The role of ABL/BCR in the leukemogenic potential of BCR/ABL in Philadelphia chromosome positive leukemia

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Dedicated to my father

and to all patients suffering from leukemia

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ABBREVIATIONS

A

a.a.	Amino acid
ABL	Abelson murine leukemia virus homology gene
ALL	Acute lymphatic leukemia
AML	Acute myeloid leukemia
AMP	Ampicillin
ARG	ABL-related genes
ATP	Adenosine triphosphate
att	Attachment

B

BCR	Breakpoint cluster region
BCR/ABL	Breakpoint cluster region/Abelson murine leukemia virus
BM	Bone marrow
BrdU	5-bromo 2-deoxyuridine
BSA	Bovine serum albumin

C

ca.	Circa
Ca^{2+}	Calcium
CC	Coiled-coil
CD	Cluster of differentiation
cDNA	Complementary DNA
CFU	Colony forming unit
CFU-GEMM	CFU-granulocyte erythroid megakaryocyte macrophage
CFU-GM	CFU-granulocyte macrophage
CLL	Chronic lymphatic leukemia
CLP	Common lymphoid progenitor
cm	Centimeter
CML	Chronic myeloid leukemia
CMP	Common myeloid progenitor
CMV	Cytomegalovirus
CR	Complete remission
Crkl	Crk-like protein

D

dATP	Deoxyadenosine triphosphate
dCTP	Deoxycytidine triphosphate
ddH_2O	Double distilled water
DEPC	Diethylpyrocarbonate
der.	Derivate
dGTP	Deoxyguanosine triphosphate
DMEM	Dulbecco's Modified Eagle Medium
DMSO	Dimethyl sulfoxide
DNA	Desoxyribonucleic acid
dNTPs	Deoxynucleoside triphosphate
DSMZ	German Collection of Microorganisms and Cell Cultures
DTT	Dithiothreitol (threo-1,4-dimercapto-2,3-butandiol)
dTTP	Desoxythymidine triphosphate

E		
	E.coli	*Escherichia coli*
	EBV	Epstein Barr virus
	ECL	Enhanced chemiluminescence
	EDTA	Ethylenediaminetetraacetate
	eGFP	Enhanced green fluorescent protein
	EGFR	Epidermal growth factor receptor
	ERK	Extracellular signal regulated kinase
F		
	FAB	French American British
	FACS	Fluorescence activated cell sorting
	Fak	Focal adhesion kinase
	FCS	Fetal calf serum
	FELASA	Federation of European Laboratory Animal Science Associations
	FITC	Fluorescein Isothiocyanate
G		
	g	Gram
	Gal	Galactose
	G-CSF	Granulocyte-colony stimulating factor
	GFP	Green fluorescent protein
	Grb2	Growth factor receptor-bound protein 2
	GTP	Guanosine triphosphate
	Gy	Gray
H		
	HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
	HRP	Horse radish peroxidase
	HSC	Hematopoietic stem cell
I		
	i.v.	Intravenous
	IFN-α	Interferon alpha
	IgG	Immunoglobulin G
	IMDM	Iscove's Modified Dulbecco's Medium
	IPTG	Isopropyl-1-thio-Beta-D-galactopyranoside
J		
	JAK	Janus kinase
	JNK	c-Jun-N-terminal kinase
K		
	Kb	Kilo base pair(s)
	KDa	Kilodalton(s)
L		
	LB Medium	Luria-Bertani Medium
	LNGFR	Low affinity nerve growth factor receptor
	LSC	Leukemic stem cell
	LT-HSC	Long-term hematopoietic stem cell
M		
	µg	Microgram
	M	Molar
	MACS	Magnetic activated cell sorting

	MAPK	Mitogen activated protein kinase
	M-BCR	Major-breakpoint cluster region
	m-BCR	Minor-breakpoint cluster region
	mg	Milligram
	ml	Milliliter
	mM	Millimolar
	MNC	Mononuclear cell
	MPD	Myeloproliferative disease
	MPP	Multipotent progenitor
	µ-BCR	Micro-breakpoint cluster region
	µl	Microliter
	µM	Micromolar
N		
	N	Amino
	ng	Nanogram
	nM	Nanomolar
	NK	Natural killer
	NLS	Nuclear localization signal sequence
O		
	OD	Optical density
	ORF	Open reading frame
P		
	$p185^{BCR/ABL}$	185 kDa fusion protein of t(9;22)
	$p210^{BCR/ABL}$	210 kDa fusion protein of t(9;22)
	$p40^{ABL/BCR}$	40 kDa fusion protein of t(9;22)
	$p96^{ABL/BCR}$	96 kDa fusion protein of t(9;22)
	PBS	Phosphate-buffered saline
	PCR	Polymerase chain reaction
	PDGFR	Platelet-derived growth factor receptor
	PE	Phycoerythrin
	pH	Reverse logarithmic representation of hydrogen ion concentration
	Ph^-	Philadelphia chromosome negative
	Ph^+	Philadelphia chromosome positive
	PI	Propidium iodide
	PI3K	Phosphatidylinositol-3-kinase
	PLZF	Promyelocytic leukemia zinc finger
	PML	Promyelocytic leukemia protein
	pmol	Picomol
R		
	RAR	Retinoic acid receptor
	rhFlt3-ligand	Recombinant human FMS-like tyrosine kinase 3-ligand
	rhIL-7	Recombinant human interleukin-7
	rhTPO	Recombinant human thrombopoetin
	rmIL-3	Recombinant murine interleukin-3
	rmIL-6	Recombinant murine interleukin-6
	rmSCF	Recombinant murine stem cell factor
	Rnase	Ribonuclease
	rpm	Revolution per minute

	RPMI	Roswell Park Memorial Institute
	RT	Reverse transcriptase
S		
	Sca1	Stem cell antigene 1
	SDS	Sodium dodecyl sulfate
	SDS-PAGE	SDS-Polyacrylamide gel electrophoresis
	Ser	Serin
	SFFV	Spleen focus-forming virus
	SHC	Src homology 2 domain-containing protein
	S/T	Serine/threonine
	STAT	Signal transducers and activators of transcription
	ST-HSC	Short-term hematopoietic stem cell
	STI	Signal transduction inhibitor
	SOS	Son of sevenless
T		
	t	Translocation
	t(9;22)	Translocation of chromosome 9 and 22
	TAE	Tris acetate EDTA
	TBS	Tris-buffered saline
	TBS-T	Tris-buffered saline plus Tween 20
	TE	Tris-EDTA
	TEMED	Tetramethylethylenediamine
	Thr	Threonine
	TK	Tyrosine kinase
	Tris	Tris(hydroxymethyl)aminomethane
	Tyr	Tyrosine
U		
	U	Enzyme unit
	UTR	Untranslated region
	UV	Ultraviolet
V		
	V	Volt
	v/v	Volume per volume
W		
	w/v	Weight per volume
	WBC	White blood cell count
	WT	Wild type
X		
	XTT	Tetrazolium salt
Y		
	Y177	Tyrosine at a.a. position 177
	Y245	Tyrosine at a.a. position 245
	+	Positive
	-	Negative
	%	Percent
	7-AAD	7-Aminoactinomycin
	°C	Degree Celsius

1 INTRODUCTION

1.1 Normal hematopoiesis

The highly regulated and complex process of blood cell production and homeostasis is designated as hematopoiesis. The human body produces each day billions of new red and white blood cells (erythrocytes and leukocytes, respectively) and platelets, in order to keep the body homeostasis in balance, while being able to respond to stress conditions, bleeding or infection (Smith, 2003).

In adult hematopoiesis occurs in the bone marrow and the cells from the hematopoietic system are continually generated from self-renewing pluripotent cells in the bone marrow called hematopoietic stem cells (HSCs)(Seita & Weissman, 2010), which have been isolated in both human and mouse (Doulatov et al, 2012; Wilson & Trumpp, 2006). The hematopoietic system is constructed in a hierarchical manner. HSCs can be divided into a long-term subset (LT-HSC), capable of indefinite self-renewal, and a short-term subset (ST-HSC) that self-renew for a defined interval. HSCs give rise to non-self-renewing multipotent progenitors (MPP), which in turn give rise to progeny that are more restricted in their differentiation potential and finally to functionally mature cells. HSCs generate the multiple hematopoietic lineages through a successive series of intermediate progenitors. These multipotent stem and/or progenitor cells then commit either to the myeloid or lymphoid lineage by differentiating to common myeloid progenitors (CMP) or common lymphoid progenitors (CLP), respectively. These progenitor cells go through a number of proliferation and commitment steps to give rise to all the myeloid and lymphoid lineages of the blood. The CLP give rise to T cells, B cells, natural killers (NK), and antigen presenting dendritic cells. The CMP give rise to granulocytes, monocytes, megakaryocytes, erythrocyte and dendritic cells (Passegue et al, 2003; Seita & Weissman, 2010). Ultimately, terminally differentiated cells are produced that cannot divide and undergo apoptosis after a period of time ranging from hours (for neutrophils) to decades (for some lymphocytes). Process of hematopoiesis is schematically shown in Figure 1 (Passegue et al, 2003).

The regulation of hematopoiesis involves a complex interplay between the intrinsic genetic processes of blood cells and their environment and this interplay determines whether HSCs remain quiescent, proliferate, differentiate, self-renew, or undergo apoptosis (Eliasson & Jonsson, 2009; Wilson & Trumpp, 2006). During the maturation process of HSCs to more

mature cells, distinct specific surface markers will be developed and expressed on the cells, which enables us to identify and hence isolate the HSCs according to their grade of differentiation. Some of these surface markers are presented in Figure 1 (Passegue et al, 2003).

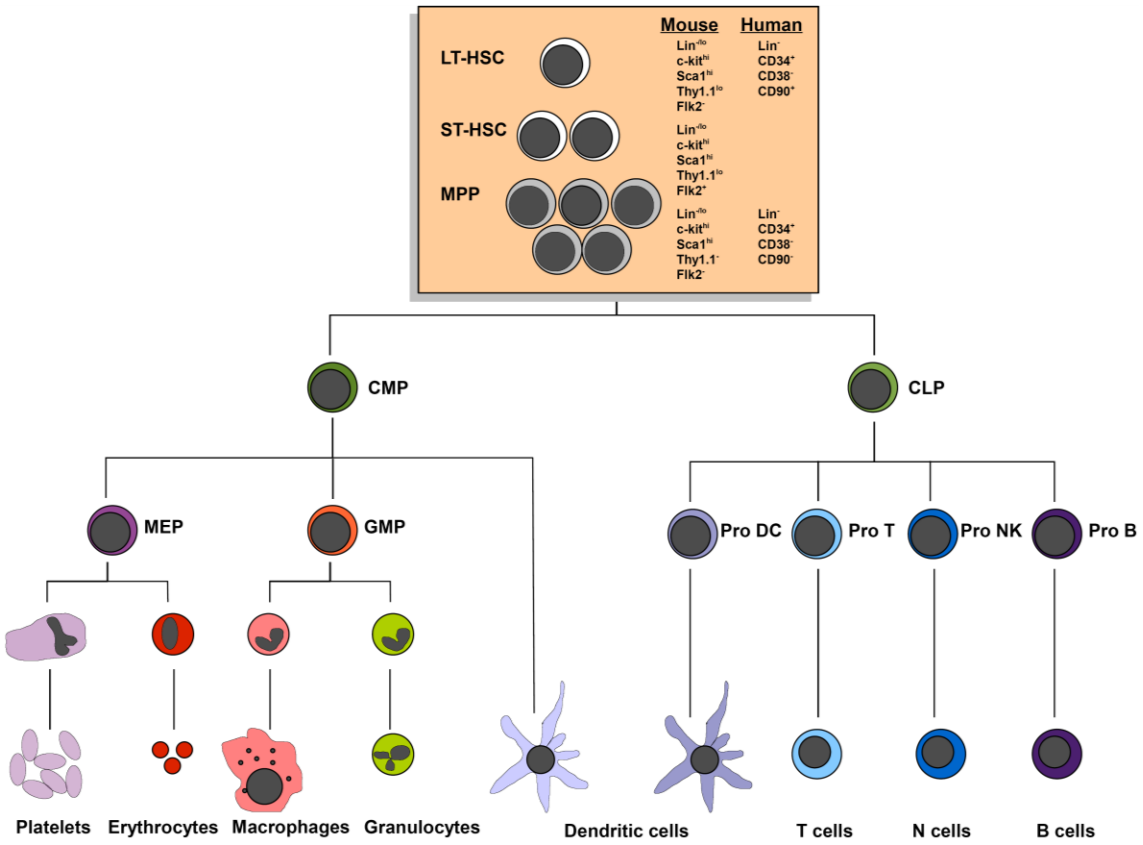

Figure 1. Hematopoietic and progenitor cell lineages. HSCs can be divided into LT-HSCs and ST-HSCs. LT-HSCs self-renew for the whole lifetime, while ST-HSCs possess this ability only for approximately 8 weeks. ST-HSCs differentiate into MPPs, which do not or briefly self-renew, and have the ability to differentiate into oligolineage-restricted progenitors that ultimately give rise to differentiated progeny through functionally irreversible maturation steps. The CLPs give rise to T lymphocytes, B lymphocytes and natural killer (NK) cells. The CMPs give rise to GMPs, which then differentiate into monocytes/macrophages and granulocytes, and to megakaryotic/erythroid progenitors (MEP), which produce megakaryocyte/platelets and erythrocytes. Both CMPs and CLPs can give rise to dendritic cells. LT: Long-term; ST: Short-term; CLP: Common lymphatic progenitors; CMP: Common myeloid progenitors. Modified from (Passegue et al, 2003; Seita & Weissman, 2010).

1.2 Hematopoietic stem cell

1.2.1 Definition

Hematopoietic stem cells are described as cells with extensive self-renewal and multilineage differentiation ability. They can rescue a lethally irradiated recipient *in vivo* for the lifetime of the recipient (Passegue et al, 2003). The first experimental evidence to indicate the existence

of HSCs was the discovery in 1961 by Till and McCulloch. Their pioneering studies proposed a population of clonogenic bone marrow cells capable of generating myelo-erythroid colonies in the spleen of lethally irradiated hosts (Till & McCulloch, 1961). Occasionally these colonies contained clonogenic cells that could be further retransplanted into secondary lethally irradiated hosts and reconstitute their whole blood system. These were suggested to be HSCs, or progenitor cells with the essential characteristic of self-renewal and differentiation potential for all types of blood cells (Becker et al, 1963; Siminovitch et al, 1963; Till & McCulloch, 1961; Wu et al, 1968).

Proliferation and self-renewal are not synonymous. Self-renewal is a unique cell division in which the capacity of one or both progeny to maintain the same properties as its parent is generated. Self-renewing cell division of a HSC results in a cell that maintains its proliferative capacity and can reconstitute the blood system for the life of an animal (Ema et al, 2006; Morrison & Weissman, 1994). HSCs are the only cells within the hematopoietic system which have both potential, namely self renewal and multi potency (Seita & Weissman, 2010). Indeed, a single HSC or a progeny that arise from a self-renewing cell division can be serially transplanted several times and restore blood production in lethally irradiated animals. It has been estimated, based on limiting dilution assays *in vivo*, that the frequency of long-term reconstituting HSCs in murine bone marrow is about 1 in 100,000 whole bone marrow nucleated cells (Harrison et al, 1993).

1.2.2 Isolation and characterization of HSCs

Characterization of multi-potent HSCs and their differentiation into diverse functional cells was widely studied using cell surface markers. The isolation of HSCs by flow cytometry and the use of functional assays attempted to characterize these cells. Most commonly, a panel of cell surface antigens, in the absence of all lineage markers (Lin^-), identifies HSCs. In the adult mouse bone marrow, HSCs are most commonly isolated as c-Kit^{hi} Thy1.1^{lo}Lin^- Sca1^{hi} (KTLS) (Spangrude et al, 1988). The LT-HSC fraction is contained within the Thy 1.1^{lo}$Flk2^-$ and ST-HSC fraction have been found in Thy 1.1^{lo}$Flk2^+$ population (Christensen & Weissman, 2001). The use of IL-7R expression within the Lin^-Thy1.1$^-$ Sca1^{lo}c-Kit^{lo} population has allowed the distinction between CLP and CMP progenitor populations. The CLP progenitor population, which possessed a rapid and prominent T, B, NK cells reconstitution activity, is found in the IL-$7R^+$ fraction, whereas the CMP progenitor population is found almost exclusively in the IL-$7R^-$ fraction (Akashi et al, 2000).

The markers used to identify human HSCs differ from the ones used for murine HSCs. One of the most important markers for human HSCs is CD34, which is expressed on 0.5–5% of human bone marrow cells, and is found on early progenitor cells but not on their mature counterparts (Civin et al, 1984; Civin et al, 1990; Doulatov et al, 2012). This surface marker has been used to provide cells that achieve clinical engraftment following transplantation (Brugger et al, 1994). Other surface markers have been used in association with CD34 to identify more primitive populations of cells, such as in the case of $CD34^{+}CD38^{-}$ cells. A higher number of long-term repopulating cells were found in the $CD34^{+}CD38^{-}$ population when compared to a $CD34^{+}CD38^{lo}$ population (Miller et al, 1999). The $CD34^{+}CD38^{lo}$ population was further differentiated and had lost both the long-term engraftment capability and the bipotent lymphomyeloid capacity (Bonnet & Dick, 1997; Brugger et al, 1994).

The co-expression of CD10 or CD7 on $CD34^{+}$ defines the earliest human lymphoid committed progenitor (Galy et al, 1995; Hao et al, 2001). The expression IL-$3R^{lo}CD45RA^{-}$ on $CD34^{+}CD38^{+}$ cells gives rise to all types of myeloid progenitor cells, defining the CMP population (Manz et al, 2002). Stem cell markers such as CD133 or KDR have been used as an alternative to, or in association with the CD34 marker (Yin et al, 1997; Ziegler et al, 1999). Although they represent valid cell surface markers, no real advantage over the CD34 marker has been found so far.

Other strategies that have been employed to purify hematopoietic stem cells using flow cytometry are based on the efflux properties of the vital dye Hoechst 33342 or rhodamine123 by HSCs. In 1996, Goodell et al reported a novel method to identify HSCs in mouse bone marrow that depends on dual-wavelength flow cytometric analysis of cells stained with Hoechst 33342 alone. By simultaneously monitoring fluorescence emission of Hoechst 33342 at 450 nm (“Hoechst Blue” fluorescence) and at >675 nm (“Hoechst Red” fluorescence) following ultraviolet (UV) excitation, a rare subset of mouse bone marrow cells (<0.1%) was observed that displayed low blue and red fluorescence. The investigators showed that these so-called “side population” (SP) cells, which expressed the Sca1 HSC antigen but were not stained by a cocktail of antibodies directed against a number of lineage markers found on mature hematopoietic cells, contained the vast majority of long-term hematopoietic repopulating activity in mouse bone marrow (Goodell et al, 1996).

Even with the most rigorous isolation protocol HSC populations remain heterogeneous. Most stem cell markers are known not to be essential for stem cell function and therefore the

expression of these proteins may not directly correlate with stem cell potential, as it has been shown in the case of the CD34 antigen (Matsuoka et al, 2001; Sato et al, 1999). Indeed several groups have provided evidence of various types of HSCs that do not express detectable levels of CD34 (Bhatia et al, 1998). Currently, the only conclusive assay for HSCs is to assess their ability to give raise to the lymphoid and myeloerythroid lineage in a lethally irradiated host following transplantation (Morita et al, 2010). For many years the enumeration of spleen colony forming unit (CFU-S) was considered to fulfill the requirements of HSCs. They had the ability to generate colonies, which contained cells of different lineages, in the spleen of a mouse 8-12 days after transplantation. A fraction of these colonies were able to repopulate an irradiated host when reinjected into mice (Till & McCulloch, 1961). However, the long-term repopulating ability of CFU-S12 is now under doubt and current opinion regards day 12 CFU-S derived from primitive MPP and day 8 CFU-S derived from late myeloerythroid committed progenitors (MEP) (Na Nakorn et al, 2002). In mice HSCs are assessed by *in vivo* competitive repopulation assays and long-term engraftment of HSCs can be assessed by secondary and tertiary transplant (Yuan et al, 2004). Assay for the detection of human HSCs consists in the engraftment of human cells in a range of xenogenic hosts, particularly the immunodeficient NOD/SCID murine model (Dick et al, 1997; Doulatov et al, 2012; Larochelle et al, 1996).

1.2.3 Regulation of self-renewal of HSCs

While the phenotypic and functional properties of HSCs have been extensively characterized, a fundamental question that remains is how self-renewal of HSCs is regulated. Ectopic expression of the transcription factors HoxB4 or HoxA9 has been shown to be able to increase the numbers of transplantable hematopoietic stem cells both *in vitro* and *in vivo* (Antonchuk et al, 2002; Thorsteinsdottir et al, 2002). Mice that overexpress the antiapoptotic gene *bcl-2* have increased numbers of HSCs, suggesting that such antiapoptotic signals may contribute to regulating stem cell numbers (Domen et al, 2000). The polycomb group protein, Bmi-1 was demonstrated to regulate the proliferation and self-renewal capacity of HSCs due to its repressive effect on p21 (Cdkn1, $p21^{WAF1/CIP1}$) and other cell cycle inhibitors (Oguro, 2006). Mice lacking the cell cycle regulator p21 display a higher rate of HSC proliferation and differentiation, and a lower self-renewal capacity suggesting that p21 is required for maintaining HSC quiescence and that, in its absence HSCs rapidly proliferate and differentiate to more committed lineages (Cheng et al, 2000). The relation between expression

of cell cycle regulators, like p53 tumor suppressor protein, and self- renewal/proliferation capacity of HSCs is under extent investigations, which would be next to cell surface markers a reliable method at the molecular level for characterization of HSCs (Insinga et al, 2012; Viale et al, 2009).

The points mentioned above are clearly important mediators of HSC development; however, the upstream signal that may control the activity of HSCs remains unclear. Signaling pathways classically studied in the context of embryonic development such as the Notch, Sonic hedgehog, and Wnt signaling pathways have emerged as candidates for regulating self-renewal of HSCs. The canonical Wnt/β-catenin pathway was shown to play an important role in proliferation, self-renewal and differentiation of HSCs (Lento et al, 2013; Wilusz & Majka, 2008). The expression of constitutively active Notch1 in hematopoietic progenitors has been shown to lead to the establishment of at least some immortalized, cytokine-dependent cell lines that retain the potential to generate both lymphoid and myeloid cells *in vitro* and in long-term mouse reconstitution assays (Varnum-Finney et al, 2000). Studies demonstrated that HSCs infected with Notch expand *in vivo* (Stier et al, 2002). Sonic hedgehog has also emerged as a potential mediator of HSC development. Human cells highly enriched for hematopoietic progenitors exhibited increased self-renewal in response to sonic hedgehog signaling *in vitro*, albeit in combination with a cocktail of six other growth factors (Bhardwaj et al, 2001). The fact that both Notch and Sonic hedgehog activation has been observed to inhibit the differentiation of progenitors in many different systems by maintaining progenitors in an undifferentiated state (Artavanis-Tsakonas et al, 1995; Dorsky et al, 1997) certainly supports the idea that these signals may promote self-renewal in a variety of tissues.

1.3 Leukemia

Leukemia is used to describe a variety of cancers of the white blood cells that have their origin in blood-forming cells of the bone marrow (Adamietz et al, 2009). This malignancy is monoclonal by definition, which means that the induction of leukemia can be traced back to a single malignant transformed cell. White blood cells evolve from the immature cells referred to as blasts, some of which are lymphoblasts and myeloblasts, depending on whether they eventually mature into lymphocytes or myeloid cells. Normally, blasts constitute less than 5% of healthy bone marrow (Ribera et al, 2007). In leukemia, however, these blasts remain abnormally immature and multiply continuously, eventually constituting from 30% to 100%

of the bone marrow. The malignant cells fill up the bone marrow and prevent production of healthy red cells, platelets, and white cells. They often leave the bone marrow and migrate into the blood stream, liver, spleen, lymphatic system and other organs and impair their function (Adamietz et al, 2009). As the number of normal cells declines, symptoms develop which, if untreated, become lethal (Adamietz et al, 2009; Lemez, 1989). Leukemias are divided into two types according to their progression: acute (which progresses quickly with many immature white cells) and chronic (which progresses more slowly and has more mature white cells) (Adamietz et al, 2009).

