing radiation
JAK	Janus kinase
Jip	JNK interacting protein

JNK	c-Jun NH2 terminal kinase
MAPK	Mitogen Activated Protein Kinase
MAPKK	Mitogen Activated Protein Kinase Kinase
MAPKKK	Mitogen Activated Protein Kinase Kinase Kinase
Mcl-1	Myeloid cell leukemia 1
MEF	Mouse embryonic fibroblast
MKK	MAPK Kinase
NES	Nuclear export signal
NF-κB	Nuclear factor kappa-B
NGF	Nerve growth factor
NLS	Nuclear localization signal
OSM	Oncostatin M
PAG/MSP23	Proliferation-associated gene
Ph	Philadelphia chromosome
PI-3K	Phosphoinositide 3-kinases
PTK	Protein tyrosine kinase
Rb	Retinoblastoma protein
SAPK	Stress Activated Protein Kinase
Ser	Serine
SH	Src homology domain

SOS	Son of sevenless
STAT	Signal transducers and activators of a transcription
Thr	Threonine
TNFα	Tumor necrosis factor α
Tyr	Tyrosine
UV	Ultraviolet light

Nomenclature

In this thesis I have used the following nomenclature:

Mouse genes are denoted in italics and first letter is capitalized (e.g. *Jnk1*).

Human genes are in italics and all in upper case (e.g. *JNK1*).

Proteins are denoted in regular font and the first letter is capitalized.

CHAPTER I

INTRODUCTION

The JNK/SAPK signal transduction pathway modulates the cell response to external cues and has many biological actions including cell survival, apoptosis and tumorigenesis. The aim of this thesis is to investigate the role of JNK in cell transformation.

I.I. JNK

The c-Jun N-terminal kinase (JNK), also known as stress-activated protein kinase (SAPK), is a family of mitogen activated protein kinases (MAPKs). As the name indicates, this group of MAPK is typically activated upon exposure of cells to environmental stresses (UV, IR and genotoxic stress) and inflammatory cytokines. This section gives an overview of the JNK/SAPK pathway and its role in diverse cellular processes.

I.I.A. The Signaling Pathway.

Like all MAPK pathways, JNK signaling includes a central "three-tiered" signaling module (Fig. 1A) in which the MAPKs are activated by phosphorylation on specific threonine (Thr) and tyrosine (Tyr) residues within a conserved Thr-

Pro-Tyr motif located in the activation loop of their kinase domain. This phosphorylation is carried out by a dual specificity (Ser/Thr and Tyr) kinase known as MAPK kinase (MAPKK). These MAPKKs are, in turn, activated by phosphorylation at serine (Ser)/threonine (Thr) residues located in their kinase domain by upstream MAPKK kinases (MAPKKK). Some insight into the biological role of the JNK pathway came with the characterization of the components of this signaling module (reviewed in Davis, 2000; Kyriakis and Avruch, 2001). At the MAPK level, the JNK protein kinases are encoded by three genes: *Jnk1*, *Jnk2* and *Jnk3* (Fig. 1B). While *Jnk1* and *Jnk2* are ubiquitously expressed, *Jnk3* transcripts are found exclusively in heart, brain and testis. In addition, each transcript can undergo two different types of alternative splicing (Gupta et al., 1996). The first type of alternative splicing is common to all three *Jnk* genes and involves the 3' coding region; this processing creates a 46 kD and a 55 kD isoform. The second type of alternative splicing is observed only in *Jnk1* and *Jnk2* transcripts; this second alternative splicing occurs between two exons within the kinase domain. The functional consequence of these splicing choices is the generation of ten different isoforms of JNK. Although a difference in substrate binding *in vitro* has been reported (Gupta et al., 1996), no dramatic distinctions among JNK isoforms are observed *in vivo.* Despite the functional complementation between the different *Jnk* genes, gene disruption studies revealed that differential tissue distribution of the different isoforms might account

for tissue-specific defects. Thus, mice deficient in JNK1 or JNK2 have severe defects in the differentiation of CD4 T helper cells into effector cells and this defect may correlate with the pattern of JNK expression in murine T cells (Dong et al., 2000; Dong et al., 1998; Yang et al., 1998).

The activity of all JNK isoforms is regulated by two competing mechanisms: JNK can be inactivated by protein phosphatases (including serine/threonine-, tyrosine- and dual specificity protein phosphatases) (Keyse, 2000) and activated by upstream MAPKK.

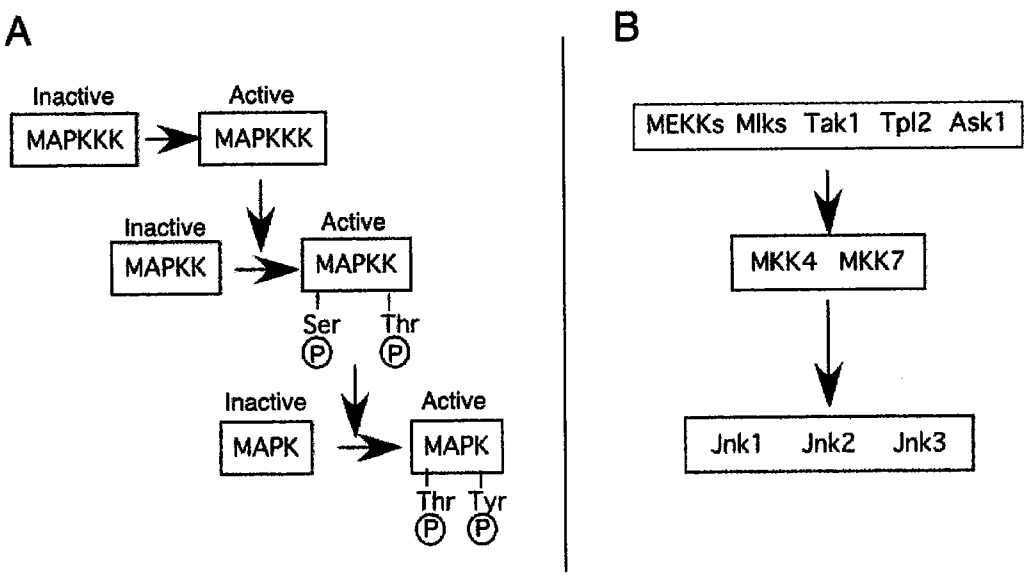


Fig.1 The MAPK signal transduction pathway. (A) The three tiered signalling module. (B)Components of the Jnk signaling pathways.

There are two characterized MAPKK that phosphorylate all JNK isoforms: MKK4 and MKK7 (Fig. 1B). While MKK4 is also involved in the activation of the p38 pathway in response to certain stimuli, MKK7 is a JNK-specific activator. Genetic studies on MAPKK null cells revealed that MKK4 and MKK7 display

different activation patterns depending on the extracellular stimuli (Tournier et al., 2001; Tournier et al., 1997; Tournier et al., 1999; Yang et al., 1997a). Despite this partial overlap in the activation pattern there is evidence that both of these MAPKKs cooperate in the phosphorylation of JNK. Thus, MKK4 and MKK7 preferentially target JNK at Tyr and Thr respectively (Lawler et al., 1998). This observation suggests a synergistic activation of JNK by these two MAPKKs.

The stress activated MAPKKs are regulated by Ser/Thr phosphorylation within a conserved region of the activation loop. This phosphorylation is carried out by a heterogeneous family of MAPKKKs (Fig. 1B) (Davis, 2000; Kyriakis and Avruch, 2001). There are several broad families of MAPKKKs responsible for the activation of the JNK signaling pathway: the MEK kinases (MEKK1-4), the mixed lineage kinases (MLK1-4, DLK and LZK), TGF-β-activated kinase-1 (Tak1), Tumor progression locus-2 (Tpl2) and the apoptosis stimulating kinases (ASK1-2). Many of these MAPKKK were identified as JNK activators using transfection assays (Fanger et al., 1997) but their role as physiological JNK activators *in vivo* and their selectivity for the JNK pathway are still unclear. Some insight into the *in vivo* function of some MAPKKKs comes from gene disruption studies (Davis, 2000; Fanger et al., 1997; Weston and Davis, 2002). Nevertheless, the interpretation of these studies is particularly complex due to the inherent redundancy among the different MAPKKKs and due to their promiscuity of function. An additional level of complexity is determined by the upstream

signaling that activates MAPKKKs. In fact, the MAPKKK receive cellular inputs from a highly diverse group of upstream kinases such as Ste20 homologs (PAK, GCK, GLK and HPK) (Fanger et al., 1997) and the Rho family of GTPases (Rac1 and Cdc42) (Coso et al., 1995; Minden et al., 1995). Thus, the important role of MAPKKK is the integration of these multiple cellular inputs into different MAPK signaling modules.

Given the large number of signaling components in the JNK pathway (especially at the MAPKKK level) a key issue for a stressed cell is the achievement of rapid signal specificity. A clever solution is the organization of the signaling components in discrete clusters through their interactions with scaffolding proteins. Among the four groups of scaffolding proteins that provide selective coordination of the JNK signaling pathway (Weston and Davis, 2002), the best characterized is Jip (JNK interacting protein). The Jip family is composed of three members (Jip-1 –3), which differentially bind to specific components of the JNK signaling module. In transfection studies, Jips facilitate JNK activation stimulated by Mlk. This indicates that one functional role of these scaffolds is the rapid and specific activation of the JNK pathway (Whitmarsh and Davis, 1998). In contrast, overexpression of Jip1 alone can inhibit JNK activation by extracellular stimuli (Dickens et al., 1997). This inhibition is probably due to the disruption of the normal stochiometry of Jip and its effectors. In this situation, no single scaffold is associated with all the necessary components of the

signaling cascade and the overexpressed Jip becomes a JNK inhibitor. Interestingly, both Jip1 and Jip2 are able to bind to kinesin light chain in addition to their functions as scaffolds for JNK signaling modules (Verhey et al., 2001; Whitmarsh et al., 2001). This interaction may contribute to the subcellular localization of JNK signaling components. Thus, Jip proteins may increase the efficacy of the cell response by increasing the local kinase concentrations at specific subcellular locations.

Because the activation of the JNK signaling module is critical for multiple cellular responses, including stress-induced apoptosis, cell survival and oncogenic transformation, the identification of potential JNK targets is essential. The current list of potential targets includes transcription factors, tumor suppressors and apoptosis regulating factors. The first characterized JNK target is the c-Jun transcription factor, which provided a link between JNK signaling and gene transcription (Kyriakis et al., 1994; Minden et al., 1994). JNK induced transcriptional regulation is particularly important for cell survival and oncogenic transformation, both of which usually rely on active transcription. The family of transcription factors regulated by JNK phosphorylation has grown substantially over the years and includes members of the ATF, Jun, NFAT, Myc and Ets families. These factors are differentially regulated by JNK phosphorylation. The first regulatory mechanism is the transcriptional activation of these factors by JNK mediated phosphorylation of specific Ser/Thr residues present in their

activation domains (Whitmarsh and Davis, 1996). Besides this common regulatory mechanism the phosphorylation by JNK can also affect protein stability. JNK phosphorylation of c-Jun increases its half-life, perhaps by inhibiting its ubiquitin-mediated degradation, and thus effects its accumulation in the cell (Musti et al., 1997). In addition, JNK phosphorylation can inhibit transcriptional activity of NFAT4 by inducing its nuclear exclusion (Chow et al., 1997). Hence, JNK can regulate gene expression through different mechanisms: by phosphorylating transcription factors, by affecting their stability or by affecting their cellular localization.

Additional JNK targets are regulatory proteins involved in the apoptotic stress response. Among these targets is the p53 tumor suppressor whose expression levels are controlled by JNK phosphorylation. This regulation is at both the transcription level (Schreiber et al., 1999) and at the protein stability level (Fuchs et al., 1998) where JNK regulates p53 ubiquitin mediated degradation. Other potential JNK targets are the anti-apoptotic proteins such as Mcl-1 and Bcl2. Despite the fact that both Mcl-1 and Bcl2 are JNK substrates *in vitro* (Maundrell et al., 1997; Yamamoto et al., 1999) the relevance of this phosphorylation *in vivo* is still controversial. In the case of Mcl-1, JNK mediated phosphorylation is thought to inactivate Mcl-1 anti-apoptotic function (Inoshita et al., 2002). In contrast, the biological effect of JNK phosphorylation of Bcl2 is unclear. Conflicting reports have shown that JNK mediated phosphorylation of

the same residues in Bcl2 can have opposing effects in modulating the apoptotic response (Breitschopf et al., 2000; Ito et al., 1997). Thus, further studies are needed to establish if Bcl2 is a physiological substrate of JNK.

Recently, novel JNK targets were identified among the BH3-only proteins (Donovan et al., 2002; Lei and Davis, 2003). The pro-apoptotic activity of the BH3-only proteins Bad, Bim and Bmf is a result of JNK phosphorylation. In the case of Bim and Bmf, JNK phosphorylation releases the proteins from the dynein and myosin motor complexes. Once free, the factors are able to associate with other members of the Bcl2 family such as Bax and Bak to trigger the stress-induced apoptotic response (Lei and Davis, 2003).

The identification of additional targets will be critical for our understanding of how JNK activity affects specific biological responses. Furthermore, the recent characterization of a JNK inhibitory molecule (SP600125) will provide a useful tool for the biochemical dissection of this signaling pathway. SP600125 is a drug that potently inhibits JNK activity reversibly, in cultured cells (Bennett et al., 2001). Since JNK may play a key role in different diseases such as cancer and inflammation, this new inhibitor may represent a potential therapeutic agent in the treatment of these pathological conditions.

I.I.B. Life or Death, a Stressful Choice.

Since the JNK pathway is activated mainly by stress, it is reasonable to think that this activation has a role in the decision a damaged cell makes between the choices of life versus death. Indeed there is evidence that JNK can mediate both apoptosis and survival, which is important not only in response to stressful conditions but also during development and tissue differentiation.

(1) Apoptosis

The involvement of JNK in the regulation of the apoptotic response was first observed in neuronal cells. Withdrawal of nerve growth factor (NGF) or other nutrients from differentiated neurons induces apoptosis and this response requires activation of JNK and p38 with concomitant suppression of the Erk survival pathway (Xia et al., 1995). Accordingly, constitutive activation of the JNK pathway (by overexpression of the upstream MAPKKK, MEKK1) induces apoptosis in neurons even in the presence of NGF. Conversely, expression of dominant negative mutants of c-Jun prevents MEKK1 induced apoptosis suggesting that this apoptotic response relies on JNK induced transcription. A possible transcriptional target for JNK induced neuronal cell death is the pro-apoptotic protein Fas-ligand (Fas-L). Indeed Fas-L expression is upregulated in neurons upon NGF withdrawal in a JNK dependent fashion (Faris et al., 1998; Kasibhatla et al., 1998; Le-Niculescu et al., 1999). Thus, a possible model for

stress-induced apoptosis in neuronal cells consists of JNK mediated up-regulation of Fas-L with the newly expressed Fas-L binding to Fas-receptor and triggering the apoptotic response. The involvement of JNK in neuronal cell death is confirmed in studies conducted in animals with targeted disruption of the *Jnk3* gene. These mice are defective in kainate-induced neuronal apoptosis suggesting a role of JNK in brain response to stress by excitotoxins (Yang et al., 1997b).

JNK contribution to neuronal apoptosis is not limited to the stress response. Analysis of mice with combined ablation of JNK1 and JNK2 revealed a potential role of JNK regulation of apoptosis during brain development. The disruption of both *Jnk1* and *Jnk2* causes embryonic lethality with prominent hindbrain exencephaly (Kuan et al., 1999; Sabapathy et al., 1999). Careful analysis of these embryos reveals opposite apoptotic defects in different brain regions. In the lateral edges of the converging hindbrain there is a substantial reduction of apoptotic degeneration, which accounts for the defect in cephalic neurulation. Thus, in the hindbrain JNK1 and JNK2 are essential for developmental apoptosis. In contrast, the forebrain regions of *Jnk1-/- Jnk2-/-* double knock out mice show a dramatic increase in TUNEL positive cells, which are indicative of apoptosis. Thus, JNK1 and JNK2 suppress cell death in the forebrain region of the brain. These contrasting observations highlight a critical issue in the analysis of JNK mediated signaling: the activation of the JNK

pathway may cause opposite biological outcomes depending on cellular context (see later).

Some insight into the mechanism underlining JNK induced apoptosis comes from the biochemical analysis of *Jnk1-/- Jnk2-/-* mouse embryonic fibroblasts (MEF). These cells are defective in apoptosis induced by specific types of stress such as UV and anisomycin (Tournier et al., 2000). Subsequent analysis showed that UV irradiated JNK null MEFs fail to induce mitochondria membrane depolarization and cytochrome c release. These results suggest a role of JNK in the regulation of the mitochondrial response to stress. Furthermore, JNK mediated cytochrome c release requires the pro-apoptotic factors Bax and Bak (Lei et al., 2002). Recently, it is been proposed that JNK induces activation of Bax/Bak through the BH3-only proteins (Donovan et al., 2002; Lei and Davis, 2003). Despite the identification of these new potential JNK targets the mechanism that accounts for Bax/Bak activation and of the mitochondrial apoptotic pathway is still unclear.

(2) Survival signaling

Although many studies clearly establish the role of the JNK pathway in cell death, there is growing evidence that JNK can contribute to cell survival. The strongest evidence supporting a role for JNK in cell survival comes from the analysis of *Jnk1-/- Jnk2-/-* embryos. As mentioned in the previous section, these

embryos exhibit increased apoptosis in the forebrain suggesting that JNK provides a necessary survival signal in this specific brain region. Additional evidence in support of JNK survival signaling derives from the characterization of the TNFα response in *Jnk1-/- Jnk2-/-* MEFs. These JNK null cells are more sensitive than their wild-type counterparts to TNFα induced apoptosis (Lamb et al., 2003). JNK survival signaling in response to TNFα treatment is dependent on gene expression and it is mediated by the transcription factor JunD. A potential target for JunD-dependent transcription in JNK survival signaling is the inhibitor of caspases c-IAP2. Since TNFα–induced c-IAP2 expression is also dependent on NF-κB activation the authors propose a model where the survival signal in TNFα treated cells is triggered by the coordinated actions of JNK/JunD and NF-κB. Thus, the presence of activated NF-κB translates TNFα-induced JNK activation into a survival signal.

The importance of cellular context in JNK mediated survival signaling is evident also in several antisense studies (Bost et al., 1999; Potapova et al., 2000a; Potapova et al., 2000b). Here down-regulation of JNK2 expression results in growth arrest and apoptosis only in tumor cell lines with mutated p53. Thus in the absence of a functional p53 these tumor cells rely on JNK signaling to promote survival.

A striking question that arises from all these studies is how JNK activation can mediate opposite biological events such as apoptosis and cell survival. There are several mechanisms that can explain this apparent paradox, none of which are mutually exclusive:

First, the biological outcome of JNK activation may be dependent on cellular context. In this case, the integration of multiple activated/suppressed signaling pathways determines the choice between life and death. Two examples of this mechanism are: the combined effects of the activation of JNK and suppression of Erk pathway in NGF withdrawal induced apoptosis (Xia et al., 1995) and the integration of JNK and NF-κB pathways in TNFα–induced survival signaling (Lamb et al., 2003). In addition to this pathway integration, the cell status may be important in the interpretation of a specific signal. For example, a change in the p53 status of a cell may alter the outcome of the same JNK mediated signal (Potapova et al., 2000b).

A second interesting mechanism is based on the correlation with the time course of JNK activation for a specific biological response. In particular, sustained activation, but not transient activation, of JNK is associated with apoptosis (Chen et al., 1996). Consistent with this model is the observation TNFα induces only transient activation of JNK and this correlates with JNK induced survival signaling.

A third possible mechanism is that different responses are mediated by different JNK isoforms. Thus, heterogeneous tissue distribution of these isoforms may account for the cell specific response to the same stimulus. In fact, despite all JNK isoforms are largely interchangeable, the disruption of individual *Jnk* genes gives rise to distinctive phenotypes (Conze et al., 2002; Dong et al., 1998; Yang et al., 1998). These observations support the hypothesis that JNK isoforms are heterogeneously expressed (either temporally or spatially) in different tissues and that this distribution can determine the biological response of the cells to a particular stimulus.

I.I.C. JNK and Cell Transformation

The process that leads to oncogenic transformation of normal cells involves the alteration of important biological responses such as apoptosis and cell survival. In fact the inhibition of apoptosis and the deregulation of growth control are common mechanisms that allow oncogenes to transform cells (see section I.II.D). Since JNK can regulate both apoptosis and cell survival it is not surprising that multiple studies have implicated JNK signaling pathway and its effectors in tumorigenesis. The first evidence of a role for JNK in cell transformation came from studies conducted in *c-jun* deficient MEFs. The transcription factor c-Jun is the best characterized effector of the JNK pathway. c-Jun deficient MEFs exhibit several phenotypes and are strikingly resistant to

transformation by the Ras oncogene (Johnson et al., 1996). Similar results were obtained using "knock-in" mice expressing mutated c-Jun in which the JNK phosphorylation sites (Ser63/73) are replaced by alanine residues (cJunAA). cJunAA MEFs derived from these mice can be transformed by activated Ras *in vitro* but are less tumorigenic in xenograft assays than wild type MEFs (Behrens et al., 2000). This observation suggests that active phosphorylation of c-Jun by JNK is required for efficient Ras transformation. Recently this hypothesis was challenged by the observation that JNK-deficiency increases Ras oncogenic potential. Thus, Ras transformed JNK null MEFs efficiently induced sub-cutaneous and lung tumors when injected into nude mice (Kennedy et al., 2003). In addition, JNK-deficiency increased the number and size of lung tumors *in vivo* suggesting that JNK suppresses Ras oncogenic potential. The discrepancy between the different Ras studies could be due to different cell context that might influence the outcome of JNK signaling (see previous section).

Additional evidence suggests a role for the JNK pathway in Bcr/Abl induced cell transformation. The Bcr/Abl oncogene constitutively activates JNK and this activation has been proposed to be required for pre-B cell transformation. In support of this hypothesis is the observation that expression of dominant negative mutants of c-Jun efficiently blocks Bcr/Abl transformation *in vitro* (Raitano et al., 1995). In accordance with this result, inhibition of JNK signaling by overexpression of Jip1 abolishes Bcr/Abl induced pre-B cell

outgrowth *in vitro* (Dickens et al., 1997). Both of these studies provide indirect evidence that JNK signaling pathway may contribute to malignant transformation by Bcr/Abl therefore this oncogene is a suitable candidate to study the role of JNK in cell transformation.

I.II. Bcr/Abl

I.II.A. The oncogene

The Bcr/Abl fusion protein is one of the first oncogenes found to be associated with a specific malignant disease in humans (Nowell and Hungerford, 1960). This oncogene is generated in hematopoietic stem cells by reciprocal translocation between chromosome 9 and chromosome 22 (t (9; 22)(q34; q11) (Rowley, 1973). This reciprocal translocation gives rise to an abnormal, shortened chromosome 22 known as the Philadelphia chromosome (Ph). The Philadelphia translocation is present in 90% of patients affected by Chronic Myelogenous Leukemia (CML)(Pasternak et al., 1998). The incidence of CML is approximately 5 cases per 100,000 individuals per year worldwide and it is slightly higher in males than in females. CML is a biphasic disease and it is usually diagnosed in the initial "chronic" phase during which granulocytes increase in number 10- to 100- fold in the blood (reviewed in Sawyers, 1999). Despite this leukocytosis, normal hematopoiesis still coexists with the leukemic clone. The chronic phase lasts typically 2-6 years and suddenly transforms into a more aggressive phase usually described as "acute" or "blast crisis". At this stage, immature cells appear and normal hematopoiesis is overcome by the malignant clone. Blast crisis patients exhibit additional chromosomal abnormalities such as trisomies 8 and 19 and isochromosome 17. These rearrangements have prognostic relevance in the identification of the shift to the

more aggressive phase. The median survival of patients in this stage is only about 1.5 years.

Two genes are engaged in the creation of the Bcr/Abl fusion protein: *ABL* on chromosome 22 and *BCR* on chromosome 9. Insights on the transformation events leading to the development of CML come from the characterization of these genes.

The *BCR* (breakpoint cluster region) gene encodes for a 160kD cytoplasmic protein that is ubiquitously expressed. The physiological role of the Bcr protein is not yet clear. *Bcr* knock-out mice do not have any obvious external phenotype and show normal hematopoiesis and viability. However, when challenged with endotoxin (Voncken et al., 1995) these mice develop severe septic shock attributed to an increased neutrophilic oxidative burst. The absence of a clear hematopoietic phenotype suggests an inherited redundancy in the cell-signaling pathway.

The translocation partner for Bcr in the Philadelphia chromosome is the *ABL* gene. This gene encodes a non-receptor tyrosine kinase, which shares considerable homology with the *v-Abl* (Abelson murine leukemia virus) oncogene. As is the case with the *Bcr* gene, the function of ABL kinase is poorly understood. Some insight comes from knock-out studies where the *Abl* gene was either truncated or completely ablated (Schwartzberg et al., 1991; Tybulewicz et al., 1991). In both cases the *Abl-/-* mice showed reduced survival

and only some exhibited a 10- 30-fold reduction in the number of B- and T- cells. It is unclear whether the observed lymphopenia is a direct effect of the disruption of the *Abl* gene or a secondary effect due to an increase in corticosteroids levels. Further analysis of Abl null mice has also revealed a defect in osteoblast maturation (Li et al., 2000). The osteopororotic phenotype displays a reduction in the rate of bone deposition and is present in approximately 50% of the *Abl-/-* homozygote mice (Li et al., 2000). The incomplete penetrance of all *Abl-/-* phenotypes could be ascribed to the presence of a compensatory kinase with redundant function. One potential candidate that could compensate Abl function *in vivo* is the *Abl*-related gene (Arg) kinase (Kruh et al., 1986). Both Arg and Abl kinases exhibit extensive conservation in sequence and architecture. Despite this high degree of identity, gene disruption studies suggest that these tyrosine kinases have different functions *in vivo.* In fact, *Arg* deficient mice are born normal and do not exhibit the defects observed in *Abl* null mice (Koleske et al., 1998) suggesting that Arg cannot compensate for Abl deficiency. In addition, the combined disruption of *Abl* and *Arg* genes causes embryonic death associated with defects in neural tube closure (Koleske et al., 1998) indicating that Arg function is necessary to sustain the development of *Abl*-deficient mice.