1.3.1 Acute leukemia

Acute leukemias are clonal hematopoietic stem cell disorders and characterized by the uncontrolled growth of the immature blast cells of bone marrow cells. The immature cells, which normally reside in the bone marrow accumulate in the peripheral blood, infiltrate other tissues and cause bone marrow failure (Adamietz et al, 2009). The clinical picture of acute leukemia is marked by anemia, which is usually severe (fatigue, malaise), an absence of functioning granulocytes (proneness to infection and inflammation), and thrombocytopenia (hemorrhagic diathesis) (Hoelzer & Seipelt, 1998). When untreated, patients have a life span of several weeks or months which can eventually proceed to death, because their normal blood cells become outnumbered by leukemic cells (Adamietz et al, 2009). Acute leukemias require immediate and aggressive treatment (Hoelzer & Seipelt, 1998). It is important to distinguish between acute lymphoid leukemia (ALL) and acute myeloid leukemia (AML) because of different therapeutic approaches which must be undertaken by these two malignancies. They can be distinguished based on a variety of morphological, cytoplasmatic, cytochemical and biochemical features (Adamietz et al, 2009).

1.3.1.1 Acute myeloid leukemia (AML)

AML represents a group of clonal hematopoietic stem cell disorders (Adamietz et al, 2009) in which both over-proliferation and failure to differentiate in the stem cell compartment result in accumulation of non-functional cells termed blasts (Hoelzer & Seipelt, 1998). AML is the most common acute leukemia in adults representing 75-80% of the cases (Adamietz et al, 2009). It is possible to distinguish between *de novo*, primary AML, and secondary AML, which arises as a consequence of myelodysplastic syndrome, exposure to mutagenic agents or chemo/radiotherapy due to treatment of another cancer (Schaich & Ehninger, 2006). There are

several other factors, which can provoke the onset of secondary leukemias. Some of these factors are exposure to benzene, different genetic abnormalities (trisomy 21) and Fancony's anemia (Ferti et al, 1996). Secondary AML, With the exception of acute promyelocytic leukemia (APL), has a poor prognosis (Licht, 2006). In AML, blast cells are blocked in their normal differentiation at very early stage in their myelopoiesis. In bone marrow and peripheral blood, AML leads to accumulation of large number of proliferating precursors, which are not able to terminally differentiate and to become functional blood cells (Bonnet & Dick, 1997). In this way space needed for normal hematopoietic processes is occupied by leukemic blasts which will finally be clinically manifested as leukemia (Adamietz et al, 2009). In order to diagnose AML, two classifications are used: the old FAB (French, American and British hematologists, 1976) classification and the new WHO (Word Health Organization) classification. According to FAB, acute myeloid leukemias are divided into 8 subtypes designated M0 to M7. The percentage of blasts, the presence of cytochemical myeloperoxidase, and the major cell types present defined by morphology and esterase cytochemistry, and the immunophenotype define the 8 FAB subtypes (Kumar, 2011) (Table 1).

Acute myeloid leukemia	Features
M0	Undifferentiated acute myeloblastic leukemia
M1	Acute myeloblastic leukemia with minimal maturation
M2	Acute myeloblastic leukemia with maturation
M3	Acute promyelocytic leukemia
M4 **M4 eos**	Acute promyelocytic leukemia Acute promyelocytic leukemia with eosinophilia
M5	Acute monocytic leukemia
M6	Acute erythroid leukemia
M7	Acute megakaryoblastic leukemia

Table 1. FAB classification of acute myeloid leukemia (Kumar, 2011)

As mentioned above, a new classification of AML was accomplished by WHO in 2008, which combines the morphology, the immunological phenotype (expression of surface markers like CD13, CD33, CDw65, CD117), genetic and clinical characteristics. This classification was renovated again in 2008 due to the new findings in research and clinical studies (Vardiman et al, 2009) (Table 2).

WHO classification of acute myeloid leukemia	Subtype	
AML with recurring cytogenic abnormalities	With t(8;21)(q22;q22), AML1/ETO	Independent of number of blasts in PB or BM PB: Peripheral blood BM: Bone marrow
	With inv (16)(p13.1;q22) or t(16;16) (p13.1;q22), CBFβ/MYH11	
	Acute promyelocytic leukemia: with t(15;17)(q22;q12), (PML/RARα) and variants	
	AML with t(9;11)(q22;q23), MLLT3/MLL or other 11q23 (MLL) abnormalities	≥ 20% blast cells in PB or BM PB: Peripheral blood BM: Bone marrow
	AML with t(6;9)(p23;q34), DEK/NUP214	
	AML with inv (3)(q21;q26.2), RPN1/EVI1	
	AML (megakaryoblastic) with t(1;22)(p13;q13), RBM15/MKL1	
	Temporary entities: AML with mutated NPM1 and AML with mutated CEBPα	
AML with myelodisplasia-related changes		
Therapy-related myeloid neoplasm		
Myeloid sarcoma		
Myeloid proliferation by Down-Syndrome		
AML, not otherwise classified	M0 AML with minimal maturation	
	M1 AML without maturation	
	M2 AML with maturation	
	M4 Acute myelomonocytic leukemia	
	M5 Acute monocytic leukemia	
	M6 Acute erythroid leukemia	
	M7 Acute megakaryoblastic leukemia	
	Acute basophilic leukemia	
	Acute panmyelose and myelofibrose	

Table 2. WHO classification of acute myeloid leukemia (Vardiman et al, 2009)

1.3.1.2 Acute lymphoid leukemia (ALL)

ALL results from the malignant transformation of immature lymphoid blast cells in the bone marrow, thymus or other lymphoid organs (Hoelzer & Seipelt, 1998). According to FAB classification ALL is divided into 3 subgroups as shown in Table 3. ALL can also be subdivided based on immunologic and cytogenetic abnormalities (Table 4). Major cytogenetic subgroups include the t(9;22) [Philadelphia chromosome-positive (Ph^+) ALL] and the t(8;14) found in the L3 or Burkitt's lymphoma.

Acute lymphoid leukemia	Features
L1	Small cells, high nuclear/cytoplasmic ratio
L2	Larger cells, lower nuclear/cytoplasmic ratio
L3	Vacuolated, basophilic blast cells

Table 3. FAB classification of acute lymphoid leukemia (Begemann et al, 1998a)

Immunologic subtype	% of cases	FAB subtype	Cytogenetic abnormality
Pre-B ALL	75	L1, L2	t(9;22), t(4;11), t(1;19)
T cell ALL	20	L1, L2	14q11 or 7q34
T cell ALL	5	L3	t(8;14), t(8;12), t(2;8)

Table 4. Immunological subtypes of acute lymphoid leukemia (Begemann et al, 1998a)

Since the morphological FAB classification is only applicable for the L3 (Burkitt's leukemia/ lymphoma), a new classification was done 2008 by WHO, which considered the cytogenetic abnormality as the criteria for the classification (Table 5).

In the last decades the outcome of ALL has improved considerably. In large multicenter studies remission rates range from 75% to 89%, and long-term leukemia-free survival from 28% to 39% (Gökbuget & Hoelzer, 2002). However, the treatment results in elderly patients with ALL are still poor. Comorbidity, poor tolerability to cytotoxic drugs, high mortality induction and the high frequency of the Ph chromosome contribute to the dismal prognosis of elderly patients (Annino et al, 2002). The Ph chromosome, resulting from a reciprocal translocation between chromosomes 9 and 22 [t(9;22)], confers an extremely poor prognosis

even in younger patients (Annino et al, 2002) and dose-intensification has not significantly affected survival despite higher rates of complete remission (CR) (Annino et al, 2002; Delannoy et al, 1990; Gleissner et al, 2002). Allogenic stem cell transplantation is potentially curative but is generally not applicable in elderly patients (Radich, 2001). Children with acute lymphoblastic leukemia are treated with combination chemotherapy with an expected cure rate of 80% (Mullighan, 2012). However a small proportion of children treated for ALL develop secondary AML.

Acute lymphoblastic leukemia	Features
Precursor lymphoid neoplasm	
B-lymphoblastic leukemia	B-ALL, NOS* B-ALL with recurrent genetic abnormalities B-ALL with t(9;22)(q34;q11.2); BCR/ABL1 B-ALL with t(v;11q23); MLL rearranged B-ALL with t(12;21)(p13;q22); TEL/AML1 (ETV6/RUNX1) B-ALL with hyperdiploidy B-ALL with hypodiploidy B-ALL with t(5;14)(q31;q32); IL3/IGH B-ALL with t(1;19)(q23;p13.3); E2A/PBX1 (TCF3/PBX1) *NOS: not otherwise specified
T-lymphoblastic leukemia	
Mature B-cell neoplasm	Burkitt's lymphoma

Table 5. WHO classification (2008) of acute lymphoid leukemia (Gökbuget & Ottmann, 2012; Vardiman et al, 2009)

For B cell ALL (B-ALL) t(12;21)/TEL/AML1 and t(1;19)/E2A/PBX1 translocations define patients with good treatment outcome, especially following dose intensified chemotherapy. Patients with t(9;22)-BCR/ABL or t(4;11)-MLL/AF4 translocations have a relatively poor prognosis (Gökbuget et al, 2000).

1.3.2 Chronic leukemia

Chronic leukemia results in the accumulation of mature granulocytes or lymphocytes. Chronic leukemia progresses slowly but can develop into an acute form. Major types include chronic lymphocytic leukemia (CLL) and chronic myeloid leukemia (CML).

1.3.2.1 Chronic lymphocytic leukemia (CLL)

90% of CLL cases are seen in people who are 50 years or older and is almost never seen in children. In CLL, lymphocytes accumulate in peripheral blood, bone marrow, lymph nodes, spleen, and liver (Kolyvanos et al, 2003).

1.3.2.2 Chronic myeloid leukemia (CML)

CML is a clonal myeloproliferative disease (MPD) that results from the neoplastic transformation of a hematopoietic stem cell and has unique biological and clinical features (Begemann et al, 1998a). The leukemic cells of >95% of CML patients have a reciprocal translocation between the long arms of chromosomes 9 and 22 and the derived chromosome 22 is termed the Ph chromosome (Faderl et al, 1999).

The initial chronic phase of this disease is characterized by a massive expansion of the granulocytic cell lineage, even though most, if not all, hematopoietic lineages can be produced from the CML stem cell. The median duration of the chronic phase is 3–5 years (Faderl et al, 1999). Acquisition of additional genetic and/or epigenetic abnormalities causes the progression of CML from chronic phase to accelerated phase and finally to blast crisis. This phase is characterized by a block of cell differentiation that results in the presence of 30% or more myeloid or lymphoid blast cells in peripheral blood or bone marrow, or the presence of extramedullary infiltrates of blast cells (Melo & Barnes, 2007). Allogenic stem-cell transplantation is the only known curative therapy for CML. However, most patients are not eligible for this therapy, because of advanced age which makes them unable to tolerate the serious side effects of the treatment or lack of a suitable stem cell donor (Goldman & Melo, 2003).

The discovery that BCR/ABL is required for the pathogenesis of CML, and that the tyrosine-kinase activity of ABL is essential for BCR/ABL mediated transformation, made the ABL kinase an attractive target for therapeutic interventions (Daley et al, 1990; Lugo et al, 1990; Quintas-Cardama et al, 2007). Imatinib mesylate (Glivec, also known as STI571), a potent inhibitor of the tyrosine kinases ABL, ARG, PDGF-R and c-KIT, has been shown to selectively induce apoptosis in BCR/ABL-positive cells, and is remarkably successful in treating patients with CML (Deininger et al, 2005; Druker, 2004). In newly diagnosed patients with CML in chronic phase, imatinib induces complete cytogenetic response (CCyR) in high

portion of patients (Hochhaus et al, 2009). Patients with more advanced disease also respond to imatinib, but this occurs much less frequently and treatment is less durable.

1.4 The Philadelphia chromosome

The Occurrence of the chromosome translocations or inversions, which leads to the generation of oncogenic fusion proteins and their aberrant expression, is linked to the pathogenesis of leukemia (Faderl et al, 1998). These genetic alterations contribute to the leukemic transformation of HSCs and their progenitors by deregulating cellular functions such as maintaining or enhancing an unlimited capacity for self-renewal, impairing the normal proliferation and differentiation or promote resistant to apoptotic signals (Pui et al, 2004). About 80-85% of AML and ALL patients and over 95% of CML patients have chromosomal translocations (Begemann et al, 1998b; Faderl et al, 1998; Faderl et al, 1999). In most cases this chromosomal translocations refer to reciprocal translocations between different chromosomes. Over 95% of CML cases involve a balanced translocation of a fragment of the long arm of chromosome 9 to the long arm of chromosome 22 (Faderl et al, 1999). The t(9;22)(q34;q11) translocation leads to the fusion between the *BCR* gene on chromosome 22 and the *ABL* gene on chromosome 9. The shortened chromosome 22 was first described in 1960 by Peter Nowell and termed as Philadelphia chromosome. The Philadelphia chromosome also occurs in 20-30% of adult patients with ALL (Faderl et al, 1999; Pui et al, 2004).

1.4.1 Abelson murine leukemia virus homology gene (ABL)

The *ABL* gene is the human homologue of the v-abl oncogene carried by the Abelson murine leukemia virus (A-MuLV), and it encodes a non-receptor tyrosine kinase localized in the nucleus and cytoplasm (Deininger et al, 2000; Ling et al, 2003). Human ABL is a ubiquitously expressed 145 KDa protein with two isoforms, Ia and Ib, arising from alternative splicing of the first exon (Ben-Neriah et al, 1986; Hantschel & Superti-Furga, 2004; Laneuville, 1995; Pendergast, 2002; Shtivelman et al, 1986). The Ib isoform is myristoylated at an amino-terminal glycine residue, which is not the case for Ia isoform (Hantschel et al, 2003; Jackson & Baltimore, 1989). ABL protein contains several structural domains (Figure 2). Three Src homology (SH) domains (SH1-SH3) are located towards the NH_2 terminus. The SH1 domain carries the tyrosine kinase function, whereas the SH2 and SH3 domains allow for interaction with other proteins (Cohen et al, 1995; Hantschel, 2012; Koch et al, 1991).

Proline-rich sequences in the center of the molecule interact with the SH3 domains of other proteins such as Crk (Feller, 2001; Feller et al, 1994). Towards the 3' end, nuclear localization signals, DNA-binding and actin-binding motifs are found (Deininger et al, 2000). The normal ABL protein exhibit a complex role as a cellular module that integrates signals from various extracellular and intracellular sources, thereby influencing cell cycle and apoptosis (Deininger et al, 2000; Sirvent et al, 2008).

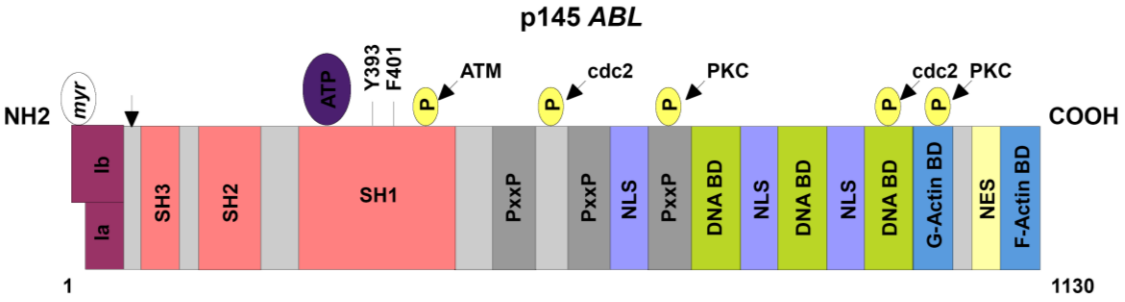

Figure 2. Structure of the Abl protein. Type Ia isoform is slightly shorter than type Ib, which contains a myristoylation (myr) site for attachment to the plasma membrane. The 3 SH domains are situated toward the NH2 terminus. Y393 is the major site of autophosphorylation within the kinase domain, and phenylalanine-401 (F401) is highly conserved in PTKs containing SH3 domains. The middle of the protein is dominated by proline-rich regions (PxxP) capable of binding to SH3 domains, and it harbors 1 of 3 nuclear localization signals (NLS). The carboxy terminus contains DNA as well as G- and F-actin-binding domains. Phosphorylation sites by Atm, cdc2, and PKC are shown. The arrowhead indicates the position of the breakpoint in the BCR/ABL fusion protein (Deininger et al, 2000).

1.4.2 Breakpoint cluster region gene (BCR)

The BCR protein is a multidomain protein of 160 KDa and is ubiquitously expressed (Laneuville, 1995; Laurent et al, 2001). The first N-terminal exon encodes a serine-threonine (S/T) kinase domain, with substrates being Bap-1 and BCR itself (Laurent et al, 2001; Maru & Witte, 1991). A coiled-coil (CC) domain at the N-terminus allows dimer formation *in vivo* (McWhirter et al, 1993). The center of the molecule contains a dbl-like pleckstrin-homology (PH) domain that stimulates the exchange of GTP to GDP on Rho guanidine exchange factors (GEF), which may activate the transcription factor NF-κB (Korus et al, 2002; Montaner et al, 1998; Zheng et al, 2006). The C-terminus has a GTPase-activating domain (GTPase activating proteins, GAPs) for Rac (regulates actin polymerisation) (Diekmann et al, 1991; Ress & Moelling, 2005). The GEFs activate members of the Ras superfamily by increasing the proportion of their GTP bound form with respect to GDP bound form (Boguski & McCormick, 1993; Jaffe & Hall, 2005), whereas the GAPs lead to hydrolysis of GTP to GDP and increasing their inactive form. Rho, cdc42 and Rac are involved in formation and

maintaining of philopodia, lamellipodia and 'stress fibers' (Scita et al, 2000; Zheng et al, 2006). BCR is a negative regulator of the small GTPase Rac (Ress & Moelling, 2005). This is demonstrated using BCR-deficient mice, where the loss of BCR up-regulates the production of a reactive oxygen species by the NADPH-oxidase complex through Rac (Voncken et al, 1995). In addition, BCR reduces via its GAP function the Rac1-dependent activation of the protein kinase Pak1, an activator of the JNK pathway (Lu & Mayer, 1999). Many GEFs contain a regulatory domain that blocks activity through an intramolecular interaction. For several, including Dbl, Vav, Asef, Tiam1, Ect2, and Net1, the removal of N-terminal sequences leads to constitutive activation when the protein is expressed *in vivo* (Katzav et al, 1991; Kawasaki et al, 2000; Miki et al, 1993; Ron et al, 1989; van Leeuwen et al, 1995). N-terminal truncated Rho-GEFs, for example, NET1, show a transforming activity in NIH3T3 fibroblasts (Alberts & Treisman, 1998; Whitehead et al, 1997). Similarly, in the case of p115RhoGEF and Lbc, removal of C-terminal sequences activates the protein (Sterpetti et al, 1999). In all these cases, it is assumed that activation of full-length GEF is through the relief of autoinhibition by phosphorylation or by binding to other proteins, but in most cases the mechanism is still unknown. The BCR related domains are shown in Figure 3.

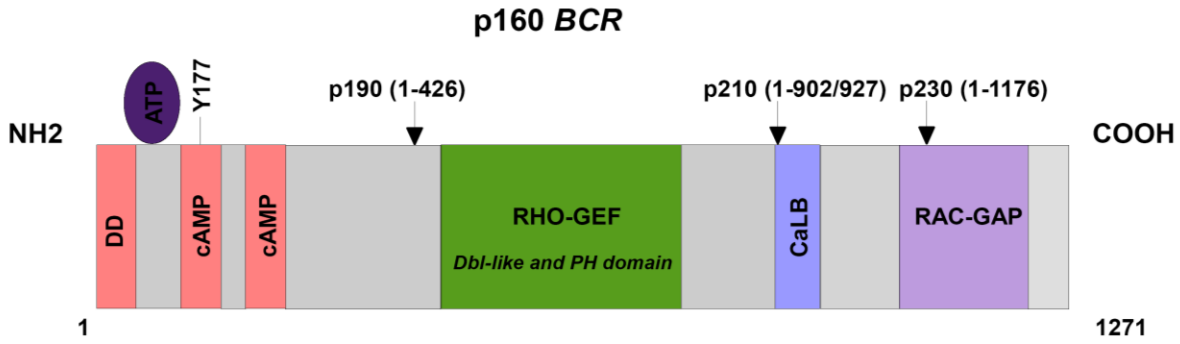

Figure 3. Structure of the BCR protein. The dimerization domain (DD) and the 2 cyclic adenosine monophosphate (cAMP) kinase homologous domains are located at the N terminus. Y177 is the autophosphorylation site crucial for binding to Grb-2. The center of the molecule contains a region homologous to Rho-GEF as well as dbl-like (DBL) and PH domains. Toward the C-terminus a putative site for calcium-dependent lipid binding (CaLB) and a domain with activating function for Rac-GAP are found. Arrows indicate the position of the breakpoints in the BCR/ABL fusion protein (Deininger et al, 2000).

1.4.3 The t(9;22)-associated translocation products

The t(9;22) leads to the generation of fusion proteins which can be associated with Ph^+ CML or ALL. The $p210^{BCR/ABL}$ and $p40^{ABL/BCR}$ are related with CML, whereas $p185^{BCR/ABL}$ and

p96$^{ABL/BCR}$ are related with ALL. The schematic representation of modular organization of the translocation partners and the fusion proteins in t(9;22) is presented in Figure 4.

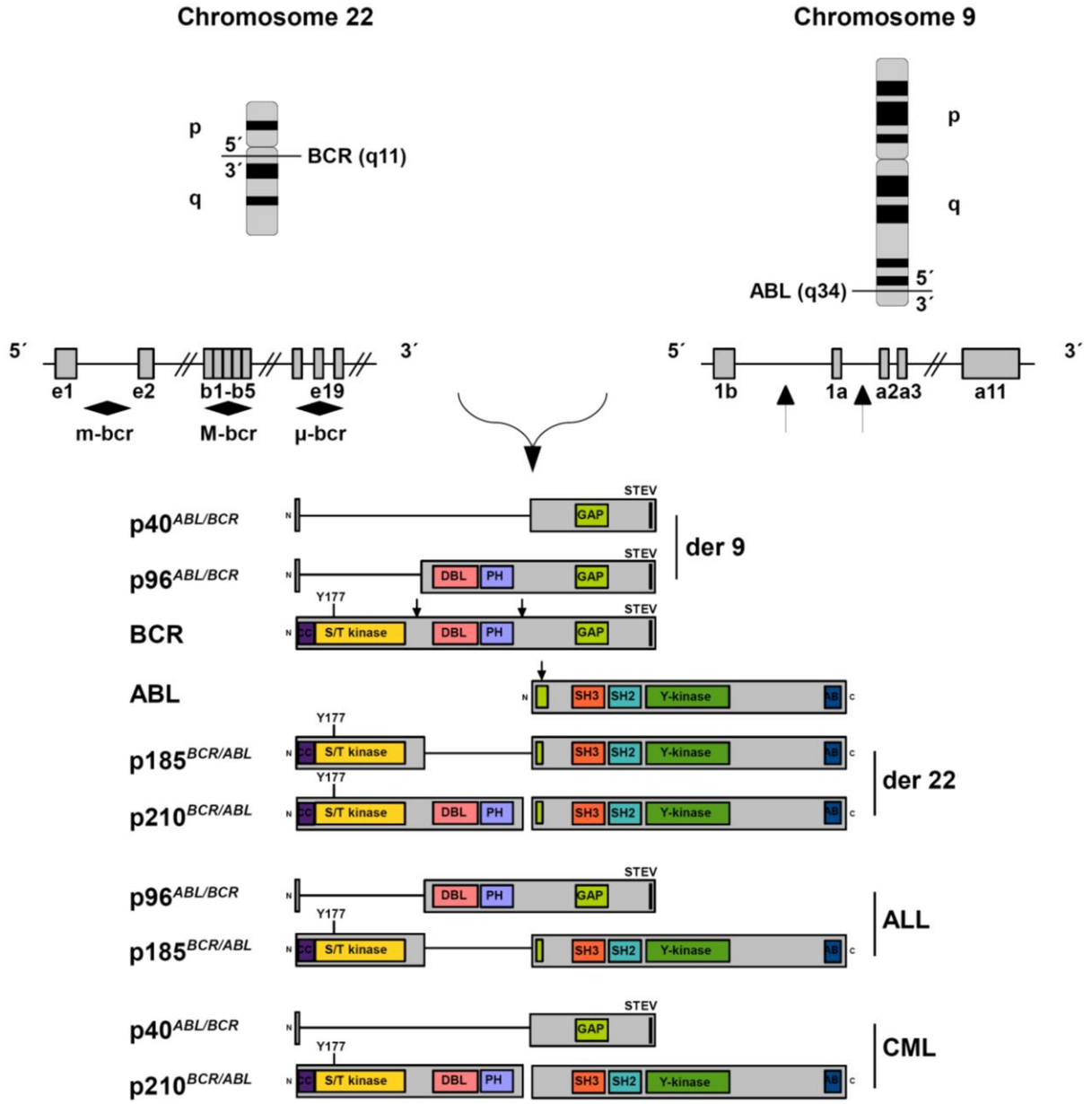

Figure 4. Four reciprocal translocation products arising from t(9;22)(q34;q11). The t(9;22) leads to the transposition of 3´ *ABL* segments on chromosome 9 to 5´ *BCR* segments on chromosome 22. Whereas breakpoint locations on chromosome 9 appear rather constant 5´ of *ABL* exon a2, several breakpoint cluster regions have been identified along the *BCR* gene on chromosome 22. A schematic representation of the fusion proteins encoded by derivative 9 (der9) and 22 (der22), as well as of their combination in m-BCR-positive Ph$^+$ ALL and M-BCR-positive CML is presented here. CC- coiled coil oligomerization interface; Y177- Tyrosine phosphorylation site at a.a. 177; S/T kinase- serine/threonine kinase domain; DH- dbl homology domain; PH- pleckstrin homology domain; GAP- Rac/GAP domain; STEV- PDZ-domain binding motif; SH2 and SH3- Src homology domains 2 and 3; Y kinase- tyrosine kinase domain; AB- actin binding domain. Modified from (Faderl et al, 1998; Zheng et al, 2009).