In vitro studies that describe the Abl kinase as a cell cycle regulator are controversial. Some studies have shown that a fraction of the Abl nuclear pool is associated with the retinoblastoma protein (Rb) in cells in G1 (Wang, 1993;

Welch and Wang, 1993). This binding inhibits the Abl kinase activity during this phase. Upon phosphorylation of the Rb protein by cyclinD-cdk4/6 the Abl kinase is released and consequently activated in S phase. This activation is thought to contribute to the stimulation of the S-phase genes through phosphorylation of the C-terminal domain of RNA polymerase II. Thus, this study envisions the Abl kinase as a growth-promoting factor. An opposite role for the Abl kinase is described in transfection studies (Van Etten, 1999) where Abl induces G1 growth arrest and subsequent apoptosis. This function of the Abl kinase is dependant on its SH2 domain and its association with p53 and Rb.

The Abl kinase is also involved in the DNA damage response (Van Etten, 1999; Wang, 2000). Ionizing radiation and genotoxic agents such as mitomycin C activate the kinase activity of nuclear c-Abl. This activation can occur either by direct phosphorylation of Abl by the ataxia telangectasia-mutated (ATM) kinase (Baskaran et al., 1997; Shafman et al., 1997) or indirectly by free radical –induced dissociation of the Abl kinase from its inhibitor Pag/MSP23 (Wen and Van Etten, 1997). Upon activation c-Abl phosphorylates multiple substrates including DNA-PK (Kharbanda et al., 1997), Rad 51 (Yuan et al., 1998), SHPTP1 (Kharbanda et al., 1996). Although these interactions suggest a role of the Abl kinase in the regulation of the DNA double-strand break repair, such a role is not evident in *Abl-/-* fibroblasts. These cells do not display defects in the DNA repair pathways or in the regulation of the G1-S checkpoint and are slightly more

radioresistant than their *Abl+/+* counterparts (Yuan et al., 1997). It is possible that failure to detect these defects is due to the intrinsic redundancy of the pathway. Recent studies on *Abl* deficient mice (Gong et al., 1999) and chick cells (Takao et al., 2000) support an alternative role for c-Abl kinase in the DNA damage response. Both studies provide evidence for a role of c-Abl tyrosine kinase in the activation of apoptosis by DNA damage. One mechanism underlying c-Abl induced cell death is through the regulation of p73 expression (Gong et al., 1999). p73 belongs to the family of p53 transcription factors and is thought to have a role in the regulation of apoptosis (Levrero et al., 1999). Indeed, ectopic expression of p73 (like p53) can induce apoptosis (Levrero et al., 1999) indicating that p73 protein level is critical for the induction of apoptosis. Interestingly, the accumulation of p73 following exposure to genotoxic stress is c-Abl dependant (Gong et al., 1999). This accumulation is due to an increase in the half-life of the p73 protein (Gong et al., 1999) and is perhaps mediated by direct phosphorylation of p73 by the c-Abl kinase (Agami et al., 1999).

The Philadelphia translocation between *BCR* and *ABL* gives rise to two hybrid genes: Bcr/Abl on chromosome 22 and Abl/Bcr on chromosome 9q+. Only the Bcr/Abl fusion protein is shown to be necessary and sufficient for malignant transformation *in vitro* as well as *in vivo* (Daley and Baltimore, 1988; Daley et al., 1990; Heisterkamp et al., 1990; Kelliher et al., 1990) while the possible role of the Abl/Bcr remains elusive.

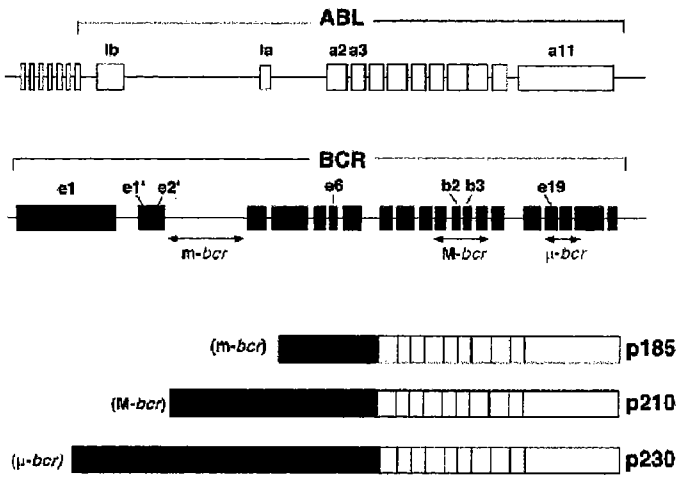


Fig.2 Genomic structure of the *ABL* and *BCR* gene (top). The Bcr/Abl isoforms generated by translocation involving the three breakpoint regions in the *BCR* gene (bottom).(Modified from Melo (1996)).

Depending on the breakpoint position in the two chromosomes different Bcr/Abl isoforms are created (Fig. 2). The breakpoint in the *ABL* gene typically occurs within intronic sequences spanning a 300Kb segment at the 5' end of the gene. This segment includes the two alternative first exons of the *ABL* gene. Thus, the fusion protein may contain exons Ib and Ia, exon Ia alone or neither one. Interestingly, the Bcr/Abl transcript never includes *ABL* exon I despite the genomic arrangement of the fusion gene. All described Bcr/Abl mRNAs consist of *BCR* sequences fused to *ABL* exon a2 (Melo, 1996). While the *ABL* contribution to the fusion oncogene is usually invariable, the translocation of different portions of the *BCR* gene determines the formation of Bcr/Abl isoforms. There are three characterized breakpoint regions in the *BCR* gene: the major (*M-bcr*), the minor (*m-bcr*) and the micro (*μ-bcr*) breakpoint cluster (Fig. 2). Each one of these breakpoint clusters and its related Bcr/Abl isoform is associated with a specific type of leukemia. Breakage within the M-*bcr* generates an 8.5-kb transcript that encodes for a 210 kD (Bcr/Abl^{p210}) fusion protein which is found in 95% of patients with Chronic

Myelogenous Leukemia (CML) and approximately one third of patients with Acute Lymphoid Leukemia (ALL). The majority of ALL patients and rare cases of CML patients express a smaller 185kD Bcr/Abl isoform (Bcr/Abl^{p185}) as a result of breakage in the m-*bcr* region. In 1990, Saglio et al. described a 230kD Bcr/Abl isoform (Bcr/Abl^{p230}) found in patients with Chronic Neutrophilic Leukemia (CNL) (Saglio et al., 1990). The breakpoint responsible for the creation of the largest Bcr/Abl isoform is the μ-*bcr* located at the 3' end of the *BCR* gene.

In addition to these well-characterized isoforms other "unusual" transcripts are observed in which the chromosomal breakpoint occurs either in introns outside the known *bcr* breakpoint clusters or within *BCR* exons or downstream the *ABL* exon a2. Interestingly, many of these atypical Bcr/Abl fusion genes can be detected by RT-PCR in leukocytes of 69% of normal individuals (Bose et al., 1998) indicating that the translocation is not, in itself, sufficient for malignant transformation.

I.II.B. Structure of the Bcr/Abl fusion protein

Both the Bcr and the Abl proteins have multiple functional domains that contribute to different transforming events induced by Bcr/Abl. These aspects include the resistance to apoptosis, growth factor independence and alterations of cell-cell and cell-matrix interactions. In addition, since Bcr/Abl isoforms differ only in the Bcr portion of the fusion oncogene, the structural characterization of

the different Bcr domains may be helpful in our understanding of the oncogenic potential of these isoforms.

(1) BCR

The *BCR* gene encodes for 160 kD cytoplasmic protein that contains several distinct domains (Fig. 3). The N-terminal region is particularly important since it is the only Bcr peptide that is retained in all three Bcr/Abl isoforms (Bcr/Abl^{p185}, Bcr/Abl^{p210} and Bcr/Abl^{p230}). This 426aa region includes: a coiled coil domain (or oligomerization domain), a serine-threonine kinase domain containing an important Tyrosine residue (Tyr177) and a Src homology 2 (SH2)-binding region.

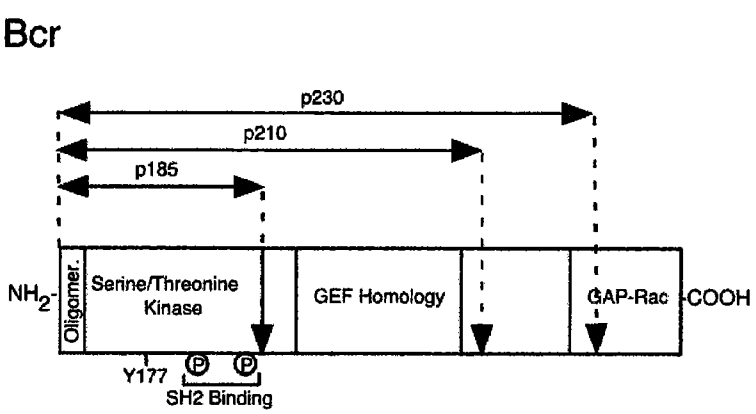


Fig.3 Domain structure of Bcr. Arrows indicate the Bcr portions comprised in the different Bcr/Abl isoforms.
(Modified from Barnes and Melo (2002)).

The coiled coil region (Fig. 3) consists of a heptad repeat typical of coiled coil amphiphatic alpha elices, which suggests a role in the oligomerization of the Bcr protein. Further studies established that both the Bcr protein and the Bcr/Abl fusions form oligomers *in vivo* (McWhirter et al., 1993). In the case of Bcr, the

crystal structure revealed the formation of a homotetramer (Zhao et al., 2002). Based on these findings the authors propose a model for the activation of the Abl kinase in Bcr/Abl based on the tetramerization of the fusion protein. The N-terminal coiled coil domain is both essential and sufficient to activate the oncogenic potential of Abl by inducing CML-like disease in mice (Zhang et al., 2001a). Deletion of the coiled coil region (ΔCC) impairs the ability of Bcr/Abl to induce myeloid leukemia *in vivo*. Interestingly, the concomitant deletion of the Abl SH3 (ΔSH3) domain rescues the ability to induce transformation in transplanted mice. This result suggests that the coiled coil region alleviates the inhibitory effect of the Abl SH3 domain.

The serine -threonine kinase (Fig. 3) domain has been shown to autophosphorylate itself as well as Bap-1 (Bcr associated protein 1) a member of the 14-3-3 family of proteins (Reuther et al., 1994). Since 14-3-3 proteins are important in the regulation of the cell cycle in yeast the authors speculate that the association and consequent phosphorylation of Bap-1 in Bcr/Abl cells may be relevant as a mitogenic signal provided by this oncogene in transformed cells (Reuther et al., 1994).

In addition, the serine/threonine kinase domain contains a very important tyrosine residue (Tyr 177) (Fig. 3). This residue is key to the activation of the Ras signaling pathway by Bcr/Abl. Upon phosphorylation, this residue binds to Grb2 adapter molecule through its SH2 domain. The SH3 domain of Grb2 binds

to proline-rich motifs on the guanine nucleotide releasing factor son-of-sevenless (Sos), which activates Ras by stimulating GTP binding. Activation of Ras leads to the activation of the mitogen-activated protein kinase (MAPK) and other signaling pathways (see later). Mutation of this single residue in Bcr/Abl impairs its ability to induce a myeloproliferative disease in mice although it still retains the ability to induce lymphoid leukemia (Million and Van Etten, 2000). The residual oncogenic activity observed is perhaps due to the ability of the Tyr177F mutant to still activate the Ras pathway through the activation of either Shc (Goga et al., 1995) or CrkL (Nosaka et al., 1999b; Senechal et al., 1996). Recently, a novel regulatory mechanism for Bcr/Abl was demonstrated which involved the phosphorylation of Grb2 on Tyr209 (Li et al., 2001a). This modification directly inhibits the binding of Sos by the Grb2 C-terminal SH3 domain. Expression of mutated Grb2 (Y209F) enhances the transformation ability of Bcr/Abl *in vitro* suggesting that the phosphorylation of Grb2 at this site exerts a negative regulatory role in Bcr/Abl -induced transformation.

Finally, the SH2-binding region (Fig. 3) within the N-terminal region of Bcr consists of two areas that are particularly rich in serine and threonine residues. Interestingly, the phosphorylated Ser/Thr residues become unusual binding partners with SH2 domains of other proteins, one of which is the Abl portion of Bcr/Abl. An intriguing hypothesis is that intra- or inter- molecular binding between these two regions might lead to the deregulation of Bcr/Abl tyrosine

kinase activity. Supporting this model are deletion studies showing that the Ser/Thr regions in the SH2-binding domain of Bcr are essential for Bcr/Abl induced transformation of rat fibroblasts (Pendergast et al., 1991).

The central region (Fig.3) of the Bcr protein is present in the two largest Bcr/Abl isoforms. This area shares considerable homology with the guanine-nucleotide exchange factor (GEF) for human CDC42: the Dbl proto-oncogene. These factors activate guanine nucleotide binding proteins by stimulating the exchange of guanosine triphosphate (GTP) for guanosine diphosphate (GDP). Domain swap analysis showed that the Dbl domain is important in inducing growth-factor independence *in vitro* (Kin et al., 2001) but the exact role of this domain is yet to be discovered.

(2) ABL

Unlike the case for Bcr, the Abl portion is constant in all Bcr/Abl isoforms (Fig. 4). The translocation breakpoint excludes the N-terminal cap that is encoded by two alternative exons (Ia and Ib). Beyond the breakpoint, the N-terminal region included in the oncogene unfolds different Src homology domains: SH1 containing the Abl tyrosine kinase, SH2 and SH3 which are non-catalytic and function as binding sites for many other signaling proteins (see later). Bcr/Abl is a constitutively active tyrosine kinase and its activity is necessary yet not sufficient for its transforming ability. In c-Abl, negative

regulation of the tyrosine kinase is achieved through its SH3 domain (Fig. 4). There are two possible regulatory mechanisms involving the SH3 domain that lead to the inhibition of c-Abl kinase activity. The first mechanism is based on the interaction of c-Abl SH3 domain with a cellular inhibitor. Several candidate inhibitors (such as Abi-1, Abi-2 and Pag/MSP23) that bind to c-Abl SH3 domain have been identified (Van Etten, 1999) but their physiological role as regulators of c-Abl kinase activity is still unclear. The second mechanism envisions the formation of an intramolecular interaction involving the SH3 domain and the linker region between the SH2 and the catalytic domain (Pluk et al., 2002). Interestingly, the N-terminal cap of c-Abl encoded by two alternative exons (Ia or Ib) is thought to be critical for this intramolecular regulation (Pluk et al., 2002). Thus, the deletion of this N-terminal cap in the Bcr/Abl translocation could contribute to the deregulation of its tyrosine kinase activity.

In addition, deletion of the SH3 domain or its substitution with *gag* viral sequence (as in the case of v-Abl) activates c-Abl transforming ability *in vivo* (Van Etten, 1999). In contrast, Bcr/Abl ΔSH3 mutants either have a milder form of the disease (Skorski et al., 1998) or develop a CML-like leukemia with a slight delay (Gross et al., 1999b). These results suggest that the presence of SH3 domain enhances (rather than inhibits) the oncogenic potential of Bcr/Abl. A possible role for the Abl SH3 domain in the Bcr/Abl fusion is to regulate the adhesion and motility of the leukemic cells. Cells transformed with Bcr/Abl ΔSH3

have a reduced ability to adhere to stromal layers and to invade bone marrow and spleen (Skorski et al., 1998). The impaired adhesion and invasion is ascribed to defective cell-cell and cell-matrix interactions rather than to reduced survival of the transformed cells.

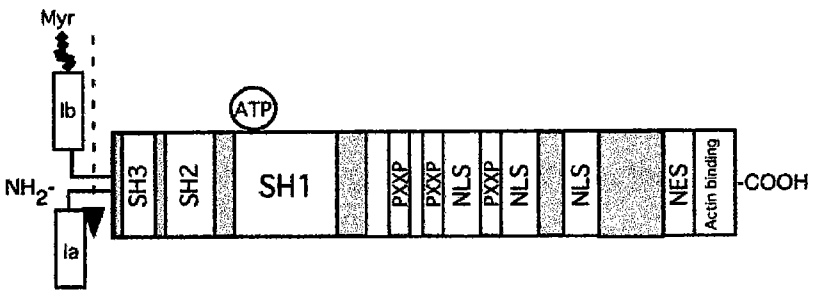


Fig.4 Domain structure of Abl. Arrow indicate the Abl portion comprised in all Bcr/Abl isoforms.
(Modified from Barnes and Melo (2002)).

Adjacent to the SH3 domain lies another important regulatory region: the SH2 domain (Fig. 4). The role of this domain in Bcr/Abl induced cell transformation is rather controversial. Initial studies using a SH2 deletion mutant showed a reduction in the extent of myeloid infiltration in the peripheral organs of transplanted mice (Skorski et al., 1997). At a molecular level, the Bcr/AblΔSH2 mutant seemed to be impaired in the activation of the PI3-Kinase and of the downstream serine/threonine kinase Akt. In accordance, coexpression of an Akt constitutively active mutant restores the ability of the SH2 deletion mutant to induce leukemia *in vivo*. Subsequent studies (Roumiantsev et al., 2001; Zhang et al., 2001b) confirmed the defect of the Bcr/Abl SH2 mutant to induce CML-like disease but observed normal induction of the PI-3K /Akt pathway with this mutant. The authors ascribe the

decrease efficiency of induction of CML disease to a dramatic reduction in the intrinsic tyrosine kinase activity of Bcr/Abl proteins. This controversy highlighted one of the fundamental challenges in Bcr/Abl research: the requirement for specific domains for Bcr/Abl-induced transformation is typically cell type- or context- dependent. Up to now it is not clear which experimental model system should be used in order to evaluate Bcr/Abl domain function and have observations relevant to the development of CML in humans.

The C-terminal region of Abl is characterized by three nuclear localization signals (NLS) as well as one nuclear-export signal (NES) (Fig. 4). In the case of c-Abl, the balance of the opposing effects of these two regions creates a cellular gradient of the Abl kinase with a higher concentration in the nucleus. This gradient can be disrupted by the use of nuclear-export inhibitory drugs such as leptomycin B (Vigneri and Wang, 2001). Despite the presence of the same NLS and NES regions in all Bcr/Abl fusions, the cellular distribution of the oncoprotein is strictly cytoplasmic. Nevertheless, inhibition of Bcr/Abl tyrosine kinase activity in the presence of leptomycin B induces Bcr/Abl nuclear import. Once trapped in the nucleus the release of kinase inhibition induces an immediate apoptotic response (Vigneri and Wang, 2001). This may explain the tight cytoplasmic localization of the oncogene. For this purpose, the C-terminus Actin-binding domain (Fig. 4) provides an important cytoplasmic anchoring domain. This domain can bind to both monomeric and filamentous actin and it is thought that

the Abl kinase can influence and be influenced by local changes of the cytoskeleton structure. The relationship between abl kinase activity and cytoskeleton is underlined by the observation that the Abl cytoplasmic pool relocates into focal adhesion once cells become exposed to fibronectin (Van Etten, 1999). The disruption of this integrin signaling is one of the many affected pathways in the Bcr/Abl fusion oncogene.

I.II.C. Activated signaling pathway by Bcr/Abl

The functional consequence of the fusion of the Bcr sequence to Abl is the conversion to a constitutive active tyrosine kinase and its localization to the cytoplasm. Here, the new deregulated kinase is exposed to a variety of new potential substrates and activates different signaling pathways which contribute to the cell transformation (Fig. 5). This section provides an overview of the signaling pathways activated in Bcr/Abl transformed cells.

Despite the identification of these newly activated signaling pathways it is difficult to link any specific signaling event to a specific biological effect due to the redundancy of many of the activated pathways. Nevertheless, the understanding of the molecular processes that stem from the deregulation of Bcr/Abl is important for future development of a therapeutic strategy.

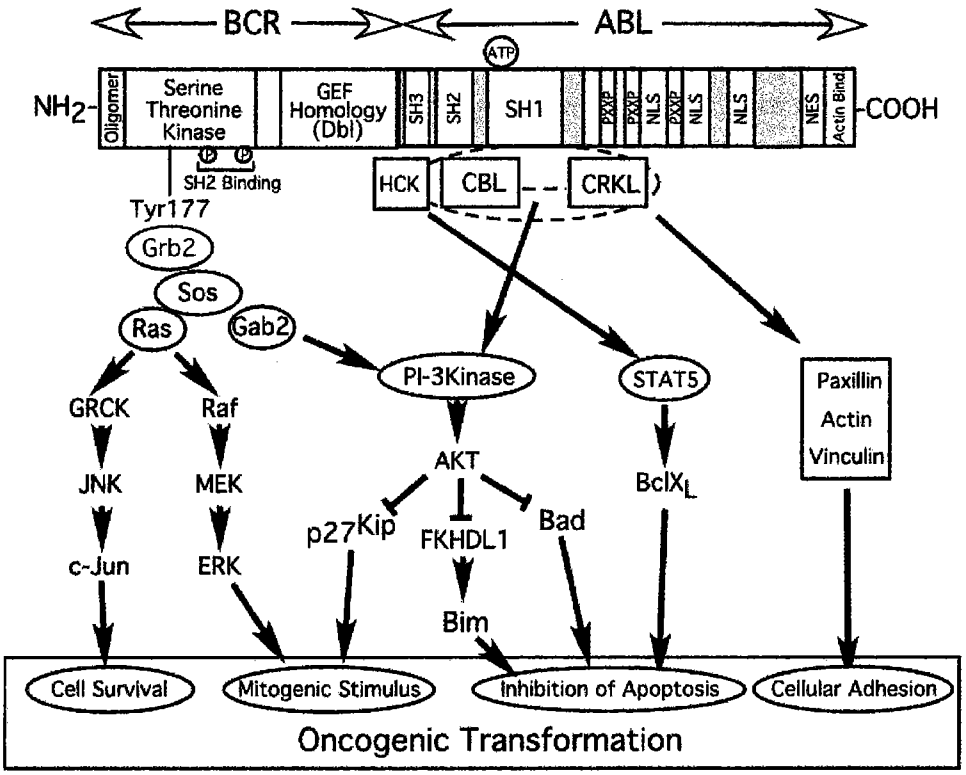


Fig.5 Bcr/Abl activated signalling pathways.

(1) Ras

Ras signaling is one of the first deregulated pathways found in Bcr/Abl transformed cells. The key to the identification of Ras activation was the finding of the Grb2/Sos association at Tyr177 on Bcr/Abl (Fig 5)(Pendergast et al., 1993). The formation of this complex stimulates the conversion of the inactive GDP-bound form of Ras to the active GTP-bound state. Later studies established that Bcr/Abl-induced activation of Ras also could be achieved

through two other adapter molecules: SHC and CrkL (Goga et al., 1995; Nosaka et al., 1999b; Senechal et al., 1996). Both these adapters are known Bcr/Abl substrates and associate with the SH2 (Shc) and SH3 (CrkL) domains respectively. Interestingly, the CRKL adapter protein is one of the most prominent tyrosine phosphorylated Bcr/Abl substrates found in CML patients (Oda et al., 1994; ten Hoeve et al., 1994). This observation suggests that CrkL plays a critical role in Bcr/Abl induced transformation (de Jong et al., 1995; Heaney et al., 1997; Hemmeryckx et al., 2001; Kardinal et al., 2000; Kolibaba et al., 1999; Oda et al., 1994; Senechal et al., 1996; Tari et al., 1997; ten Hoeve et al., 1994). In fact, inhibition of complexes mediated by CrkL SH3 domain can block proliferation of CML cell lines (Kardinal et al., 2000). In contrast, transgenic expression of CrkL accelerates the onset of Bcr/Abl^{p185} induced disease (Hemmeryckx et al., 2001). Despite the critical role of CrkL in Bcr/Abl signaling, CrkL is not required for Bcr/Abl^{p185} induced leukomogenesis (Hemmeryckx et al., 2002). Since Bcr/Abl activates Ras through multiple pathways, it is possible that these redundant pathways can compensate for CrkL deficiency.

Two different MAPK are activated through Ras in Bcr/Abl transformed cells: the extracellular signal-related kinase (Erk) and the Jun NH_2-terminal kinase (JNK). Activation of Erk is mediated by Raf activation of the serine/threonine kinases Mek1/Mek2 (Fig. 5) while in the case of JNK the signal is transduced by the germinal center kinase –related (GCKR) (Shi et al., 2000;

Shi et al., 1999). The functional importance of Ras in Bcr/Abl induced cell transformation was demonstrated by dominant negative studies (Sawyers et al., 1995). Coexpression of Bcr/Abl with either GAP or dominant negative Ras hinders its oncogenic potential while coexpression of c-H-Ras accelerates its transforming ability (Sawyers et al., 1995). Ras has a central role in mediating a growth-stimulatory signal in different human leukemias. Deregulation of the Ras pathway through specific activating mutations is observed with high frequency in patients with acute myeloid leukemia (AML) or Ph-negative CML (which thereby lack the Bcr/Abl fusion protein) while Ras mutations are noticeably absent in Ph+ CML patients (Cogswell et al., 1989; Watzinger et al., 1994). This apparent paradox could be explained by the fact that in Bcr/Abl expressing leukemias the Ras pathway is constitutively activated, consequently further activating mutations may not be required.