1.4.3.1 Chromosome der. 9: p40$^{ABL/BCR}$ and p96$^{ABL/BCR}$

The *ABL/BCR* fusion gene is placed on the 9+ chromosome, which differ according to the breakpoint on chromosome 22. Fusion between M-*BCR* (exons b3 or b4) and exon a1 of *ABL* result in production of ABL/BCR protein, p40$^{ABL/BCR}$, which can be detected in ca. 65% of examined CML patients (Melo et al, 1993a). ALL-associated ABL/BCR, p96$^{ABL/BCR}$, is detected in 100% of the patients (Melo et al, 1993b; Zheng et al, 2009). Both p40$^{ABL/BCR}$ and p96$^{ABL/BCR}$ represent mutated BCR, which have altered function as compared to the wild-type (wt) BCR (Zheng et al, 2006). In both p40$^{ABL/BCR}$ and p96$^{ABL/BCR}$, the BCR coiled-coil region and S/T kinase domain of BCR are missing. The difference between ALL and CML-specific ABL/BCR is related to the presence of the potentially oncogenic DH and PH domains. CML-associated ABL/BCR lacks DH and PH domain, which is contained in p210$^{BCR/ABL}$. In the ALL-specific ABL/BCR, this domain is conserved, which makes it a truncated Rho-GEF member (Figure 4). Earlier study demonstrated that the deletions of functional domains of wt BCR in ABL/BCR fusion proteins leads to a deregulated activation pattern of Rho-like GTPases in Rat-1 cells, since the BCR pattern of activated Rho, inhibited Rac and unmodified cdc42 was shown to shift to an ABL/BCR pattern of unmodified Rho and Rac, and activated cdc42 (Zheng et al, 2006). The ABL/BCR proteins were shown to have their own leukemogenic potential, since both were able to induce a myeloid leukemia-like disease in mice (Zheng et al, 2009) and increased the proliferation of early progenitors and enhanced the short term stem cell capacity of HSCs (Zheng et al, 2009).

1.4.3.2 Chromosome der. 22: p185$^{BCR/ABL}$ and p210$^{BCR/ABL}$

The breakpoint in the *ABL* gene locus is constantly found between exons 1 and 2 (a1 and a2). On chromosome 22, t(9;22) involves three different breakpoints in the *BCR* locus: (minor) m-*bcr*, which maps in the first intron of *bcr*; (major) M-*bcr*, which spans between exons 12 to 16 (also referred to as b1 to b5) and (micro) µ-*bcr* which was found between exon e19 and e20 (Faderl et al, 1999). These different breakpoints give rise to three distinct fusion proteins of molecular mass 190, 210, and 230 KDa, respectively, which contain the same portion of the c-ABL tyrosine kinase in the COOH terminus but include different amounts of BCR sequence at the NH2 terminus (Melo, 1997). The p210$^{BCR/ABL}$ protein is highly specific for CML. Different p210$^{BCR/ABL}$ has been found depending on the exon involved: b2a2- p210$^{BCR/ABL}$ or b3a3- p210$^{BCR/ABL}$. The p210$^{BCR/ABL}$ constructs were tested from many groups, but without

paying special attention to the breakpoint of the translocation. Due to the fact that in Ph^+ ALL related $p185^{BCR/ABL}$ the m-bcr breakpoint maps within an intron, the $p185^{BCR/ABL}$ transcript is constant (Faderl et al, 1999; Goldman & Melo, 2003; Melo, 1997). Based on the observation that the ABL part in the chimeric protein is constant, while the BCR portion varies, it can be concluded that ABL is likely to carry the transforming principle, whereas different sizes of the BCR sequence may determine the phenotype of the disease (Deininger et al, 2000).

1.4.3.2.1 Regulation of ABL kinase

The activity of ABL kinase is regulated by complex intramolecular interactions that impinge on the ABL kinase domain and lead to inhibition of tyrosine kinase activity *in vitro* and *in vivo* (Hantschel, 2012). It can be conclusively demonstrated that ABL is autoinhibited and there is no need for any other cellular inhibitors (Pluk et al, 2002). The SH2 and SH3 domains are key regulators of ABL autoinhibition. In unstimulated cells ABL SH3 domain interact with its own SH2-kinase domain and the N-terminal lobe of the kinase domain causing a conformational change that inhibits interaction with substrates (Barila & Superti-Furga, 1998; Goga et al, 1993). The interaction of SH2 with the C-terminal lobe of the kinase domain occludes access of phosphotyrosine ligands to the phosphotyrosine-binding pocket (Nagar et al, 2003). Another mechanism involved in regulating ABL kinase activity is ABL myristoylation and the mutants of ABL Ib that lack the myristoyl group showed strongly deregulated *in vitro* tyrosine kinase activity (Hantschel et al, 2003). Binding of myristoyl group to a hydrophobic pocket in the C-lobe of the kinase enables the assembly of the autoinhibited conformation of ABL Ib and mutations that block access to the myristate pocket strongly increase the kinase activity (Hantschel et al, 2003).

1.4.3.2.2 BCR/ABL-dependent signaling

Under unstimulated conditions ABL tyrosine kinase activity is tightly regulated by the mechanisms pointed above. However, the fusion of *BCR* gene to the *ABL* gene and the expression of BCR/ABL fusion protein leads to the deregulation of ABL kinase activity and phosphorylation of a variety of different substrate proteins. A schematic representation of BCR/ABL-dependent signaling pathways is illustrated in Figure 5.

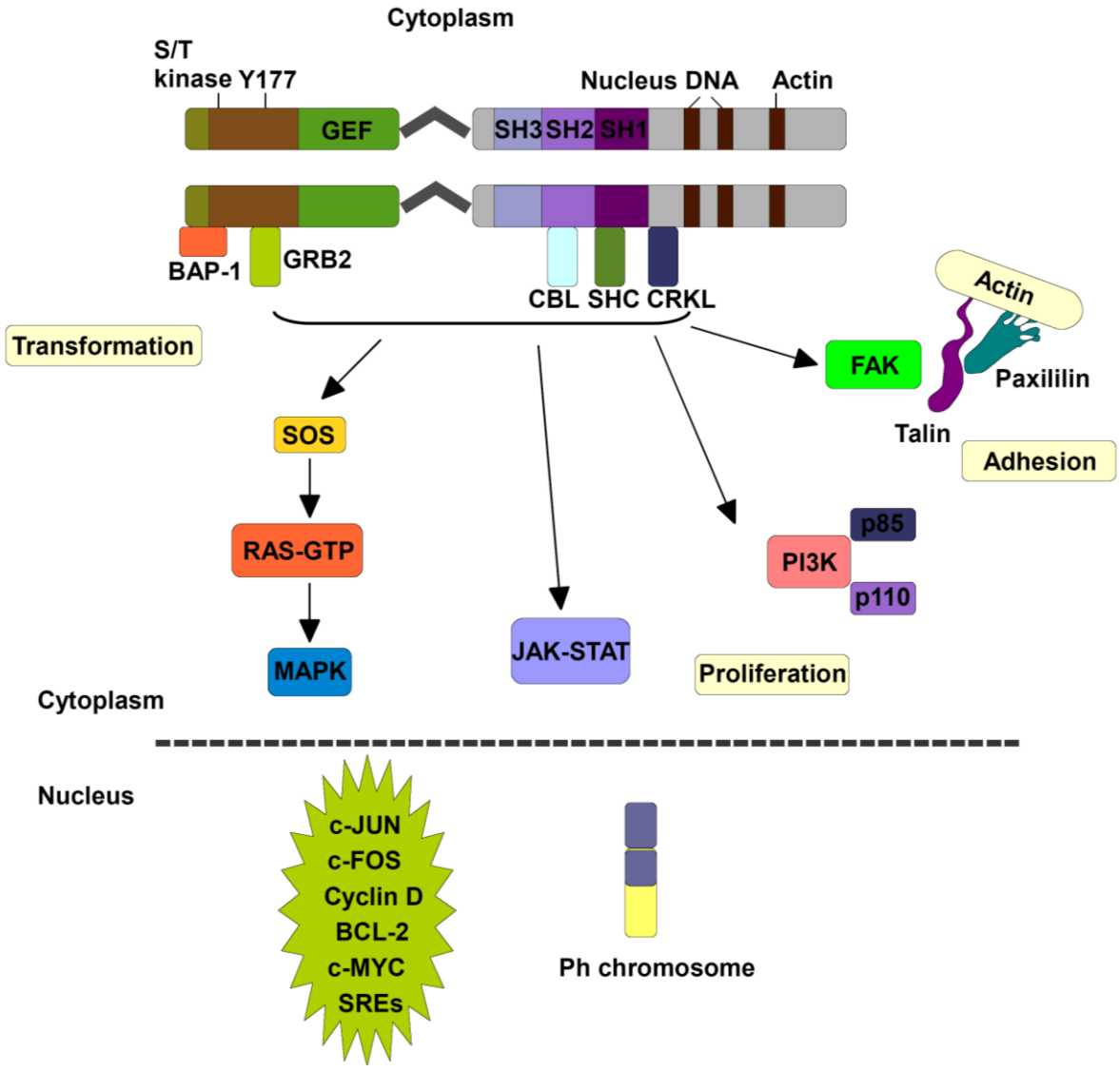

Figure 5. Schematic representation of the main BCR/ABL-activated pathways. A simplified diagram of pathways that regulate the proliferation and survival of BCR/ABL (p210)-transformed cells is presented here. Dimerization of BCR/ABL triggers autophosphorylation events that activate the kinase and generate docking sites for intermediary adapter proteins, such as GRB2. The activation of different signaling pathways lead to transformation of the cells and aberrant proliferation and adhesion. BAP1- BCR-associated protein 1, GRB2- growth factor receptor-bound protein 2, CBL- casitas B-lineage lymphoma protein, SHC- SRC homology 2-containing protein, CRKL- CRK oncogene–like protein, JAK-STAT- Janus kinase-signal transducers and activators of transcription, FAK- focal adhesion kinase, SOS- son of sevenless, GDP- guanosine diphosphate, GTP- guanosine triphosphate, SRE- stimulated response element, S/T- serine–threonine, Y177 a conserved tyrosine residue, GEF- GDP-GTP exchange factor, SH- SRC homology domain. Modified from (Faderl et al, 1999; O'Hare et al, 2011)

The N-terminal coiled coil domain of BCR/ABL facilitates dimerization and trans-autophosphorylation (McWhirter et al, 1993; Zhao et al, 2002). Autophosphorylation of tyrosine-177 of BCR/ABL promotes formation of a Grb2 complex with Gab2 and SOS triggering activation of Ras and recruitment of PI3-Kinase and the tyrosine phosphatase SHP2 (Chu et al, 2007; Sattler et al, 2002a). Signaling from Ras activates mitogen-activated protein

kinase (MAPK) and enhances proliferation. PI3-kinase activates the S/T kinase AKT (Markova et al, 2010). An additional critical role of BCR/ABL is STAT5 activation through direct phosphorylation or indirectly through phosphorylation by HCK or JAK2 (Ilaria et al, 1999; Klejman et al, 2002) and lack of STAT5 was reported to abrogate both myeloid and lymphoid leukemogenesis (Hoelbl et al, 2010). Other biological properties of BCR/ABL include: (i) altered adhesion to bone marrow stromal cells and extracellular matrix by expression of an adhesion inhibitory variant of α1 integrin not found in normal progenitors and (ii) degradation of inhibitory proteins Abi-1 and Abi-2 (ABL interactor proteins 1 and 2) in acute leukemias (Lewis et al, 1996).

1.4.3.2.2.1 STAT signaling

STATs are a seven member family of cellular proteins that function as both cytoplasmic signaling molecules and nuclear transcription factors (Aaronson & Horvath, 2002; Darnell, 1997). STATs are phosphorylated by receptor tyrosine kinases, or various cytoplasmic kinases such as Jaks or SFKs, in response to numerous cytokines and growth factor signals (Wilson et al, 2002).

BCR/ABL may also directly activate STAT1 and STAT5, and there seems to be specificity for STAT6 activation by $p185^{BCR/ABL}$, as opposed to $p210^{BCR/ABL}$ (Ilaria & Van Etten, 1996). STAT5 is activated by BCR/ABL via the SFK, HCK (Klejman et al, 2002). On interaction with the SH3 and SH2 domains of BCR/ABL, this kinase is activated and phosphorylates STAT5, leading to its translocation to the nucleus, where it functions as a transcription factor (Klejman et al, 2002). A STAT5 target gene involved in BCR/ABL leukemogenesis is the antiapoptotic gene Bcl-X_L. The expression of this protein is down-regulated in Imatinib treated BCR/ABL-expressing cells (Horita et al, 2000). Two other STAT5 targets are the antiapoptotic proteins A1 (member of the Bcl-2 family) and pim-1 (a S/T kinase and a proto-oncogene) (Calabretta & Perrotti, 2004).

STAT5 was reported to be essential for the leukemia initiation mediated by $p185^{BCR/ABL}$ (Hoelbl et al, 2006) and indispensable for the maintenance of $p210^{BCR/ABL}$-positive leukemia (Hoelbl et al, 2010). Evidence for the role of STAT5 in BCR/ABL leukemogenesis is supported by the following observations that BCR/ABL mutants (ΔSH2+ΔSH3 and P1013L+ΔSH2 BCR/ABL mutants) defective in STAT5 activation were less efficient than the wild-type form in transformation of myeloid precursor cells. In addition to this, a

constitutively active STAT5 mutant (H295R+S715F) rescued the leukemogenic potential of STAT5 activation-deficient BCR/ABL mutants (Nieborowska-Skorska et al, 1999).

1.4.3.2.2.2 Ras/MAPK

Autophosphorylation of tyrosine-177 provides a docking site for the adaptor molecule Grb2 (Hantschel, 2012). Grb2 then binds to Gab2, and the phosphorylation of SHC leads to enhanced activity of the protein SOS, which promotes the accumulation of the active GTP bound form of Ras (Hantschel, 2012; Pendergast et al, 1993). Crkl, which is a direct substrate of BCR/ABL, can also activate Ras (Calabretta & Perrotti, 2004). Activation of Ras leads to the subsequent recruitment of the S/T kinase Raf to the cell membrane. Raf initiates a signaling cascade through the S/T kinases Mek1/Mek2 and Erk, which ultimately leads to activation of gene transcription (Deininger et al, 2000; Johnson & Lapadat, 2002).

Other pathways activated are the Jnk/Sapk, p38, Dok-1 (forms complexes with Crkl, Ras, Gab2, and BCR/ABL). In any case, signals are transduced to the transcriptional machinery of the cell. The importance of Ras-dependent signaling for the phenotype of BCR/ABL-expressing cells is supported by the observation that, down-regulation of this pathway by antisense strategies, expression of dominant negative molecules, or chemical inhibitors, suppresses proliferation and sensitizes cells to apoptotic stimuli (Calabretta & Perrotti, 2004).

1.4.3.2.2.3 PI3K/AKT

BCR/ABL interacts via various adapter proteins including Grb2/Gab2 and c-Cbl with the p85 regulatory subunit of PI3K (Adams et al, 2012; Sattler et al, 2002b). PI3K activates the S/T kinase AKT triggering an AKT-dependent cascade, which regulates the subcellular localization or activity of several targets such as BAD, MDM2, IκB-kinase-α and members of the forkhead family of transcription factors (Vivanco & Sawyers, 2002). Phosphorylation of BAD by AKT suppresses its pro-apoptotic activity, because when phosphorylated, BAD is sequestered in the cytoplasm, in a complex with 14-3-3 proteins and this prevents the binding of this protein to Bcl-2 family members, Bcl-2 and Bcl-XL (Kharas & Fruman, 2005). Phosphorylation of MDM2 enhances its nuclear-cytoplasmic export, inducing a more efficient degradation of p53 (transcription factor that regulates the cell cycle and functions as a tumor suppressor). Phosphorylation of IκB-kinase-α enhances the activity of this protein towards its substrate IκB. On phosphorylation, IκB is subjected to ubiquitination and proteasome-dependent degradation allowing the translocation of nuclear factor κB (NF-κB) into the

nucleus, where it functions as a transcription factor (Silverman & Maniatis, 2001). Phosphorylation of the transcription factor FKHRL1 prevents its translocation to the nucleus due to binding to 14-3-3 family of proteins and by this blocking the transactivation of genes, which promotes apoptosis (for example TRAIL), or inhibiting cell cycle progression (for example p27) (Komatsu et al, 2003). Consistent with the effects of AKT, inhibition of the PI3K/AKT pathway suppresses *in vitro* colony formation and *in vivo* leukemogenesis of BCR/ABL expressing cells (Skorski et al, 1997). Bone marrow cells with inactivated PI3K/AKT are resistant to BCR/ABL transformation (Badura et al, 2013; Calabretta & Perrotti, 2004).

1.4.4 Molecular therapy of Ph^+ leukemia

Given the fact that BCR/ABL is the causative agent for Ph^+ leukemia (Daley et al, 1990; Lugo et al, 1990), it is obvious that targeting the function of the fusion protein itself would provide an appropriate therapeutic approach. It was shown that the transforming capacity of BCR/ABL is strictly dependent on its tyrosine kinase (TK) activity (Lugo et al, 1990). Thus, a selective ABL kinase inhibitor called imatinib was developed, which competes with ATP for the binding to the TK domain and inhibits oncogenic ABL proteins like v-ABL, BCR/ABL and TEL/ABL (Deininger et al, 1997; Druker et al, 1996; Weisberg & Griffin, 2000). Imatinib proved to be clinically very effective: 95% of CML-chronic phase patients achieve a complete hematologic and cytogenetic remission upon imatinib treatment (Druker et al, 2006; O'Dwyer et al, 2003). 70% of CML blast crisis or Ph^+ ALL patients achieve a transient complete remission, but relapse frequently within 4-6 months and become refractory towards further treatment with imatinib. Recently, much effort has been undertaken to solve the problem of imatinib resistance. There are two major obstacles in imatinib-based therapies for patients with CML. One is the persistence of BCR/ABL-positive cells; this is known as ‘residual disease’ and is detected by a sensitive nested reverse-transcriptase PCR assay. Suppression of the disease, therefore, relies on continuous imatinib therapy. The other major problem is relapse of the disease due to the emergence of resistance to imatinib. Several mechanisms of resistance have been described, the most frequent of which are the appearance of point mutations in BCR/ABL that impair the drug binding (Ren, 2005). Many point mutations are inhibited by increased doses of imatinib, as the achievable serum levels of imatinib are below the concentrations necessary to overcome resistance (O'Hare et al, 2005b; Weisberg et al, 2006; Weisberg et al, 2005).

Treatment failure with imatinib led to the development of second-generation of ABL-kinase inhibitors: Nilotinib (Novartis, Basel, Switzerland), Dasatinib (Bristol-Myers-Squibb, New York, USA) and Bosutinib (Pfizer, New York, USA).

Nilotinib, like imatinib, is a selective ABL kinase inhibitor and has a 20-fold higher affinity for the ABL kinase domain than imatinib (O'Hare et al, 2005a). Nilotinib inhibits most imatinib-resistant point mutations (such as F317L/C/V, Q252H, M244V) although some P-loop mutations are only partially inhibited (E255K, Y253F/H) and is unable to inhibit the gate-keeper mutation, T315I (Weisberg et al, 2005).

The dual SRC-ABL kinase inhibitor dasatinib takes advantage of a characteristic difference between SRC and ABL kinase domain conformations to inhibit imatinib-resistant mutations: The inactive conformations of SRC and ABL are different, while the active conformations are closely related (Schindler et al, 2000). Imatinib and nilotinib bind to the inactive ABL kinase conformation, which is the reason for their selectivity, while dasatinib binds to active ABL and active SRC (Schindler et al, 2000; Weisberg et al, 2006). Therefore the binding of dasatinib is dependent on another choice of amino acid residues as imatinib or nilotinib (Schindler et al, 2000). Dasatinib has a 325-fold higher affinity towards ABL than imatinib (O'Hare et al, 2005a) and inhibits most mutations identified in imatinib resistant patients (for instance: F317L, E255K/V, Y253F, Q252R/H), but is still unable to target the T315I mutation (O'Hare et al, 2005a; Shah et al, 2004).

Another dual SRC and ABL kinase inhibitor, Bosutinib, was demonstrated to possess antiproliferative activity in BCR/ABL-positive cell lines like LAM84R, K562R and KCL22R, where the resistance mechanism is not due to mutations in BCR/ABL and in the case of K562R and KCL22R even not defined (Puttini et al, 2006). This inhibitor was shown to have no effect on PDGFR or KIT, which is supposed to have lower toxicity *in vivo* (Puttini et al, 2006), but again not able to inhibit cells transfected with BCR/ABL harboring T315I mutation (Puttini et al, 2006; Remsing Rix et al, 2009).

Efforts in developing more potent ABL inhibitors led to the development of INNO-406 (NS-187), which was shown to inhibit ABL and LYN kinases and is 25-55 times more potent than imatinib and able to inhibit a wide range of BCR/ABL mutants *in vitro* (for example: M244V, Q252H, Y253F, G250E), but still unable to conquer T315I resistance (Naito et al, 2006).

The introduction of AP24534 (ponatinib) (Ariad, Cambridge, Massachusetts), a pan-BCR/ABL inhibitor, was a breakthrough in the treatment of T315I mutation, since ponatinib was shown to have the potential to inhibit all the BCR/ABL mutants, covering T315I mutation (O'Hare et al, 2009). In contrast to developed compounds like MK-0457, which were able to some extent conquer the resistance of T315I mutation in advanced phase CML patients (Giles et al, 2007), but were clinically rather unsuccessful due to toxicity difficulties, ponatinib reached even the phase 2 trial in the treatment of Ph^+ leukemia (Cortes et al, 2013).

Another possibility to cope with imatinib resistance is targeting sites out of the ATP pocket. Since the ABL N-terminal region is absent in BCR/ABL, the vestigial myristate-binding pocket is unoccupied in the fusion protein and the activity of allosteric inhibitors is through binding to the myristate-binding pocket of BCR/ABL (Adrian et al, 2006). This class of inhibitors including GNF-2 and GNF-5 poses excellent selectivitiy toward BCR/ABL transformed cells and were reported to maintain potency against clinically relevant imatinib-resistant BCR/ABL mutants, however none of them were able to inhibit T315I mutation. Beside BCR/ABL-dependent mechanisms for resistance against imatinib and other ABL inhibitors, BCR/ABL-independent factors like drug efflux (up-regulation in the expression of P-glycoprotein) (Mahon et al, 2000), drug import (Thomas et al, 2004), binding (Gambacorti-Passerini et al, 2000), concentration (Picard et al, 2007) and also the activation of alternative signaling pathways (Aceves-Luquero et al, 2009; Wu et al, 2008) were postulated to describe the resistance mechanism.

Aim of the study

The aberrant kinase activity of BCR/ABL is sufficient to induce a leukemia phenotype in mice (Kelliher et al, 1990; Lugo et al, 1990); however the progression of the disease and the appearance of mutational and non-mutational-dependent resistance to the kinase inhibitors indicate requirement for additional factors. Previous studies demonstrated that ABL/BCR proteins (ALL-associated protein p96 and CML-associated protein p40), in contrast to BCR, activate Rac, which is a key player in the leukemogenesis of Ph^+ leukemia (Skorski et al, 1998; Zheng et al, 2006). Both ABL/BCR proteins showed leukemogenic potential in transduction-transplantation model and increased the proliferation of early progenitors and short term stem cell capacity of $Sca1^+/lin^-$ cells (Zheng et al, 2009). In this way ABL/BCRs could play an important role in the grade of response to kinase inhibitors that target BCR/ABL such as imatinib, nilotinib and dasatinib. This could be of great clinical importance, since therapy mediated mutations in BCR/ABL clearly demonstrate an urgent need for new therapy strategies. Due to the fact that the effect of $p96^{ABL/BCR}$ was more prominent in all performed experiments with regards to leukemogenic potential and effect on HSCs (Zheng et al, 2009), and that this protein was detected in 100% of Ph^+ ALL cell lines (Zheng et al, 2009) and patient cells (Melo et al, 1993b), the focus of this study was the Ph^+ ALL-related ABL/BCR protein, $p96^{ABL/BCR}$.

The aim of this study was to disclose the contribution of Ph^+ ALL-related t(9;22) fusion proteins, $p96^{ABL/BCR}$ and $p185^{BCR/ABL}$, to the biological features of Ph^+ ALL. The role of $p96^{ABL/BCR}$ fusion protein in the pathogenesis of Ph^+ ALL, where this protein can be detected in all the cases, has still to be elucidated. The presence of $p96^{ABL/BCR}$ in all Ph^+ ALL cell lines and patient cells might influence the transformation and leukemogenic potential of its reciprocal fusion protein, $p185^{BCR/ABL}$. Hence it would be of great importance to figure out the effect of $p96^{ABL/BCR}$ on the leukemogenic potential of $p185^{BCR/ABL}$. The activation of BCR/ABL-related signaling pathways in the presence of $p96^{ABL/BCR}$ will lead to a better understanding of the effect of the t(9;22) products on the pathogenesis of Ph^+ ALL and impact the drug development strategies downstream of BCR/ABL.

In addition to this, since the Ph^+ stem cell was reported to be insensitive to imatinib treatment (Barnes & Melo, 2006; Bhatia et al, 2003; Graham et al, 2002; Wong et al, 2004) and due to the fact that $p96^{ABL/BCR}$ increases the stem cell capacity of HSCs (Zheng et al, 2009), one can assume that the primitive Ph^+ stem cell is independent of BCR/ABL kinase activity and the

ABL/BCR protein plays an important role in this scenario. Therefore this study will investigate the question of how the t(9;22) fusion proteins influence the biology of primary HSCs alone or in combination and whether these proteins regulate the clonal leukemic stem cell population in a hierarchical manner. The effect of the possible interplay between $p96^{ABL/BCR}$ and $p185^{BCR/ABL}$ fusion proteins on biological features of stem cell such as self-renewal capacity, differentiation and apoptosis will be investigated.

These investigations will give new insights into the pathogenesis of Ph^+ ALL and might improve the treatment strategies concerning Ph^+ leukemia.

2 MATERIALS AND METHODS

2.1 Materials

2.1.1 Apparatus

Autoclave	Hp Medizintechnik, Oberschleissheim, Germany
Centrifuges	
Centifuge and Biofuge 13R	Heraeus, Hanau, Germany
Cytocentrifuge	Thermo Shandon, Pittsburgh, USA
Megafuge 1.0 with Rotor BS4402/A	Heraeus, Hanau, Germany
Rotina 48 RS Table top centrifuge	Hettich, Tuttlingen, Germany
Sorvall RC-5B refrigerated superspeed	Hettich, Tuttlingen, Germany
Table top centrifuge 5415C/D	Eppendorf, Hamburg, Germany
Gel electrophoresis system	
DNA-sub cell and Mini-Sub cell System	Gibco, Karlsruhe, Germany
SDS-polyacrylamide gel electrophoresis	Bio-Rad, Munich, Germany
Incubators	
Bacteria incubator	Heraeus, Hanau, Germany
Benchtop gyrotory incubator	New Brunswick Scientific, Edison, USA
Cell culture incubator	Heraeus, Hanau, Germany
Irradiation of mice	
Betatron 500A	Siemens, Munich, Germany
Microscopes	
Axioplan 2-imaging Fluorescence microscope	Zeiss, Goettingen, Germany
Binocular stereo microscope (SZ)	Olympus, Munich, Germany

Leitz Labovert inverted Microscope	Leitz, Wetzlar, Germany
SZ40 Zoom Stereo Microscope	Olympus, Munich, Germany
Olympus BX50	Olympus, Munich, Germany
Zeiss ID03 inverted microscope	Zeiss, Goettingen, Germany
Magnetic cell separation	
MACS separation columns: LD and LS	Miltenyi, Bergisch Gladbach, Germany
MidiMACS separator	Miltenyi, Bergisch Gladbach, Germany
MiniMACS separator	Miltenyi, Bergisch Gladbach, Germany
PCR machines	
Mastercycler® Pro	Eppendorf, Hamburg, Germany
DNA Engine Opticon®2	Bio-Rad, Munich, Germany
Spectrophotometer	
Gene Quant II	Pharmacia Biotech, Freiburg, Germany
Biophotometer	Eppendorf, Hamburg, Germany
Protein transfer	
Novex blotting apparatus	Invitrogen, Karlsruhe, Germany
Trans-Blot SD Semi-dry Transfer cell	Bio-Rad, Munich, Germany
Shakers	
Bacterium shaker	New Brunswick Scientific, Edison, USA
Horizontal shaker	Heidolph, Schwabach, Germany
Miscellaneous	
FACS Canto II	Beckton Dickinson, Heidelberg, Germany
Fluorchem™ imaging system	Alpha Innotech, Miami, USA
Fridge	Liebherr, Stuttgart, Germany
Freezer	Liebherr, Stuttgart, Germany

Magnet mixer	Heidolph, Schwabach, Germany
pH meter	Hanna Instruments, Kehl, Germany
Power PAC 2000	Bio-Rad, Munich, Germany
Rotating shaker	Heidolph, Schwabach, Germany
Scale	Sartorius, Goettingen, Germany
Steril bank	Heraeus, Hanau, Germany
Tecan Spectra Microplate reader	Tecan, Switzerland
Thermoblock	Eppendorf, Hamburg, Germany
Thermomixer	Eppendorf, Hamburg, Germany
Vortex	Eppendorf, Hamburg, Germany
Water bath	Julabo Labortechnik, Seelbach, Germany

2.1.2 Instruments

3MM Whatman paper	Whatman, Dassel, Germany
Cell culture flasks/dishes	Greiner, Heidelberg, Germany
Cell scraper	Corning, Wiesbaden, Germany
Cell strainer (40 µM)	Becton Dickinson, Heidelberg, Germany
Cryotubes	Nalgene, Rochester, NY , USA
Cuvette	Sarstedt, Nuembrecht, Germany
Cytofunnel	Shandon, Pittsburgh, USA
Micropipettes 10/20/200/1000 µL	Gilson, Middletown USA
Nitrocellulose membrane (0.2 µM)	Bio-Rad, Munich, Germany
Pipetteboy	Brandt, Lemgo, Germany
Steril filter	
0.22 µM	Millipore, Eschborn, Germany
0.45 µM	Millipore, Eschborn, Germany

2.1.3 Chemicals

10x FACS lysing solution	BD Biosciences, Heidelberg, Germany
10x Restriction buffer 1,2,3,4	New England Biolabs, Frankfurt, Germany
Acetic acid glacia	Sigma, Steinheim, Germany
Acetone	Roth, Karlsruhe, Germany
Agarose	Sigma-Aldrich, Munich, Germany
Ammonium persulfate	Fluka, Deisenhofen, Germany
Ampicillin	Sigma, Steinheim, Germany
Antioxidant	Invitrogen, Karlsruhe, Germany
Bacterial cultur LB medium	Roth, Karlsruhe, Germany
Biorad protein dye	Bio-Rad, Munich, Germany
Bromophenol blue	Sigma, Steinheim, Germany
Calcium chloride	Sigma, Steinheim, Germany
Chloroform	Fluka, Deisenhofen, Germany
Cumaric acid	Sigma, Steinheim, Germany
ddH_2O	Sigma, Steinheim, Germany
DEPC	Sigma, Steinheim, Germany
dGTP/dATP/dTTP/dCTP-Nucleotides	Roche, Basel, Switzerland
Dithiothreitol	Sigma-Aldrich, Munich, Germany
DMSO	Sigma, Steinheim, Germany
DNA marker	New England Biolabs, Frankfurt, Germany
EDTA, disodium, dihydrate	Sigma, Steinheim, Germany
Ethanol	Merck, Darmstadt, Germany
Formaldehyde	Merck, Darmstadt, Germany
Gel Red	Biotium, Hayward, USA

Glucose	Sigma, Steinheim, Germany
Glycerol	Sigma, Steinheim, Germany
Glycine	Merck, Darmstadt, Germany
H_2O_2	Sigma-Aldrich, Munich, Germany
HCL	Merck, Darmstadt, Germany
Isobutanol	Sigma, Steinheim, Germany
Isopropanol	Fluka, Deisenhofen, Germany
Kanamycine	Sigma, Steinheim, Germany
KCl	Sigma-Aldrich, Munich, Germany
LiCl	Sigma-Aldrich, Munich, Germany
Lineage cell depletion kit	Miltenyi, Bergisch Gladbach, Germany
Low Fat Milk	Roth, Karlsruhe, Germany
Low Melting Agarose	Sigma-Aldrich, Munich, Germany
Luminol	Fluka, Deisenhofen, Germany
Methanol	Merck, Darmstadt, Germany
Methylene blue	Sigma, Steinheim, Germany
$MgCl_2$	Sigma, Steinheim, Germany
$MgSO_4$	Fluka, Deisenhofen, Germany
NaCl	Merck, Darmstadt, Germany
NaH_2PO_4	Sigma, Steinheim, Germany
NaN_3	Sigma, Steinheim, Germany
NaOH	Roth, Karlsruhe, Germany
Paraformaldehyde	Merck, Darmstadt, Germany
p-Coumaric acid	Sigma, Steinheim, Germany
Phenol	Fluka, Deisenhofen, Germany

Polybrene	Sigma, Steinheim, Germany
Ponceau S	Sigma, Steinheim, Germany
Pre-stained SDS-PAGE standard	Bio-Rad, Munich, Germany
Protease-inhibitors	Roche, Basel, Switzerland
SDS	Sigma, Steinheim, Germany
Sodium carbonate	Sigma, Steinheim, Germany
Sodium thiosulfate	Fluka, Deisenhofen, Germany
Sodium acetate	Fluka, Deisenhofen, Germany
Sodium citrate	Roth, Karlsruhe, Germany
S.O.C medium	Invitrogen, Karlsruhe, Germany
Stripping buffer	Thermo Scientific, Bonn, Germany
TEMED	Sigma, Steinheim, Germany
Triton X-100	Sigma, Steinheim, Germany
Trizma HCl	Sigma, Steinheim, Germany
Tween20	Sigma, Steinheim, Germany
Xylene cyanol	Sigma, Steinheim, Germany
ZnSO4	Sigma, Steinheim, Germany
β-Mercaptoethanol	Fluka, Deisenhofen, Germany

2.1.4 Special reagents and materials

2.1.4.1 Cell culture medium and reagents

7-aminoactinomycin D (7-AAD)	Sigma, Steinheim, Germany
Bovine Serum Albumin	Sigma, Steinheim, Germany
CD34 multisort kit	Miltenyi, Bergisch Gladbach, Germany
Chloroquine	Sigma, Steinheim, Germany

DMEM medium	Sigma, Steinheim, Germany
DMSO	Sigma, Steinheim, Germany
Fetal Bovine Serum	Gibco, Karlsruhe, Germany
Fetal Calf Serum	Hyclone, Logan UT, USA
Ficoll separating solution	Pan-biotech, Aidenbach, Germany
Gelatin	Sigma, Steinheim, Germany
GNF-2	Sigma, Steinheim, Germany
HBS	Gibco, Karlsruhe, Germany
HEPES solution	Gibco, Karlsruhe, Germany
Horse serum	Gibco, Karlsruhe, Germany
Imatinib	Novartis, Basel, Switzerland
IMDM medium	Merck, Darmstadt, Germany
L-Glutamine	Gibco, Karlsruhe, Germany
Lineage Cell Depletion Kit	Miltenyi, Bergisch Gladbach, Germany
MethoCult™ GF M3534	Stem cell Technologies, Vancouver, Canada
PBS	Gibco, Karlsruhe, Germany
Penicillin-Streptomycin solution	Gibco, Karlsruhe, Germany
Retronectin®	Takara Bio Inc., Otsu, Japan
RPMI 1640	Sigma, Steinheim, Germany
Sca-1 Isolation Kit	Miltenyi, Bergisch Gladbach, Germany
Trypan blue stain (0.4%)	Gibco, Karlsruhe, Germany
Trypsin EDTA, 0.25% solution	Gibco, Karlsruhe, Germany
X-VIVO 10 medium	Lonza, Basel, Switzerland

2.1.4.2 Cytokines

rhFlt3-ligand	Miltenyi, Bergisch Gladbach, Germany
rhIL-3	Miltenyi, Bergisch Gladbach, Germany
rhSCF	Miltenyi, Bergisch Gladbach, Germany
rhTPO	Miltenyi, Bergisch Gladbach, Germany
rmFlt3-ligand	Peprotech, Offenbach, Germany
rmIL-3	Peprotech, Offenbach, Germany
rmIL-6	Peprotech, Offenbach, Germany
rmSCF	Peprotech, Offenbach, Germany

2.1.4.3 Enzymes

Antartic phosphatase	New England Biolabs, Frankfurt, Germany
Gateway LR clonase enzyme mix	Invitrogen, Karlsruhe, Germany
Klenow-Fragment DNA-polymerase I	New England Biolabs, Frankfurt, Germany
Proteinase K	Stratagene, La Jolla, USA
Restriction endonucleases	New England Biolabs, Frankfurt, Germany
RNAse	Sigma, Steinheim, Germany
Superscript® II reverse transcriptase	Invitrogen, Karlsruhe, Germany
T4 DNA ligase	New England Biolabs, Frankfurt, Germany

2.1.4.4 PCR reagents

dNTPs	Bioline, Luckenwalde, Germany
MgCl2	Bioline, Luckenwalde, Germany
PCR Buffer	Bioline, Luckenwalde, Germany
PfuTurbo polymerase	Stratagene, La Jolla, USA
Primers	Sigma, Steinheim, Germany

Taq DNA polymerase	Bioline, Luckenwalde, Germany

2.1.4.5 Antibodies

2.1.4.5.1 Primary antibodies used for western blotting

Mouse anti-β tubulin (Ab-2)	Neo Markers, Asbach, Germany
Rabbit anti-p-ABL-Tyr-245	Cell Signaling, Boston, USA
Rabbit anti-p-ABL-Tyr-412	Cell Signaling, Boston, USA
Rabbit anti-p-BCR-Tyr-177	Cell Signaling, Boston, USA
Rabbit anti-BCR (C-20)	Santa Cruz, Heidelberg, Germany
Mouse anti-phospho-STAT5 (Y694)	Cell Signaling, Boston, USA
Rabbit anti -STAT5 (C-17)	Santa Cruz, Heidelberg, Germany
Rabbit anti-phospho-CrkL (Y207)	Cell Signaling, Boston, USA
Mouse anti-CrkL (H-62)	Santa Cruz, Heidelberg, Germany
Rabbit anti-phospho-Erk (T202-Y204)	Cell Signaling, Boston, USA
Mouse anti-Erk	Cell Signaling, Boston, USA
Rabbit anti-c-ABL (24-11)	Santa Cruz, Heidelberg, Germany

2.1.4.5.2 Secondary antibodies

Anti-mouse IgG-HRP	Santa Cruz, Heidelberg, Germany
Anti-rabbit IgG-HRP	Santa Cruz, Heidelberg, Germany

2.1.4.5.3 FACS antibodies

Mouse-anti-PE-IgG 2a, K	Beckton Dickinson, Heidelberg, Germany
Mouse-anti-PE-IgG 2b, K	Beckton Dickinson, Heidelberg, Germany
Rat-anti- mouse-PE-B220	Beckton Dickinson, Heidelberg, Germany
Rat-anti- mouse-PE-Gr-1	Beckton Dickinson, Heidelberg, Germany

Rat-anti- mouse-PE-Mac-1	Beckton Dickinson, Heidelberg, Germany

2.1.4.6 Buffers

Phosphate-buffered saline (PBS)

NaCl	137 mM
KCl	2.7 mM
Na_2HPO_4	10 mM
KH_2PO_4	2 mM

The above components were dissolved in water and adjusted pH to 7.4 with HCl.

Tris-HCl (1 M)

121.1g of Tris-base was dissolved in 800 ml of water. The pH was adjusted to the desired value by adding concentrated HCl as follows:

pH	HCl
7.4	70 ml
7.6	60 ml
8.0	42 ml

The solution was allowed to cool down to room temperature before making final adjustments to the pH. The volume of the solution was adjusted to 1 L with water.

EDTA (0.5 M, pH 8.0)

186.1 g of disodium EDTA was added to 800 ml of H_2O. The mixture was stirred vigorously on a magnetic stirrer. The pH was adjusted to 8.0 with NaOH. The disodium salt of EDTA did not go into solution until the pH of the solution was adjusted to 8.0 by the addition of NaOH.

10x Tris EDTA (TE) buffer

pH 7.4	100 mM Tris-Cl (pH 7.4)
	10 mM EDTA (pH 8.0)
pH 7.6	100 mM Tris-Cl (pH 7.6)
	10 mM EDTA (pH 8.0)
pH 8.0	100 mM Tris-Cl (pH 8.0)
	10 mM EDTA (pH 8.0)

0.1% Diethylpyrocarbonate (DEPC)

1 g DEPC was dissolved in 1 L water and mixed it vigorously. It was left to stand with loose lid overnight at room temperature or at 37°C for 1hour and autoclaved for 15 minutes on liquid cycle.

2.1.4.7 Plasmids and vectors

pCDNA 3.1 vector	Invitrogen, Karlsruhe, Germany
pENTR1A	Invitrogen, Karlsruhe, Germany
PINCO	Retroviral hybrid vector with LTR derived from Moloney murine Leukemia virus M-MLV). The main characteristics of this vector are the presence of the EBV origin of replication and the EBNA-1 gene and the presence of the cDNAs that encodes for the eGFP, controlled by a cytomegalovirus promoter (CMV). This vector allows high-efficiency gene transfer (Grignani et al, 1998)
PAULO	This vector is a modified PINCO vector in which GFP is replaced by LNGFR
PSIEW	Lentiviral vector containing SFFV promoter and eGFP expression through IRES (M. Grez, Georg Speyer Haus, Frankfurt, Germany)
PLKO.1	Lentiviral vector with hPGK (Human phosphoglycerate kinase eukaryotic promoter) allow for transient or stable transfection of the shRNA (Sigma Aldrich, Munich, Germany).
pMD2.G	Expression vector for the vesicular stomatitis vector G-protein (VSV-G) which yields a pantropic envelope which enables the virus to infect any vertebrate cell (M. Grez, Georg Speyer Haus, Frankfurt, Germany)
pCMVDR8.91	Expression vector for the group-specific antigen (GAG) and polymerase (POL) of the virus (M. Grez, Georg Speyer Haus, Frankfurt, Germany)

2.1.4.8 Bacterial *E.coli* Strain and genotype

E. coli – HB101 Invitrogen, Karlsruhe, Germany

2.1.4.9 Cell lines

2.1.4.9.1 Ph^+ cells

Ph^+ ALL

In Ph^+ ALL cells, the translocation product encodes for p185 $^{BCR/ABL}$.

Sup-B15: Human B cell precursor leukemia cell line, established from the bone marrow of a patient with acute lymphoblastic leukemia, carrying the ALL-variant (m-bcr) of the BCR/ABL fusion gene.

Patient-derived long-term cultures (PD-LTCs): BV (Ph^+ ALL), PH (Ph^+ ALL), HP (Ph^- ALL), CM (Ph^+ CML) and VG (TEL/ABL-positive). These cultures were established in our laboratory (Badura et al, 2013; Nijmeijer et al, 2009).

2.1.4.9.2 Other Cell lines

Ba/F3: IL-3 dependent murine pro B cell line established from peripheral blood; derived from BALB/c mouse.

Rat-1: Rat fibroblast cell line.

293T: Transformed human embryonal kidney cell line.

Phoenix: Based on the 293T cell line and expresses the retroviral structural genes *gag*, *pol* und *env*. It is also known as a packaging cell line. The ecotropic packaging cell line delivers genes only to dividing murine or rat cells.

$CD34^+CD38^-$ human cells: Obtained from blood of G-CSF stimulated healthy donors by immunomagnetically isolation using CD34 multisort kit.

All cells were obtained from the German Collection of Microorganisms and Cell Cultures (DSMZ), with the exception of the ecotropic Phoenix cells which were obtained from Nolan lab (Stanford, USA), PD-LTCs, which were kindly provided by Prof. Dr. Oliver Ottmann (Frankfurt, Germany) and $CD34^+CD38^-$ cells were obtained from blood donation organization (Blutspendediesnst, Frankfurt, Germany).

2.1.4.10 Medium for Cell culture

Medium for Baf3 cells

L-glutamine	1% (v/v)
FBS	10% (v/v)
Penicillin/Streptomycin	1% (v/v)
rmIL-3	10 ng/ml

Added the above to RPMI medium.

Medium for Sup-B15

L-glutamine	1% (v/v)
FBS	20% (v/v)
Penicillin/Streptomycin	1% (v/v)

Added the above to RPMI medium.

Medium for PD-LTCs

L-glutamine	1% (v/v)
Leidenbrew (Nijmeijer et al, 2009)	4% (v/v)
Penicillin/Streptomycin	1% (v/v)

Added the above to IMDM medium.

Medium for Rat-1, 293T and Phoenix cells

L-glutamine	1% (v/v)
FBS (Hyclone)	10% (v/v)
Penicillin/Streptomycin	1% (v/v)

Added the above to DMEM medium.

Medium for $Sca1^+$ and $Sca1^+/Lin^-$ mouse BM cells

L-glutamine	1% (v/v)
FBS (Hyclone)	10% (v/v)
Penicillin/Streptomycin	1% (v/v)
rmIL-3	20 ng/ml

rmIL-6	20 ng/ml
rmSCF	100 ng/ml

Added the above to DMEM medium.

Medium for $CD38^+CD38^-$ human cells

L-glutamine	1% (v/v)
FBS (Hyclone)	10% (v/v)
Penicillin/Streptomycin	1% (v/v)
rhTPO	25 ng/ml
rhSCF	50 ng/ml
rhFlt3-ligand	50 ng/ml
rhIL-3	20 ng/ml

Added the above to X-VIVO 10 medium.

2.1.5 Materials for animal experiments

2.1.5.1 Mice

The C57BL/6J mice (age between 6-12 weeks) were purchased from Janvier Laboratories (St. Berthevin, France) and used for all animal experiments.