(2) JNK

Bcr/Abl transformed hematopoietic cells show increased JNK activity and substantial activation of jun-responsive promoters (Raitano et al., 1995). Interestingly, among the MAPK pathways Bcr/Abl preferentially activates JNK and this activation is Ras-dependent. In fact, use of dominant negative Ras mutants block Bcr/Abl induced JNK activation (Shi et al., 1999). The link connecting Bcr/Abl, Ras and JNK is thought to be the Germinal Center Kinase

Related (GCKR) (Fig. 5). GCKR kinase associates and is activated by Bcr/Abl in a Ras-dependent manner. Inhibition of the GCRK activity by either a dominant negative mutant or by antisense blocks Bcr/Abl induced activation of JNK (Shi et al., 1999). These results identify GRCK kinase as the transducer of Bcr/Abl signaling to JNK. The main downstream effector of JNK is the c-Jun transcription factor. JNK phosphorylation of c-Jun at Ser63 and Ser73 leads to the increase of *jun*-responsive gene transcription including the *c-jun* gene itself. c-Jun-mediated transcription is essential for Bcr/Abl cell transformation. Expression of dominant negative mutants of c-Jun can inhibit Bcr/Abl oncogenic potential *in vitro* (Raitano et al., 1995). Interestingly, substantial JNK activity and subsequent Ap-1-dependent gene transcription is observed in blast cells derived from patients with acute myeloid leukemia (Burgess et al., 1998). In addition, there is an intriguing correlation between constitutive JNK activity and patients that show either relapse or secondary leukemia whereas young patients with *de novo* acute leukemia lack JNK activity and fail to express c-Jun protein (Burgess et al., 1998; Cripe et al., 2002). Very high levels of JNK activity are especially found in acute myeloid leukemia patients that are refractory to chemotherapy. This observation supports the hypothesis that JNK might induce resistance to chemical agents by c-jun-dependent transcriptional upregulation of multidrug resistance genes such as *MDR1* (Cripe et al., 2002). These findings are fundamental for the

development of therapeutic strategies in AML patients that fail conventional treatment.

(3) Jak/Stat

Many studies have reported the phosphorylation of the signal transducer and activator of transcription (STAT) family in CML cell lines. The STAT transcription factors are important mediators of cytokine and growth factor signaling. Activation of these transcription factors is achieved through tyrosine phosphorylation and is usually mediated by the Janus kinases (JAKs). STATs are activated in Bcr/Abl expressing cell lines. In these lines there is substantial phosphorylation of STAT5A and STAT5B and modest activation of STAT1, STAT3 and STAT6 (Ilaria and Van Etten, 1996; Nieborowska-Skorska et al., 1999; Shuai et al., 1996). Interestingly, the phosphorylation of STAT6 is detected in cell lines transformed with either v-Abl or Bcr/Abl^{p185} and not with Bcr/Abl^{p210} (Ilaria and Van Etten, 1996). Since both v-Abl and Bcr/Abl^{p185} induce primarily lymphoid leukemias, STAT6 phosphorylation might be a hallmark for lymphoid transformation.

Although JAK2 is activated in Bcr/Abl expressing cell lines, the activation of STAT5 is JAK-independent (Carlesso et al., 1996; Chai et al., 1997; Ilaria and Van Etten, 1996; Xie et al., 2001). Several studies identified the involvement of Hck as the tyrosine kinase that couples Bcr/Abl to STAT5 activation in myeloid

cells (Fig. 5) (Klejman et al., 2002; Lionberger et al., 2000). Hck is recruited to the SH2 and SH3 domains of Bcr/Abl, becomes activated and directly phosphorylate STAT5 on Tyr699 (Klejman et al., 2002). Inhibition of STAT5 activation by expression of a truncation mutant lacking the transcriptional activation domain (ΔSTAT5) reduces cell viability in Bcr/Abl$^+$ cell lines (Gesbert and Griffin, 2000). The reduced viability is due to increased apoptosis rather than cell cycle blockage. On the other hand, expression of a constitutively active STAT5 mutant (STAT51*6) results in growth factor-independent proliferation and enhanced viability. STAT5-induced cell survival is mediated by transcriptional upregulation of anti-apoptotic $BclX_L$. Again, dominant negative ΔSTAT5 mutant reduces $BclX_L$ levels in Bcr/Abl$^+$ cell lines (Horita et al., 2000) while STAT51*6 upregulates them.

The importance of STAT5-mediated cell survival in Bcr/Abl transformation depicted by these *in vitro* studies has been challenged by recent work carried out in STAT5A/B-deficient mice (Sexl et al., 2000). Results from this study show that Bcr/Abl can induce myeloproliferative disease in STAT5A/B- deficient mice. This indicates that STAT5A/B signaling is not essential or necessary for Bcr/Abl-induced transformation. In addition, the authors found no expression of STAT5-dependent genes in cell lines derived from tumors from STAT5A/B-deficient mice indicating that no redundant pathway is compensating for the absence of STAT5 signaling.

(4) PI3 Kinase

PI3 Kinases (PI3K) are pleiotropic regulators of many biological function including apoptosis, proliferation and integrin activation. Bcr/Abl constitutively activates PI-3 Kinase through multiple pathways. The first identified pathway involved the formation of a multimeric complex with the regulatory subunit of the PI3K (p85), p120Cbl, Crk and CrkL (Fig. 5) (Sattler et al., 1996). The association of the regulatory p85 subunit with the activated tyrosine kinase activates the p110 catalytic subunit of the PI3-kinase and its downstream effectors. Recently, a second model was described in which Bcr/Abl induced PI3K activation through the recruitment of the scaffolding adaptor Gab2 (Sattler et al., 2002). The recruitment is mediated by the formation of a Grb2/Gab2 complex that associates with Bcr/Abl at Tyr177. Since Tyr177 is known to be important in the activation of Ras signaling pathway (see previous section), the authors speculate that Gab2 could be responsible for the regulation of the Ras/Erk pathway.

The downstream effector that transduces PI3K-signal in Bcr/Abl transformed cells is the serine/threonine kinase Akt (Fig.5). PI3K-mediated activation of Akt is essential for Bcr/Abl-induced leukomogenesis: a dominant negative Akt mutant or the PI3K inhibitory drug wortmannin both inhibit Bcr/Abl-dependent transformation *in vitro* and *in vivo* (Skorski et al., 1997). In CML cells, activated Akt has multiple downstream signaling targets, which contribute to different aspects of cell transformation. Among these targets there is a key cell

cycle regulator $p27^{Kip1}$. $p27^{Kip1}$ is an inhibitor of the cell cycle kinase cdk2. Binding of $p27^{Kip1}$ to the cdk2/cyclinE complex prevents the phosphorylation of effectors (such as Rb) essential for entry in S phase. In Bcr/Abl expressing cell lines, Akt-mediated phosphorylation of $p27^{Kip1}$ induces its downregulation perhaps through proteasome–mediated degradation (Gesbert et al., 2000). The resulting low levels of $p27^{Kip1}$ lead to an accelerated entry to the S phase suggesting that the PI-3K/Akt pathway mediates one of Bcr/Abl mitogenic signals.

Another key substrate of the PI-3K/Akt pathway is the pro-apoptotic protein Bad. Bad induces apoptosis by sequestering Bcl2 and $BclX_L$ anti-apoptotic factors. Bad phosphorylation by Akt induces its association with cytoplasmic 14-3-3 proteins, which prevents the formation of the inactivating complex with Bcl2 and $BclX_L$. Bad phosphorylation by PI-3K/Akt is an important survival signal in Bcr/Abl transformed cells (Neshat et al., 2000). Interestingly, other pro-apototic factors such as Bim are downregulated by PI-3K/Akt pathway in CML cell lines. In this case, the regulation is thought to be mediated by Akt-mediated repression of FKHR-L1 transcriptional activity (Komatsu et al., 2002).

Taken together these studies provide evidence that the PI-3K/Akt pathway mediates many aspects of Bcr/Abl induced cell transformation including inhibition of apoptosis and mitogenic stimulation of the transformed cells.

I.II.D. Mechanisms of transformation

Several lines of evidence indicate that tumorigenesis is a multistep process. A normal cell undergoes essential alterations of important cellular functions that collectively lead to malignant transformation. Like many other oncogenes, Bcr/Abl accelerates the rate of accumulation of these physiological alterations by deregulating multiple cellular processes through the activation of multiple signaling pathways. Each signaling pathway activated by Bcr/Abl contributes to one or more of these cellular alterations. These important cellular alterations include: growth factor independence, inhibition of apoptosis, mitogenic stimulus, altered adhesion and tissue invasion, limitless replicative potential and genome instability.

(1) Growth factor independence

Proliferation and differentiation of hematopoietic cells is tightly regulated by several growth factors (GF) and cytokines such as interleukin 3, oncostatin M and GM-CSF. These factors activate specific signaling pathways through their association with cell surface receptors. While normal cells depend on the activation of these pathways for their survival, Bcr/Abl transformed cells can survive in the absence of these factors. In transformed cells different strategies can be used to achieve growth factor independence including the autocrine

production of the growth factors and the aberrant activation of cytokine signal transduction pathways.

Many cancer cells acquire the ability to produce the growth factors to which they are responsive. This self-supporting production creates a positive feedback signaling loop often described as autocrine stimulation. Bcr/Abl expressing myeloid cell lines synthesize granulocyte-macrophage colony-stimulating factor (GM-CSF) and/or interleukin 3 (IL-3). The conditioned media from these cell lines can sustain growth of non-transformed parental cell lines (Anderson and Mladenovic, 1996). The presence of an autocrine loop in Bcr/Abl transformed cells is also found *in vivo*. Leukemic cells derived from Bcr/Abl transplanted mice produce excess IL-3 and GM-CSF, this autocrine loop contributes to CML progression in these animals (Zhang and Ren, 1998). Although autocrine loops clearly contribute to Bcr/Abl transformation they are not required for the establishment of GF independence. In fact, the two pathways leading to GF independence or to the establishment of autocrine loops can be uncoupled in cells transformed by a Bcr/Abl mutant lacking the SH2 domain (Bcr/AblΔSH2). Thus, expression of Bcr/AblΔSH2 mutant in a factor-dependent myeloid cell line induces growth factor independent proliferation but prevents the production of IL-3 and GM-CSF and conditioned media from Bcr/AblΔSH2 lines fail to sustain growth of the parental cell line (Anderson and Mladenovic, 1996). In agreement with these observations, the use of neutralizing antibodies against

IL-3 or GM-CSF in Bcr/Abl transformed cells does not affect GF independence. Taken together these results suggest that GF independence and establishment of an autocrine loop are two uncoupled pathways and both of these alterations contribute to Bcr/Abl cell transformation.

Another strategy to develop autonomous growth is to deregulate cytokine signaling pathways in order to bypass the requirement of ligand-receptor interaction. In leukemic cells the most affected cytokine signaling pathway is JAK/STAT. As described in the previous section, Bcr/Abl constitutively activates STAT5 and this activation is JAK independent. Interestingly, STAT5 activation in CML lines is not a consequence of IL-3 or GM-CSF autocrine loop but is directly correlated to Bcr/Abl tyrosine kinase activity (Chai et al., 1997). A possible mechanism by which STAT5 mediates malignant proliferation is through the transcriptional regulation of different genes such as *oncostatin M* and *pim-1*. Oncostatin M (OSM) is a cytokine that is critical for the development of multipotential hematopoietic progenitors (Grenier et al., 1999). Mice reconstituted with bone marrow expressing OSM develop myeloproliferative disease indicating that STAT5 mediated expression of this cytokine might contribute to malignant myeloid expansion (Schwaller et al., 2000). Pim-1 is a serine/threonine kinase that is upregulated in Bcr/Abl expressing cell lines (Amson et al., 1989; Nosaka et al., 1999a) and whose expression is STAT5 dependent (Nieborowska-Skorska et al., 2002). In addition, exogenous

expression of Pim-1 in hematopoietic cell lines allows GF autonomous proliferation (Nosaka and Kitamura, 2002). Recently, a Bcr/Abl mutant (Bcr/Abl ΔSH2SH3) that does not activate STAT5 was shown to be defective in Pim-1 upregulation. This Bcr/Abl mutant has impaired leukemogenic capacity, which cannot be restored by overexpression Pim-1 alone but required coexpression of multiple STAT5 anti-apoptotic targets (Nieborowska-Skorska et al., 2002). In addition, expression of a dominant negative mutant of Pim-1 does not affect Bcr/Abl induced growth factor independent proliferation (Nosaka and Kitamura, 2002). Taken together these results suggest that Pim-1 might play a critical role but it is dispensable for Bcr/Abl induced transformation.

(2) Inhibition of apoptosis

The ability of the Bcr/Abl oncogene to inhibit apoptosis is thought to be important for survival of CML differentiated granulocytes and for the induction of clonal expansion in the initial phase of the disease (Bedi et al., 1994). Although the role of Bcr/Abl as an inhibitor of apoptosis has been studied intensively, the mechanisms by which this inhibition is accomplished are still controversial. The majority of the proposed mechanisms involve the regulation of Bcl-2 family members and pathways that lie upstream of procaspase-3 activation (Dubrez et al., 1998). There are three main protagonists in Bcr/Abl anti-apoptotic events that have been reported: Bad, Bcl-2 and $BclX_L$.

Bcl-2 is the founder of a large family of apoptotic regulators. Both Bcl2 and $Bcl-X_L$ carry out their anti-apoptotic function by acting at the level of the mitochondrial membrane. The expression of the anti-apoptotic factor Bcl-2 is up-regulated in Bcr/Abl expressing cells (Cirinna et al., 2000; Sanchez-Garcia and Martin-Zanca, 1997). This induction is mediated by the combined action of the PI3K/Akt (Skorski et al., 1997) and the Ras pathways (Sanchez-Garcia and Martin-Zanca, 1997). Suppression of either Bcl-2 expression or Ras activation abolishes Bcr/Abl anti-apoptotic properties and blocks tumorigenicity *in vivo* (Cortez et al., 1996; Sanchez-Garcia and Martin-Zanca, 1997). On the other hand, Bcl-2 overexpression restores the transforming potential of a mutated Bcr/Abl defective in transformation (Cirinna et al., 2000). The mechanism by which Bcr/Abl induced overexpression of Bcl-2 prevents apoptosis is still poorly understood. One hypothesis is based on the observation that Bcl-2 might associate with the serine/threonine kinase Raf-1 localizing it to the mitochondrial membrane (Wang et al., 1994). Here, Raf-1 is able to negatively regulate the pro-apoptotic factor Bad by phosphorylation of an important serine residue (see later).

Bad is a pro-apoptotic member of the Bcl-2 family which lacks the mitochondrial insertion signal and therefore is mainly localized in the cytoplasm (reviewed in (Gross et al., 1999a). In normal cells, Bad is found in a phosphorylated inactive form usually associated with cytoplasmic 14-3-3

proteins. In response to an apoptotic stimulus, Bad becomes unphosphorylated, it dissociates from 14-3-3 and translocates to the mitochondria. Here, active Bad interacts with membrane bound anti-apoptotic factors Bcl-2 and $BclX_L$. This association interferes with the anti-apoptotic properties of these two proteins and triggers cell death. Different studies suggest that Bcr/Abl exerts its anti-apoptotic activity in part by modulating different steps of Bad-induced death pathway. One level of regulation is achieved by activating the kinases responsible for Bad phosphorylation. Constitutively phosphorylated Bad is unable to translocate to the mitochondria and thus induce apoptosis under stress conditions. Akt and Raf-1 are likely candidates to accomplish Bad inactivation and indeed both of these kinases are constitutively activated in Bcr/Abl transformed cells (Neshat et al., 2000; Skorski et al., 1997). A second strategy to block Bad-induced apoptosis is to inhibit the phosphatase activity of PP-1α thus blocking Bad dephosphorylation (Salomoni et al., 2000). An interesting observation is that Bcr/Abl transformed cells are more resistant to death induced by a mutated form of Bad in which the regulatory serines are replaced by alanines (Salomoni et al., 2000). This particular mutant is resistant to phosphorylation-dependent inactivation. A proposed mechanism envisions Bcr/Abl quenching Bad apoptotic activity through the overexpression of the anti-apoptotic factors Bcl-2 and $BclX_L$ (Salomoni et al., 2000).

As research in the Bcr/Abl field advances additional mechanisms for the anti-apoptotic activity of this oncogene have been identified. Recently Gelfanov and coworkers showed a cooperative response between the Ras and the PI-3K/Akt pathway in Bcr/Abl cells leading to the activation of p65NF-κB and the transcriptional upregulation of c-IAP2 (Gelfanov et al., 2001). This clearly indicates that other mechanisms are likely to participate in this important aspect of Bcr/Abl oncogenic activity.

(3) Mitogenic stimulus

The ability to activate mitogenic signaling pathways is one of the important steps that allow transformed cells to expand rapidly. During the chronic phase, CML cells are still able to differentiate normally but they exhibit increased proliferative capacity since they undergo extra cycles of cell division as compared to normal myeloid cells. This cycling ability confers a growth advantage to the transformed cells allowing the initial expansion of the malignant clone (Clarkson and Strife, 1993). The subsequent accumulation of secondary mutations contributes to the increased proliferation rate of CML cells observed in blast phase. Thus, Bcr/Abl might increase the cellular proliferation in two ways: by accumulating mutations in important components of the cell cycle machinery; or direct activation of mitogenic signaling pathways that drive cell cycle progression. While mutations of cell cycle regulators are frequently found in blast crisis CML

patients, in chronic phase CML these mutations are uncommon. Thus, in this phase, the ability of Bcr/Abl to activate mitogenic signaling pathways is particularly important. This mitogenic ability is evident in experiments where inducible expression of Bcr/Abl stimulated cell cycle re-entry of starved normal myeloid cells (Cortez et al., 1997). In these cells the G1-S transition stimulated by Bcr/Abl is associated with the activation of specific components of the cell cycle machinery including Cdk2 (Cortez et al., 1997) and cyclin D2 (Cortez et al., 1997; Jena et al., 2002). In accordance with these observations, deficiency in cyclin D2 inhibits Bcr/Abl-induced cell proliferation *in vitro* and transformed cells are arrested predominantly in G1 phase (Jena et al., 2002). Interestingly, the block in Bcr/Abl-induced cell cycle proliferation is observed in bone marrow derived from either hemizygous *cyclin D2+/-* or homozygous *cyclin D2-/-*. This result might suggest that a threshold amount of cyclin D2 is required in order to induce G1-S progression in CML cells.

Bcr/Abl induces G1-S transition also through the downregulation of cdk inhibitors such as $p27^{Kip1}$. Regulation of $p27^{Kip1}$ is achieved through the activation of the PI3K/Akt pathway (see previous section) and is mediated by proteasome-dependant degradation (Gesbert et al., 2000).

Besides increasing malignant proliferation, Bcr/Abl induced deregulation of the cell cycle machinery also serves a different function in transformed cells.

Recent reports have shown that Bcr/Abl induces a prolonged G2/M arrest following irradiation (Nishii et al., 1996). This delay allows transformed cells to recover from genotoxic stress and prevents mitotic catastrophe. Thus Bcr/Abl induced growth arrest observed under these conditions may serve an unusual anti-apoptotic function.

(4) Altered Cellular Adhesion

In CML, Bcr/Abl$^+$ progenitor cells have selective growth advantage over normal progenitors cells. This advantage determines the abnormal clonal expansion that is observed in the initial chronic phase of the disease. Interestingly, at this initial stage CML progenitors lack responsiveness to signals from the bone marrow microenvironment. This effect is correlated to the failure of CML progenitors to adhere to bone marrow stromal layer and to the extracellular matrix (reviewed by Salesse and Verfaillie, 2002). In normal progenitors, adherence to the stromal layer is believed to be essential for the regulation of hematopoiesis since it brings the anchored progenitors to close proximity of cytokine producing cells. The pluripotent progenitors will determine their fate depending on the type of extracellular signals they receive from the neighboring cells. In CML cells, failure to adhere would allow the cells to escape the differentiation program and to migrate from the marrow into capillaries at an immature stage of development. Direct contact of the progenitor cells with the

stromal layer is not required for proliferation, which is stimulated by soluble factors. However, a substantial increase in myeloid proliferation is observed in the absence of stromal contact indicating that the stroma mediates an inhibitory signal (Salesse and Verfaillie, 2002). The adhesion studies that followed these initial observations are contradictory. The major shortcoming is due to the variability in adhesion capacity of primary CML cells versus cultured cell lines and to culture conditions. So while primary CML cells show decreased adherence, Bcr/Abl expressing myeloid cell lines are tightly anchored to stromal layers.

In primary CML progenitors (which resemble more closely the adherence characteristics observed in CML) the decreased adherence is correlated with a defect in integrin-mediated signaling. Integrins are transmembrane proteins that form heterodimers consisting of α and β subunits. The intracellular domain of the β subunit is linked to the cytoskeleton. Upon binding to its ligand the integrins initiate a signaling cascade that ultimately affects the cytoskeleton structure. In CML cells, incubation with a β1 integrin-activating antibody restores stromal and fibronectin adhesion and inhibits cell proliferation (Salesse and Verfaillie, 2002). These results suggest that reduced binding of the integrin extracellular domain results in defective intracellular signaling and loss of cell adhesion. Interestingly, IFN-α induces a significant dose-dependent increase in the adhesion of CML progenitors to the stroma. This effect is inhibited by the addition of blocking

antibodies against different integrins (Salesse and Verfaillie, 2002). The normalization of progenitor-stroma interaction is probably the reason why IFN-α is successfully used as alternative treatment of CML patients that cannot undergo allogenic bone marrow transplantation.

The molecular mechanism that links Bcr/Abl to the deregulation of integrin signaling is still unclear. Among the proteins that tyrosine phosphorylated in Bcr/Abl expressing cells there are important focal adhesion components such as CrkL (ten Hoeve et al., 1994), Fak (Gotoh et al., 1995; Salgia et al., 1995a), p130Cas (Salgia et al., 1996), Cbl (Andoniou et al., 1994) and Paxillin (Salgia et al., 1995b). Inhibition of Bcr/Abl tyrosine kinase activity with PTK inhibitors (Tyrphostin AG957) reduces the level of tyrosine phosphorylation of these focal adhesion molecules and increases stromal adhesion (Bhatia et al., 1998). These results suggest that constitutive phosphorylation of focal adhesion components may impair their ability to mediate integrin signaling leading to decreased adhesion in CML progenitors.

(5) Limitless replicative potential

One hallmark of oncogenic transformation is the ability of tumor cells to replicate indefinitely, a process known as immortalization. In contrast, normal human cells have the capacity for a definitive number of cell duplications (usually 60-70 doublings). The "counting device" that controls the number of completed

doublings is the telomeric ends of chromosomes. Telomeres are composed of several thousands of repeated 6bp sequence, portions of these stretches are systematically lost at each replicative event due to the inability of conventional DNA polymerases to complete synthesis of chromosomal ends (reviewed in Maser and DePinho, 2002). The progressive erosion of telomeres ends with the induction of cellular checkpoints that block cell division. This first level of regulation, known as senescence, prevents the exposure of the unprotected chromosome ends that could cause karyotipic rearrangements. This first regulatory mechanism can be breached by the inactivation of cell cycle regulators such as p53 and Rb. As proliferation continues further telomere erosion contributes to genomic instability and peaks with massive cell death known as "crisis". The subset of cells that survive this second proliferation block usually display massive genetic instability and most probably have acquired the combination of genetic alterations required for malignant transformation. The limitless replicative potential is achieved usually by activating telomere maintenance mechanisms. Among these mechanisms is the activation of a telomerase reverse transcriptase that *de novo* synthesizes the 6bp repeats at the ends of chromosomes. Interestingly, transformed cells exhibit short telomeric ends compared to the adjacent "normal cells" since they undergo more cell division that allow the accumulation of the array of oncogenic alteration prior telomerase activation (Hanahan and Weinberg, 2000; Maser and DePinho,

2002). In this model, the advent of oncogenic transformation occurs before the establishment of telomeric maintenance. In accordance to this model is the finding that telomerase is not required for tumorigenesis and telomerase knock out mice are more prone to develop tumors. In this new view of the oncogenic process the telomere dysfunction and subsequent crisis represents a mechanisms by which a cell accumulates the transforming alterations.

In CML cells, telomere length drastically shortens with progression between chronic and blast phase. In this latter phase, the acquisition of cytogenetic aberration is also associated with an increase of telomerase activity (Brummendorf et al., 2000). Different studies have suggested a correlation between telomere length in blast phase and the onset of blast crisis whereas shorter chronic phase telomeres are associated with shorter latency to blast phase and a high frequency of chromosomal rearrangements (Boultwood et al., 2000; Brummendorf et al., 2000). Therefore, telomeric measurement in chronic phase patients could be an invaluable diagnostic tool for the selection of patients at high risk of disease transformation.

(6) Genomic instability

The progression of CML to blast phase is usually accompanied by the appearance of chromosomal defects including duplication of the Ph chromosome. These macroscopic rearrangements suggest that Bcr/Abl can

induce genomic instability. The ability to increase the mutation rate is a powerful mechanism that allows oncogenes to accelerate the process of tumor progression. One way Bcr/Abl achieves genomic instability is by disabling key components responsible for the surveillance of genomic integrity. A typical targeted member of this "surveillance team" is the tumor suppressor protein p53. This protein is a DNA damage sensor that elicits either a cell cycle arrest to allow DNA repair or apoptosis if damage is excessive. The p53 DNA damage signaling pathway is lost in most CML cells that reach blast crisis (Chopra et al., 1999). Besides disabling DNA damage sensors (as in the case of p53), Bcr/Abl prevents the elimination of cells with excess DNA damage and/or with irreparable lesions by inhibiting apoptotic pathways (see previous section). The ability to elude elimination is also useful for transformed cells to survive genotoxic damage induced by treatment and allows CML cells to tolerate higher levels of DNA damage compared to normal cells (Skorski, 2002). Interestingly, the level of DNA damage in CML cells is higher than in normal cells despite the fact that CML cells can repair most of DNA lesions faster. This facilitated repair is a direct effect of Bcr/Abl catalytic activity and it may be mediated by the upregulation of Rad51. Rad51 is functionally similar to the *E.coli* RecA protein, it is a key player in the homologous recombination DNA repair responsible for the elimination of double strand breaks (DSB). Bcr/Abl regulates Rad51 at multiple levels: at the mRNA level by inducing STAT5-mediated transcription and at a post-translational

level by activating Rad51 ability to associate with DSB through direct phosphorylation of specific tyrosine residues and by inhibiting caspase 3-mediated Rad51 degradation (Skorski, 2002). Although Bcr/Abl enhances the cell's repair ability, the efficiency of the repair mechanisms may be diminished. For example, in Bcr/Abl expressing cell lines there is enhanced expression of DNA polymerase β, which has intrinsic low fidelity. In addition Bcr/Abl^{p210} is thought to hinder the repair capability of the xeroderma pigmentosum group B protein (XPB) (Takeda et al., 1999). This effect in combination with the inhibition of apoptosis and mitogenic stimulation allows the accumulation of mutations that could result in blastic transformation.

I.II.E. Therapeutic strategies for treatment of Bcr/Abl positive leukemias

Currently allogenic bone marrow transplantation is the only successful way to eradicate CML. Unfortunately, only 20-25% of CML patients are eligible for this procedure due to age restraints or lack of a suitable donor. Even in transplant cases the risk of morbidity and mortality is very high. Other therapeutic strategies were developed based on the observation that the driving force of CML is Bcr/Abl tyrosine kinase activity. This activity is not only required for the initial development of the disease but also for the maintenance of leukemia. This can be observed in a transgenic mice model where the expression of Bcr/Abl is regulated by a tetracycline inducible promoter. In these

mice, the increase in Bcr/Abl expression determines the onset of leukemia. After the disease is established a subsequent inhibition of Bcr/Abl expression induces remission in all mice indicating that expression of Bcr/Abl is required for the maintenance of the disease (Huettner et al., 2000). Different therapies developed to lower Bcr/Abl expression at a translational level by either antisense oligonucleotide (Gewirtz et al., 1998) or ribozyme (James and Gibson, 1998) strategies; unfortunately these approaches failed to fulfill their initial promise. However, direct inhibition of Bcr/Abl tyrosine kinase activity turned out to be a successful strategy. The development of a specific signal transduction inhibitor (STI571) drastically changed the prognosis of many CML patients (Druker et al., 1996). Selective inhibition by STI-571 is achieved by binding to the inactive conformation of the tyrosine kinase. This reversible inhibitor competes for the Bcr/Abl ATP binding site and blocks the phosphorylation of downstream effectors (Schindler et al., 2000). The drug is specific at micromolar concentrations although it does affect other tyrosine kinase such as c-Abl and c-Kit. The therapeutic power of STI-571 is evident in clinical trials where it induced hematological remission in nearly all chronic phase patients treated with appropriate doses (Sawyers, 2002). Unfortunately the drug is not as powerful in blast crisis cases; after an initial hematological response the majority of patients relapse with a drug resistant form of the disease. Molecular characterization of CML cells from these patients revealed different drug-resistance mechanisms.

The majority of these mechanisms are Bcr/Abl dependent and include gene amplification of the oncogene and specific mutations of Bcr/Abl kinase domain that hinder STI-571 binding (Nimmanapalli et al., 2002; Sawyers, 2002). These observations suggest that blast phase.

CML cells are genetically unstable and normally accumulate mutations during disease progression. In this context, STI-571 selects for the drug resistant alleles that cause the observed relapse. In order to circumvent the occurrence of resistance novel therapies combine STI-571 with traditional chemotherapy. Although there are some encouraging results (Nimmanapalli et al., 2002; Thiesing et al., 2000) the search for the optimal drug combination is still in progress. The elucidation of critical steps in the intracellular signaling pathways will be pivotal in the identification of additional molecular targets for effective therapeutic compound.

CHAPTER II

MATERIALS AND METHODS

(1) Plasmids

A bicistronic MSV retroviral vector, and vectors expressing v-Abl, Bcr/Abl^{p185} or Bcr/Abl^{p210} or JBD have been described (Dickens et al., 1997; Muller et al., 1991). An MSCV-IRES-GFP retroviral vector (Zindy et al., 1998) was used for *in vivo* bone marrow reconstitution assays. Bcr/Abl isoforms (EcoRI fragment from the corresponding MSV vectors) were cloned in the corresponding Eco RI restriction site of the MSCV-IRES-GFP vector. The Ψ^{eco} plasmid expressing retroviral packaging proteins was provided by Charles Sawyers.

The Dbl domain of Bcr/Abl^{p210} (aa 413-789) was amplified by polymerase chain reaction (PCR) using the following primers 5'-GCGAGTGAATTCGACTTGG-AAAAGGGCTTGGAG-3' and 5'-GCTGTGCTCGAGCTGGAGTTTCACACACGAGTTG-G-3'. The amplicon was digested with EcoRI and XhoI and ligated into the corresponding restriction sites of pCMV-Tag2B (Stratagene) in frame with the Flag epitope tag. Bcl2 promoter reporter plasmid (LB334; (Wilson et al., 1996) was provided by Linda Boxer and was used in cotransfection assays with the *Renilla reniformis* luciferase plasmid pRLnull (Promega).

(2) Mice

C57BL6 mice expressing Bcl-2 under the μ enhancer IgH promoter (Strasser et al., 1991) and wild-type C57BL6 mice were obtained from The Jackson Laboratories. *Jnk1 -/-* mice were described previously (Dong et al., 1998). The animals were housed in a facility accredited by the American Association for Laboratory Animal Care.

(3) Cell lines: Culture, Transfection protocols and Viral Production.

293T and NIH3T3 cells were cultured in Dulbecco modified Eagle medium (DMEM) supplemented with 10% Fetal Bovine Serum (FBS)(Omega scientific, Hyclone) 2mM L-Glutamine (Life Technologies), 100 U/ml penicillin and 100μg/ml Streptomycin (Life Technologies). For the analysis of the Dbl domain described in section III.II.C subconfluent 293T cells were transfected with 1μg of either Flag-Dbl plasmid or pcDNA3 as a empty vector control using Superfect (Gibco) following the manufacturer's protocol. The endogenous JNK activity of the transfected cells was analyzed 48 hours post transfection. 293T cells were also used to produce viral supernatants. For this purpose subconfluent plates (80%) were cotransfected using Superfect with equal amounts of the retroviral plasmid of choice and the Ψ^{eco} plasmid. Cells were incubated in growth media for 24 hours post transfection and then shifted to Virus Collecting medium (VCM)

(Iscove's Modified medium with 10% FBS, 2mM L-Glutamine (Life Technologies), 100 U/ml penicillin and 100μg/ml Streptomycin (Life Technologies)). Culture supernatants containing virus were collected starting 40 hours up to 72 hours post transfection. After each collection the cultures were replenished with fresh VCM medium. All collected aliquots of viral supernatant were pooled, cleared by centrifugation and filtered through a 0.45μ filter. The filtered aliquots were stored at –80°C. Viral titers for the MSV retroviruses were measured by infecting murine cells (NIH3T3) with serial dilutions of viral supernatants. The infections were carried our by incubating exponentially growing cells overnight at 37°C 5% CO_2 with retroviral supernatants in the presence of 8μg/ml hexadimethrine bromide (Polybrene) (Sigma). 48 hours post infections cells were transferred to 150mm^2 dishes and cultured in selection media (growth media containing 200μg/ml of Geneticin (G418, Gibco). Media was routinely replaced every 48 hours. Two weeks post infection plates were analyzed for the presence of G418-resistant colonies in the different viral dilutions. Viral titers for the MSCV retroviruses were estimated by infecting equal numbers of Baf3 cells with serial dilutions of viral supernatant. Infected cells were analyzed by flow cytometry 48 hours post infection for the presence of GFP$^+$ cells. The percentage of GFP$^+$ cells in a specific dilution would give an estimate of the viral titer. All infected cells were analyzed by immunoblot for Bcr/Abl expression.

Baf3 were cultured in growth media (RPMI medium supplemented with 0.5ng/ml recombinant mouse Interleukine-3 (IL-3) (R&D System), 10% Fetal Bovine Serum (FBS)(Omega scientific, Hyclone) 2mM L-Glutamine (Life Technologies), 100 U/ml penicillin and 100μg/ml Streptomycin (Life Technologies)). Baf3 stable cell lines expressing Bcr/Abl^{p185}, Bcr/Abl^{p210} or empty vector control were generated by retroviral infection using MSV retroviral constructs. Equal number (10^6 cells) of exponentially growing cells were incubated overnight at 37°C 5% CO_2 with retroviral supernatants in the presence of 0.5ng/ml IL-3 and 8μg/ml Polybrene (Sigma). After 16 hours cells were washed and resuspended in fresh growing media at a concentration of $2x10^5$ cells/ml. 48 hours post infection cells were transferred to selection media (growth media containing 400μg/ml Geneticin (Gibco)), uninfected cells were also plated as negative controls. After two week of selection samples from each culture were analyzed by western blot for Bcr/Abl expression. Stable cell lines were routinely cultured in selection media. For the SP600125 experiments described in Results (section III.I.B) $2x10^6$ cells (Baf3/p185 or Baf3/Vector control) were cultured in 10ml of growth media with or without 0.5ng/ml IL-3 in the presence of either 20μM SP600125 (Biomol) or dimethyl sulfoxide (DMSO) (Sigma). The DMSO concentrations in all samples was 0.2%. Cells were treated for either 16 hours (for BrdU and trypan blue viability assays) or 24 hours (for Annexin V labeling) and cell viability was evaluated by counting trypan blue

negative cells in each culture. Equal number of cells were analyzed by flow cytometry. For the transient transfection experiments described in section III.II.B equal numbers of exponentially growing Baf3 cells (Baf3/p210 and Baf3/Vector) ($5x10^6$cells/sample) were washed twice in serum-free media (RPMI with no supplements) and resuspended at a concentration of 10^7 cells/ml. Cells were aliquoted (0.5 ml) in electroporation chambers and incubated for 10min at room temperature with a DNA mixture containing 4μg of Bcl2 promoter reporter plasmid, 0.5μg of pRLnull and 8μg JBD (when indicated) plus varying amounts of pBluescript II KS^+ (Stratagene) as a carrier in order to reach total DNA content of 30 μg. Cells were electroporated (250V, 1180μF, low Ω) using an Invitrogen Cellporator and immediately placed on ice for 10 min. Following this incubation cells were allowed to recover in growth media for 48 hours before analyzing luciferase activity.

S17 cells (Collins and Dorshkind, 1987) were routinely cultured in αMEM supplemented with 20% FBS (Omega scientific, Hyclone) 2mM L-Glutamine (Life Technologies), 100 UI/ml penicillin and 100μg/ml Streptomycin (Life Technologies). For the production of stromal layers, S17 cells were allowed to reach confluency and then they were placed in Lymphoid media (RPMI 5% FBS $5x10^{-5}$ M 2-mercaptoethanol (βME) 2mM L-Glutamine (Life Technologies), 100 U/ml penicillin and 100μg/ml Streptomycin (Life Technologies)) for two days to

prepare conditioned media to sustain the growth of primary lymphoblast (see primary cultures section).

(4) Primary cells: cultures, Bone marrow assay

For bone marrow assays described in section III.I.A, I followed the protocol for high density bone marrow long term culture described in (Whitlock et al., 1984). Bone marrow cells were isolated by flushing tibias and femurs of 4 week-old male mice. Cells were resuspended in Lymphoid media and cell density was determined by counting cells in 0.2% methylene blue and 3% acetic acid which preferentially lyses erythrocytes. Equal numbers of cells ($1.5x10^7$cells) were infected with the desired virus in the presence of 8μg/ml Polybrene for 3 hours at 37°C 5%CO_2. After infection cells were washed with Lymphoid media and plated in three 60mm^2 dishes ($5x10^6$cells/dish). Culture plates were set on a tray wrapped with aluminum foil and placed in 37°C 5%CO_2 incubator. Cells were routinely fed every three-four days by either adding a 2 ml of fresh lymphoid media (on day 3-4 post infection) or by removing 75% of medium without removing non-adherent cells and adding 4 ml of fresh media (on day 7 post infection). The routine feeding schedule was repeated every week for the entire duration of the assay. Starting 12 days post infection aliquots were taken from each culture every two days and cell density was measured by counting trypan-negative cells with a hemocytometer.

(5) Leukemogenic transformation assays and "ex vivo" cultures

We used two different methods of *in vivo* reconstitution that would give rise to either Bcr/Abl induced lymphoid or myeloid disease in recipient mice. For the "lymphoid" reconstitution protocol, bone marrow cells were isolated from tibias and femurs of donor male mice (4 week-old) and transduced with MSCV retroviruses (Bcr/Abl^{p185}, Bcr/Abl^{p210} and empty vector control) following the same infection protocol as for the bone marrow assay described above. Three hours post infection, the transduced bone marrow was washed once in lymphoid media and resuspended in sterile Phosphate buffered saline (PBS) at a concentration of $2x10^6$ cells/ml. Transduced bone marrow cells (10^6) were injected into the tail vein of lethally irradiated (2x525 Rads each dose administered 4 hours apart) C57BL6 recipient mice (8 week-old females). Secondary transplantation assays were performed using non-lethally irradiated (1 x 450 Rads) mice (8 week old C57BL6 females). All recipient mice were monitored daily for signs of morbidity and weight loss. Premoribund mice were euthanized by cervical dislocation.

For the "myeloid" reconstitution protocol donor mice (4 week-old males) were treated with a single dose of 10mg/ml 5-Fluorouracil (5-FU) (0.008ml/gr of body weight) 3 days prior harvest of the bone marrow. The bone marrow was transduced with MSCV retroviruses (Bcr/Abl^{p185}, Bcr/Abl^{p210} and empty vector control) and C57BL6 mice were reconstituted using the same protocol as

described for the "lymphoid" reconstitution assay. All recipient mice were monitored daily for signs of morbidity and were euthanized 15 days post reconstitution.

White blood counts were performed on all recipient mice twice a week using the Unopette microcollection system (Becton Dickinson). After euthanization the organs from the recipient mice were harvested, weighed, fixed in 4% paraformaldehyde, processed for paraffin-embedded sectioning, and stained with hematoxylin and eosin. Femurs were treated with Decalcifier-1 (Surgipath Medical Ind.) overnight prior to fixation. Whole bone marrow was harvested from tibias and femurs and treated with red blood cell lysis solution ACK (0.15M NH_4Cl, 1mM $KHCO_3$ and 0.1mM disodium EDTA [ph7.3]) (Zhang and Ren, 1998). Cells were counted and aliquots were cytocentrifuged onto slides and stained with Diff Quik (Dade Behring AG) following manufacturer's recommendations. Bone marrow was also analyzed by flow cytometry for the expression of surface markers (see later).

Cells isolated from the bone marrow of the recipient premoribund mice were cultured *in vitro* following the bone marrow long term culture protocol described for the bone marrow assay (Whitlock et al., 1984). After one week non-adherent cells were harvested and plated onto confluent S17 stromal layers. The cells obtained were routinely passaged on stromal layers. To examine the dependence of the cells on the stromal layer, the cells were washed with media

and plated in the presence and absence of the stromal layer (10^6 cells/10ml). BrdU labeling and Annexin V assays were performed 3 days post plating.

(6) Immunoblot Analysis

Cells were lysed in Triton Lysis Buffer (TLB) containing 20mM Tris (pH7.4), 137mM NaCl, 2mM sodium pyrophosphate, 1% Triton X-100, 10% glycerol, 2mM EDTA, 25mM β-glycerophosphate, 1mM sodium orthovanadate, 1mM phenylmethylsulfonyl fluoride, 0.5mM dithiothreitol and 5μg/ml of aprotinin and leupeptin. Lysates were incubated for 10 min on ice and then they were cleared by centrifugation (13,000rpm for 15 min at 4°C). The protein content of the lysates was quantified by Bradford Assay (Biorad) and equal amount of total protein was resolved on SDS-PAGE. Proteins were transferred electrophoretically onto polyvinylidene difluoride membrane (Immobilon). The membrane was blocked following the antibody manufacturer's protocols. Immunoblots were probed with antibodies to Abl (Goga et al., 1995), JNK (Pharmingen), Flag epitope (Kodak and Sigma), Akt (Cell Signaling), phospho-Ser473 Akt (Cell Signaling), Bcl-2 (Pharmingen) and phosphotyrosine (Py-99; Santa Cruz). Immune complexes were detected by enhanced chemiluminescence (NEN).

(7) Protein Kinase Assays

Two different procedures were used to measure JNK kinase activity: GST-pull down and immunocomplex kinase assay. The GST pull-down assay was performed to analyze JNK activity in primary cells. These cells were lysed in TLB and then quantitated by Bradford. Lysates (50μg) were incubated with GST-cJun (aa 1-223) bound to Glutathione-Sepharose 4B beads (Amersham Pharmacia) for 2 hours at 4°C. Beads were washed twice with TLB and twice with Kinase Buffer (25mM Hepes [pH 7.4], 25mM β-glycerophosphate, 25mM MgCl2, 0.1mM sodium orthovanadate, 0.5mM dithiothreitol). The kinase reaction was carried out at 30°C for 15 min in a final volume of 25μl kinase buffer containing 50μM ^{32}P-γ-ATP (370 kBq/nmol) and 20μM ATP. Laemmli buffer was added to stop the kinase reaction. Immunocomplex kinase assay were used to evaluate JNK kinase activity in transfection assays (Fig.12). In this assay, equal amounts of TLB lysates were immunoprecipitated by incubation for 3 hrs at 4°C with 1μg of α-JNK antibody pre-bound to Protein-G Sepharose beads (Amersham Pharmacia). Immunocomplexes were washed twice with TLB and twice with Kinase Buffer. The kinase reaction was carried out at 30°C for 15 min in a final volume of 25μl kinase buffer containing 50μM ^{32}P-γ-ATP (370 kBq/nmol) and 20μM ATP and 2μg of GST-Jun (aa1-223). All kinase reactions were resolved on SDS-PAGE 12% gel and the phosphorylation of GST-cJun was quantitated using a Phosphoimager (Molecular Dynamics).

(8) Luciferase reporter gene assays

Bcl2 promoter activity described in section II.B was analyzed using a dual-luciferase reporter assay (Promega) following manufacturer's protocol. Transfected cells were lysed in passive lysis buffer (PLB; provided by the manufacturer). Cell lysates were subjected to three freeze/thaw cycles and then spun for 10min 15,000xg 4°C. Aliquots of the cell lysates were added to luciferase substrate (LARII) luminescence were immediately quantitated using a luminometer (Monolight 2010; Analytical Luminescence Lab.). The firefly driven reaction was quenched and the substrate for the Renilla luciferase was added by supplementing the reaction with Stop&Glo reagent (Promega). *Renilla* induced luminescence was quantitated and these values were used to normalize the transfection efficiency between samples.

(9) Immunofluorescence analysis

Whole bone marrow was harvested from tibias and femurs and spleen were dissected from the euthanized mice. Dissociated cells from the spleen and bone marrow cells were treated with red blood cell lysis solution ACK. For immunophenotyping by flow cytometry, equal numbers (10^6/sample) of trypan negative cells were blocked with anti-mouse CD16/CD32 (FcγIII/II receptor; Pharmingen). Cells were stained with allophycocyanine (APC) conjugated anti-CD45R/B220 and phycoerythrin (PE)-conjugated anti- Thy1.2, CD43, CD11b

(Mac-1α chain), Ly-6G (Gr-1), CD19, Ly-6AE (Sca-1), IgG, IgM, Ly-51 (6C3/BP-1) or Ter119 (Pharmingen). Cell cycle analysis was performed using cells pulse-labeled (15 min for primary cells and 1 hour for Baf3 cell lines) with 10μM BrdU, fixed in 70% ethanol, and stained with DAPI (Molecular Probes) and an antibody to BrdU (FITC- or PE-conjugated) (Pharmingen) following manufacturer's protocol. Cell death was examined by co-staining with either a combination of Annexin V FITC-conjugated and Propidium iodide (PI) (Pharmingen) or AnnexinV PE-conjugated (Pharmingen) and 7-aminoactinomycin D (7-AAD; Sigma). Staining was performed following manufacturers' recommendations. Flow cytometry was performed using a MoFlo FACS machine (Cytomation) and a FACScan (Becton Dickinson). The extent of apoptosis was calculated as a ratio between AnnexinV$^+$ (apoptotic) cells and Annexin V$^-$ (live) cells in the 7AAD- population (apoptotic index). In the experiments described in Fig. 11 the apoptotic index was normalized to the basal death observed in untreated samples in the absence of IL-3. TUNEL assays (Roche) were performed according to the manufacturer's protocol and were examined by conventional Immunofluorescence microscopy.

(10) Ribonuclease protection assays

Total RNA was extracted from ex-vivo cultures exponentially growing on S17 stromal layers utilizing the RNeasy Mini kit (Qiagen) and following the

manufacturer's protocol. Cells were lysed in a guanidinium isothyocyanate buffer. Lysates are passed through a silica based column that traps total RNA. Following serial washes with ethanol based solutions the total RNA was eluted with 30μl of diethylpyrocarbonate-treated (DEPC) water. The RNA was quantified and the purity determined by measuring the absorbance at 260 and 280nm using an Ultraspec (Pharmacia). Samples were aliquoted and stored at –80°C until use. Multi-probe ribonuclease protection assays were performed with the Riboquant RPA kit by Becton Dickinson-Pharmingen and following the manufacturer's protocol. Multi-probe templates were radiolabeled with 10μl of α-^{32}P-Uridine triphosphate (10μCi/μl; NEN) by T7 RNA polymerase for 1 hour at 37°C. The reaction was treated with DNAse and extracted with phenol: chloroform (1:1 vol/vol). The resulting aqueous phase was chloroform extracted and total RNA was precipitated by adding ammonium acetate (final concentration of 0.57M) and 100% cold Ethanol. Precipitation were incubated for 30 min at -80°C. Samples were spun 15,000xg for 15 min at 4°C and pellets were washed once with ice cold 90% Ethanol. Dried pellets were resuspended in 8ml of hybridization buffer (supplied by the manufacturer). Heat denaturated samples were hybridized overnight to the labeled probe (2μl of 4x10^{5}cpm/μl) by slow temperature shift (from 90°C to 56°C). The hybridized RNA was digested with RNAse (250U/ml), phenol: chlorophorm extracted and precipitated as before. Dried pellets were resuspended in 5ml of loading buffer (supplied by the

manufacturer). $1.5x10^5$ cpms of the unhybridized template set(s) and the total volumes of the hybridized samples were resolved on a 6% denaturating polyacrylamide gel. Gels were exposed to a Phosphoimager (Molecular Dynamics) for quantification and to film for autoradiography.

(11) Statistical Analysis

Calculation of mean and standard deviation (SD) and standard error of the mean (SEM) was performed using Microsoft Excel 98. The statistical analysis of organ weights and survival curve data was performed using Survival Tools for StatView5 (Abacus Concepts, CA) using the Mantel-Cox (log-rank) test and Kaplan-Meier survival analysis. The statistical analysis of the *in vivo* data was carried out by Joanne Wu and Qui Liu.

CHAPTER III

RESULTS

III.I. *In vitro* Approach

Initially I investigated the role of JNK in cell transformation using an *in vitro* approach. For this purpose I used Bcr/Abl as a transforming agent since previous studies indirectly linked the JNK pathway to Bcr/Abl-induced cell transformation. I examined the effect of JNK gene disruption in Bcr/Abl transformation using *in vitro* bone marrow assays (McLaughlin et al., 1987) to study the effect of Bcr/Abl on lymphoid malignant proliferation. Preliminary experiments described in section III.I.A show that JNK1 is the predominant isoform in Bcr/Abl transformed lymphoblast. This finding allowed me to use *Jnk1-/-* mice for my *in vitro* assays and thus circumvent the unavailability of *Jnk1-/- Jnk2-/-* mice which die at an early embryonic stage. Interestingly, the results obtained from the *in vitro* bone marrow assays suggest that while JNK1 plays a critical role in Bcr/Abl^{p210} lymphoid expansion it is dispensable for Bcr/Abl^{p185} induced transformation (section III.I.B). One possible explanation for this result is that JNK2 compensates for the absence of JNK1 in Bcr/Abl^{p185} transformed lymphoblast. To test this hypothesis I used a pharmacological

approach to deplete JNK activity in pro-B cells transformed by Bcr/Abl^{p185}. The results of these experiments are described in section III.I.C.

In addition, the results of the bone marrow assays provide evidence of a functional difference between Bcr/Abl^{p185} and Bcr/Abl^{p210}. These two isoforms have defined structural differences which include a GEF domain with high homology to the Dbl proto-oncogene present in the Bcr/Abl^{p210} and not in the Bcr/Abl^{p185} isoform. Thus, I investigated whether this domain had a role in the activation of the JNK pathway by testing the induction of JNK activity by transient expression of the isolated Dbl domain (section III.I.D).

III.I.A. JNK1 is the predominant isoform in transformed pre-B cells

To investigate the role of JNK in leukemogenesis, I examined the effect of JNK gene disruption in experiments using the oncogene Bcr/Abl^{p210}. Lethally irradiated mice were reconstituted with wild-type (WT) and *Jnk1-/-* bone marrow cells transduced with a retrovirus that expresses Bcr/Abl^{p210}. The cells from the reconstituted bone marrow were isolated and levels of JNK activity were measured. High levels of JNK protein

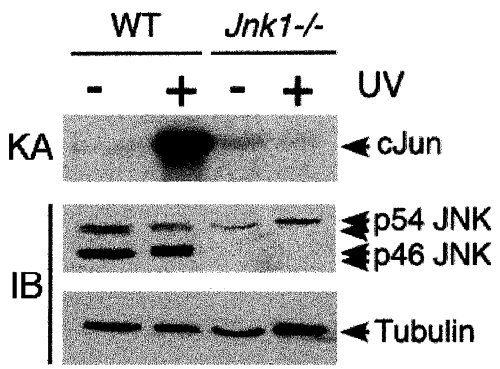


Fig. 6 Measurement of JNK activity in cell lines derived from reconstituted mice. These cells were cultured *in vitro* and exposed in the presence or absence of 60J/m^2 of UV light and JNK kinase activity was measured by *in vitro* kinase assay (KA) using c-Jun as a substrate. Cell extracts were examined by immunoblot (IB) analysis using antibodies to JNK and Tubulin. JNK1 and JNK2 are expressed as groups of isofororms of approximately 46-kD and 54-kD. The major JNK1 and JNK2 isoforms are 46-kD.

kinase activity was observed in extracts prepared from wild-type cells after exposure to UV light (Fig. 6). In contrast, a severe reduction in JNK activity was detected in *Jnk1-/-* cells. This observation correlates with the results of immunoblot analysis that indicated high levels of JNK1 and low levels of JNK2 in wild-type cells (Fig. 6). The residual JNK2 levels found in *Jnk1-/-* cells did not seem to contribute substantially to the JNK kinase activity after UV exposure (Fig. 6). These data demonstrate that JNK1 is the major JNK in these cells. Since *Jnk1-/-Jnk2-/-* mice are not viable (Kuan et al., 1999; Sabapathy et al., 1999), I focused our analysis of Bcr/Abl^{p210} on *Jnk1-/-* mice.