2.1.5.2 Materials for preparation of mice

Micro isolator cages	EBECO, Germany
Preparation set	Roth, Karlsruhe, Germany
Animal food	SSNIFF Spezialdiäten GmbH, Soest, Germany
Forene (Isofluran)	Abbott GmbH, Wiesbaden, Germany

2.1.6 Miscellaneous

Zyppy™ Plasmid Miniprep Kit	Zymo Research, Freiburg, Germany

PCR cleaning kit	Qiagen, Duesseldorf, Germany
Qiagen gel extraction Kit	Qiagen, Duesseldorf, Germany
Qiagen Plasmid kit Mini, Midi and Maxi	Qiagen, Duesseldorf, Germany
Qiagen PCR purification Kit	Qiagen, Duesseldorf, Germany
Quick change site-directed mutagenesis kit	Stratagene, La Jolla, USA
Cell proliferation (XTT) kit	Roche, Basel, Switzerland
RNAeasy kit	Qiagen, Duesseldorf, Germany
Tetro cDNA synthesis kit	Bioline, Luckenwalde, Germany
qPCR Mastermix NO ROX	Eurogentec, Cologne, Germany

2.2 Methods

2.2.1 Molecular biology techniques

2.2.1.1 Preparation of plasmid DNA

2.2.1.1.1 Bacterium growth in liquid media

Laura-Bertani (LB) medium

Bacto-Tryptone	1% (w/v)
Bacto-Yeast-Extract	0.5% (w/v)
NaCl	1.5% (w/v)

Adjusted the pH to 7.4 with NaOH and autoclaved.

To make an overnight saturated culture of *E.coli*, 2 ml liquid LB medium containing antibiotics was inoculated with a single colony quickly to minimize contact of the tube with the possibly contaminated air. The culture grew at 37°C with vigorous agitation (～220 rpm) until the culture was saturated, which normally takes at least 6 hours. Larger cultures were inoculated with overnight cultures diluted 1:100 and grew at 37°C with vigorous agitation (～300 rpm) to ensure proper aeration.

2.2.1.1.2 Transformation of *E.coli*

LB agar plates

Bacto-Agar	1.5% (w/v)

A frozen vial of competent *E.coli* bacteria was thawed on ice. The transforming DNA (up to 25 ng per 50 ul competent bacterium or 10 ul ligation product) was pipetted directly to competent *E.coli* bacteria and mixed by swirling the tubes gently. Bacteria were left on ice for 30 minutes and then exposed to 42°C heat shock for exactly 2 minutes. The vial was removed from 42°C and put on ice for 2 minutes, 500 µl of S.O.C medium (without antibiotic) was added and the tube was transferred to a shaking incubator set at 37°C. The culture was incubated for 45 minutes to allow the bacterium to recover and express antibiotic resistant marker encoded by plasmid. 1/10 of the transformed cells were spread

on a LB agar plate containing antibiotics. The plate was incubated for 16 hours at 37°C and individual colonies were identified, picked with a steril pipette tip and given to liquid culture to grow.

2.2.1.1.3 Freezing of bacterial culture

Bacterium freezing solution

Glycerin	65% (v/v)
$MgSO_4$	0.1 M
Tris-HCl, pH 8.0	0.025 M

900 µl bacterial culture and the same volume of freezing solution were mixed in a freezing vial and were kept by -80°C.

2.2.1.1.4 Plasmid preparation: mini-, midi- and maxiprep

Solutions for plasmid minipreparation

Resuspension solution (*Sol I*)

Glucose	50 mM
Tris-HCl pH 8.0	25 mM
EDTA pH 8.0	10 mM

Autoclaved and stored the solution at 4°C.

Alkaline lysis solution (*Sol II*)

NaOH	0.2 M
SDS	1% (w/v)

Sol II was prepared fresh and used at room temperature.

Neutralization solution (*Sol III*)

5 M Potassium acetate	60 ml
Glacial acetic acid	11.5 ml

ddH_2O 28.5 ml

The solution was stored at 4°C and transferred to ice before use.

Preparation of the plasmids was performed using Zyppy Plasmid Miniprep Kit (Zymo Research) or Maxi- and Midiprep kits (Qiagen) according to the manufacturer's instructions. Both protocols are based on an alkaline lysis of bacterial cells, followed by

binding of plasmid DNA to silica membrane column (Zymo) or a resin column (Qiagen) under appropriate salt concentrations and pH conditions. The isolation of plasmid DNA is based on the interaction between negatively charged DNA through its phosphate groups and the positively charged groups on a resin or silica membrane. In order to remove contaminations like RNA, protein or small molecules, the membrane is washed with a low salt concentrated buffer. For eluting the DNA, the membrane is washed with a high salt concentrated buffer and precipated with Isopropanol. The DNA will be washed with 70% Ethanol and eventually eluated in distilled water.

2.2.1.1.5 Determination of DNA yield and quality

Plasmid yield is measured by UV spectroscopy using the following relationship: 1 OD at 260 nm (10 cm path length) is equivalent to 50 µg plasmid DNA/ml. Plasmid quality is checked initially by running on a 1% agarose gel. This will give information on percentage of super coiled form of isolated plasmid DNA. Plasmid quality is further checked by UV spectroscopy (quotient 260 nm/ 280 nm). A ratio of 1.80-1.90 is an indication for pure plasmid DNA.

2.2.1.2 Enzymatic modification of nucleotide acids

2.2.1.2.1 Restriction digestion of plasmid DNA

The restriction endonucleases used for restriction of plasmid DNA belong to the type II endonucleases, which can recognize short DNA sequences and cleave double stranded DNA at specific sites. Thereby either blunt ends or sticky ends are produced. Restriction endonuclease cleavage is accomplished by incubating the enzymes with DNA in the appropriate reaction conditions. 0.5 µg-1 µg plasmid DNA was used for one restriction reaction. The DNA was incubated together with 5-fold excess of enzyme (5-10 U enzyme/µg DNA) and appropriate restriction buffer for 1-2 hours at 37°C (or appropriate temperature recommended by the manufacturer).

2.2.1.2.2 Dephosphorylation of linear plasmid DNA by Antarctic phosphatase

This method was used to decrease self-ligation of the cut-plasmid DNA. The treated fragments lack the 5´phosphoryl-termini, which are required for proper ligation by ligases. The reaction was performed by incubating cut-plasmid DNA with antarctic phosphatase buffer and 1 µl (0.5 U/µg vector) antarctic phosphatase at 37°C for 1 hour. The reaction

was stopped by heating at 70°C for 5 minutes or gel extraction was performed to purify the DNA fragments (2.2.1.4).

2.2.1.2.3 Fill-in of 5´-Overhangs to form blunt ends by Klenow-reaction

The 5´overhang of DNA ends were filled up by Klenow-fragment, a large subunit of the DNA polymerase I of *E.coli*. The enzyme is active in all NEB buffers and was incubated with the restricted DNA (1 U/µg DNA) and dNTPs at 25°C for 15 minutes. The enzyme activity was stopped by adding EDTA to a final concentration of 10 mM and heating at 75°C for 20 minutes.

2.2.1.2.4 Ligation of DNA fragments

DNA fragments and vector were ligated with T4 DNA ligase. T4 DNA ligase catalyses the formation of a phosphor-diester bonds between juxtaposed 5´phosphate and 3´hydroxyl termini in duplex DNA. 15 µl reaction was prepared with 25-50 ng vector, 3 fold molar excess of insert, 1.5 µl 10x ligase buffer and 1 U of T4 DNA ligase overnight at 16°C. The whole reaction was used for the transformation with chemically competent *E.coli* .To control for self-ligation of the vector an additional ligation reaction was included using only vector.

2.2.1.2.5 Quick change site-directed mutagenesis

The *in vitro* site-directed mutagenesis protocol was performed according to Stratagene's Quick-change site-directed mutagenesis kit protocol. This technique is used for vector modifications. With this technique, point mutations are introduced. The mutagenic polymerase chain reactions (PCRs) are performed using *PfuTurbo* DNA polymerase. *PfuTurbo* DNA polymerase replicates both plasmid strands with high fidelity and without displacing the mutant oligonucleotide primers. The basic procedure utilizes a supercoiled double-stranded DNA (dsDNA) vector with an insert of interest and two synthetic oligonucleotide primers containing the desired mutation (Figure 6). The oligonucleotide primers, each complementary to opposite strands of the vector, are extended during temperature cycling by *PfuTurbo* DNA polymerase. Incorporation of the oligonucleotide primers generates a mutated plasmid containing staggered nicks. Following temperature cycling, the product is treated with *Dpn*I. The *Dpn*I endonuclease (target sequence: 5´-Gm6ATC-3´) is specific for methylated and hemimethylated DNA and is used to digest the

parental DNA template and to select for mutation-containing synthesized DNA. DNA isolated from almost all *E.coli* strains is dam methylated and therefore susceptible to *Dpn*I digestion. The nicked vector DNA containing the desired mutations is then transformed into *E.coli* HB101 competent cells. The small amount of starting DNA template required to perform this method, the high fidelity of the *PfuTurbo* DNA polymerase, and the low number of thermal cycles all contribute to the high mutation efficiency and decreased potential for generating random mutations during the reaction.

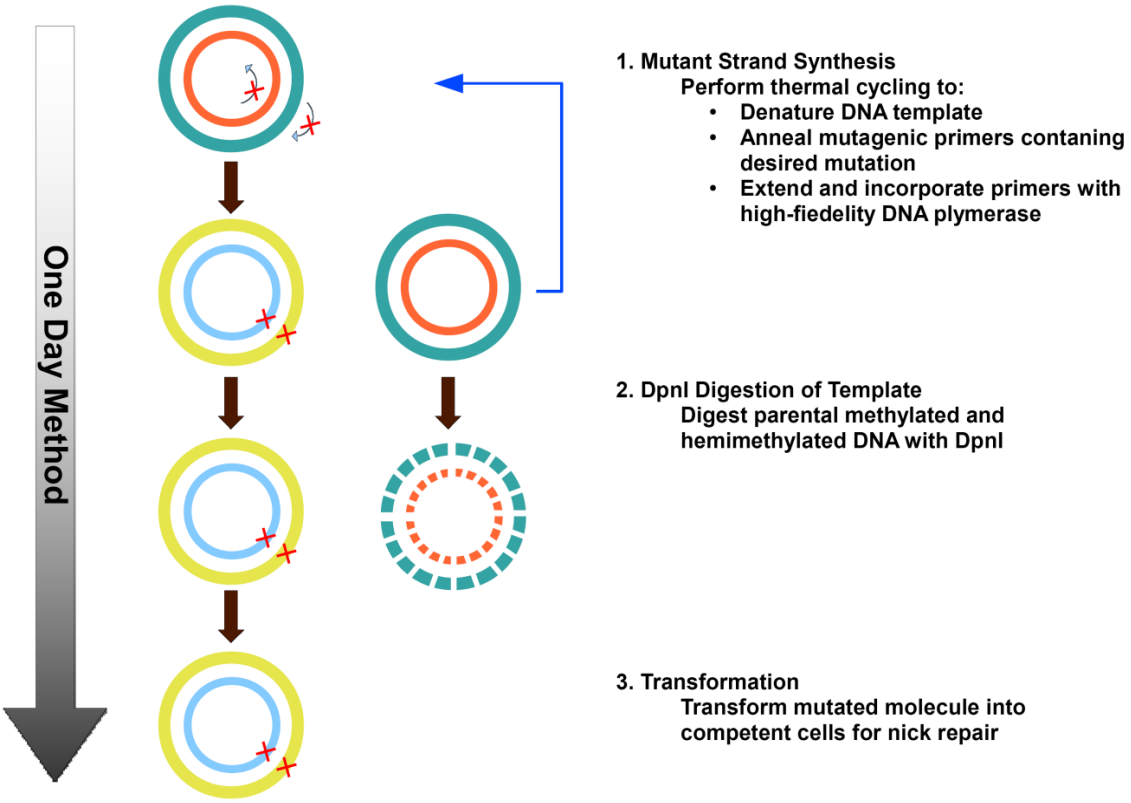

Figure 6. Overview of the Site-directed mutagenesis method (adapted from Stratagen protocol).

2.2.1.3 Electrophoretic separation of DNA

1x TAE Electrophoresis buffer

Tris-acetate	0.04 M
EDTA, pH 8	1 mM

DNA was separated electrophoretically on agarose gels for the following reasons: to control the progression of a restriction enzyme digestion, determine the yield and purity of the DNA preparation, check the products of the PCR reaction, or to purify the DNA

fragments. Concentration of agarose gels ranged from 0.5% to 3%, depending on the size of the desired fragment. Agarose was mixed with 1x TAE buffer and heated in a microwave until completely dissolved. This solution was cooled down to 50°C, 1x Gel Red was added and poured into a gel tray. Samples were mixed with 6x DNA loading buffer and electrophoresis was carried out in 1x TAE buffer at 5 V/cm. Following electrophoresis, gel was exposed to UV light (λ=320 nm) to visualize the Gel Red-stained DNA fragments, and fragment length determined by comparing to DNA marker (Sambrook & Russel, 1989).

2.2.1.4 Purification of DNA

Purification of PCR or restriction digestion products, oligonucleotides ≥17 nucleotides from enzymatic reactions, extract and purification of DNA fragments from agarose gels were performed with QIAquick PCR Purification Kits, QIAquick Nucleotide Removal Kits and QIAquick Gel Extraction Kits, respectively. The principle of the method based on a silica membrane assembly for binding of DNA in high-salt buffer and elution with low-salt buffer or water. The purification of the DNA fragment from the agarose gel is performed as following: Gel slices containing DNA fragment are cut from the agarose gel under UV-light (302 nm) using a scalpel and given to 1.5 ml microcentrifuge tube. A high-salt concentrated buffer is given to the gel 3x the gel volume and kept by 50°C for 10 minutes, in order to solve the agarose gel. According to the size of the desired fragment (<500 bp and >4 kb) one gel volume isopropanol is given to the mixture and mix. The mixture is applied to the spin column. Nucleic acids adsorb to the silica membrane in the high-salt conditions provided by the buffer. Another wash step with a low-salt buffer is performed and pure DNA is eluted eventually with water.

2.2.1.5 Recombination („gateway LR clonase enzyme kit'' from Invitrogen)

This technique is based on the recombination reactions of the bacteriophage λ vector and makes use of a special Enzyme Mix (Integrase and Excisionase of the bacteriophage λ; and Integration Host Factor from *E.coli*). The Gateway cloning mechanism includes a BP Reaction [PCR fragment (contains attB sites) + Donor vector (contains attP sites) = Entry Clone (gene of interest flanked by attL sites), followed by an LR reaction (Entry Clone (gene of interest flanked by attL sites) + Destination Vector (contains attR sites) = Expression Clone]. Sequences known as attachment sites (att sites) are the points were

excision and integration occurs. The recombination reaction generates two molecules. One molecule contains the DNA segment of interest, the other molecule is a by-product.

The Recombination reactions are carried out with the Gateway LR-clonase II enzyme kit (Invitrogen, Karlsruhe, Germany). For this, 150 ng Entry clone DNA (for example: pE $p185^{BCR/ABL}$ plasmid), 150 ng Destination vector DNA (for example: PINCO vector) were given into a 1.5 ml microcentrifuge tube and filled up with TE buffer to 8 µl (10 mM Tris-HCl, pH 7.4; 1 mM EDTA, pH 8.0) and mixed by pipetting. 2 µl LR-Clonase II Enzyme Mix (composition not mentioned by manufacturer), was added, mixed and incubated for 1 hour at room temperature. The reaction was stopped using 1 µl Proteinase K solution and Finally, *E. coli* HB101 competent cells were transformed using the whole reaction mixture and plasmid DNA was prepared for transfection and infection.

2.2.1.6 Cloning of the used plasmids

All cDNAs encoding $p40^{ABL/BCR}$, $p96^{ABL/BCR}$, as well as $p185^{BCR/ABL}$ and $p210^{BCR/ABL}$ have been described previously (Beissert et al, 2008; Beissert et al, 2003; Zheng et al, 2006). All retroviral expression vectors used in this study were based on the bi-cistronic vectors PINCO or PAULO converted into Gateway®-destination vectors by the introduction of a Gateway® cassette according to the manufacturer's instructions (Invitrogen, Karlsruhe, Germany). All related inserts were available in the Gateway® entry-vector (pENTR1A) for recombination into the destination vectors using the "LR clonase" enzyme kit (Invitrogen, Karlsruhe, Germany). The mutant constructs were prepared using the following strategies and the modular organization of these constructs is shown in Figure 7. The simultaneous expression of $p96^{ABL/BCR}$ and $p185^{BCR/ABL}$ transgenes was performed using p2a peptide-linked bicistronic retroviral vectors. The 2a sequences are 18-19 amino acid long peptides found in many picornaviruses (Agol & Gmyl, 2010; Donnelly et al, 2001). These viral peptides are placed between polyproteins and function as cleavage sites during ribosomal translation. As a result, two protein products are generated from one open reading frame (ORF) at the approximate ratio of 1:1 (Szymczak et al, 2004).

pE $p96^{ABL/BCR}$-p2a-$p185^{BCR/ABL}$ construct was generated by the amplification of p2a fragment using *Taq*-polymerase by PCR using following primers:

Fw 5´-GCGGCCGCGAGCCACGAACTTCTC-3´

Rev 5´-GGTCAGTAAATTGGATATCGGCC-3´.

The PCR fragment was inserted into PCR2.1 vector via TA cloning (invitrogen, Karlsruhe, Germany). After cutting this fragment at EcoRV and NotI restriction sites, ligation with pEp96$^{ABL/BCR}$ vector was performed. For producing one open reading frame starting at p96$^{ABL/BCR}$ to the end of p2a fragment, the stop codon of *ABL/BCR* gene was deleted using quick change site-directed mutagenesis kit (Stratagen, La Jolla, USA) using the following primers:

Fw 5´-TTCTCCACCGAAGTCAAGAATTCGCGGCCG-3´
Rev 5´-CGGCCGCGAATTCTTGACTTCGGTGGAGAA-3´.

Subsequently the p96$^{ABL/BCR}$-p2a fragment was amplified using PCR while introducing FseI and SacII at each site. The PCR primers used were as following:

Fw 5´-ACCCGCGGATGTTGGAGATCTGC-3´
Rev 5´-GGCCGGCCTTCGGCCCGGGGTTTT-3´.

The resulting PCR product was controlled by Sanger sequencing (Seqlab, Goettingen, Germany) and cloned into the FseI/SacII-digested pE p185$^{BCR/ABL}$. The pE p96$^{ABL/BCR}$-p2a-p210$^{BCR/ABL}$ was made using pE p96$^{ABL/BCR}$-p2a-p185$^{BCR/ABL}$ vector and substitute p185$^{BCR/ABL}$ with p210$^{BCR/ABL}$ using FseI and XbaI cutting sites. The pE p40$^{ABL/BCR}$-p2a-p210$^{BCR/ABL}$ was constructed by substitution of p96$^{ABL/BCR}$ with p40$^{ABL/BCR}$ using EcoRI restriction site.

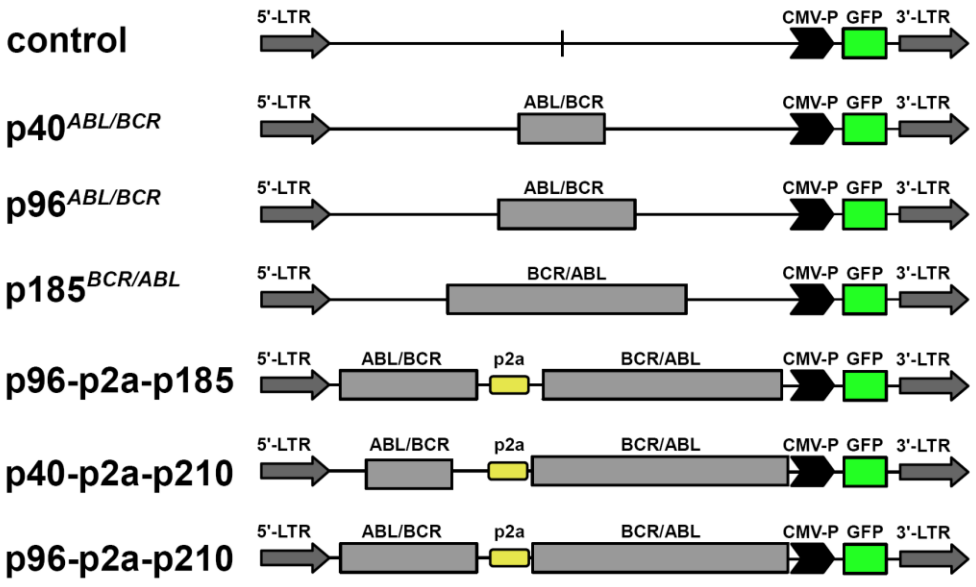

Figure 7. Schematic representation of constructs used in this study.

2.2.1.7 Reverse transcription-PCR (RT-PCR)

2.2.1.7.1 Isolation of total RNA from animal tissue (spleen)

The extraction of RNA from the spleens obtained from CFU-S12 experiment was done using RNeasy Mini Kit (Qiagen, Duesseldorf, Germany). During the isolation of the RNA sterile and disposable plastic ware was used, all reagents prepared with RNase free-H_2O (DEPC-treated water), in order to prevent the activity of RNases. The spleens were disrupted and homogenized by passing the tissue through a 40 µM nylon filter. The cells were washed twice with ice cold sterile PBS and supernatant was removed. Subsequently 350 µl RLT buffer (guanidine-isothiocyanate lysis buffer) was added to the cells and gently resuspended by mixing up and down with the pipette. The mixture was given to a 1.5 ml microcentrifuge tube and one volume 70% ethanol was added to the lysates to provide ideal binding condition of RNA to silica membrane spin columns. RNA binds efficiently to the silica membrane and all contaminants are washed away. For sensitive applications (for example micro array analysis) 10 µl DNase was added in order to remove the residual amounts of DNA. After centrifugation (≥8000 x g, 15 seconds) the membrane was washed 3 other times and the pure, concentrated RNA was eluted in 30-50 µl RNase-free water and stored at -80°C. All steps mentioned above were operated according to the manufacturer's instructions.

2.2.1.7.2 RT-PCR

Due to the fact that DNA is less prone to the degradation by contaminating nucleases and can be fractioned by electrophoresis more accurately compared to RNA, first strand cDNA was synthesized from total RNA and used for PCR amplification. For the production of cDNA the Tetro cDNA Synthesis Kit (Bioline, Luckenwalde, Germany) was used. Total RNA was extracted from the spleen of CFU-S12 experiment using RNeasy kit as described in chapter 2.2.1.7.1. First-strand cDNA was synthesized from total RNA primed with random hexamers and using Moloney Murine Leukemia Virus (M-MLV) reverse transcriptase. 3-5 µg of total RNA was given to an RNase-free reaction tube on ice and mixed with 1 µl random hexamers, 1 µl 10 mM dNTP mix, 4 µl 5x RT buffer, 1 µl RNase inhibitor and 1 µl reverse transcriptase. The reaction was filled with DEPC-treated water to 20 µl, incubated at 25°C for 10 minutes followed by 45°C for 30 minutes. The reaction

was terminated by incubating at 85°C for 5 minutes and chilled on ice. All the described steps were performed according to the manufacturer's instructions.

The cDNA can now be used as a template for amplification in PCR. For performing the PCR following reagents were added to a PCR tube:

10xPCR buffer	5 µl
50 mM MgCl	21.5 µl
10 mM dNTP	1 µl
Forward primer (10 µM)	1 µl
Reverse primer (10 µM)	1 µl
*Taq*DNA polymerase (5U/µl)	0.4 µl
cDNA from first-strand reaction	2 µl

Steril-filtered ddH_2O was added to 50 µl.

The PCR program was as following:

5 minutes by 95°C for 1 cycle,
30 seconds by 95°C, 30 seconds by 60°C, 1 minute per 1 Kb by 72°C for 30 cycles,
10 minutes by 72°C for 1 cycle.

2.2.1.8 Real time PCR

Total RNA (from spleens from the CFU-S12 experiment) and first strand cDNA were obtained as described in chapters 2.2.1.7.1 and 2.2.1.7.2, respectively. For the quantification of p53, GADD45α and p21 mRNAs, gene expression quantification was performed using TaqMan® gene expression assay. The TaqMan® gene expression assay (primer and probe mixture) of the corresponding transcripts was purchased from Taqman (Applied Biosystems, CA, USA) (Hs01034249-m1: Mtbp (p53); Hs00355782-m1: Cdkn1a (p21); Hs00169255: GADD45α and Hs02758991-g1: GAPDH). The Real Time PCR was conducted in triplicates with the following components:

cDNA	3-5 µl
2x Mastermix	10 µl
Primer and Probe mix	1.25 µl

Steril-filtered ddH_2O was added to 20 µl.