III.I.B. *In vitro* transformation by Bcr/Abl^{p210} is impaired in *Jnk1-/-* bone marrow cells

To examine the potential role of JNK in transformation, I investigated the effect of the Bcr/Abl oncogene using bone marrow assays *in vitro* to measure the outgrowth of transformed pre-B cells (McLaughlin et al., 1987). Comparison of bone marrow from wild-type and *Jnk1-/-* mice demonstrated no differences in the relative abundance of cell lineages monitored by flow cytometry (Fig. 7A). The bone marrow was transduced with retroviruses that express Bcr/Abl^{p210} or Bcr/Abl^{p185}. Control experiments were performed using a retrovirus without Bcr/Abl (Vector). Both Bcr/Abl isoforms caused outgrowth of transformed pre-B cells in cultures of wild-type (WT) bone marrow (Fig. 7B). Bcr/Abl^{p185} caused

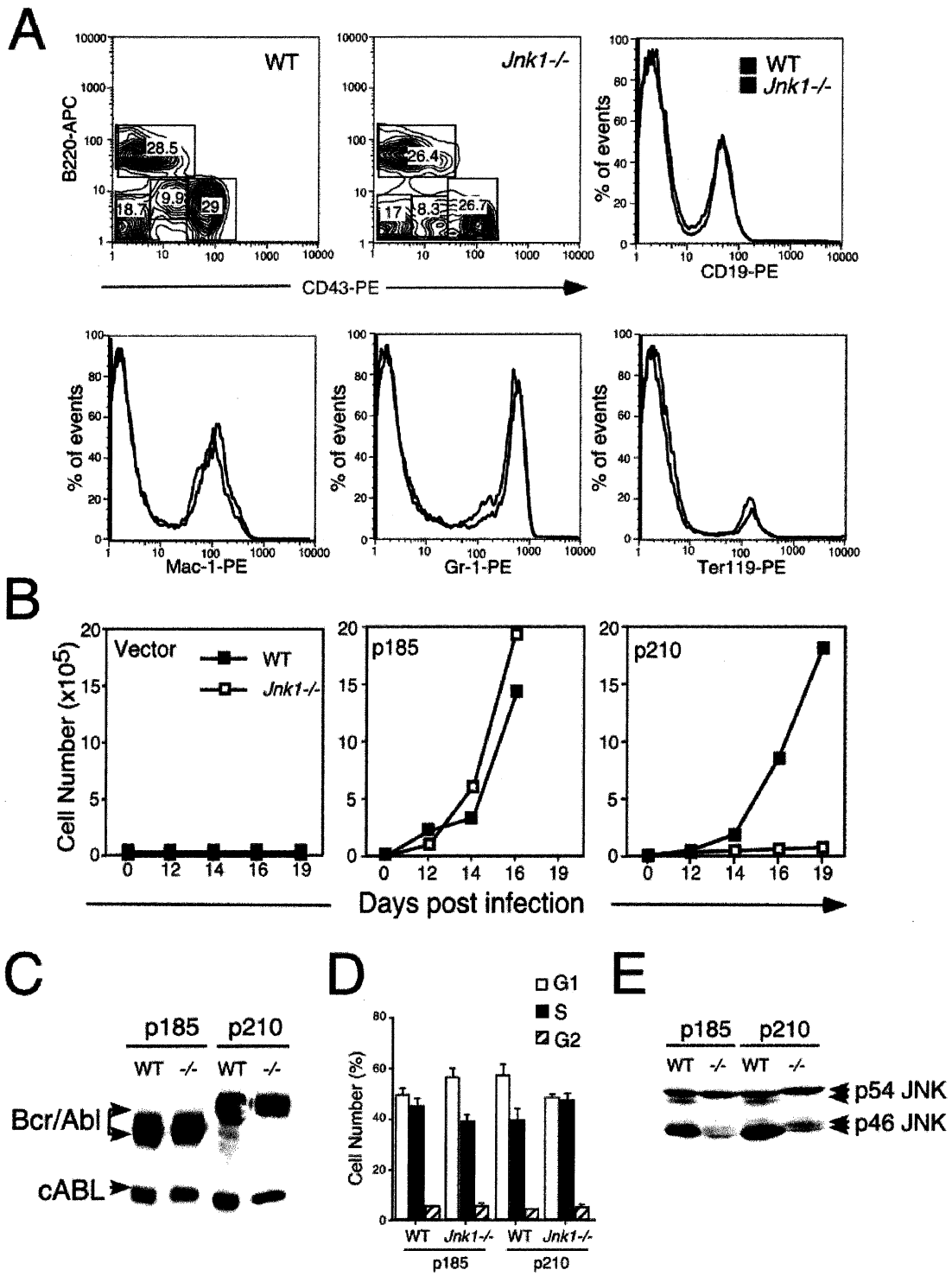


Fig. 7 JNK1 is required for Bcr/Abl^{p210} induced cell transformation *in vitro*.
(A) Analysis of whole bone marrow derived from WT(blue) and *Jnk1-/-* (red) mice by flow cytometry. Cells were analized for the indicated surface markers. (B) *In vitro* bone marrow assay performed using mouse bone marrow derived from WT and *Jnk1-/-* mice transduced with retroviruses (Vector, Bcr/Abl^{p185}, Bcr/Abl^{p210}). Non-adherent cells were harvested and the mean number of tryptan blue negative cells was measured. Similar data were obtained in three independent experiments. (C) and (E) Immunoblot analysis of WT and *Jnk1-/-* transformed cells using antibodies to Abl(C) and JNK(E). (D) Non-adherent pre-B cells (14 days post-infection) were pulse labeled (15 min) with BrdU and stained with DAPI and a FITC-conjugated antibody to BrdU. The percentage of cells (mean±SD, n=3) in G1, S and G2 phases of the cell cycle was measured by flow cytometry.

rapid outgrowth of pre-B cells in *Jnk1-/-* cultures. In contrast, the outgrowth of pre-B cells in *Jnk1-/-* cultures transduced with Bcr/Abl^{p210} was severely reduced (Fig. 7B). This reduced outgrowth caused by Bcr/Abl^{p210} in *Jnk1-/-* was not due to differences in Bcr/Abl expression (Fig. 7C), nor to a cell cycle defect (Fig. 7D). Furthermore, transformation by Bcr/Abl did not increase JNK2 expression in *Jnk1-/-* cells (Fig. 7E). The absence of a detected cell cycle defect suggested that the failure of the *Jnk1-/-* Bcr/Abl^{p210} cells to accumulate might be caused by increased cell death (Section III.III.A). Together these data indicate that JNK1 plays a critical role in Bcr/Abl^{p210} transformed cells. The proliferation defect in *Jnk1-/-* Bcr/Abl^{p210} cells may be caused by an inability of the *Jnk1-/-* stroma to support the growth of the transformed cells. To test this hypothesis, Bcr/Abl^{p210} transformed cells were harvested from the *in vitro* bone marrow cultures

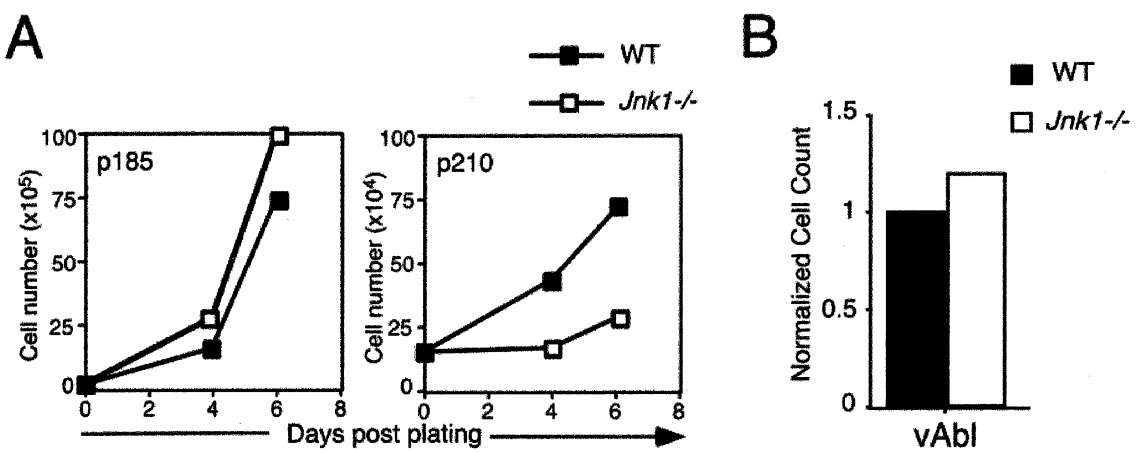


Fig. 8 The lack of proliferation in *Jnk1-/-* Bcr/Abl^{p210} is not due to a defect in stromal support (A) or in intrinsic transduction efficiency (B).
(A) Equal numbers of Bcr/Abl transformed cells were plated onto stromal layers formed by S17 cell line. Proliferation at different times was measured by counting trypan blue negative cells. Similar data were obtained in three independent experiments.
(B) Primary mouse bone marrow cells derived from WT and *Jnk1-/-* mice were infected with a retroviral vector that expresses v-Abl. Non-adherent cells were collected and the mean number of trypan-blue negative cells was measured on day 15 after infection.

and equal numbers were plated on wild-type and *Jnk1-/-* stromal layers or on stromal layers formed by the S17 cell line. Rapid proliferation of wild-type cells and Bcr/Abl^{p185} transformed *Jnk1-/-* lymphoblasts was observed on each of these stromal layers. In contrast, the Bcr/Abl^{p210} transformed *Jnk1-/-* cells failed to proliferate (Fig. 8A). These data indicated that the effect of JNK-deficiency on the proliferation of Bcr/Abl^{p210} transformed cells was an autonomous defect in the transformed cells and was not related to the genotype of the stromal cells.

One possible explanation of the observed transformation defect is that the number of targeted cells transduced by the Bcr/Abl retrovirus was reduced in the *Jnk1-/-* bone marrow. However, the absence of cell lineage and cell number defects (Fig. 7A) in *Jnk1-/-* bone marrow is inconsistent with this hypothesis. Indeed, the observation that Bcr/Abl^{p185} (Fig. 7B) and v-Abl (Fig. 8B) caused similar transformation of bone marrow isolated from wild-type and *Jnk1-/-* mice demonstrated that differences in transduced target cell number does not account for the *Jnk1-/-* specific defect in transformation caused by Bcr/Abl^{p210}.

III.I.C. Both JNK1 and JNK2 activities are required for Bcr/Abl^{p185} induced growth factor-independent growth of Baf3 cells.

The results in the previous section showed that Bcr/Abl^{p185} was able to induce transformation in the absence of JNK1 in bone marrow assays. This observation led to the hypothesis that in the absence of JNK1 this oncogene

might rely on the presence of JNK2 to induce transformation *in vitro*. To verify this hypothesis I tested the ability of Bcr/Abl^{p185} to induce transformation in the

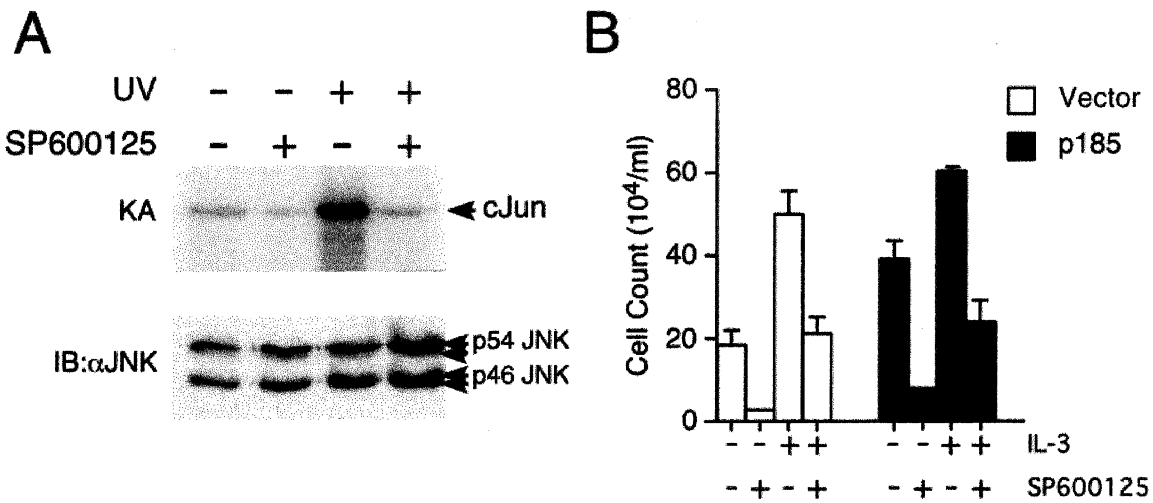
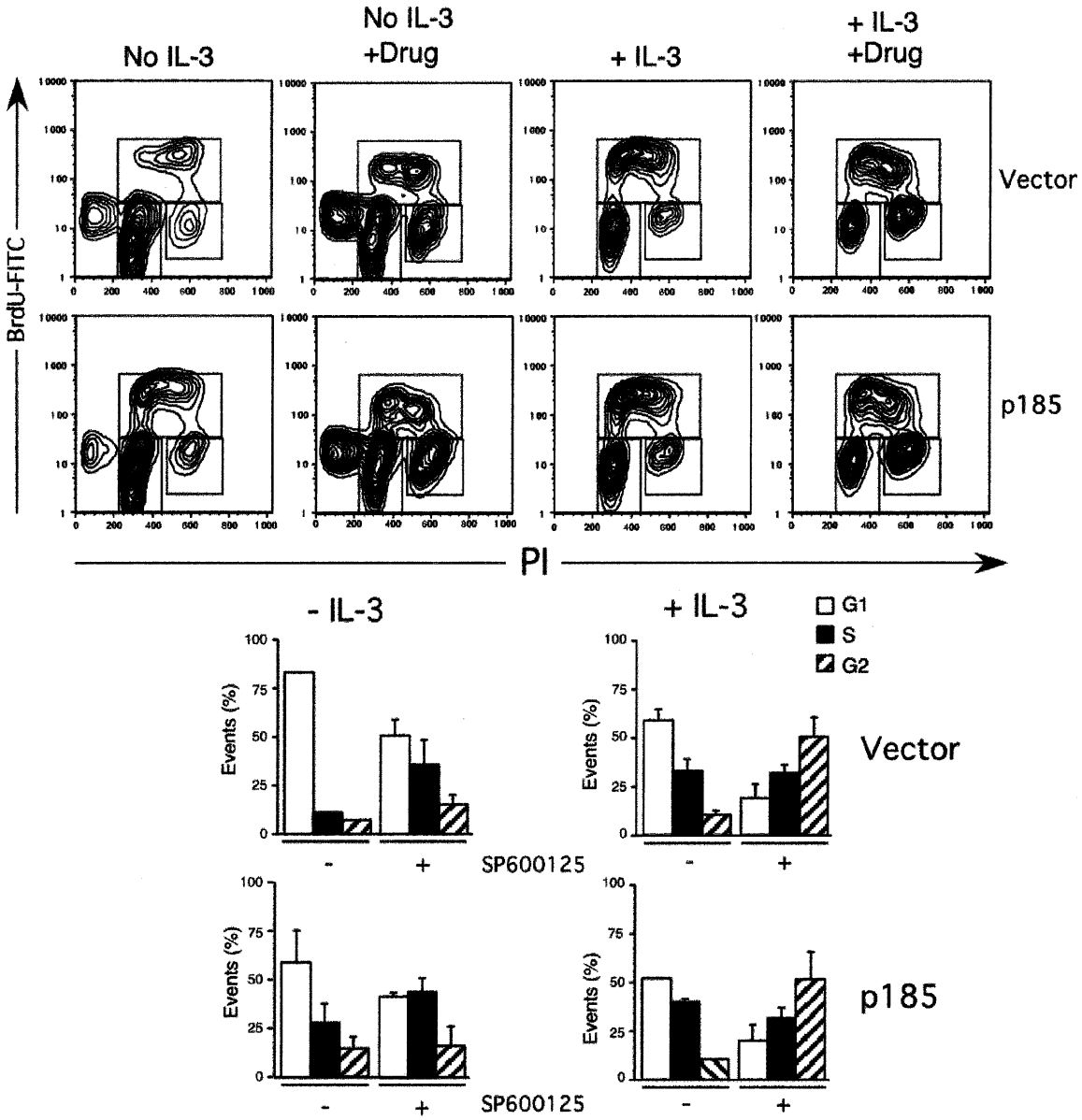


Fig. 9 Inhibition of JNK activity by SP600125 blocks IL-3 independent proliferation in Bcr/Abl^{p185} transformed Baf3 cells. (A) The JNK inhibitor SP600125 was tested in Baf3 cells exposed to 60 J/m^2 UV light. Cell lysates were harvested after 45 min of incubation. JNK activity was measured by *in vitro* kinase assay and lysates were analyzed by immunoblot using JNK antibodies.(B) Equal numbers (2x10^5) of Baf3 cells stably expressing Bcr/Abl^{p185}(p185) or vector control (Vector) were plated in the presence or absence of IL-3 and treated with either 20μM SP600125 or DMSO. Trypan blue negative cells were counted 16 hours after plating (mean±SD, n=3).

absence of both JNK1 and JNK2 kinase activities. In order to circumvent the unavailability of *Jnk1-/-Jnk2-/-* double knock out mice, which die *in utero*, I took advantage of the JNK inhibitory drug SP600125. This drug was shown to specifically inhibit all JNK isoforms in the micromolar range (Fig. 9A Bennett et al., 2001). As a model system I used the pro-B cell line, Baf3. This cell line is normally IL-3 dependent but upon Bcr/Abl expression it becomes IL-3 independent. This acquired growth factor independence is a hallmark of Bcr/Abl induced transformation. Baf3 lines stably expressing either empty vector (Vector) or Bcr/Abl^{p185} (p185) were made by retroviral transduction and cells were



Flg. 10 Baf3 cell lines expressing Bcr/Abl^{p185} (p185) or vector control (Vector) were treated with 20μM SP600125 (Drug) in the presence or absence of IL-3 for 16 hours. Cells were then pulse labeled (one hour) with BrdU and stained with propidium iodide (PI) and FITC-conjugated antibody to BrdU. The distribution of cells in different phases of the cell cycle was analyzed by flow cytometry (top panel) and percentage of cells (mean±SD, n=3) in G1, S and G2 phases was measured (bottom panel).

routinely cultured in the presence of IL-3. Upon removal of IL-3 cell proliferation was inhibited in the Baf3/Vector line while Baf3/p185 growth was unaffected and these cells underwent a single doubling during the 16 hours experimental time

course (Fig. 9 and 10). The combination of IL-3 removal and treatment with 20μM SP600125 drastically inhibited cell proliferation in Baf3/p185 and further exacerbate the reduction in cell number in Baf3/Vector cell line (Fig. 9B). This reduction in cell proliferation induced by SP600125 in the absence of IL-3 was due predominantly to a substantial increase in apoptosis as shown by the relative

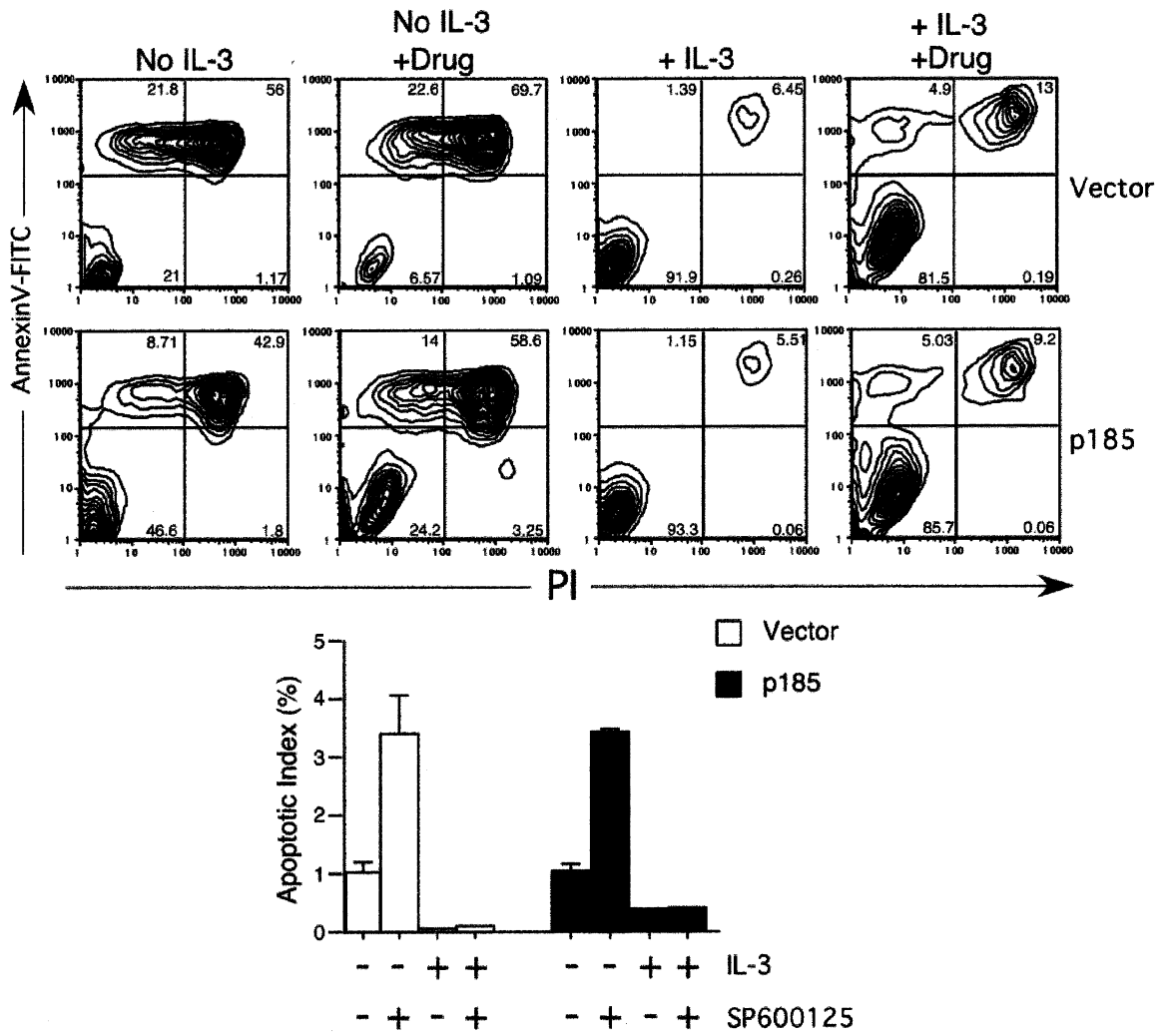


Fig.11 Cell death was analyzed by Annexin V assay of Baf3 stable cell lines (Vector and p185). Cells were stained with Annexin V-FITC and propidium iodide (PI) after 24 hour treatment with 20μM SP600125 (Drug) in the presence or absence of IL-3 and analyzed by flow cytometry (upper panels). The normalized ratio between the number of annexin-positive (apoptotic) cells and annexin-negative (live) cells in the PI negative population (apoptotic index) is presented (mean±SD, n=3;lower panel).

amount of Annexin V^+ cells in the drug treated samples compared to the untreated controls (Fig. 11). This increase in cell death induced by treatment with SP600125 in the absence of IL-3 also correlated with the expansion of the subG1 population observed in our cell cycle experiments (Fig 10 upper panels). These experiments provided evidence that the drug treatment in the absence of IL-3 did not affect the overall cell cycle distribution in either Baf3/Vector or Baf3/p185 cell lines (Fig. 10). The observed SP600125-induced apoptosis was rescued by the addition of IL-3 in both cell lines (Fig. 11). Taken together these data suggest that JNK is required for Bcr/Abl^{p185} induced growth-factor independent cell proliferation.

Although IL-3 completely rescued SP600125-induced apoptosis, it was interesting that both cell lines, Vector and p185, still showed reduced proliferation after drug treatment compared to DMSO-treated counterparts (Fig. 9B). Careful analysis of Baf3/Vector and Baf3/p185 cells revealed that SP600125 induced a G2 cell cycle arrest in the presence of IL-3 (Fig. 10). This observation suggested that SP600125 has dual effect in Baf3 cell lines: in the presence of IL-3 it arrests the cell cycle in G2 while in the absence of IL-3 it causes cell death. The possible causal relation between these two events needs further investigation.

III.I.D. The Dbl domain of Bcr/Abl^{p210} is sufficient to activate JNK

The transformation defect described in section III.I.A is the first reported functional difference between the two isoforms Bcr/Abl^{p210} and Bcr/Abl^{p185}. This prompted the analysis of the two isoforms in search of structural differences that would correlate with the observed phenotype. As previously mentioned in the Introduction (section I.II.B), Bcr/Abl^{p185} and Bcr/Abl^{p210} differ exclusively in the Bcr portion of the fusion oncogene. In particular, only Bcr/Abl^{p210} contains a region of the Bcr gene that includes a GEF domain with high homology to the Dbl proto-oncogene (Dbl domain). This "Dbl domain" includes two adjacent regions: a Dbl homology domain (DH) followed by a Pleckstrin homology domain (PH). The isolated Dbl domain of Bcr/Abl^{p210} has been shown to activate CDC42, a member of the Rho family of small GTPases (Korus et al., 2002). Since CDC42 can activate JNK (Coso et al., 1995; Minden et al., 1995) I sought to verify whether the Dbl domain derived from Bcr/Abl^{p210} could trigger the JNK signaling pathway. Overexpression of the Dbl domain in 293T cells induced

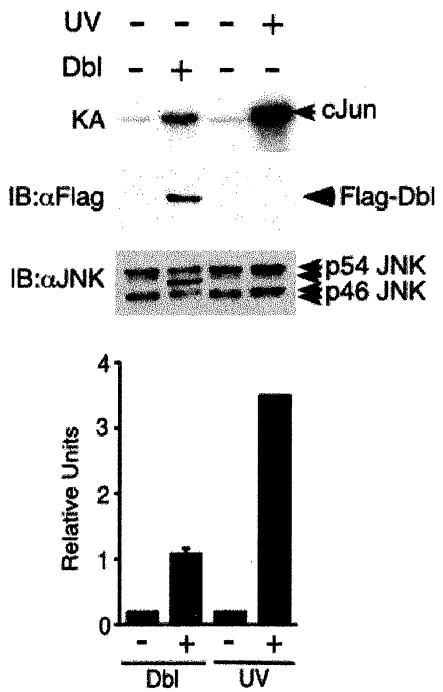


Fig.12 Expression of /BcrAbl^{p210} Dbl domain in 293T cells induces JNK activation.
JNK activity of cells expressing the Dbl domain of Bcr/Abl^{p210} (Flag-Dbl) was measured by *in vitro* kinase assay (KA) (mean±SD, n=3). Cells exposed in the absence or presence of 60 J/m^2 UV light were used as positive controls. Cell lysates were examined by immunoblot analysis using antibodies to the Flag epitope and JNK.

a 6.7 fold increase in JNK activity compared to vector alone (Fig.12). This result suggests that Bcr/Abl^{p210} and not Bcr/Abl^{p185} could activate JNK through its Dbl domain.

III.I.E. Conclusions

In this section I analyzed the requirement for JNK activity for Bcr/Abl induced transformation *in vitro*. The conclusions drawn from the data presented in this section can be summarized as follows:

- Both Bcr/Abl^{p210} and Bcr/Abl^{p185} require JNK activity to induce lymphoid transformation. While JNK1 alone plays a critical role in Bcr/Abl^{p210} induced pro-B cell growth *in vitro* (Fig. 7), JNK activity is required to elicit Bcr/Abl^{p185} stimulated GF-independent proliferation of Baf3 cells (Fig. 9 and Fig. 10). These results suggest that JNK might have a role in eliciting GF-independent proliferation in primary pro-B cells transformed by Bcr/Abl^{p185}
- The depletion of JNK activity does not affect the cell cycle in either Bcr/Abl^{p210} or Bcr/Abl^{p185} transformed lymphoblasts (Fig. 7D and Fig. 10), but it clearly induces apoptosis in Bcr/Abl^{p185} cell lines upon IL-3 removal (Fig. 11). Collectively, these data suggest a potential role of JNK in mediating a survival signal in Bcr/Abl transformed cells.

- The two Bcr/Abl isoforms have different dependency on JNK activity for transformation. This could be due to the presence in the Bcr/Abl^{p210} of the Dbl domain. I show that this domain can trigger JNK activation *in vitro* (Fig. 10). Thus, the presence of the Dbl domain may link the JNK pathway to the pathogenesis caused by the Bcr/Abl^{p210} oncogene. In contrast, Bcr/Abl^{p185} might compensate the lack of the Dbl-activated pathway through the engagement of alternative pathways. Thus complete depletion of JNK activity impairs Bcr/Abl^{p185} ability to transform cells.

III.II. *In vivo* Approach

The *in vitro* experiments described in the previous section indicated a potential role for JNK in sustaining survival of Bcr/Abl transformed cells. I next sought to examine the physiological relevance of the *in vitro* results using two mouse models of Bcr/Abl induced disease that would give rise to either lymphoid or myeloid leukemia. These experiments could not be performed in a JNK null background since *Jnk1-/- Jnk2-/-* mice are not viable and *in vivo* treatment with the JNK inhibitory drug (SP600125) might induce secondary toxic effects. Nevertheless, based on the observation that JNK1 is the predominant isoform in Bcr/Abl transformed lymphoblast (section III.I.A) and it is critical for Bcr/Abl^{p210} induced transformation *in vitro* (section III.I.B) I used *Jnk1-/-* cells to examine the role of JNK in Bcr/Abl induced leukemia. In section III.II.A, I present the results obtained using an *in vivo* model for Bcr/Abl induced lymphoid leukemia. These experiments indicated that JNK1 has a role in Bcr/Abl^{p210} and not Bcr/Abl^{p185} induced lymphoid leukemia. In particular, Bcr/Abl^{p210} transformed lymphoblasts were unable to infiltrate peripheral organs in the absence of JNK1. Additional analysis suggested that JNK1 might have a role in providing a survival signal in Bcr/Abl^{p210} transformed lymphoblasts (section III.II.B). This signal seemed to be mediated in part by the upregulation of Bcl2 expression. Based on these observations I tried to rescue the lymphoid transformation defect caused by

JNK1 deficiency using transgenic mice that overexpress Bcl2 in the B cell compartment. The results of these experiments are described in section III.II.B. Finally, since Bcr/Abl^{p210} is mainly associated with human myeloid leukemia, I examined the role of JNK1 in Bcr/Abl^{p210} induced myeloid leukemia using a mouse model for Bcr/Abl-induced myeloproliferative disease (section III.II.C).

III.II.A. Analysis of the effect of JNK1 disruption on Bcr/Abl induced lymphoid leukemia

To test the role of JNK1 in transformation of pre-B cells by Bcr/Abl *in vivo*, I used a method that has been previously described to cause pre-B cell transformation in mice (Daley et al., 1990; Van Etten, 2001a; Van Etten, 2001b). I reconstituted lethally irradiated mice with bone marrow derived from wild-type and *Jnk1-/-* donors transduced with retroviral vectors. The effect of Bcr/Abl^{p210} and Bcr/Abl^{p185} expression was examined. Control studies were performed with the empty vector which expresses GFP from an internal ribosome entry site. The development of leukemia occurred with an initial expansion of a homogeneous population of immature blasts evident in the femur sections of mice transplanted with Bcr/Abl transduced bone marrow, but not vector transduced bone marrow (Fig. 13A). At the terminal stage of leukemia the bone marrow of these animals showed increased stromal cells and vascularity with extensive fibrosis (Fig. 13B)

resembling the spent phase typical of myeloproliferative diseases. Mice transplanted with bone marrow transduced with empty vector (GFP retrovirus) did not develop leukemia during the period of experimental analysis (100 days) and normal reconstitution of the bone marrow was observed (Fig. 13A, B). No marked differences in the histology of femur (Fig. 13B) or the number of proviral integrations in bone marrow genomic DNA was detected between mice transplanted with wild-type and *Jnk1-/-* bone marrow cells by Bcr/Abl *in vivo.*

Immunophenotyping of bone marrow from mice transplanted with Bcr/Abl^{p210} transduced *Jnk1-/-* marrow revealed the presence of two distinct populations that express GFP (Fig. 13C). The population with higher GFP intensity expressed exclusively B-cell markers (B220 and CD19) while the lower intensity GFP cells expressed myeloid and lymphoid markers (Gr-1, CD11b/Mac-1, CD19 and B220)(Fig. 13C). Both populations were negative for the erythroid lineage marker Ter119 and for the T cell surface antigen Thy1.2, and the B cell antigens Sca-1, IgA IgG and IgM. This analysis indicated that Bcr/Abl caused leukemia with both lymphoid and myeloid components. Similar results were obtained from the analysis of wild-type and *Jnk1-/-* bone marrow in experiments using Bcr/Abl^{p210} and Bcr/Abl^{p185} (Fig. 13C and data not shown). I confirmed that the disease observed was leukemia by secondary transplantation assays. Together, these data demonstrate that Bcr/Abl^{p210} and Bcr/Abl^{p185} caused a

similar bone marrow disease (mixed lymphoid and myeloid leukemia) in mice transplanted with wild-type and *Jnk1-/-* bone marrow (Fig. 13). This conclusion

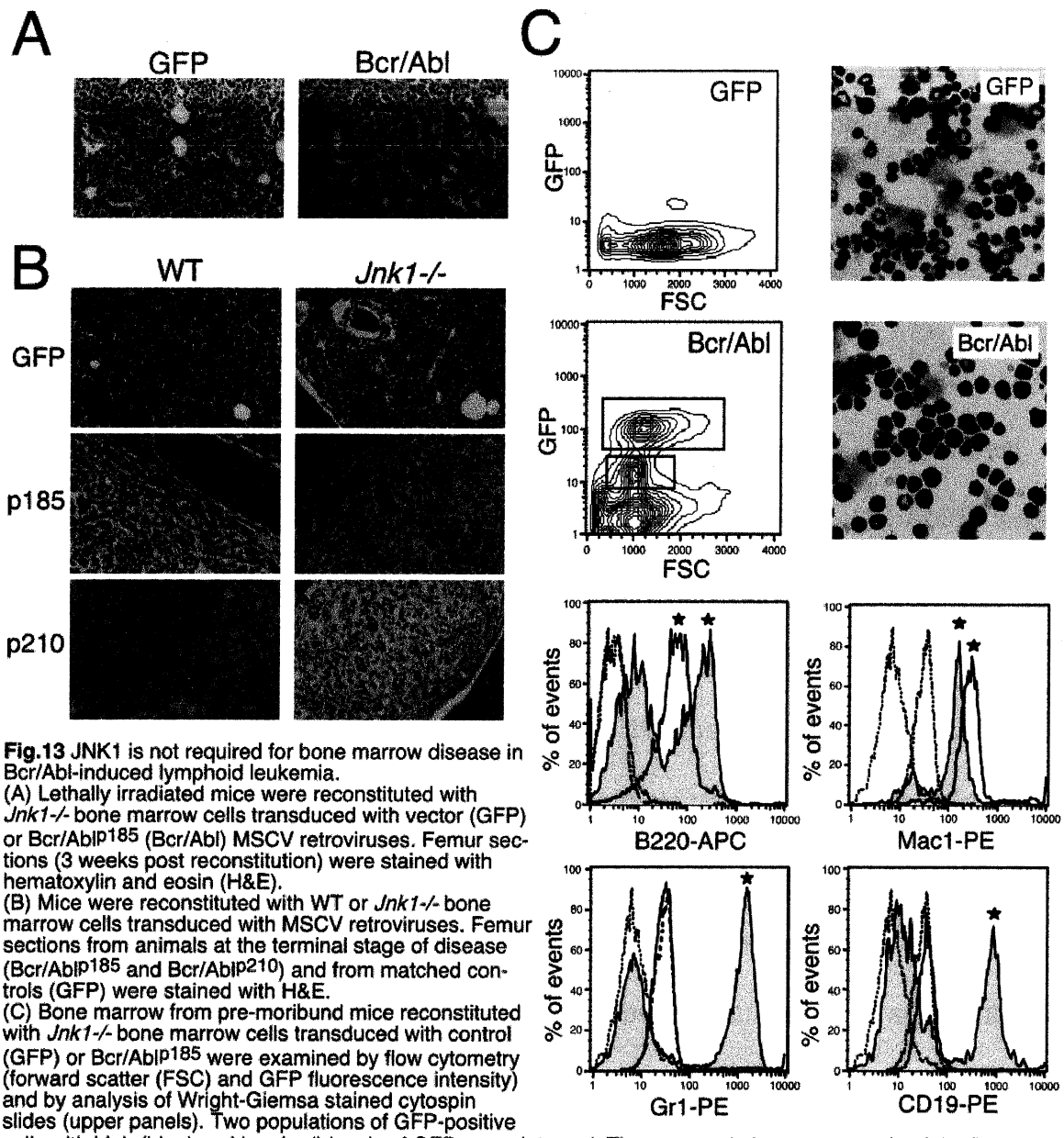


Fig.13 JNK1 is not required for bone marrow disease in Bcr/Abl-induced lymphoid leukemia.
(A) Lethally irradiated mice were reconstituted with *Jnk1-/-* bone marrow cells transduced with vector (GFP) or Bcr/Abl^{p185} (Bcr/Abl) MSCV retroviruses. Femur sections (3 weeks post reconstitution) were stained with hematoxylin and eosin (H&E).
(B) Mice were reconstituted with WT or *Jnk1-/-* bone marrow cells transduced with MSCV retroviruses. Femur sections from animals at the terminal stage of disease (Bcr/Abl^{p185} and Bcr/Abl^{p210}) and from matched controls (GFP) were stained with H&E.
(C) Bone marrow from pre-moribund mice reconstituted with *Jnk1-/-* bone marrow cells transduced with control (GFP) or Bcr/Abl^{p185} were examined by flow cytometry (forward scatter (FSC) and GFP fluorescence intensity) and by analysis of Wright-Giemsa stained cytospin slides (upper panels). Two populations of GFP-positive cells with high (blue) and low (red) levels of GFP were detected. These popoulation were examined by flow cytometry for the surface markers B220, Mac-1, Gr-1 and CD19 (lower panels).Cell populations positive for these cell surface markers(☆)were identified by comparison with unstained cells (dotted lines).
Cells expressing low levels of GFP(red) were positive for B220, Mac-1,Gr-1and CD19. Similar data were obtained in experiments with Bcr/Abl^{p185} and Bcr/Abl^{p210} and also experiments using WT and *Jnk1-/-* cells.

markedly contrasts with the results of *in vitro* assay that demonstrate an essential requirement of JNK1 for pre-B cell transformation (Fig.7).

Bcr/Abl-induced leukemia is an invasive disease. Malignant cell expansion in the bone marrow precedes increased white blood cell number and the subsequent infiltration of peripheral organs, including the spleen and liver. A consequence of this malignant infiltration is organomegaly. Thus, mice reconstituted with wild-type marrow transduced with Bcr/Abl^{p210} or Bcr/Abl^{p185} or *Jnk1-/-* marrow transduced with Bcr/Abl^{p185} showed a marked enlargement of the spleen (n=28) compared to GFP vector controls (n=22) (Fig. 14A). In contrast, the spleen mass of animals with *Jnk1-/-* marrow transduced with Bcr/Abl^{p210} was similar to GFP vector control animals (Fig. 14A). These data indicate that JNK1-deficiency caused a selective defect in splenomegaly caused by Bcr/Abl^{p210}.

The absence of splenomegaly suggests that there might be a defect in the malignant infiltration of peripheral organs of animals transplanted with *Jnk1-/-* bone marrow transduced with Bcr/Abl^{p210}. To test this hypothesis, I performed histological examination of the spleen and liver. Malignant infiltration in the white and red pulp of the spleen was classified as follows: Infiltrated, with substantial infiltration of both the white and red pulp; Partial, with infiltration of either the white or red pulp; and Minimal, with small foci or no malignant cells. Malignant infiltration in the liver was classified as follows: Infiltrated, which showed

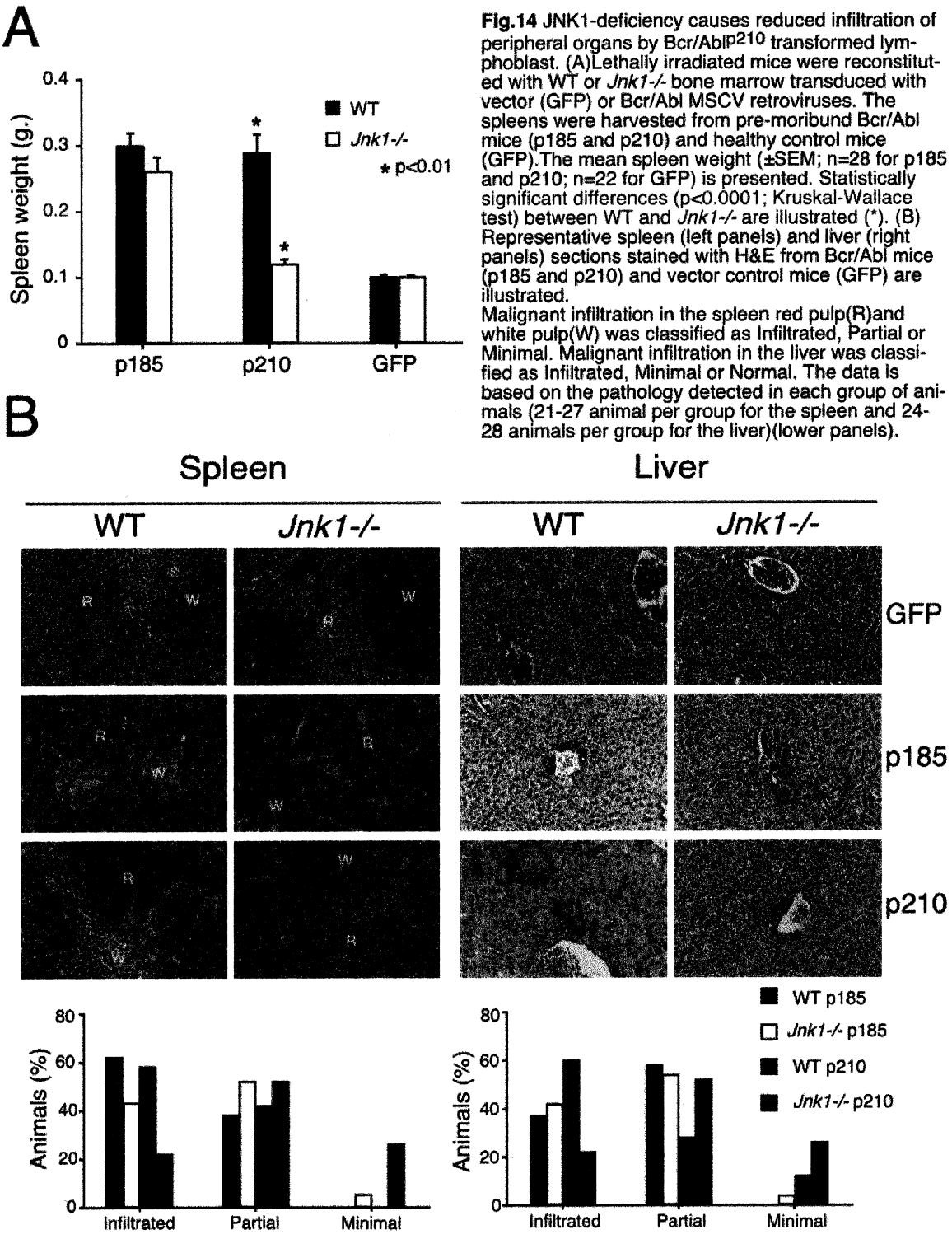


Fig.14 JNK1-deficiency causes reduced infiltration of peripheral organs by Bcr/Abl^{p210} transformed lymphoblast. (A)Lethally irradiated mice were reconstituted with WT or *Jnk1-/-* bone marrow transduced with vector (GFP) or Bcr/Abl MSCV retroviruses. The spleens were harvested from pre-moribund Bcr/Abl mice (p185 and p210) and healthy control mice (GFP).The mean spleen weight (±SEM; n=28 for p185 and p210; n=22 for GFP) is presented. Statistically significant differences ($p<0.0001$; Kruskal-Wallace test) between WT and *Jnk1-/-* are illustrated (*). (B) Representative spleen (left panels) and liver (right panels) sections stained with H&E from Bcr/Abl mice (p185 and p210) and vector control mice (GFP) are illustrated.
Malignant infiltration in the spleen red pulp(R)and white pulp(W) was classified as Infiltrated, Partial or Minimal. Malignant infiltration in the liver was classified as Infiltrated, Minimal or Normal. The data is based on the pathology detected in each group of animals (21-27 animal per group for the spleen and 24-28 animals per group for the liver)(lower panels).

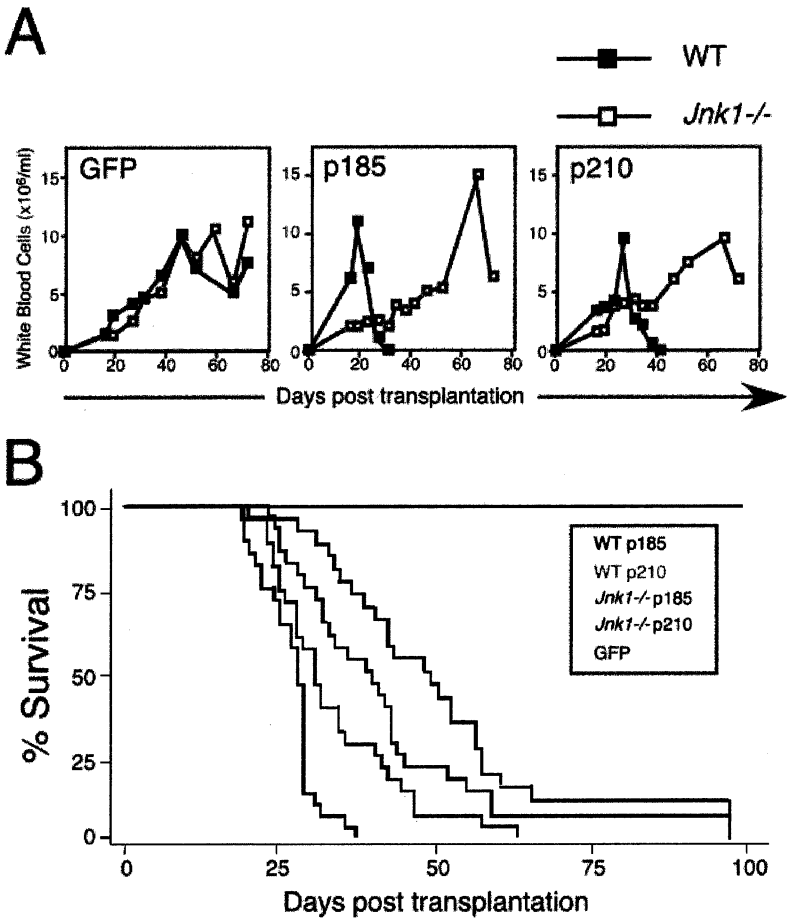


Fig. 15 JNK1-deficiency increases Bcr/Abl lymphoid disease latency. (A) Lethally irradiated mice were reconstituted with WT or *Jnk1-/-* bone marrow cells transduced with vector (GFP) or Bcr/Abl MSCV retrovirus. Peripheral white blood cell (WBC) numbers in Bcr/Abl (p185 and p210) and vector control (GFP) reconstituted mice were measured. The data represent the mean count determined in each group (ten mice per group at day 0). Similar results were obtained in two independent experiments. (B) Survival of lethally irradiated mice receiving transduced bone marrow cells (Kaplan-Meier). The survival data are cumulative from three independent experiments. There were 28 animals in each Bcr/Abl experimental group and 22 animals in each control group (WT GFP and *Jnk1-/-* GFP). The prolonged survival of mice reconstituted with Bcr/Abl (p210 and p185) transduced *Jnk1-/-* bone marrow is statistically significant compared to the survival of mice with WT bone marrow transduced with Bcr/Abl (log-rank test $p<0.0002$).

perivascular and sinusoidal infiltration; Minimal, with small perivascular foci; and Normal, with no malignant cells detected. The histological analysis confirmed that there was reduced infiltration of the spleen and liver (Fig. 14B) of mice transplanted with *Jnk1-/-* marrow transduced with Bcr/Abl^{p210}. However, JNK1-deficiency did not alter the extent of malignant infiltration caused by Bcr/Abl^{p185}. These data indicated that JNK1-deficiency alters the leukemic disease load caused by Bcr/Abl^{p210}, but not that caused by Bcr/Abl^{p185}. This altered form of leukemia was characterized by the

proliferation of malignant cells in the bone marrow, but failure of these malignant cells to efficiently infiltrate peripheral organs.

To examine the cause of the reduced infiltration of peripheral organs by malignant *Jnk1-/-* Bcr/Abl^{p210} cells. I investigated the presence of these cells in the blood. The number of white blood cells (WBC) in the peripheral blood of lethally irradiated mice reconstituted with bone marrow transduced with the empty vector increased gradually with time and was not affected by JNK-deficiency (Fig. 15A). In contrast, Bcr/Abl^{p185} and Bcr/Abl^{p210} caused a rapid increase in the number of WBC within 2-3 weeks after reconstitution (Fig. 15A). A rapid decline in WBC number was observed during the terminal phase of the disease (Fig. 15A). This decrease correlates with bone marrow failure (Fig. 13B). JNK-deficiency delayed the increase in WBC number caused by Bcr/Abl^{p185} and by Bcr/Abl^{p210} (Fig. 15A). These data are consistent with the hypothesis that JNK1 –deficiency selectively affects Bcr/Abl^{p210} malignant cells in the peripheral organs of mice.

Although JNK-deficiency caused changes in the observed leukemia, all animals reconstituted with Bcr/Abl transduced bone marrow developed leukemia and died (Fig. 15B). JNK-deficiency caused a statistically significant delay in disease mortality (log-rank test $p<0.0002$). This delay correlates with the delayed kinetics of WBC accumulation in the peripheral blood (Fig. 15A). The major cause of death was most likely due to bone marrow failure (Fig. 13B and 15A).

III.II.B. JNK1 provides a survival signaling in Bcr/Abl^{p210} lymphoblast

JNK-deficiency was found to cause no apparent defects in the cell cycle of Bcr/Abl^{p210} transformed cells *in vitro* (Fig. 7D). Nevertheless, these cells failed to accumulate (Fig. 7B). This *in vitro* defect correlates with the observation that *Jnk1-/-* Bcr/Abl^{p210} cells did not accumulate in peripheral organs *in vivo* (Fig. 14). One mechanism that could account for the failure to proliferate is increased apoptosis. Thus, JNK1 may provide a necessary survival signal.