The PCR program was performed following the 2-step protocol as suggested by Mastermix provider (Eurogentec). The PCR program was as following:

2 minutes by 50°C for 1 cycle,
10 minutes by 95°C for 1 cycle,
15 seconds by 95°C, 1 minute by 60°C for 40 cycles.

The PCR was done using the ABI PRISM 7700. Normalization to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was done for each sample. CT values were exported into a Microsoft Excel worksheet for calculation of fold changes according to the comparative CT method (Bustin, 2000). The amount of target, normalized to an endogenous GAPDH is given by $2^{-\Delta\Delta Ct}$.

2.2.1.9 Short hairpin RNA (shRNA)

Lentiviral vectors expressing short hairpins against human $p96^{ABL/BCR}$ and non targeting control (NTC) lentiviral vectors were based on PLKO-1 vector and were purchased from Sigma Aldrich (Sigma, Steinheim, Germany). The shRNAs were designated as siR961 and siR962 and their sequences were as following:

siR961:CCGGCAGATCCAGATACCTAATAAGCTCGAGCTTATTAGGTATCTGGATCTGTTTTTTG (TRCN000000792)

siR962:CCGGCAAGAGTTACACGTTCCTGATCTCGAGATCAGGAACGTGTAACTCTTGTTTTT (TRCN000000789)

The efficiency of the vectors was confirmed by western blotting in the target cells.

2.2.2 Immunoblot

2.2.2.1 Lysis of cells (Sambrook & Russel, 1989)

E1A lysis buffer

NaCl	250 mM
HEPES, pH 7	50 mM
EDTA, pH 8	5 mM

The following compounds were added to the freshly made buffer:

Triton X-100	1% (v/v)
50x Protease inhibitor	1x (v/v)

Na_3VO_4	2 mM

Cells were washed twice with cold PBS, collected by centrifugation (1200 rpm for 10 minutes at 4°C) and lysed by adding approximately 100 µl of cold E1A lyses buffer (2.1.4.6) per million cells. After 30 minutes incubation on ice, lysates were cleared by centrifugation at 14000 rpm for 15 minutes at 4°C. The supernatants were transferred to the fresh tubes and stored by –20°C.

2.2.2.2 Determination of protein concentration

Protein concentrations were determined by Bradford-assay with Bio-Rad protein detection kit according to the manufacturer's instruction and quantified with a spectrophotometer at 595 nm. The standards for protein concentration were prepared with different concentrations of bovine serum albumin (BSA) (0.3 to 10 mg/ml).

2.2.2.3 SDS-polyacrylamide gel electrophoresis (SDS-PAGE)

A very common method for separating proteins by electrophoresis uses a discontinuous polyacrylamide gel as a support medium and SDS to denature the proteins. The method is called sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). SDS is an anionic detergent. A polypeptide chain binds to SDS in proportion to its relative molecular mass. The negative charges on SDS destroy most of the complex structure of proteins, and are strongly attracted toward an anode (positively-charged electrode) in an electric field. Their net migration through gel is not determined by the electrical charge but by molecular weight.

2.2.2.3.1 SDS-PAGE using Bio-Rad system

10x SDS running buffer

Tris-base	25 mM
Glycin	192 mM
SDS	0.1%
pH 8.3	

Mini-PROTEAN® TGX Stain-Free ™ Gels	Bio-Rad, Munich, Germany
Precision plus protein (PPP)	Bio-Rad, Munich, Germany
NuPAGE LDS Sample Buffer	Invitrogen, Karlsruhe, Germany
NuPAGE Reducing Agent	Invitrogen, Karlsruhe, Germany

For the Bio-Rad System 4-20% Tris-HCl ready-to use gradient gels were used. Tris-HCl gels are run in the 1x SDS running buffer. Thioglycolat is used as antioxidant and is given directly to the buffer (500 µl from 2 mM thioglycolat in 200 ml buffer). The samples are diluted in 4x LDS sample buffer and reduced with 10x Reducing agent for 10 minutes by 70°C. Electrophoresis was run for approximately 1 hour, at a constant voltage of 150 V.

2.2.2.3.2 Transfer of proteins onto a nitrocellulose membrane using NOVEX® NuPAGE Wet Blot system (Invitrogen)

10x SDS transfer buffer

Tris	480 mM
Glycine	390 mM
SDS	0.375%
pH 7.2	

Ponceau S staining solution

Ponceau S	2 % (w/v)
Trichloroacetic acid	30 % (v/v)
Sulfosalicylic acid.	30 % (v/v)

Following electrophoresis, proteins were transferred to a 0.2 µm nitrocellulose membranes in a process called Western blotting. This transfer was carried out between the two horizontal metal plates in a wet-blot system (Invitrogen). The membrane was soaked in a transfer buffer for 10 minutes, placed on top of the 3 MM Whatman® paper immersed in the same buffer, and laid the gel over. This was covered by another sheet of wet 3 MM Whatman® paper, all dimensions being slightly brighter then the gel. Transfer was carried

out in excess of blotting buffer (1x transfer buffer + 10% Methanol) by 300-400 mA current (45 V) for 90 minutes. Following Western blotting, membranes were usually stained with Ponceau-S for 5 minutes and protein bands were visualized. The dye was removed by washing with TBS-T and the membrane was dried on a Whatman® paper and stored at 4°C until further analysis.

2.2.2.3.3 Immunodetection of specific proteins

10x TBS

Tris-HCl, pH 7.5	24.23 g
NaCl	80.06 g

Above materials were mixed in 800 ml water, and pH was adjusted to 7.6 with pure HCL. The volume was adjusted to 1 L with water.

TBS-T

1x TBS + 0.05% Tween 20

Blocking solution for immunoblot

5% non-fat dry milk in TBS-T

Enhanced chemo luminescence (ECL) solution I:

Tris-HCl, pH 8.5	100 mM
p-Coumaric acid (in DMSO)	0.396 mM
Luminol (in DMSO)	2.5 mM

Water was added up to 100 ml; aliquots were made and stored in the dark at -20°C.

ECL solution II:

30% H_2O_2	64 µl
1 M Tris-HCl, pH 8.5	10 ml

Water was added up to 100 ml, aliquot solution and store in the dark at -20°C.

Proteins immobilized on a membrane were identified by using specific antibodies. The membrane was first incubated in TBS-T containing 5% (w/v) non-fat dry milk in order to saturate nonspecific protein binding sites, and after one hour or overnight incubation by

4°C on a rocking platform the membrane was washed three times for 10 minutes in TBS-T. Primary antibody was diluted to appropriate concentration (1:200-1:10000) in TBS-T and the membrane was incubated for 3 hours by room temperature or overnight by 4°C. Incubation was followed by three washing steps for 10 minutes in TBS-T and secondary antibody was added (as recommended). After one hour, membrane was again repeatedly washed in TBS-T to remove unbound secondary antibody. Signals were detected in the dark by rinsing the membrane shortly in water and then incubating with ECL solutions I and II in 1:1 ratio for one minute. Visualizing the signals took 30 seconds to 5 minutes depending on the intensity of the signal detected and antibody-bound proteins visualized. The visualization was performed using Fluorchem™ imaging system (Alpha Innotech, Miami, USA). In order to incubate the same membrane with a new antibody, the membrane was washed 3 times 10 minutes in TBS-T and incubated 20 minutes in 20 ml stripping buffer, which contains reagents that remove primary and secondary antibodies from the membrane (Thermo Scientific, Bonn, Germany). The membrane was washed again 3 times 10 minutes with TBS-T and the immunodetection was performed as described above.

2.2.2.4 Fluorescence activated cell sorting (FACS) - Fluorescence coupled antibodies

FACS wash buffer

PBS + 1% FCS + 1% NaN_3

FACS fixing solution

PBS + 2% formaldehyde solution

0.1-1 x 10^6 cells were given into a polypropylene tube and centrifuged by 1200 rpm, 4°C for 5 minutes. The supernatant was discarded and the cells were washed with FACS wash buffer by 1200 rpm, 4°C for 5 minutes. The supernatant was discarded again, however some drops of FACS wash buffer were left and 2 µl antibody was given to the tube. The cells were incubated 20 minutes by 4°C in the dark. After washing once with FACS wash buffer, the cells were fixed in 150 µl of FACS fixing solution. The cells can be kept now for maximal 1 week by 4°C in the dark. Detection of the fluorescent-labeled cells was performed using the FACS CantoII system and FACS Diva software (Becton Dickinson,

Heidelberg, Germany). For detection of retroviral plasmid constructs (for example: PINCO vector) which contains green fluorescence protein (GFP) no extracellular staining is needed, since under excitation with appropriate wavelength, the protein is able to emit the light and can be detected using FACS. This method was used to measure the transfection and infection efficiency of the viral plasmid constructs.

2.2.3 Cell biology techniques

2.2.3.1 Cell cultures

Cells were grown in culture flasks in a humidified atmosphere at 37°C and by 5% CO_2.

2.2.3.1.1 Used Cell lines

SupB15, Ba/F3 and PD-LTC cells were cultivated as mentioned before (2.1.4.10). Cultures were seed out in 1:3 to 1:5 ratios every 2-3 days in order to keep constant log phase population growth at about 1 x 10^6 cells/ml.

Adherent Rat-1, Phoenix and 293T cells were grown in the medium mentioned before (2.1.4.10). Upon reaching sub confluent state cells were washed with PBS and 1 ml of trypsin-EDTA (Gibco, Karlsruhe, Germany) was added. After 1-2 minutes, trypsin was inactivated by addition of FCS-containing medium and detached cells reseeded in the fresh plates at approximately 1:10 ratios. This was done 3 times a week.

2.2.3.2 Cell counting and determination of cell viability

Cell number and viability were controlled by trypan blue exclusion. 10 µl cell suspension was mixed with 40 µl of 0.4% trypan blue in 0.9% NaCl. Only unstained cells were considered viable, and their number was calculated as following:

Cell concentration [10^6/ml] = Number of counted cells in 5 quadrants x 10^4 / ml.

2.2.3.3 Freezing and thawing

Cell freezing medium

Solution I

RPMI/DMEM	70% (v/v)
FBS	30% (v/v)

Solution II

RPMI/DMEM	80% (v/v)
DMSO	20% (v/v)

Freezing

For storing in liquid nitrogen cells were collected at 1200 rpm for 5 minutes and washed once with PBS. After centrifugation, the cells were resuspended in 0.9 ml freezing solution I, transferred into cryovials, and mixed with dropwise added 0.9 ml of freezing solution II. The vials were immediately placed in cryobox containing isopropanol and stored at -80°C, and after 1 day transferred to liquid nitrogen freezer.

Thawing

Cryo-preserved cells were taken out of the liquid nitrogen, thawed rapidly in a 37°C incubator and resuspended in 10 ml culture medium. Following one washing step to remove residual DMSO, cells were resuspended with fresh medium and transferred into culture flasks.

2.2.3.4 Genetic modification of mammalian cells

2.2.3.4.1 Transfection of Phoenix and 293T cells using $CaPO_4$ method

2 M $CaCl_2$ solution

The component was dissolved in ddH_2O and sterile filtered with 0.22 µm filter and stored at -20°C in aliquots until use.

2x HBS solution

Na_2HPO_4	0.315 g (1.5 mM final)
HEPES	19.5 g (0.05 M final)
NaCl	24 g (0.28 M final)

Water was added up to 1200 ml, three clean bottles were filled with exactly 400 ml of the solution and the pH was adjusted to 6.95/ 7.0/ 7.05. Each bottle was filled to 500 ml and pH was controlled again. In order to select the best HBS for transfection, Phoenix and 293T cells were transfected using the HBS solutions with three different pH values and the

HBS which resulted in the best transfection efficiency was selected for further use, filter-sterilized and stored at -20°C in aliquots.

A day prior to transfection, Phoenix cells were plated in fresh medium. After 14-16 hours, cells were sub confluent (60-70% confluency), which is the stage when they are most transfectable and give the highest possible virus titer.

For transfection, following premix was made:

2 M $CaCl_2$ 62 µl
DNA 5 µg
Steril-filtered H_2O was added to make final volume of 500 µl.

While vortexing the premix, 0.5 ml of 2x HBS was added dropwise. The mix was kept for 10-20 minutes at room temperature. Next, medium was aspired, DNA/HBS solution was gently spread across the cell layer and plates were transferred to 37°C incubator. After 30 minutes, fresh pre-warmed medium with 25 µM chloroquine was added, and incubation continued for the next 8-12 hours (DNA delivered by $CaPO_4$ transfection is transit through lysosomes, and chloroquine acts to inhibit lysosomal DNases by neutralizing vesicle pH). Following this time, medium was replaced and cells were left overnight. Next day medium was changed and the cells were incubated for another 24 hours. Transfection efficiency was estimated according to the level of expression of GFP using FACS. Only after reaching the transfection efficiency of at least 70%, experiment was proceeded and retroviral supernatant was collected, filtered and cryo-frozen in liquid nitrogen. Next day again the virus supernatant was collected, stored and used for further infection of target cells.

Amphotrophic lentiviral supernatant was obtained by transfection of 293T cells with desirable lentiviral vectors, pCMVDR8.91 vector to encode *gag* and *pol* and pMD2.G vector to encode VSV-G pseudotyped envelope protein. Lentiviral supernatant was collected at days 2 and 3 after transfection.

2.2.3.4.2 Retroviral infection

For infection of target cells, retronectin (Takara Bio, Otsu, Japan) was used to enhance the infection efficiency following the manufacturer's instructions. Non tissue culture 24-well plates were coated with 0.2 mg retronectin per well in 200 µL volume. The plate was incubated at 4°C overnight. Retronectin was removed, washed with PBS and blocked with

2% BSA for 30 minutes. 200 µL of viral supernatant was plated in each well and centrifuged at 4000 rpm for 15 minutes at 15°C. Viral supernatant was discarded and the procedure was repeated 2-3 times. The wells were washed with PBS and target cells (10^5 cells/ml) were plated in the viral coated plate. Infection efficiency was measured by FACS analysis of GFP-positive cells after 48 hours.

2.2.3.5 Cell growth and proliferation assay

Cell growth was assessed by dye exclusion using Trypan-blue. Ba/F3 cells transduced with either empty vector or vectors harboring $p96^{ABL/BCR}$, $p185^{BCR/ABL}$ or $p96^{ABL/BCR}$-p2a-$p185^{BCR/ABL}$ were plated at 10^5 cells/ml. The IL-3 independent growth was monitored for 6 days using dye exclusion assay. Proliferation was assessed using proliferation kit XTT (Roche, Basel, Switzerland) according to the manufacture instructions and was measured after 72 hours.

2.2.3.6 Apoptosis measurement using 7-aminoactinomycin D (7-AAD)

7-AAD is used in the staining of apoptotic cells, whereby it binds to the DNA. The 7-AAD staining was carried out as following: 2.5 to 5 x 10^5 cells were taken in 1 ml FACS binding buffer (Becton Dickinson, Heidelberg, Germany) and centrifuged for 5 minutes at 1200 rpm. Supernatant was completely discarded. The pellet was resuspended in 200 µl of 7-AAD solution (diluted in PBS at a ratio of 1:10 to have a final concentration of 20 µg/ml). The cells were then incubated in the dark for 20 minutes at 4°C. The Tubes were filled with PBS and the cells were pelleted by centrifugation. Supernatant was discarded, and cells were resuspended in an appropriate volume (200 to 500 µl) of FACS fixing solution. Cells were analyzed immediately by FACS.

2.2.3.7 Classical transformation assays

Many fibroblastic cell lines that must attach to a solid surface before they can divide fail to grow when suspended in a viscous fluid or gel (for example: agar or agarose). However when these cells are transformed, they are able to grow in a viscous fluid or gel and become anchorage-independent. This can be observed and detected using colony forming assay in soft agar. Another property of transformed cells is the ability to from foci, since cells that contain a transforming oncogene will grow without contact inhibition and form dense, raised foci which can be visualized by fixing and staining the cells.

2.2.3.7.1 Soft agar anchorage-independent growth assay

For soft-agar assay Rat-1 fibroblasts were transduced with PINCO or PINCO vector harboring $p96^{ABL/BCR}$, $p185^{BCR/ABL}$ or $p96^{ABL/BCR}$-p2a-$p185^{BCR/ABL}$. Six-well plates were filled with 2 ml/well DMEM+10%FCS+0.5% bactoagar (DIFCO Laboratories, Detroit, USA). 5000 Rat-1 cells were plated in 1 ml/well top agar (DMEM+10% FCS+0.25% bactoagar). Rat-1 colonies were counted after 15 days incubation at 37°C and 5% CO_2.

2.2.3.7.2 Focus formation assay

For focus-formation assay Rat-1 cells were transduced with PINCO or PINCO vector harboring $p96^{ABL/BCR}$, $p185^{BCR/ABL}$ or $p96^{ABL/BCR}$-p2a-$p185^{BCR/ABL}$ and 40,000 cells/well were plated in a 24-well plate. After 15 days incubation at 37°C and 5% CO_2, the Foci were photographed using AxioCam HRc (Zeiss, Goettingen, Germany) with 10x magnification.

2.2.4 Stem cell biology techniques

2.2.4.1 Enrichment of $Sca1^+$ and $Sca1^+/Lin^-$ HSCs from murine bone marrow and fetal liver cells

MACS buffer

20% (w/v) BSA	12.5 ml
EDTA	2 ml
Penicillin/Streptomycin	5 ml
Add PBS up to	500 ml

$Sca1^+$ and $Sca1^+/Lin^-$ HSCs were isolated from 8 to 12 week-old female C57Bl/6J mice After anesthetizing the animals using isofluran by inhalation, the mice were killed by cervical dislocation. Bone marrow was harvested from the femura and tibiae by flushing the bones with a syringe and 26-gauge needle. The cells were ‘‘lineage depleted’’ by labeling the cells with biotin-conjugated lineage panel antibodies against B220, CD3e, Gr-1, Mac-1 and Ter-119 (Miltenyi, Bergisch-Gladbach, Germany). Labeled cells were removed using ‘‘MACS’’ cell separation columns (Miltenyi, Bergisch-Gladbach, Germany). $Sca1^+$ cells were purified by immunomagnetic beads using the ‘‘MACS’’ cell separation columns according to the manufacturer’s instructions (Miltenyi, Bergisch-Gladbach, Germany). Prior to further use, the purified cells were pre-stimulated for 2 days

in medium containing rmIL-3 (20 ng/ml), rmIL-6 (20 ng/ml) and rmSCF (100 ng/ml). For the isolation of Sca^+/Lin^- from mouse fetal liver, the C57Bl/6J breeders were put together late in the afternoon and checked for vaginal plugs the following morning. The livers were dissected from the fetuses 14 days after observation of the plug, meshed in a 40 µM nylon cell strainer and washed once with PBS. The PBS was discarded and 1x red blood cell (RBC) lysis buffer (Becton Dickinson, Heidelberg, Germany) was given to the cells for 5 minutes. The reaction was stopped by washing the cells with PBS. The isolation of Sca^+/Lin^- cells was proceeded as above.

2.2.4.2 Retroviral infection of $Sca1^+$ and $Sca1^+/Lin^-$ primary murine bone marrow cell and fetal liver cells

24-well plates were first coated with 200 µl of retronectine/well (0.2 mg retronectin). After one hour by room temperature (or overnight by 4°C) the plates were washed with PBS and blocked with 2% BSA in PBS. After 30 minutes the plates were washed again with PBS. Each well was covered with 0.5 ml retrovirus supernatant and plates were spinned (4000 rpm, 15 minutes, 15°C). This was repeated 3-4 times. $Sca1^+/Lin^-$ primary murine bone marrow cell or fetal liver cells were plated at 200,000 cells/well and placed in the 37°C incubator over night. On the next day the supernatant was discarded, 0.5 ml retrovirus supernatant was given to the wells and the plates were spinned (2200 rpm, 45 minutes, 32°C). This procedure was repeated after 6 hours and afterwards cells were incubated overnight in the 37°C incubator. Infection efficiency was estimated on FACS as intensity of GFP expression.

For retroviral transduction of $Sca1^+$ cells, 24-well plates were first coated with 200 µl of retronectine/well (0.2 mg retronectin) overnight by 4°C. The plates were washed with PBS on the next day and $Sca1^+$ cells were plated at 200,000 cells/well and placed in the 37°C incubator over night. On the next day the supernatant was discarded, 0.5 ml retrovirus supernatant was given to the wells and the plates were spinned (2200 rpm, 45 minutes, 32°C). This procedure was repeated after 6 hours and on the next day previous to transplantation into recipient mice.

2.2.4.3 Enrichment and lentiviral infection of $CD34^+CD38^-$ human cells

The $CD34^+CD38^-$ human cells were isolated immunomagenetically from blood of G-CSF stimulated healthy donors using CD34-Multisort kit. $CD34^+$ cells were purified by

immunomagnetic beads using the ‘‘MACS’’ cell separation columns according to the manufacturer’s instructions (Miltenyi, Bergisch-Gladbach, Germany). Depletion of the second marker, CD38, was achieved by labeling the cells with Anti-FITC antibody (Miltenyi, Bergisch-Gladbach, Germany) and eventually removing the $CD38^+$ labeled cells through a second ‘‘MACS’’ cell separation column. Prior to further use, the purified cells were pre-stimulated for 2 days in medium containing rhIL-3 (20 ng/ml), rhTPO (25 ng/ml), rhSCF (50 ng/ml) and rhFlt3-ligand (50 ng/ml) (Miltenyi, Bergisch-Gladbach, Germany). The lentiviral infection of $CD34^+CD38^-$ cells was done similar to retroviral infection of $Sca1^+/Lin^-$ mouse cells (2.2.4.2).

2.2.4.4 Colony assay (Colony forming unit- CFU) in semi solid medium and replating efficiency

At day 5 after infection, 5000 cells/ml $Sca1^+/Lin^-$ cells were plated in triplicates into methylcellulose supplemented with rmIL-3 (20 ng/ml), rmIL-6 (20 ng/ml), and rmSCF (100 ng/ml). On day 10 after plating, the colony number was counted and colony morphology was evaluated using AxioCam HRc camera (Zeiss, Goettingen, Germany) with 20x magnification. After washing out the cells from methylcellulose, 5000 cells/plate were plated again in methylcellulose for the determination of replating efficiency by serial plating. The replating of the cells was repeated so often until no colony formation was observed in any of the groups.

2.2.5 Animal experiments

2.2.5.1 Keeping/breeding of animals

Minimal of 7 days before transplantation, the C57BL/6J inbred female mice were purchased from Janvier Laboratories, St. Berthevin, France. All mice used in the experiments were kept/bred in the animal center of the Johann Wolfgang Goethe University in Frankfurt am Main. They were kept in standard sterile cages (maximal 5 mice /cage) and feed with standard rodent diets and water. The health status of the animal center was monitored based on the guidelines of the Federation of European Laboratory Animal Science Associations (FELASA). All experiments were performed out according to the German Animal Protection Law (Tierschutz: Genehmigungsaktenzeichen: F39/08).

2.2.5.2 Transduction/transplantation model of leukemia

Recipients were sublethally irradiated with 4.5 Gy. Transduced $Sca1^+$ HSCs were inoculated into anesthetized mice by retro-orbital injection. The mice were killed at the first appearance of signs of morbidity, loss of weight ≥ 10% and neurological abnormalities. Bone marrow was harvested from the femura and tibiae by flushing the bones with a syringe and 26-gauge needle. Spleen cells were isolated by passing the tissue through a 40 µM nylon filter. $2x10^5$ whole bone marrow and spleen cells were then cytospinned on glass slides by centrifugation at 500 rpm for 8 minutes and stained with May-Gruenwald-Giemsa staining. For FACS analysis, murine bone marrow or spleen cells were washed one time in FACS wash buffer and incubated with phycoerythrin (PE)-conjugated B220, Gr-1, and Mac-1 antibodies, kept 20 minutes by 4°C and subsequently 1 ml 1x RBC lysis buffer was given to the mixture in order to eliminate erythrocytes. After washing the samples once with FACS wash buffer, samples were fixed with FACS fixing solution. As negative control PE-conjugated anti-murine IgG1 was used. 10,000 events were counted on FACS CantoII (Becton Dickinson, Heidelberg, Germany) using FACS Diva software (Becton Dickinson, Heidelberg, Germany).