The activation of anti-apoptotic pathways, including the Akt pathway and the expression of Bcl2-related proteins, is closely linked to the oncogenic potential of Bcr/Abl. I found similar protein levels of the pro-survival kinase Akt in wild-type and *Jnk1-/-* cells but detected a modest reduction in Akt activation in *Jnk1-/-* bone marrow transduced with Bcr/Abl^{p210}. No difference in Akt activation was observed in Bcr/Abl^{p185} transduced cells (Fig. 16A). The anti-apoptotic Bcl2 family member Bcl-X_L is implicated in several hematopoietic malignancies and it is known that Bcr/Abl increases Bcl-X_L expression, in part by the Stat5 pathway (see Introduction section I.II.C). However, immunoblot analysis failed to show any differences in the expression of Bcl-X_L protein or mRNA (Fig. 16C). In contrast, JNK-deficiency caused marked decrease in Bcl2 (Fig. 16B) and BclW expression (Fig.16C). No change in the expression of Bax, Bak or Bad was detected (Fig. 16C). Decreased expression of Bcl2 and BclW together with a modest reduction in Akt activity, may therefore contribute to the decreased

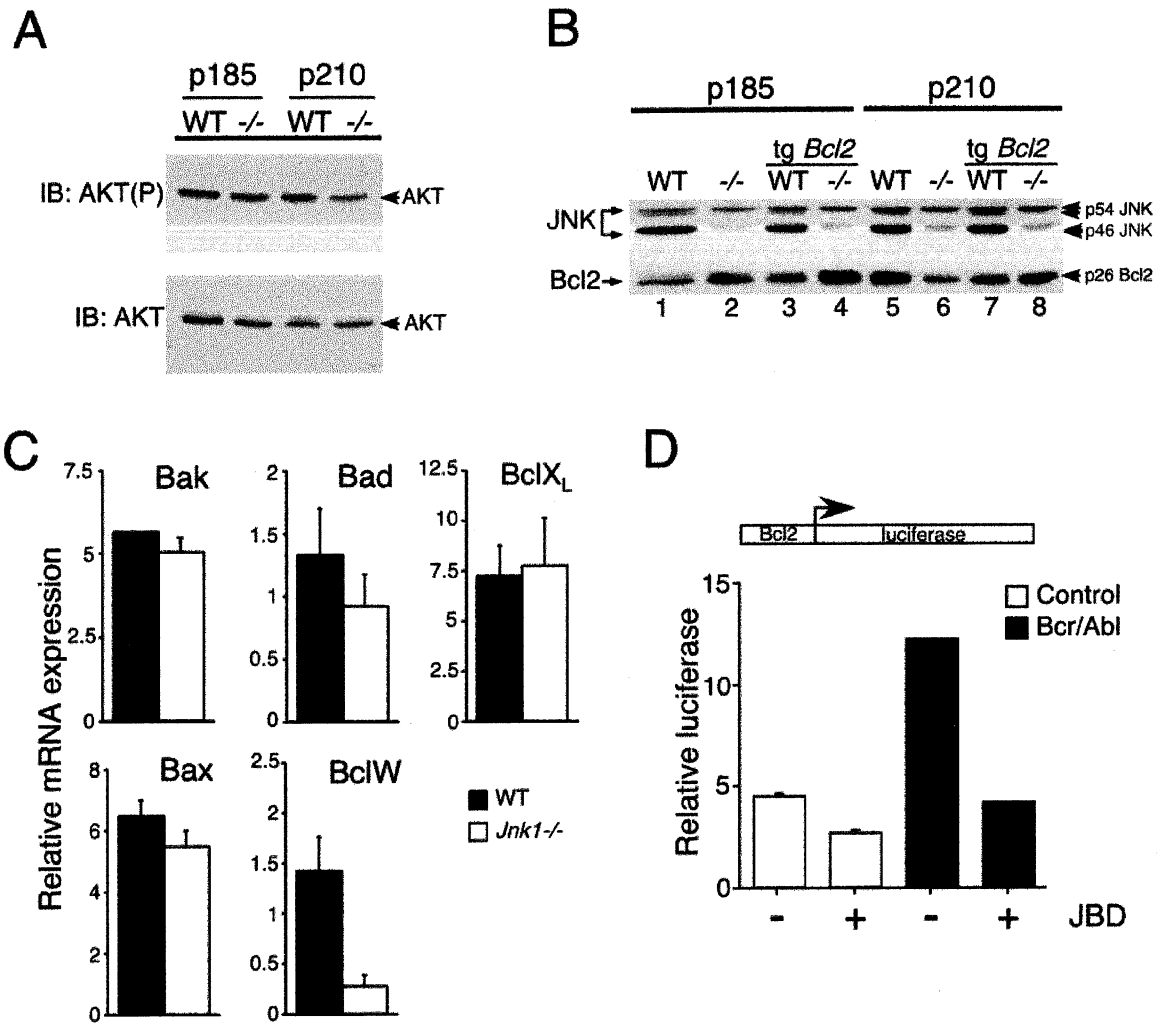


Fig.16 JNK1 deficiency causes decreased expression of Bcl2.
(A) Bone marrow from WT and *Jnk1-/-* and (B) WT and *Bcl2* transgenic mice was transduced *in vitro* with Bcr/Abl retroviruses (p210 and p185). Transformed lymhoblast were examined by immunoblot (IB) analysis with antibodies to (A) AKT, phospho-Ser473 AKT (AKT(P)), (B) JNK and Bcl2. (C) Ribonuclease protection assays were carried out to measure the gene expression of the Bcl2-related family members and glyceraldehyde 3-phosphate dehydrogenase (GADPH). The mRNA expression profile compared with the GADPH is presented as the mean±SD (n=3). (D) *Bcl2* promoter activity was examined in firefly luciferase reporter assays in Baf3 cells. Transfection efficiency was monitored using a *R.reniformis* luciferase reporter plasmid. The cells were cotrasfected with an expression vector encoding the JNK inhibitor JBD and the *Bcl2* promoter firefly luciferase reporter plasmid. The data presented represent the mean±SD (n=3).

survival of Bcr/Abl^{p210} transformed *Jnk1-/-* lymphoblast.

To examine the mechanism of reduced Bcl2 expression caused by JNK-deficiency, I investigated *Bcl2* promoter activity with a luciferase reporter gene assay. Expression of Bcr/Abl^{p210} caused increased *Bcl2* promoter activity (Fig.

16D) this increase was greatly attenuated by expression of JBD, a dominant inhibitor of JNK. These results indicate that Bcr/Abl^{p210} causes a JNK-dependant increase in *Bcl2* promoter activity. The JNK protein may also increase *Bcl2* expression by inhibiting the ubiquitin-mediated degradation of Bcl2 (Breitschopf et al., 2000).

The observed decrease in *Bcl2* expression caused by JNK-deficiency in Bcr/Abl^{p210} transformed cells (Fig. 16B) may be functionally significant. To test this hypothesis, I examined the effect of transgenic expression of Bcl2 in mice. Immunoblot analysis demonstrated that the transgene restores the expression of Bcl2 protein in *Jnk1-/-* Bcr/Abl^{p210} cells (Fig. 16B; compare lanes 7 and 8). Control studies indicated that the expression of JNK2 was unaffected by the *Bcl2* transgene, JNK-deficiency, and whether Bcr/Abl^{p210} or Bcr/Abl^{p185} was expressed (Fig. 16B). Together, these data indicate that JNK1-deficiency causes decreased *Bcl2* expression in Bcr/Abl^{p210} transformed cells and that this decrease can be compensated by transgenic expression of *Bcl2 in vivo*.

Bone marrow transplantation assays were performed to examine the effect of the transgenic expression of *Bcl2* on Bcr/Abl induced disease. Lethally irradiated mice were transplanted with bone marrow isolated from non-transgenic and *Bcl2* transgenic mice. Mice transplanted with bone marrow transduced with a GFP retrovirus (Vector) did not develop leukemia and histological examination demonstrated no pathology. These data demonstrated that the level of

transgenic *Bcl2* expression was not sufficient to cause proliferative disease during the period of experimental analysis (100 days). In contrast, all animals transplanted with bone marrow transduced with retroviruses expressing Bcr/Abl (p210 or p185) developed disease. No statistically significant difference in splenomegaly was observed between mice transplanted with WT or *Jnk1-/-* bone marrow from *Bcl2* transgenic animals (Fig. 17A). Histological examination demonstrated a similar amount of malignant infiltration by *WT Bcl2* and *Jnk1-/- Bcl2* cells caused by Bcr/Abl^{p210} or Bcr/Abl^{p185} in the liver (Fig. 17B) and spleen. The extent of infiltration by the transformed *Bcl2* transgenic cells was greater than non –transgenic cells (compare Fig. 14B and 17B). However no significant difference between WT and *Jnk1-/-* groups of *Bcl2* transgenic cells was detected. Thus, JNK-deficiency caused no change in liver infiltration by *Bcl2* transgenic Bcr/Abl^{p210} cells (Fig. 17B). This observation is in marked contrast to the severe defect in malignant infiltration caused by JNK-deficiency in non-transgenic cells caused by Bcr/Abl^{p210} (Figure 14B). Together, these data demonstrated that the *Bcl2* transgene rescues the defect in the infiltration of peripheral organs caused by JNK-deficiency.

I next examined the white blood cell (WBC) number in the peripheral blood of mice transplanted with *Bcl2* transgenic bone marrow. Compared with non-transgenic WBC (Fig. 15A), the number of *Bcl2* transgenic WBC in the transplanted mice was larger (Fig. 17C). This is consistent with the anti-

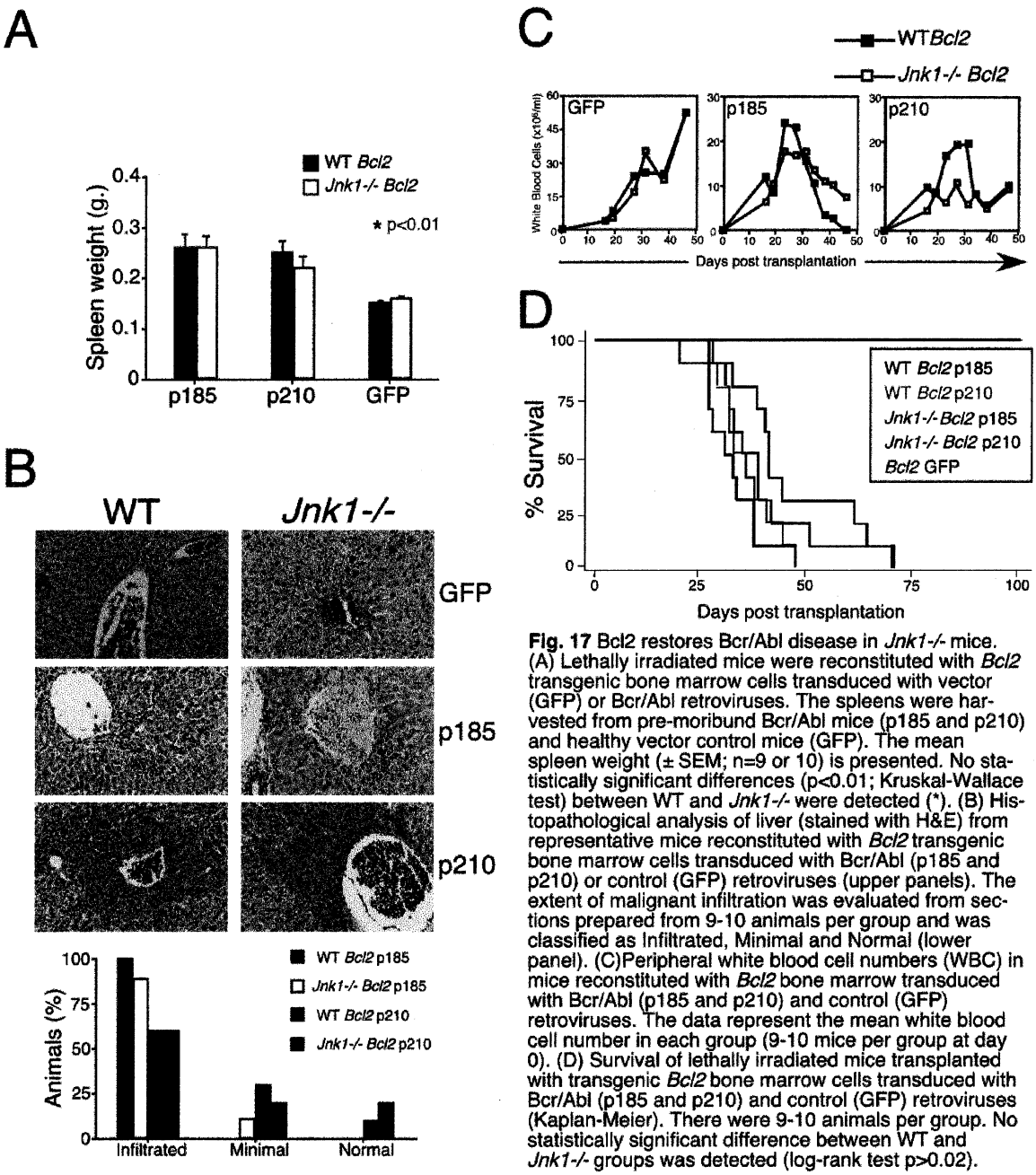


Fig. 17 Bcl2 restores Bcr/Abl disease in *Jnk1-/-* mice. (A) Lethally irradiated mice were reconstituted with *Bcl2* transgenic bone marrow cells transduced with vector (GFP) or Bcr/Abl retroviruses. The spleens were harvested from pre-moribund Bcr/Abl mice (p185 and p210) and healthy vector control mice (GFP). The mean spleen weight (± SEM; n=9 or 10) is presented. No statistically significant differences ($p<0.01$; Kruskal-Wallace test) between WT and *Jnk1-/-* were detected (*). (B) Histopathological analysis of liver (stained with H&E) from representative mice reconstituted with *Bcl2* transgenic bone marrow cells transduced with Bcr/Abl (p185 and p210) or control (GFP) retroviruses (upper panels). The extent of malignant infiltration was evaluated from sections prepared from 9-10 animals per group and was classified as Infiltrated, Minimal and Normal (lower panel). (C)Peripheral white blood cell numbers (WBC) in mice reconstituted with *Bcl2* bone marrow transduced with Bcr/Abl (p185 and p210) and control (GFP) retroviruses. The data represent the mean white blood cell number in each group (9-10 mice per group at day 0). (D) Survival of lethally irradiated mice transplanted with transgenic *Bcl2* bone marrow cells transduced with Bcr/Abl (p185 and p210) and control (GFP) retroviruses (Kaplan-Meier). There were 9-10 animals per group. No statistically significant difference between WT and *Jnk1-/-* groups was detected (log-rank test $p>0.02$).

apoptotic function of Bcl2. Time course analysis demonstrated that the delayed Bcr/Abl stimulated increase in peripheral WBC number caused by JNK-deficiency (Fig. 15A) was not detected in mice transplanted with *Bcl2* transgenic bone marrow (Fig. 17D). This observation indicated that Bcl2 complements the effect

of JNK-deficiency on Bcr/Abl disease. Indeed, the effect of JNK-deficiency on delayed disease mortality (Fig. 15B) was not observed in mice transplanted with *Bcl2* transgenic bone marrow transduced with Bcr/Abl (Fig. 17D). These data provide further confirmation that the restoration of *Bcl2* expression in JNK-deficient cells rescues the *Jnk1-/-* defect in Bcr/Abl leukemogenesis.

III.II.C. JNK1 is required for Bcr/Abl^{p210} lymphoid leukemia but is dispensable for the development of myeloid disease.

In humans, the presence Bcr/Abl^{p210} is principally correlated with the onset of CML, a myeloproliferative disease. Since the results described in the previous section were obtained in a "mixed type" leukemia (lymphoid and myeloid), I sought to investigate the role of JNK1 in Bcr/Abl transformation using an experimental procedure that would induce the development of a myeloproliferative disease in the transplanted mice (Ren, 2002; Van Etten, 2002). Mice reconstituted with bone marrow transduced with Bcr/Abl^{p210} using this new procedure developed leukemia within 15 days. Immunophenotyping of whole bone marrow from the transplanted animals showed the expansion of a single population of GFP^{+} cells that were positive for the myeloid markers CD43, Mac-1 and Gr-1 and negative for B220, CD19 and Thy1.2 (Fig.18A). These results confirmed the onset of a myeloproliferative disease. All recipients

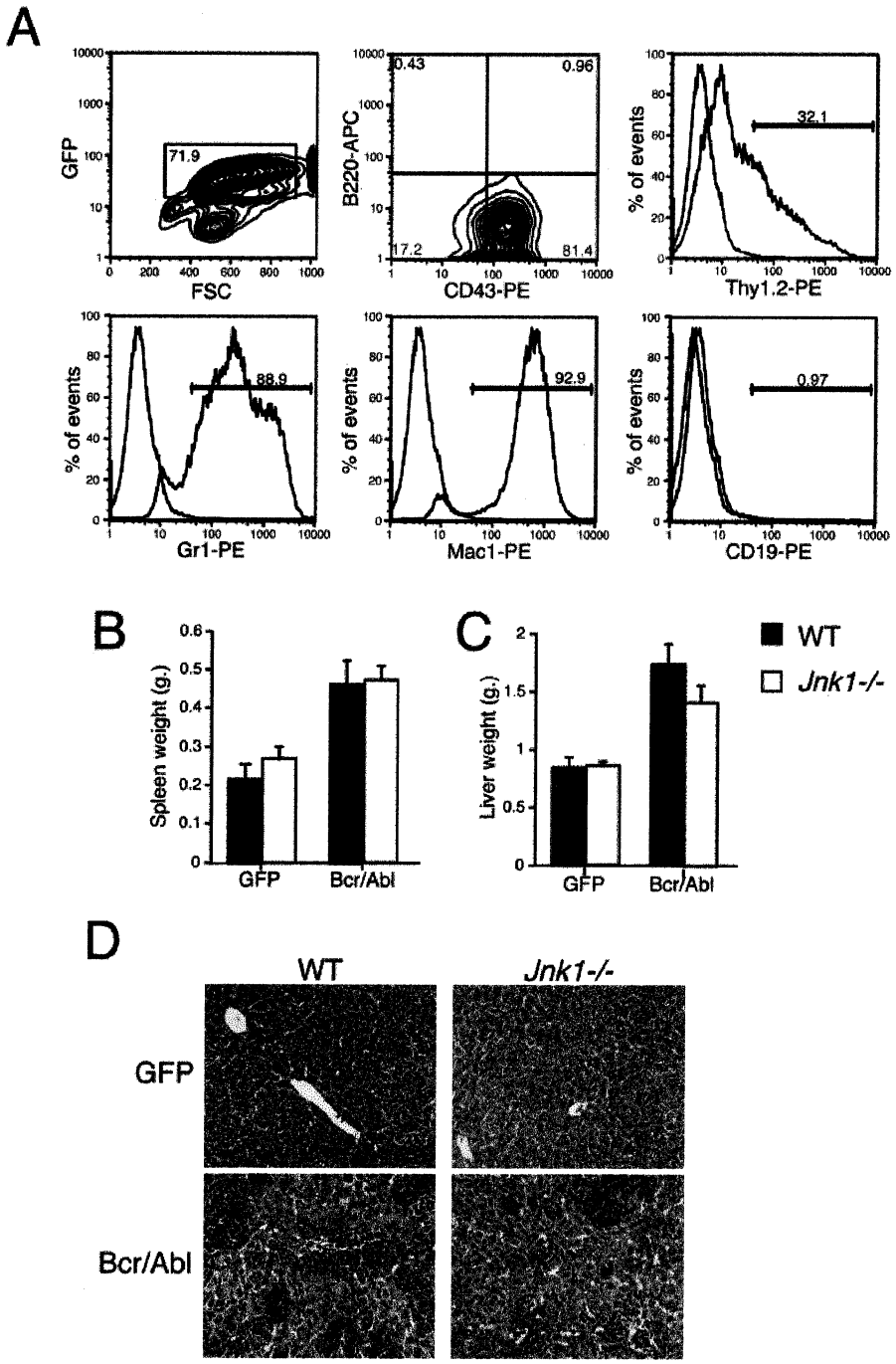


Fig. 18 JNK1 is not required for Bcr/Abl-induced myeloproliferative disease.
(A) Bone marrow cells from mice reconstituted with 5-fluorouracil (5-FU) treated *Jnk1-/-* bone marrow transduced with Bcr/Abl^{p210} retrovirus were examined by flow cytometry. A single GFP-positive population was detected and examined for the indicated surface markers (B220, CD-43, Thy1.2, Gr-1, Mac-1 and CD-19). Cell populations positive for these surface markers (blue lines) were identified by comparison with unstained cells (red lines). Similar data was obtained in experiments with WT and *Jnk1-/-* cells. (B, C) Lethally irradiated mice were reconstituted with 5-FU treated WT or *Jnk1-/-* bone marrow transduced with vector (GFP) or Bcr/Abl^{p210} retroviruses. Spleens and livers were harvested from all recipients (GFP and Bcr/Abl) 15 days post reconstitution. The mean weight (±SD; n=10 for each group) of these organs is presented.(D) Representative liver sections (stained with H&E) from Bcr/Abl^{p210} and vector control (GFP) mice are illustrated.

transplanted with bone marrow transduced with Bcr/Abl^{p210} showed a marked enlargement of the spleen and liver (n=10) compared to GFP vector controls (n=10) (Fig.18B). No significant difference in splenomegaly (Fig. 18B) or hepatomegaly (Fig.18C) was observed between WT and *Jnk1-/-* transplanted animals. Histological examination demonstrated a similar amount of malignant infiltration by WT and *Jnk1-/-* cells caused by Bcr/Abl^{p210} in the liver (Fig. 18D) and lungs, while GFP vector controls showed no infiltration in these tissues. In contrast, infiltrated cells were found in the spleen of all recipients reconstituted with bone marrow transduced with GFP vector control. Due to this background infiltration it was impossible to reliably evaluate the extent of Bcr/Abl^{p210} induced malignant infiltration in this organ. However no significant difference in liver and lung infiltration was detected between the WT and *Jnk1-/-* groups. Taken together these results indicate that JNK1 is not required for Bcr/Abl^{p210} induced myeloproliferative disease.

III.II.D. Conclusions

In this section I examined the requirement of JNK1 in Bcr/Abl induced leukemia. For this purpose, I used two different experimental procedures to induce either lymphoid or myeloid disease in mice. The results obtained in the experiments using the mouse model for Bcr/Abl-induced lymphoid leukemia support the following conclusions:

- JNK1 plays a critical role in Bcr/Abl^{p210} induced lymphoid leukemia. Mice reconstituted with Bcr/Abl^{p210} transformed *Jnk1-/-* bone marrow cells are defective in peripheral organ infiltration.
- This defect in malignant infiltration might cause the delay in disease mortality observed in recipients transplanted with Bcr/Abl^{p210} transduced *Jnk1-/-* cells.
- Despite the defect in malignant infiltration *Jnk1-/-* lymphoblast transformed with Bcr/Abl^{p210} showed normal proliferation in the bone marrow indicating that while JNK1 is not required for Bcr/Abl^{p210} initial transformation event *per se* it is critical for the maintenance of the malignant lymphoblasts.
- The defect in malignant infiltration in the peripheral organs is evident in transplanted animals receiving Bcr/Abl^{p210} but not in Bcr/Abl^{p185} transduced *Jnk1-/-* bone marrow cells. This observation supports the hypothesis obtained in our *in vitro* studies that JNK1 plays a critical role in the transforming ability of Bcr/Abl^{p210}.
- Biochemical analysis of Bcr/Abl^{p210} *Jnk1-/-* lymphoblasts showed reduced expression of several anti-apoptotic factors including Bcl2. Indeed, JNK can mediate Bcr/Abl^{p210}-induced Bcl2 transcriptional upregulation (Fig. 16D). Accordingly, transgenic expression of Bcl2 in the lymphoid compartment rescues the defect in the malignant infiltration of peripheral organs and also abolishes the delay in disease mortality (Fig.17).

- The fact that Bcl2 efficiently rescues JNK1 defect in Bcr/Abl^{p210} induced leukemogenesis suggests that JNK1 is providing a critical survival signal in Bcr/Abl^{p210} transformed lymphoblasts.

The results obtained using the mouse model for Bcr/Abl-induced myeloid leukemia suggest that:

- JNK1 is dispensable for the development of Bcr/Abl^{p210} induced myeloproliferative disease. All recipient mice reconstituted with bone marrow transduced with Bcr/Abl^{p210} retrovirus develop comparable organomegaly (Fig. 18) within the same time frame.
- JNK1 is not essential for organ invasion by Bcr/Abl^{p210} transformed myeloid cells (Fig. 18). Indeed, all mice reconstituted with bone marrow transduced with Bcr/Abl^{p210} show similar extent of malignant infiltration in the liver (Fig.18) and lungs.
- Taken together these observations suggest that JNK1 has an essential role in Bcr/Abl induced lymphoid disease while it is dispensable for development of "myeloid" leukemia.

III.III. *Ex vivo* Approach

The observations obtained with the "lymphoid leukemia" model described in the previous section indicated that Bcr/Abl^{p210} transformed *Jnk1-/-* lymphoblasts were able to proliferate and expand in the bone marrow but are absent in the blood and failed to invade peripheral organs. The inability of Bcr/Abl^{p210} transformed lymphoblasts to leave the bone marrow microenvironment was rescued by the ectopic expression of the anti-apoptotic Bcl2 protein. Taken together these observations suggested that JNK1 is providing an essential survival signal that enables the Bcr/Abl^{p210} transformed lymphoid cells to proliferate outside the bone marrow microenvironment. One critical step that allows transformed lymphoblast to survive in the bloodstream and to invade peripheral organs is the ability to proliferate in the absence of growth factors normally provided by the bone marrow. This prompted me to examine the growth factor dependency of Bcr/Abl transformed lymphoblast derived from the transplanted mice described in section III.II.A and B. The derived "ex vivo" cell lines were cultured in the presence or absence of S17 stromal layers which mimics the bone marrow microenvironment. Under these conditions I examined Bcr/Abl induced growth factor independent proliferation. The results of these experiments are illustrated in this section.

III.III.A. JNK1 is required for Bcr/Abl^{p210} –induced stromal layer independence

Wild-type and *Jnk1-/-* cells expressing Bcr/Abl^{p185} or Bcr/Abl^{p210} were obtained from mice transplanted with non–transgenic and Bcl2-transgenic bone marrow. Flow cytometry analysis demonstrated that the cells obtained were B-lymphoblast expressing cell surface B220, CD19, BP-1 and negative for Gr-1, Mac-1/CD11b, Sca-1, Ter119, Thy1.2 (Fig. 19A). No significant difference between wild-type and *Jnk1-/-* was detected by flow cytometry. Immunoblot analysis demonstrated that these cells expressed similar levels of total Bcr/Abl, tyrosine phosphorylated Bcr/Abl and JNK2 (Fig. 20D). As expected, JNK1 was detected in all WT cells, but not in *Jnk1-/-* cells.

We investigated the proliferation of these cells in the presence and absence of stroma. DNA synthesis was examined by measurement of the incorporation of BrdU. There was no marked difference detected between cell lines with different *Jnk1* genotype grown in the presence or absence of stroma (Fig. 19B). Similar results were obtained for *Bcl2* transgenic cells (Fig. 19C). The incorporation of BrdU was also similar in experiments using cells transformed with Bcr/Abl^{p210} and Bcr/Abl^{p185} (Fig. 19B and C). These observations indicated that JNK1-deficiency and transgenic *Bcl2* expression did not alter DNA synthesis by these cells.

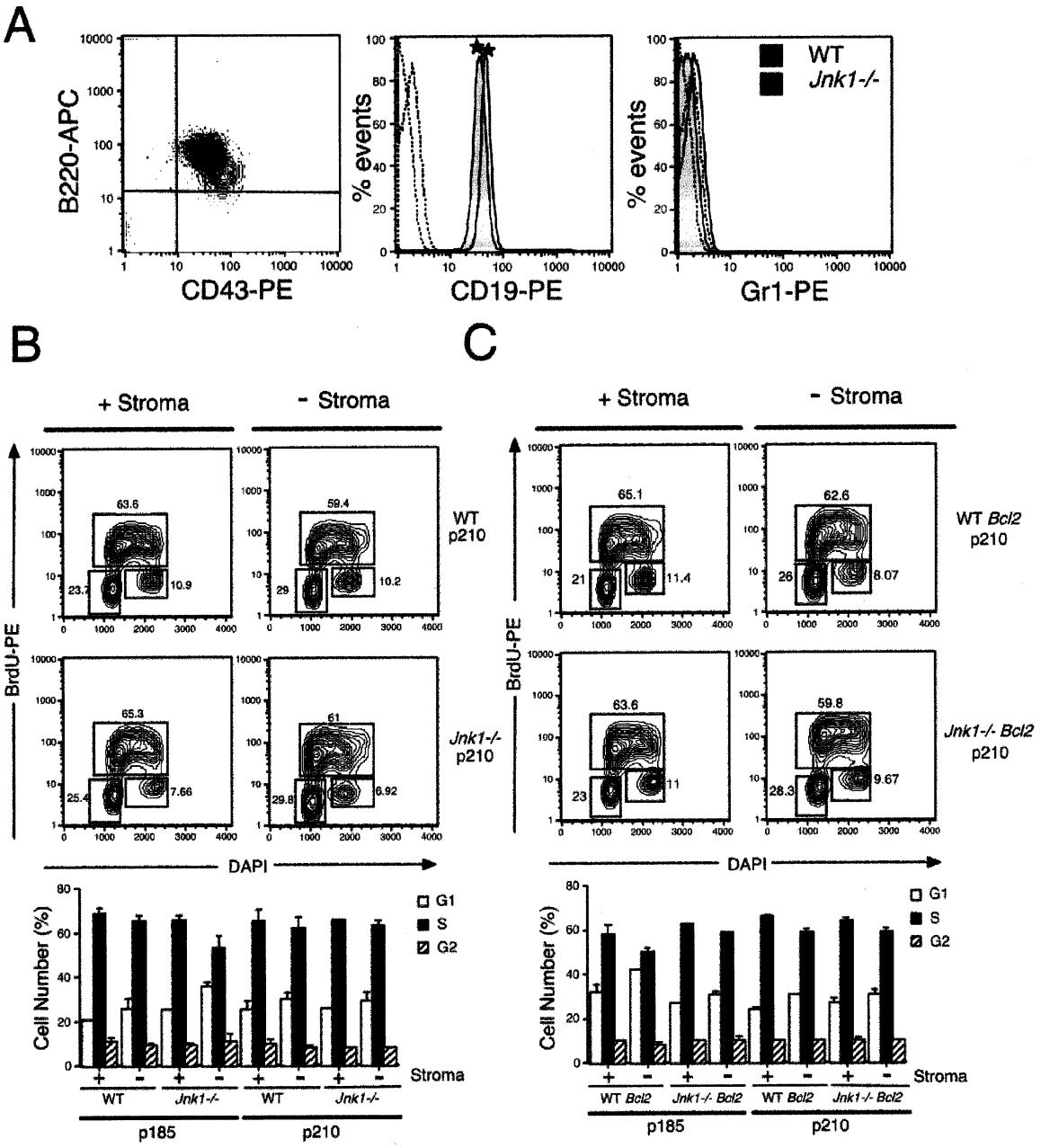


Fig.19 Bcr/Abl^{p210} transformed *Jnk1-/-* lymphoblast have the same cell cycle distribution as WT cells.
(A) Lymphoid cell lines were derived from mice reconstituted with WT (blue) and *Jnk1-/-* (red) bone marrow transduced with Bcr/Abl (p185 and p210) retroviruses. These lines were then examined by flow cytometry for the surface markers B220, CD43, CD19 and Gr-1. Cell populations positive for these cell surface markers (☆)were identified by comparison with unstained cells (dotted lines). Similar data was obtained in experiments using recipients of *Bcl2* transgenic bone marrow transduced with Bcr/Abl (p185 and p210) retroviruses. (C, D) Cell cycle analysis of BrdU- labeled cells was performed by flow cytometry of fixed cells stained with DAPI and an antibody to BrdU. Representative data for Bcr/Abl^{p210} cells are illustrated (upper panels). The G1, S and G2 cell cycle populations of Bcr/Abl^{p185} and Bcr/Abl^{p210} cells were quantitated (mean±SD, n=3; lower panels).

Cell death was examined by flow cytometry using Annexin V and 7-Aminoactinomycin D (7-AAD) staining. Studies of wild-type Bcr/Abl^{p210} cells demonstrated a small increase in cell death in the absence of stroma (Fig. 20A) but a dramatic increase in the apoptosis of *Jnk1-/-* Bcr/Abl^{p210} cells (Annexin V$^+$, 7-AAD$^-$) in the absence of stroma. The markedly increased apoptosis of *Jnk1-/-* Bcr/Abl^{p210} cells was also detected by TUNEL assays (Fig. 20C). In contrast, JNK-deficiency did not cause increased apoptosis of Bcr/Abl^{p185} transformed cells in the absence of stroma (Fig. 20A). Similarly, no increase in the number of apoptotic cells (Annexin V$^+$, 7AAD$^-$) was detected when *Bcl2* transgenic cells were cultured in the absence of stroma (Fig.20B). Furthermore, the apoptotic defect caused by JNK1 deficiency in transformed lymphoblasts was not detected in studies of B lymphoblasts derived from normal bone marrow cultured *in vitro*. Thus the role of JNK1 in mediating survival seems selectively relevant to transformed cells.

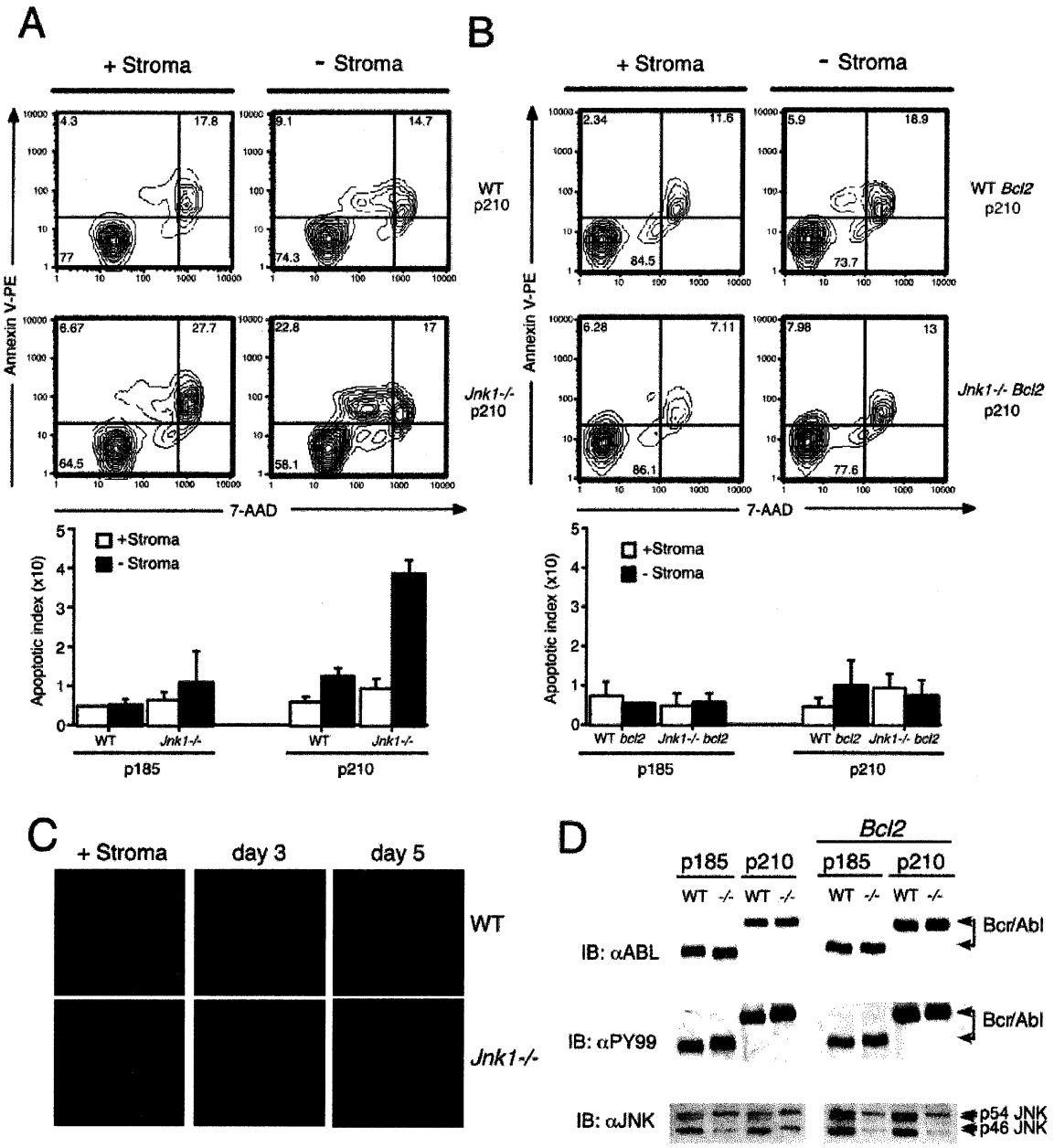


Fig. 20 *Jnk1-/-* cells transformed by Bcr/Abl^{p210} are stromal dependent.
(A, B) Bcr/Abl transformed cell derived from bone marrow recipients were plated in the presence or absence of stroma for 3 days. At this time, apoptosis was measured by flow cytometry using cells labeled with Annexin V and 7-AAD. Representative data for Bcr/Abl^{p210} cells are illustrated (upper panels). The ratio between the number of annexin- positive (apoptotic) cells and annexin- negative (live) cells in the 7-AAD negative population (apoptotic index) is presented (mean±SD, n=3; lower panels). (C) Apoptosis was examined by TUNEL assay of Bcr/Abl^{p210} cells (WT and *Jnk1-/-*)plated on stroma or in the absence of stroma (3 or 5 days). The cells were stained with DAPI (blue) and TUNEL (red) and examined by fluorescence microscopy. (D) Bone marrow from reconstituted animals were cultured *in vitro* on stromal layers. *Bcl2* transgenic and non-transgenic cells expressing Bcr/Abl (p185 and p210) were cultured from animals reconstituted with WT and *Jnk1-/-* bone marrow cells. Lysates prepared from these cells were examined by immunoblot (IB) analysis using antibodies to Abl, phosphotyrosine (PY99) and JNK.

III.III.B. Conclusions

The "*ex vivo*" data presented in this section demonstrate that:

- Disruption of the *Jnk1* gene did not affect DNA synthesis in Bcr/Abl^{p210} transformed lymphoblasts.
- JNK-deficiency caused markedly increased apoptosis when Bcr/Abl^{p210} transformed lymphoblasts were cultured in the absence of stroma.
- The transgenic expression of *Bcl2* rescued the increased apoptosis caused by JNK-deficiency in Bcr/Abl^{p210} lymphoblast. Thus, the ability of Bcr/Abl^{p210} transformed cells to become stromal independent requires JNK1. This role of JNK1 can be replaced by Bcl2 *in vivo* (Fig. 17) and *in vitro* (Fig. 20).
- Together, these data indicate that JNK1 can promote growth factor independent survival of transformed B lymphoblasts. Thus JNK1 contributes to the leukemogenic potential of Bcr/Abl^{p210} by promoting survival of Bcr/Abl^{p210} transformed lymphoblast in the blood and in the peripheral organs

CHAPTER IV

DISCUSSION AND FUTURE DIRECTIONS

IV.I. JNK and transformation by Bcr/Abl

In humans, the expression of Bcr/Abl is associated with malignancies that affect either lymphoid (ALL) or myeloid cells (CML) (see Introduction). For this reason, in our quest to investigate the potential role of JNK in Bcr/Abl induced transformation I took advantage of different mouse models that would give rise to either lymphoid or myeloid leukemia. Interestingly, our data suggest that while JNK activity is required for the Bcr/Abl induced lymphoid transformation (Fig. 11 and 14) it is dispensable for the development of myeloid disease (Fig.18). This differential requirement for JNK in transformation involving lymphoid or myeloid cells could be ascribed to several reasons.

First the relative abundance of the JNK isoforms in either the myeloid or lymphoid compartment might affect the importance of this signaling pathway in the overall transformation process triggered by Bcr/Abl. Indeed the Bcr/Abl oncogene activates redundant pathways leading to overlapping biological outcomes that collectively induce transformation. The preferential use of a specific pathway to induce a transforming event (such as growth-factor

independent proliferation) might be influenced by the presence or absence of the components of the JNK signaling pathway. Thus cell context may play a role in the choice of signaling pathways that Bcr/Abl employs to transform cells. Another reason is that the biological outcome mediated by JNK activation is not required for the transformation process of myeloid cells. Thus, JNK mediates a cell specific signal that is needed in lymphoid but not myeloid transformation. This hypothesis entails that myeloid and lymphoid cells require different transforming programs mediated by the same oncogene. Further studies are needed to elucidate the mechanisms underlying this discrepancy in JNK requirement between Bcr/Abl induced myeloid and lymphoid disease.

Two separate mechanisms are employed by Bcr/Abl to transform cells. First, Bcr/Abl is an activator of several signal transduction pathways that mediate mitogenesis (Cortez et al., 1997). Second, Bcr/Abl is a potent inhibitor of cell death (McGahon et al., 1994). The proliferation and survival functions of Bcr/Abl can be uncoupled. Expression of Bcr/Abl^{p210}with a deletion in the Bcr region (Δ176-427) in myeloid cells causes resistance to apoptosis in the absence of increased proliferation (Cortez et al., 1995). These cells fail to form tumors in nude mice. In contrast, wild-type Bcr/Abl^{p210} causes increased proliferation and does cause tumors in nude mice (Cortez et al., 1997). Thus, both proliferation and inhibition of apoptosis are essential for transformation.

The anti-apoptotic signaling pathways that are activated by Bcr/Abl include Akt (Neshat et al., 2000; Skorski et al., 1997), Stat5 (Gesbert and Griffin, 2000; Horita et al., 2000; Shuai et al., 1996) and JNK (this study). The relative roles of these anti-apoptotic pathways are unclear. Gene disruption studies indicate that Stat5 is not required for transformation by Bcr/Abl (Sexl et al., 2000) but is required for transformation by other leukemogenic oncogenes, including TEL-JAK2 (Schwaller et al., 2000). The effect of *Akt* gene disruption on Bcr/ Abl disease has not been reported, but dominant-negative approaches indicate an important anti-apoptotic role of the Akt pathway (Skorski et al., 1997). Here I demonstrate that JNK contributes to the survival of Bcr/Abl transformed cells (Fig. 11 and 20). However, JNK does not appear to contribute to mitogenesis because JNK-deficiency caused no defects in DNA synthesis (Fig. 7D and 19B,C) and because JNK-deficiency caused no marked defect in blast expansion in femur sections of mice transplanted with Bcr/Abl-transduced bone marrow (Fig. 13).

Three lines of evidence indicate that JNK plays an important role in Bcr/Abl cell survival. First, JNK-deficiency causes a severely reduced number of Bcr/Abl^{p210} cells in the peripheral organs of leukemic animals *in vivo* (Fig. 14). Second, depletion of JNK activity causes apoptosis of Bcr/Abl cells cultured in the absence of growth factors *in vitro* (Fig. 11 and 20). Third, expression of the anti-apoptotic protein Bcl2 can rescue the defects caused by JNK-deficiency *in*

vivo and *in vitro* (Fig. 17 and 20B). The survival defect of cells depleted of JNK activity appears to be caused by the failure of these cells to proliferate independently of stroma and stroma-derived cytokines. The growth factor-dependence observed *in vitro* (Fig. 20) may account for the inability of Bcr/Abl^{p210} transformed *Jnk1-/-* cells to accumulate in the blood and peripheral organs *in vivo*, although these cells were able to proliferate in the stromal environment of the bone marrow. Alternatively, the defect in malignant infiltration could be attributed to the inability of the *Jnk1-/-* cells to efficiently migrate and metastasize peripheral organs.

IV.II. Survival signaling mediated by JNK

The mechanism that accounts for the requirement of JNK1 for stroma-independent growth of Bcr/Abl^{p210} cells is unclear. Here I provide evidence that JNK1 can regulate Bcl2 expression in Bcr/Abl^{p210} transformed cells and that a *Bcl2* transgene rescues the survival defect of *Jnk1 -/-* Bcr/Abl^{p210} cells (Fig. 16 and 20). The effect of JNK1 on Bcl2 expression may be mediated, in part, by an ATF2 responsive element in the promoter (Wilson et al., 1996). Indeed, Bcr/Abl^{p210} causes increased *Bcl2* promoter activity in reporter gene assays (Fig. 16D) and also increases the expression of Bcl2 mRNA and protein (Fig. 16C and

(Sanchez-Garcia and Martin-Zanca, 1997). Loss of Bcl2 in Bcr/Abl^{p210} transformed cells contributes to increased apoptosis (Sanchez-Garcia and Grutz, 1995). Interestingly, the survival function of Akt is also mediated, in part, by increased Bcl2 expression in Bcr/Abl^{p210} transformed cells (Pugazhenthi et al., 2000; Skorski et al., 1997). Thus, the JNK1 and Akt pathways may co-operate to induce expression of Bcl2 and the survival of Bcr/Abl^{p210} transformed cells.

In previous studies, Bcl-X_L has been implicated as an important mediator of anti-apoptotic signaling in leukemic cells (Packham et al., 1998) and in some cultured cell lines (e.g. HL60) the expression of Bcl-X_L rather than Bcl2 appears to be dominant (Amarante-Mendes et al., 1998). It is most likely that anti-apoptotic signaling is mediated, in part, by the combined actions of both Bcl2 and Bcl-X_L. In primary mouse bone marrow cells, JNK1 is required for the normal expression of Bcl2; no effect of JNK1-deficiency on Bcl-X_L expression was detected (Fig. 16C).

The acquisition of stromal-independent growth by Bcr/Abl^{p210} cells may require the accumulation of secondary mutations. Increased expression of cytokines may also contribute to stromal independent growth; recent studies indicate that while IL-3 and GM-CSF are not required (Li et al., 2001b), oncostatin-M may contribute to myelo- and lympho-proliferative disease (Schwaller et al., 2000). Studies of Bcr/Abl^{p210} in p53-deficient mice demonstrate that the loss of p53 decreases apoptosis, increases the malignant infiltration of

peripheral organs, increases stromal-independent proliferation, and decreases the latency of disease mortality (Honda et al., 2000; Skorski et al., 1996). A change in p53 status also correlates with the transition from the chronic to the acute phase of disease in humans. Thus, it is possible that JNK1 influences the accumulation of secondary mutations by Bcr/Abl^{p210} cells. This represents an additional mechanism by which JNK1 may contribute to the leukemogenic potential of Bcr/Abl.

IV.III. Apoptosis and survival signaling

Why does JNK activation mediate survival rather than apoptosis in Bcr/Abl transformed cells? The decision to survive might result from a high level of survival signaling mediated by other pathways (e.g. ERK or Akt) that are known to suppress JNK-mediated apoptosis (Davis, 2000). This appears to be unlikely because, if true, JNK1-deficiency would not lead to increased apoptosis. There are two alternative possible mechanisms that could account for the actions of JNK. First, the decision to survive may be the result of altered gene expression in the tumor cells and therefore the presence of novel targets of the JNK signaling pathway. This hypothesis implies that the tumor cells re-program the response to JNK activation. Second, it is possible that it is the duration and amplitude of JNK activation that is critical for the decision to survive or die (Chen et al., 1996). Although transient expression of Bcr/Abl leads to rapid activation of

JNK, chronic expression of Bcr/Abl causes a high basal level of JNK activity. It is therefore possible that the survival function of JNK is mediated by the sustained basal activity. This hypothesis implies that the loss of high basal JNK activity would lead to apoptosis. Similarly, increased levels of JNK activation would cause apoptosis (Lei et al., 2002). These two alternative hypotheses (re-programming and extent/duration of JNK signaling) are not mutually exclusive.

IV.IV. Different JNK requirement between Bcr/Abl^{p210} and Bcr/Abl^{p185}

Several studies have implicated JNK in transformation caused by the leukemogenic oncogene Bcr/Abl (Burgess et al., 1998; Dickens et al., 1997; Raitano et al., 1995; Shi et al., 2000; Shi et al., 1999). The role of JNK in survival signaling was not anticipated in these studies. In addition, these studies did not anticipate that Bcr/Abl^{p210} and Bcr/Abl^{p185} might have different JNK requirements. Here I demonstrate that while depletion of JNK activity affects growth-factor independent proliferation in both Bcr/Abl^{p185} and Bcr/Abl^{p210} transformed cells, the latter cells rely primarily on JNK1 activity. Thus, disruption of *Jnk1* gene causes a selective defect in transformation by Bcr/Abl^{p210} while it has no effect on Bcr/Abl^{p185} induced leukemia. It is possible that the selective effect of JNK1-deficiency on Bcr/Abl^{p210} (which is associated with human chronic myelogenous leukemia) results from the finding that it is a less potent oncogene than

Bcr/Abl^{p185} (which is associated with human acute lymphoid leukemia) (McLaughlin et al., 1987). Alternatively, the selective defect in Bcr/Abl^{p210} transformation caused by JNK1-deficiency may reflect a qualitative difference in function between these Bcr/Abl isoforms (Ilaria and Van Etten, 1996; Li et al., 1999; Quackenbush et al., 2000). Bcr/Abl^{p210} differs from Bcr/Abl^{p185} because of the presence of Cdc24/Dbl and plekstrin homology domains (collectively referred to as Dbl domain) in the Bcr region of the Bcr/Abl^{p210} fusion protein. These domains are found in tandem in many exchange factors for small GTP binding proteins of the Rho family. Two members of the Rho family, Rac1 and Cdc42, are known to regulate JNK (Coso et al., 1995; Minden et al., 1995). Indeed, I show that transient expression of Bcr/Abl^{p210} Dbl domain can induce JNK activation (Fig.12). This observation provides a potential Ras-independent pathway for JNK activation in Bcr/Abl^{p210} transformed cells. These Bcr/Abl^{p210} cells may rely on this privileged pathway for JNK activation in order to achieve the deregulation of specific cellular processes that contribute to malignant transformation. Decreased levels of JNK activity might hinder the biological outcome of this specific pathway leading to a defect in oncogenic transformation. In contrast, Bcr/Abl^{p185} might compensate for the lack of the Dbl-activated pathway through the engagement of alternative pathways. Nevertheless, in Bcr/Abl^{p185} transformed cells JNK is still activated through the Ras pathway. Thus

complete depletion of JNK activity impairs the ability of Bcr/Abl^{p185} to transform cells.

FUTURE DIRECTIONS

The findings described in this thesis suggest JNK provides a survival signal in Bcr/Abl^{p210} transformed lymphoblast. This signal may be mediated by transcriptional regulation of key effectors (such as Bcl2 or BclW) that are critical for maintenance of the malignant cells. The identification of critical effectors and the subsequent understanding of how specific gene expression programs translate into malignant transformation could lead to the manipulation of JNK signaling for therapeutic benefit. One possible approach to identify these effectors is the utilization of microarray technology. This technique allows the analysis of gene transcription in a specific tissue or cell population. The analysis is achieved by hybridizing total mRNA to an array of oligonucleotide probes. These probes represent most of the identified ORFs sequenced in the mouse genome project. Data arrays obtained with different samples can than be compared using Affymetrix specialized software. The combination of microarray techniques with genetically modified cells can provide a powerful tool for a comprehensive analysis of the global changes

mediated by JNK during transformation by Bcr/Abl. In particular, gene expression between wild-type and JNK-deficient cells could be compared by taking advantage of the "ex vivo" lymphoid cell lines that were isolated from the reconstituted animals. In addition, this analysis could be performed using different growing conditions to identify potential effectors that mediate JNK-induced growth factor independent proliferation in Bcr/Abl^{p210} lymphoblast.

Another novel observation reported in this thesis is the differential requirement for JNK in myeloid versus lymphoid transformation and between the two isoforms of the Bcr/Abl oncogene (Bcr/Abl^{p185} and Bcr/Abl^{p210}). While JNK1 alone is critical for Bcr/Abl^{p210} lymphoid but not myeloid transformation, inhibition of both JNK1 and JNK2 activities is required to prevent Bcr/Abl^{p185} malignant proliferation. Thus, in order to verify if JNK has a role in myeloid and Bcr/Abl^{p185} induced leukomogenesis it would be essential to use either *Jnk1 Jnk2* deficient mice or a pharmacological inhibitor of these kinases. Unfortunately, the inactivation of *Jnk1Jnk2* genes results in early embryonic lethality and pharmacological inhibitors available for JNK kinases would probably have high toxicity *in vivo*. Thus, a "conditional" gene targeting strategy could be used to circumvent these issues. Conditional gene targeting refers to a gene modification in the mouse that is restricted to either certain cell types (tissue specific) or to a specific stage within development (temporally specific) or both. Compared to "conventional" gene targeting, the

conditional strategy will test the function of a widely expressed gene in a particular tissue without being influenced by gene loss in adjacent tissues, as the rest of the embryo is genetically wild-type. There are two systems to achieve conditional gene inactivation in mice: the *cre-loxP* system (Lakso et al., 1992; Orban et al., 1992; Sauer, 1998) and the *Flp* recombinase from *Saccharomyces cerevisiae* (Dymecki, 1996). Both of these systems are based on the use of site-specific recombinases, which allow targeted excision of specific genes flanked by recognition sites (such as *loxP* sites). Thus, the gene of choice can be universally targeted with the *loxP* sites throughout the animal and tissue specific excision can be achieved by crossing these animals with transgenic mice, which ectopically express the heterologous recombinase in specific tissues. By using this approach it would be possible to obtain viable mice which are *Jnk* null in the lymphoid or myeloid compartment. These mice can be used as donors in reconstitution assays designed to investigate the role of JNK both in Bcr/Abl^{p185} induced transformation and in Bcr/Abl induced myeloproliferative disease.

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