2.2.5.3 Colony forming unit spleen-day12 assay (CFU-S12 assay)

Tellesniczky's fixative

70% Ethanol	100 ml
Formaldehyde	4.5 ml
Glacial acetic acid	4.5 ml

$Sca1^+/Lin^-$ cells were purified from female donor mice retrovirally and transduced after prestimulation and cultured for 9 days in medium containing rmIL3, rmIL6 and rmSCF (2.1.4.10). The cells were washed 2 times with PBS and 10000 cells/200µl per mouse were injected i.v. into lethally (11Gy) irradiated recipient mice. 12 days later, transplanted animals were anesthetized and scarified by cervical dislocation. The spleens were removed through a left lateral incision and immediately fixed in Tellesniczky's fixative for 5 minutes, then transferred to 70% Ethanol for future preservation. The spleen colonies were enumerated under the binocular microscope.

2.2.6 Gene expression array

RNA was isolated from CFU-S12 Spleens as described before (2.2.1.7.1). The cDNA synthesis was performed using standardized protocols (Ovation Pico WTA, NuGEN, Bemmel, Holland). Microarray hybridization to GeneChip MoGene-1.0-ST-V1 arrays (Affymetrix, Santa Clara, USA), washing steps and scanning of the microarray were performed according to Affymetrix protocol.

2.2.6.1 Statistical analysis

The statistical analysis was done with the statistical computing environment R version 2.12 (R-Development-Core-Team, 2005). Additional software packages were taken from the Bioconductor project (Gentleman et al, 2004).

2.2.6.2 Microarray preprocessing

2.2.6.2.1 Normalization

Probe level normalization was conducted using the variance stabilization method (Huber et al, 2002). This method renders the variance of probe intensities approximately independent of their expected expression levels. Parameters (offset and a scaling factor) are estimated for each microarray, in consideration of the fact that a fair fraction of probes is not differentially expressed across the samples. In view of computational complexity of the algorithm parameters are estimated on a random subset of probes and are then used to transform the complete arrays.

2.2.6.2.2 Probeset summary

Probeset summarization was calculated using the medial polish method (Tukey, 1977) on the normalized data. For each Probeset a robust additive model was fitted across the arrays, considering the different sensitivity of the Probeset via the probe effect.

2.2.6.2.3 Heatmap

A heatmap is a two dimensional plot, where the same signals display with the same colors. It is not only a visually impression of the gene expression. Heatmaps are done with the spotfire software (Spotfire Decision Site 9.1.2).

2.2.6.2.4 Differentially expression

Many of the genes on the microarray won't be expressed, or might have only a small variability across the samples. First an expression intensity filter is used to reduce the dimension of the microarray data: The data are filtered with an intensity (the intensity of a gene should be above 100 in at least 0.25 percent of the samples, if the group size is equal) and a variance filter (the interquartile range of $\log_2$ intensities should be at least 0.5, if the groups size is equal). After the expression intensity filtering p values were calculated with two sample t-test (variance=equal) to identify genes that are differentially expressed between two groups. For the multiple testing problems a False Discovery Rate (FDR) (Hochberg & Benjamini, 1990) was used. Also Fold changes (FC) between the two groups was calculated for each gene. The lists of differentially expressed genes were filtered with FDR and FC criteria.

Gene expression array was performed in the group of Prof. Dr. Hansmann, Senckenbergischen institute for pathology, university hospital, Frankfurt, Germany.

2.2.7 Statistical Analyses

All statistical analyses were performed using Student's-*t*-test and $p \leq 0.05$ was considered as significant. All experiments were performed at least 3 times and the results were taken only if the replicates of independent experiments indicated the same results. The data are presented as mean values of triplicates from one of the representative experiments with corresponding standard error. GaphPad Prism 5.0 was used to provide the statistical calculations.

3 RESULTS

3.1 Both t(9;22) fusion proteins are required for the pathogenesis of Ph^+ ALL

3.1.1 Down-regulation of $p96^{ABL/BCR}$ using shRNA decreases the proliferation of Ph^+ ALL cell line, SupB15

In 1993 Melo *et al.* demonstrated that all patients carrying $p185^{BCR/ABL}$ (minor breakpoint) also exhibit $p96^{ABL/BCR}$ (Ia/Ib-e2) transcripts (Melo et al, 1993b). In agreement with this it was shown that 100% of ALL cell lines and patient cells translate this gene to a functional protein (Zheng et al, 2009). This was not the case for the CML-associated protein, $p40^{ABL/BCR}$, since this protein was detected in only 60% of CML patient cells (Melo et al, 1993a). The presence of $p96^{ABL/BCR}$ protein in all Ph^+ ALL cells indicates that there must be an indispensable role of this fusion protein in the transformation capacity of Ph^+ ALL cells. The use of RNA interference (RNAi) is an established method, which gives the possibility to sequence-specific and post-transcriptionally silence a gene (Elbashir et al, 2001). The shRNAs (short hairpin RNAs) are 21-22 nucleotide double stranded RNAs (dsRNAs), which provide a useful tool for studying the function of a gene. Therefore, Ph^+ SupB15 cell line was selected and transduced with lentiviral vectors encoding shRNA against $p96^{ABL/BCR}$ or non-target control (NTC) shRNA. The NTC contains a shRNA insert that does not target any known genes from any species, making it useful as a negative control in experiments using shRNA. Two different shRNAs, siR961 and siR962, were used and the effective down-regulation of $p96^{ABL/BCR}$ was controlled by western blotting (Figure 8A). In order to investigate the effect of $p96^{ABL/BCR}$ on the transformation potential of $p185^{BCR/ABL}$ in these cells, proliferation was assessed using XTT assay. As shown in Figure 8B, knocking down $p96^{ABL/BCR}$ reduced the proliferation rate of SupB15 cells dramatically. In contrast, no effect on the proliferation of NTC shRNA-transduced cells was observed.

Taken together these data indicate that $p96^{ABL/BCR}$ plays an important role in the proliferation of SupB15 cell line as an example for Ph^+ ALL model.

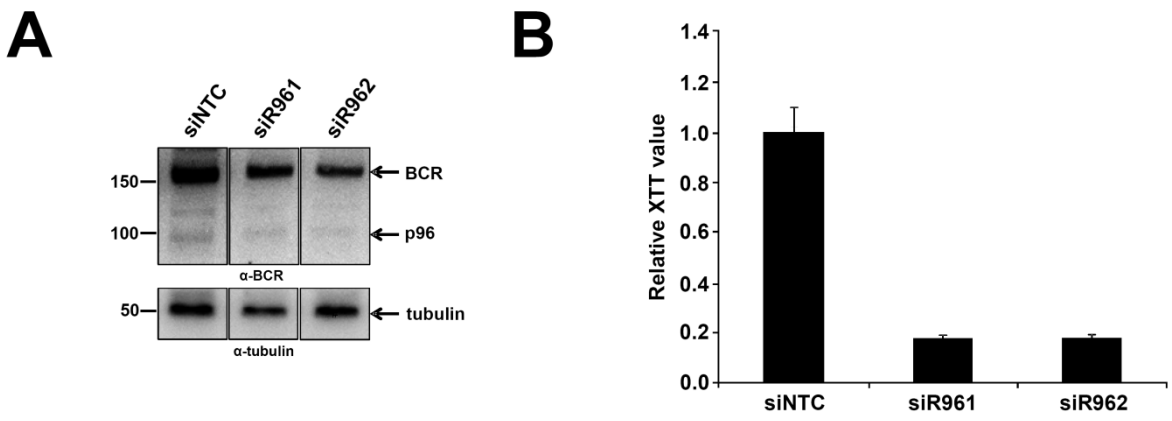

Figure 8. Down-regulation of p96$^{ABL/BCR}$ in SupB15 cell line as a Ph$^+$ ALL. (A) SupB15 cell line was lentivirally transduced with shRNAs against p96$^{ABL/BCR}$ (siR961-siR962)or NTC shRNA and the expression of endogenous BCR and p96$^{ABL/BCR}$ was followed by western blotting using anti-BCR antibody. Tubulin was taken as loading control; **(B)** SupB15 cells were transduced with either NTC shRNA or shRNAs against p96$^{ABL/BCR}$ and proliferation of the cells was measured using XTT assay after 3 days. The experiment was performed in triplicate a total of three times with similar results. The mean of triplicates ± SD from one representative experiment is given.

3.1.2 Targeting p96$^{ABL/BCR}$ fusion gene using shRNA in patient-derived long-term cultures (PD-LTCs) of Ph$^+$ ALL decreases the proliferation of the cells by induction of apoptosis

The heterogeneity of Ph$^+$ ALL in adults is not fully represented by cell lines. Therefore a recently established unique culture system for primary cells from Ph$^-$ and Ph$^+$ ALL patients or lymphatic CML-BC patients was taken for further experiments. This system allows long term cultures of directly patient derived leukemic cells, which remain genetically as well as immunophenotypically stable for at least 6 months, without entering senescence or passing the typical crisis of cell lines (Nijmeijer et al, 2009). There are 7 Ph$^+$ PD-LTCs, which exhibit different response levels towards tyrosine kinase inhibitors (TKIs) (Badura et al, 2013; Mian et al, 2012a; Nijmeijer et al, 2009). Two PD-LTCs express p210$^{BCR/ABL}$ (VB and CM) and three express p185$^{BCR/ABL}$ (PH, DW and KW). Also the non mutational TKI-resistant PD-LTC, BV harbors the p185$^{BCR/ABL}$. Three different PD-LTCs were selected in order to investigate the response of the cells to the down-regulation of p96$^{ABL/BCR}$: HP as a Ph$^-$ ALL patient cell, BV and PH, both positive for Philadelphia chromosome.

Five days after transducing the cells with NTC shRNA or p96$^{ABL/BCR}$ shRNAs (siR961, siR962), cells were lysed and the efficiency of shRNAs in down-regulation of p96$^{ABL/BCR}$ expression was determined by western blotting (Figure 9A). The proliferation rate of the cells was measured using XTT-proliferation assay.

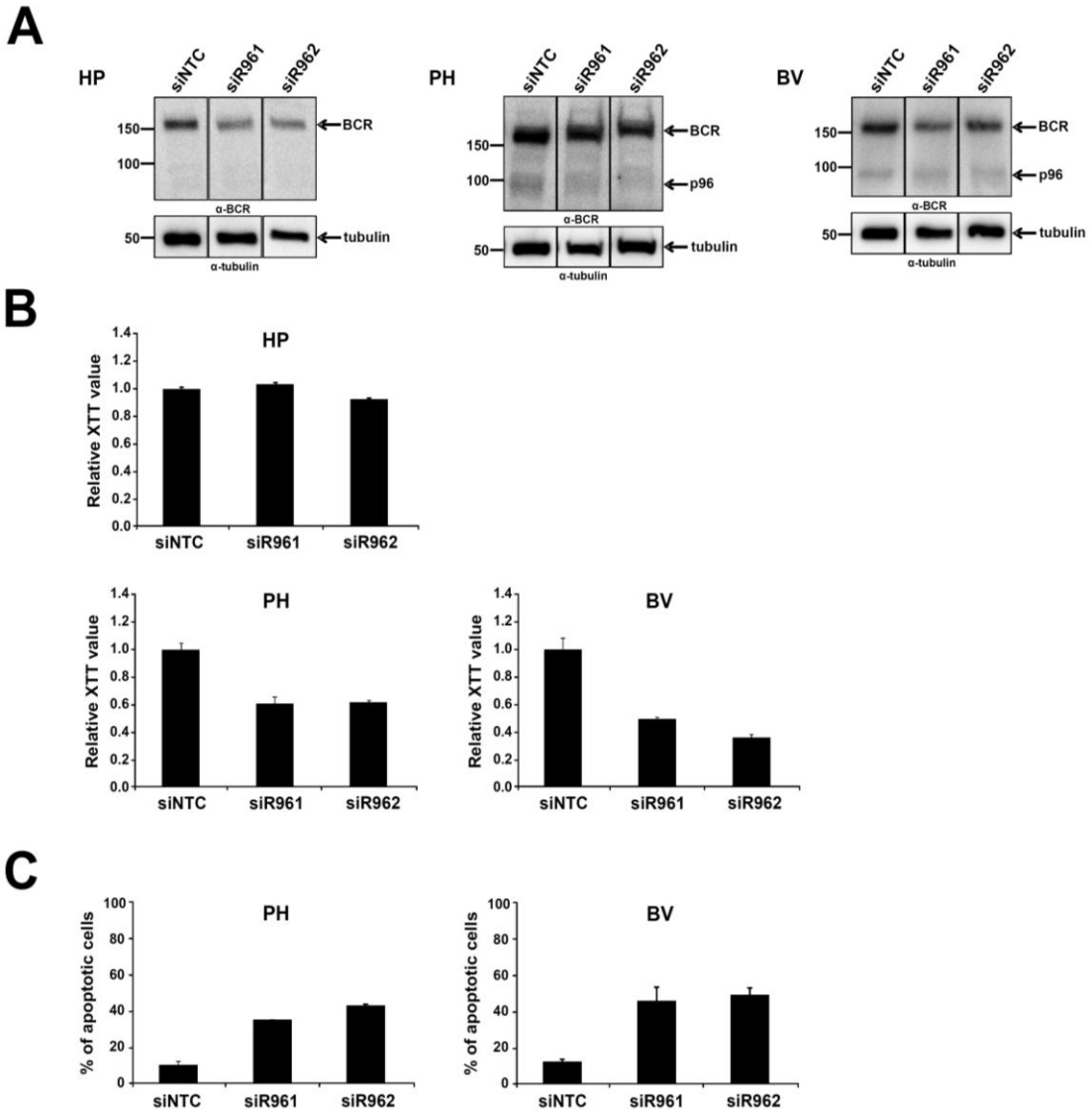

Figure 9. Effect of down-regulating p96$^{ABL/BCR}$ on the proliferation capacity and apoptosis rate of PD-LTCs of Ph$^+$ ALL. (A) The selected PD-LTCs, HP (Ph$^-$ ALL), BV and PH (Ph$^+$ ALL), were lentivirally transduced with specific shRNAs against p96$^{ABL/BCR}$ or NTC shRNA and the down-regulation of p96$^{ABL/BCR}$ was investigated using BCR-antibody. Tubulin was taken as loading control; **(B)** The proliferation of shRNA transduced HP, PH and BV cells was detected using XTT assay; **(C)** BV and PH cells were transduced lentivirally with shRNAs against p96$^{ABL/BCR}$ or NTC shRNA and apoptosis rate was measured using 7-AAD by FACS. All experiments were performed in triplicate a total of three times with similar results. The mean of triplicates ± SD of one representative experiment is given.

As presented in Figure 9A, the expression of p96$^{ABL/BCR}$ was down-regulated in the presence of p96$^{ABL/BCR}$ specific shRNAs and this inhibited the proliferation of BV and PH cells, but had no effect on HP cells (Figure 9B).

In order to investigate if the inhibition of proliferation is associated with the induction of apoptosis, the cells were stained with 7-AAD dye and the rate of apoptosis was measured

using FACS. As indicated in Figure 9C, apoptosis rate was increased when the expression of $p96^{ABL/BCR}$ was down-regulated using shRNA against this gene.

The double strand siR961 and siR962 used in the experiments were directed against the 3'-UTR of BCR. In order to exclude that the observed effects on the proliferation of Ph^+ ALL PD-LTCs was due to targeting of $p96^{ABL/BCR}$ and not that of endogenous BCR, a PD-LTC (VG) harboring the t(12;9)(p13;q34), which encodes for the TEL/ABL fusion protein (Nijmeijer et al, 2009) was lentivirally transduced with siR961, siR962 or NTC shRNA. Since the biology of TEL/ABL was reported to be similar to that of BCR/ABL (Malinge et al, 2006; Okuda et al, 1996; Pecquet et al, 2007), the effect of targeting the endogenous BCR was investigated in this PD-LTC. As presented in Figure 10A, both shRNAs efficiently down-regulated the endogenous BCR, which was accompanied by only a slight reduction of proliferation rate in VG cells (Figure 10B). The reduction of proliferation in VG cells was less prominent in comparison to BV and PH cells, where the shRNA mainly targeted the $p96^{ABL/BCR}$ fusion transcript.

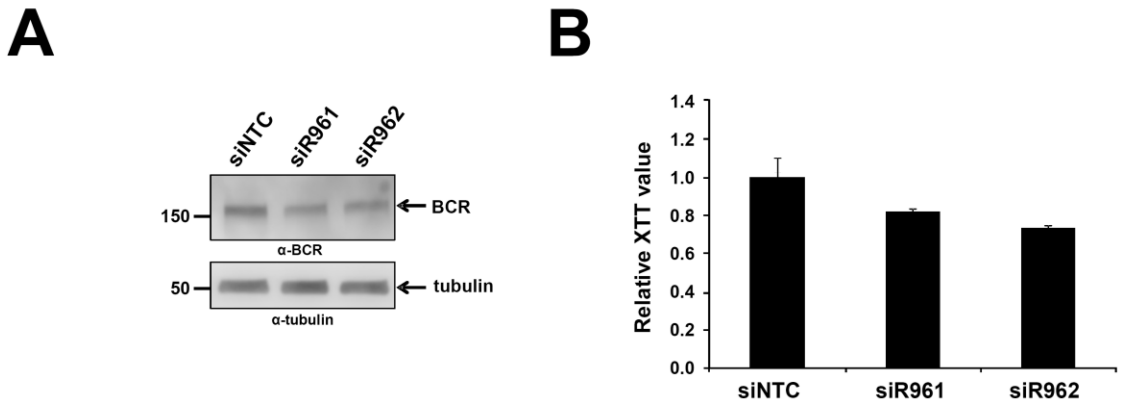

Figure 10. Down-regulation of endogenous BCR in a TEL/ABL-positive PD-LTC. (A) VG cells positive for t(12;9)(p13;q34) were lentivirally transduced with siR961, siR962 or NTC shRNA and the down-regulation of endogenous BCR was investigated using BCR-antibody. Tubulin was taken as loading control; **(B)** VG cells positive for TEL/ABL fusion protein were lentivirally transduced with siR961, siR962 or NTC shRNAs and proliferation was assessed using XTT assay. This experiment was performed in triplicate a total of two times with similar results. The mean of triplicates ± SD from one representative experiment is given.

This indicates that the role of endogenous BCR in the leukemogenic potential of TEL/ABL, and possibly BCR/ABL, is minimal and confirm the reported role of BCR delivered by several studies, since BCR was reported to have no prominent effect on BCR/ABL activation and its oncogenic potential (Lin et al, 2001; Liu et al, 1996a; Liu et al, 1996b; Perazzona et al, 2008).

In summary these data suggest that $p96^{ABL/BCR}$ is indispensible for the pathogenesis Ph^+ ALL PD-LTCs, which confirms the logic that 100% of ALL patients harbor both t(9;22) translocation products.

3.1.3 Silencing $p96^{ABL/BCR}$ fusion gene using shRNA sensitizes the PD-LTCs of Ph^+ ALL to kinase and allosteric inhibitors

The majority of CML-CP patients treated with the tyrosine kinase inhibitor, imatinib, are now anticipated to have a nearly normal life expectancy (Hoglund et al, 2013), but its impact on outcome remains dismal in patients with CML-BC or Ph^+ ALL (Giles et al, 2008; Giles et al, 2012; Ottmann et al, 2012; Talpaz et al, 2002). In Ph^+ leukemias, resistance attributable to either kinase domain mutations in BCR/ABL or non-mutational mechanisms remains the major clinical challenge. This corroborates the necessity for new therapeutic strategies in the treatment of Ph^+ leukemia, in order to overcome the resistance to ABL inhibitors and seek for new targets in advanced disease stages.

To investigate the effect of down-regulation of $p96^{ABL/BCR}$ in the presence of BCR/ABL specific inhibitors on the proliferation of Ph^+ ALL PD-LTCs, BV and PH cells were selected since they exhibit different responses to imatinib treatment. BV cells were reported to be resistant to imatinib and PH cells indicated good response to imatinib (Nijmeijer et al, 2009). In order to confirm this, the cells were treated with different concentrations of imatinib ranging from 0.1 to 2 µM and the proliferation of the cells was measured using XTT assay. The proliferation of PH cells was strongly reduced when 0.5 µM imatinib was used (Figure 11A). BV, in contrast, showed resistance even at high concentrations of imatinib (Figure 11B).

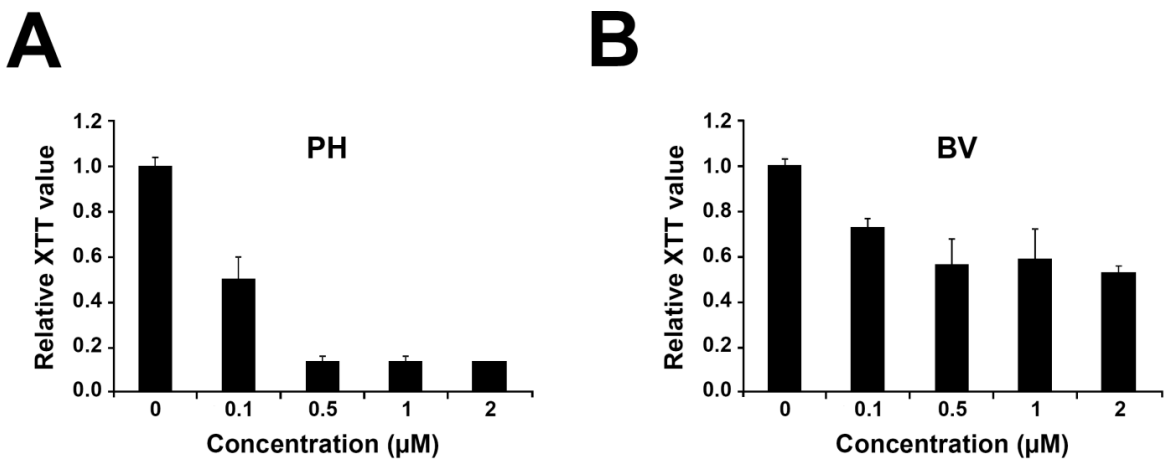

Figure 11. Responsiveness of two Ph$^+$ PD-LTCs to the kinase inhibitor imatinib. (A) PH cells were treated with different concentrations of imatinib (0.1, 0.5, 1 and 2 µM) and the response to the inhibitor was measured using XTT-assay; **(B)** Response of BV cells to different concentrations of the kinase inhibitor imatinib (0.1, 0.5, 1 and 2 µM) was measured using XTT proliferation assay. Both experiments were performed in triplicates a total of three times with similar results. The mean of triplicates ± SD from one representative experiment is given.

Next, BV and PH cells were transduced with NTC shRNA or shRNAs against p96$^{ABL/BCR}$ and treated with different concentrations of imatinib and the allosteric inhibitor, GNF-2, ranging from 0.1 to 2 µM. GNF-2 was reported to inhibit the proliferation of Ph$^+$ ALL PD-LTCs, BV and PH, and had no effect on the Ph$^-$ ALL cells, HP (Mian et al, 2012b).

The proliferation of the cells was measured after five days using XTT proliferation assay. As indicated in Figure 12A-B, comparison between cells positive for shRNA against p96$^{ABL/BCR}$ and the NTC shRNA transduced PD-LTC cells revealed that down-regulation of p96$^{ABL/BCR}$ in Ph$^+$ ALL PD-LTCs reduces the proliferation capacity of the cells and that BV cells are more sensitive to the down-regulation of p96$^{ABL/BCR}$ than PH cells.

The combination of the treatment with selective ABL inhibitors and shRNAs against p96$^{ABL/BCR}$ indicated that down-regulation of p96$^{ABL/BCR}$ sensitizes the PH cells to the ABL inhibitors and overcome the resistance of BV cells (Figure 12A). This effect did not depend on the BCR/ABL inhibitor used, since no significant difference between the kinase inhibitor, imatinib, and the allosteric inhibitor, GNF-2, was observed (Figure 12A-B).

Collectively, these data indicate that simultaneous application of BCR/ABL specific inhibitors and down-regulation of p96$^{ABL/BCR}$ fusion gene sensitizes the Ph$^+$ ALL cells and reduces their proliferation capacity.

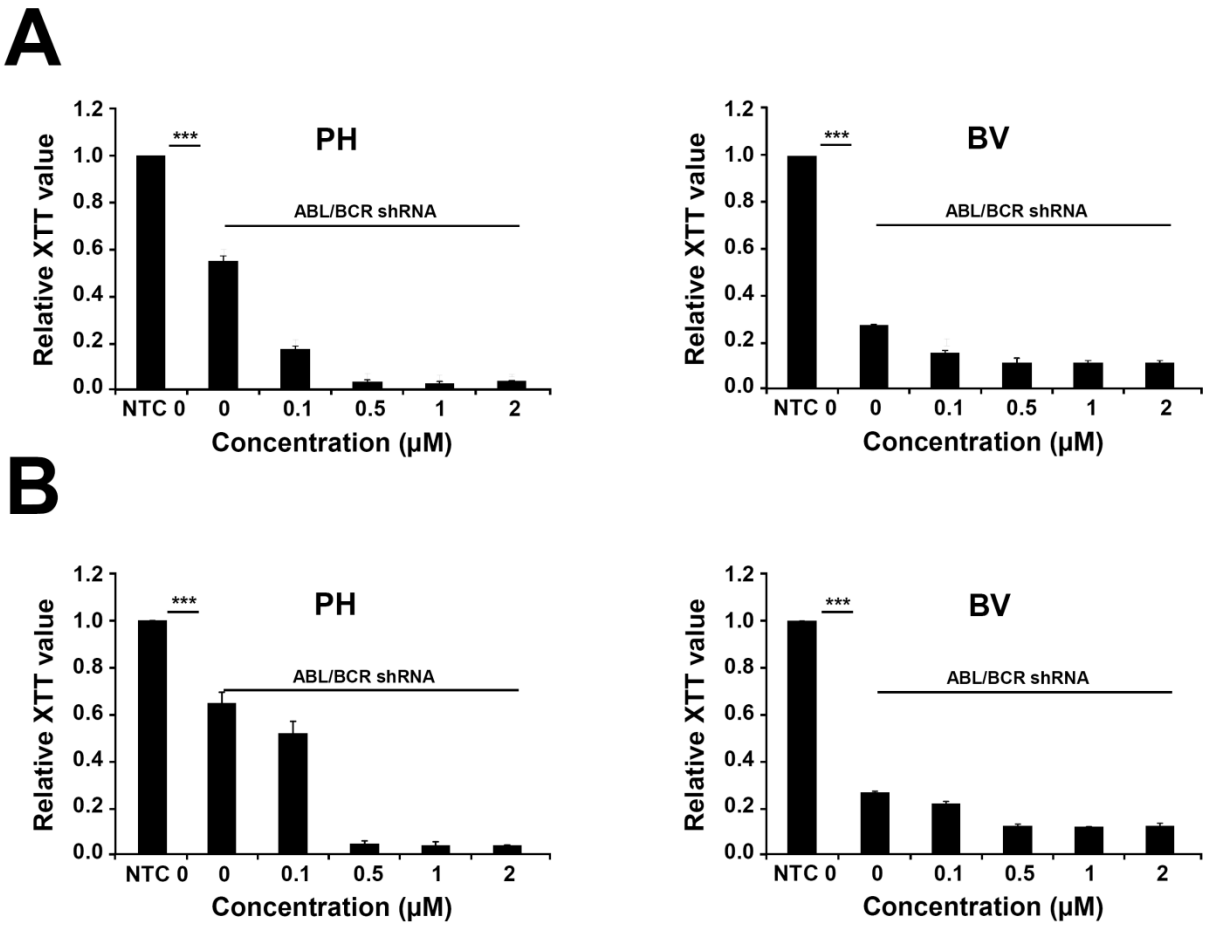

Figure 12. Role of p96$^{ABL/BCR}$ in responsiveness of PD-LTCs to selective BCR/ABL inhibitors. (A) PH and BV cells were transduced lentivirally with shRNAs against p96$^{ABL/BCR}$ or NTC shRNA and treated with different concentrations of imatinib (0.1, 0.5, 1 and 2 µM). The proliferation of these cells was measured using XTT assay; **(B)** PH and BV cells were transduced lentivirally with shRNAs against p96$^{ABL/BCR}$ or NTC shRNA and treated with different concentrations of GNF-2 (0.1, 0.5, 1 and 2 µM). All experiments were performed in triplicates a total of three times with similar results. The mean of triplicates ± SD from one representative experiment is given.

3.2 The fusion protein p96$^{ABL/BCR}$ enhances the transformation potential of p185$^{BCR/ABL}$-positive cells

3.2.1 Simultaneous expression of t(9;22) fusion proteins increases the proliferation rate of p185$^{BCR/ABL}$-positive Ba/F3 murine cells

In order to investigate the effect of p96$^{ABL/BCR}$ on the transformation potential of p185$^{BCR/ABL}$, p96$^{ABL/BCR}$ and p185$^{BCR/ABL}$ were co-expressed in factor-dependent Ba/F3 cell line. The IL3-dependent Ba/F3 cells are factor independent upon expression of BCR/ABL (Daley et al, 1992). Ba/F3 cells were retrovirally transduced with either empty vector or

vectors harboring p96$^{ABL/BCR}$, p185$^{BCR/ABL}$ or a construct harboring the simultaneous expression of both t(9;22) fusion proteins (p96$^{ABL/BCR}$-p2a-p185$^{BCR/ABL}$). This construct allows the expression of more than one gene from a single promoter using p2a peptide-linker and avoid the bias of differences in expression. The expression of both transgenes was controlled by western blotting in Ba/F3 cells (Figure 13B). Schematic representation of the transgenes is given in Figure 13A. The proliferation of the cells was assessed by dye exclusion using trypan blue and XTT proliferation assay.

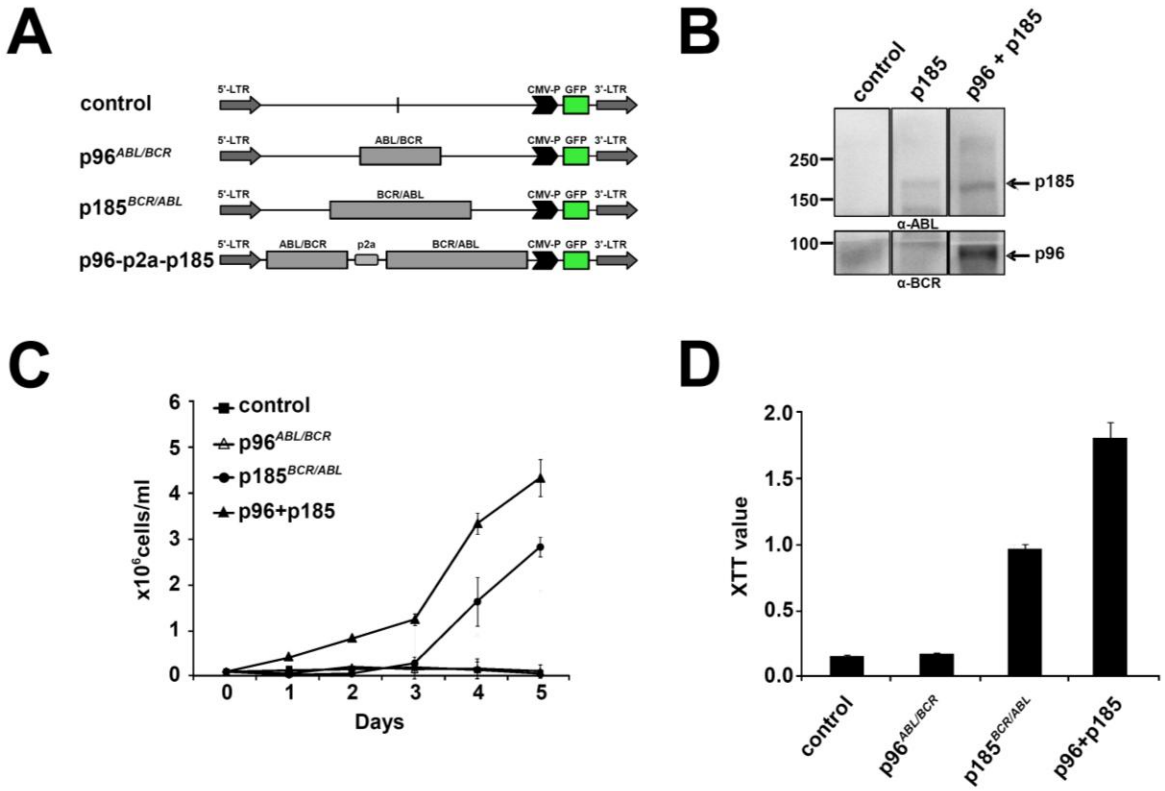

Figure 13. Effect of p96$^{ABL/BCR}$ on the proliferation rate of p185$^{BCR/ABL}$-positive Ba/F3 cells. (A) Schematic representation of the used constructs in this experiment; **(B)** Ba/F3 murine cells were infected with the indicated retroviruses and the simultaneous expression of p96$^{ABL/BCR}$ and p185$^{BCR/ABL}$ fusion proteins from p96-p2a-p185 construct was detected by western blotting using the indicated antibodies; **(C)** Ba/F3 murine cells were infected with the indicated retroviruses and IL-3 independent growth of cells expressing the fusion proteins was measured by dye exclusion using trypan blue (mean of 3 independent experiments ± SD); **(D)** The proliferation capacity of the transduced Ba/F3 cells was measured using XTT assay (mean of 3 independent experiments ± SD).

As presented in Figure 13C, the empty vector transduced cells were unable to grow under IL-3 withdrawal. This was also the case for p96$^{ABL/BCR}$-positive cells, since the expression of this fusion protein was unable to confer factor independence to Ba/F3 cells and did not influence the proliferation capacity of the cells. In contrast, cells transduced with p185$^{BCR/ABL}$ grew under withdrawal of IL-3. The presence of p96$^{ABL/BCR}$, however,

enhanced the proliferation rate of the p185$^{BCR/ABL}$-positive cells upon factor withdrawal. These results were confirmed using XTT proliferation assay. As shown in Figure 13D, Ba/F3 cells transduced with empty vector or vector harboring p96$^{ABL/BCR}$ fusion protein were unable to proliferate upon IL-3 withdrawal. The expression of p185$^{BCR/ABL}$ oncogene apparently substituted the IL-3 signaling, as indicated by the ability of Ba/F3 cells to grow without IL-3. The proliferation potential of Ba/F3 cells positive for p96$^{ABL/BCR}$-p2a-p185$^{BCR/ABL}$ was enhanced in comparison to p185$^{BCR/ABL}$-positive cells. These data reveal that p96$^{ABL/BCR}$ fusion protein increases the proliferation capacity of p185$^{BCR/ABL}$ in Ba/F3 murine cells.

3.2.2 The p96$^{ABL/BCR}$ fusion protein enhances the transformation potential of p185$^{BCR/ABL}$-positive Rat-1 fibroblasts

Due to the fact that the presence of p96$^{ABL/BCR}$ enhanced the proliferation capacity of p185$^{BCR/ABL}$-positive Ba/F3 cells and in order to investigate the effect of p96$^{ABL/BCR}$ on the transformation potential of p185$^{BCR/ABL}$, classical transformation assay was performed in untransformed fibroblasts: focus formation assays for the determination of contact inhibition and colony assay in semi-solid medium for the determination of anchorage-dependent growth. Rat-1 cells were retrovirally transduced with empty vector, p96$^{ABL/BCR}$, p185$^{BCR/ABL}$ or p96$^{ABL/BCR}$-p2a-p185$^{BCR/ABL}$. Schematic representation of the constructs is indicated in Figure 14A. The expression of the transgenes was controlled by flowcytometry analysis and the differences in the infection efficiency between samples did not exceed 10%.

As presented in Figure 14B, p96$^{ABL/BCR}$ alone, similar to the empty vector, was not able to transform the Rat-1 cells. The presence of p185$^{BCR/ABL}$ in Rat-1 cells led to the transformation of fibroblasts, which was detected by the ability to form foci (Figure 14B) or to make colonies in semi-solid medium (Figure 14C). The presence of both t(9;22) fusion proteins significantly increased the number of foci and colonies (Figure 14B-C).

Taken together these data indicate that p96$^{ABL/BCR}$ significantly enhances the transformation potential of p185$^{BCR/ABL}$-positive fibroblasts.

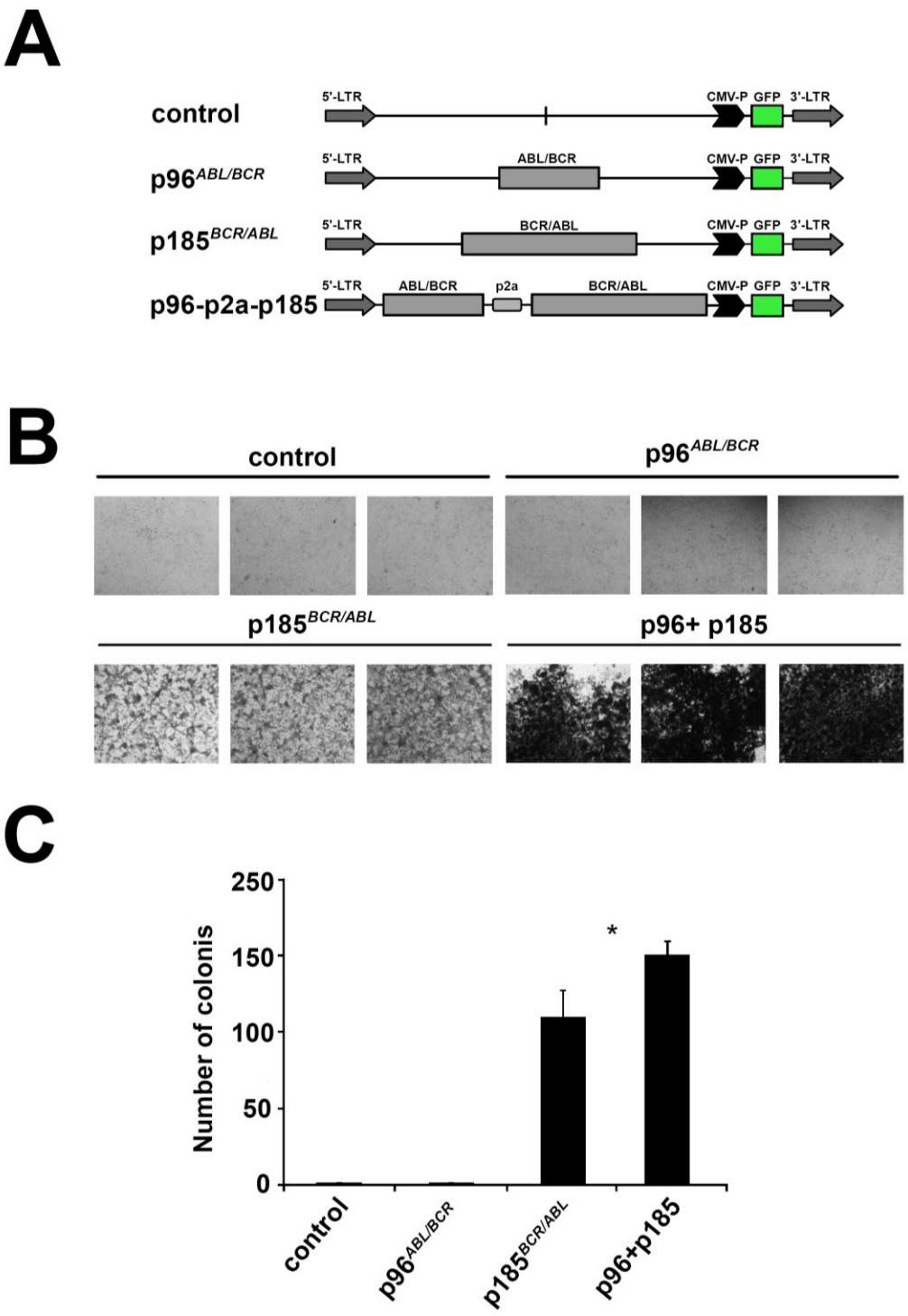

Figure 14. Effect of p96$^{ABL/BCR}$ fusion protein on the transformation potential of p185$^{BCR/ABL}$ in Rat-1 fibroblast cells. (A) Schematic representation of the transgenes used in this experiment; **(B)** Focus formation assay- For the determination of transformation potential of p185$^{BCR/ABL}$ in the presence of p96$^{ABL/BCR}$, Rat-1 cells were retrovirally transduced with the indicated transgenes and incubated for 15 days in 24-well plates and eventually photographed using 10x magnification; **(C)** Colony formation assay- Rat-1 cells were retrovirally transduced with the indicated constructs and seeded at 5x10^3 cells/well in soft-agar in 6-well-plates. After 15 days, the colonies were counted. Both experiments were performed a total of three times with similar results. For colony formation assay the mean of triplicates ± SD from one representative experiment is given.

3.2.3 The presence of both t(9;22) fusion proteins in $CD34^+CD38^-$ primary human cells increases the proliferation of the cells and reduces apoptosis

In order to determine the effect of $p96^{ABL/BCR}$ on the proliferation of $p185^{BCR/ABL}$-positive human cells, $CD34^+CD38^-$ primary human cells were isolated immunomagenetically from blood samples obtained from GSC-F stimulated healthy individuals and lentivirally transduced with $p96^{ABL/BCR}$, $p185^{BCR/ABL}$ or the construct harboring both fusion proteins ($p96^{ABL/BCR}$-p2a-$p185^{BCR/ABL}$). The schematic representation of the used constructs is illustrated in Figure 15A. Cells transduced with empty vector were used as negative control. Transduction efficiency was measured by FACS. The proliferation potential of the cells was measured using XTT proliferation assay after 72 hours. The rate of apoptosis was measured by 7-AAD staining.

As shown in Figure 15B, there was no significant effect on the proliferation of $CD34^+CD38^-$ human cells when the cells were transduced with empty vector or vectors harboring $p96^{ABL/BCR}$ or $p185^{BCR/ABL}$. This was confirmed by measuring apoptosis in these cells, since as indicated in Figure 15C the rate of apoptosis in cells positive for $p96^{ABL/BCR}$ or $p185^{BCR/ABL}$ fusion proteins was similar to the one of empty vector transduced cells. However the presence of both t(9;22) translocation products led to an enhanced proliferation and a reduction of apoptosis in $CD34^+CD38^-$ human cells.

Collectively these date indicate that the collaboration between t(9;22) fusion proteins leads to the enhanced proliferation capacity of $CD34^+CD38^-$ primary human cell, which is accompanied by the reduction of apoptosis.

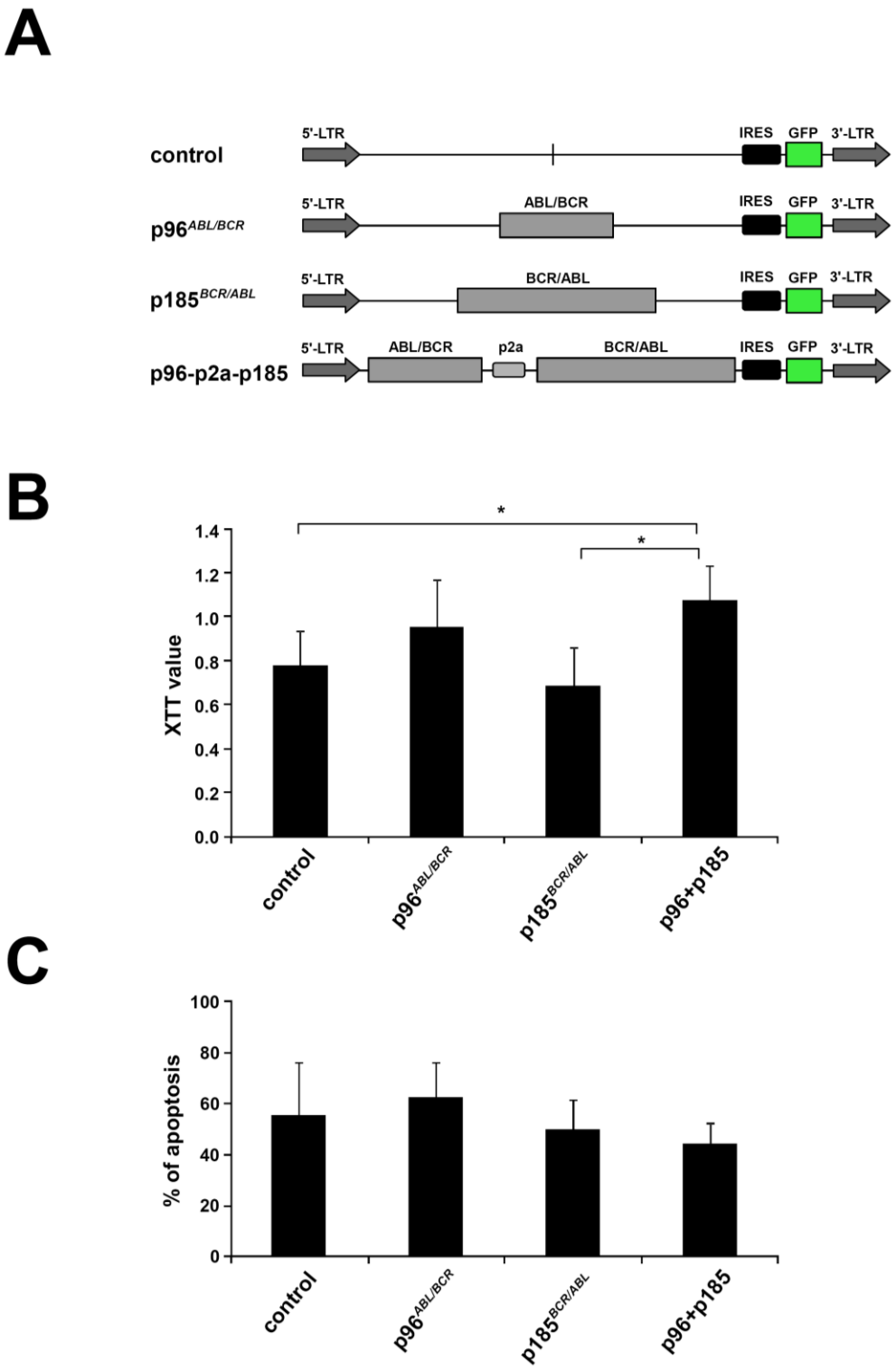

Figure 15. Effect of simultaneous expression of t(9;22) fusion proteins on the proliferation and apoptosis rate of CD34$^+$CD38$^-$ primary human cells. (A) Schematic representation of the transgenes used in this experiment; **(B)** CD34$^+$CD38$^-$ primary human cells were lentivirally transduced with the indicated transgenes and the proliferation potential of the cells was measured using XTT assay. Apoptosis rate was measured using 7-AAD staining. This experiment was performed a total of three times with similar results. Mean of triplicates ± SD from one representative experiment is given.

3.2.4 The enhanced transformation potential of p185$^{BCR/ABL}$ in the presence of p96$^{ABL/BCR}$ is accompanied by its higher kinase activity

BCR/ABL, in contrast to c-ABL, is a constitutive active kinase. Tyrosine 177 (Y177) and tyrosine 245 (Y245) are important phosphorylation sites for docking of adaptor proteins and for BCR/ABL autophosphorylation, respectively. Activation of BCR/ABL leads to the aberrant activation of downstream signaling cascades, such as STAT5, MAP-kinase and PI3K/AKT pathway, which leads to enhanced proliferation capacity and inhibition of apoptosis (Deininger et al, 2000). Since the co-expression of p185$^{BCR/ABL}$ with p96$^{ABL/BCR}$ protein increased the transformation potential of p185$^{BCR/ABL}$, the autophosphorylation status of p185$^{BCR/ABL}$ at Y245 and Y177 was investigated in Ba/F3 cell line. For this, the cells were retrovirally transduced with constructs indicated in Figure 13A (empty vector, p96$^{ABL/BCR}$, p185$^{BCR/ABL}$ or p96$^{ABL/BCR}$-p2a-p185$^{BCR/ABL}$) and the phosphorylation status of p185$^{BCR/ABL}$ and its downstream signaling pathways, like STAT5 and MAP-kinase pathways were investigated by western blotting.

The introduction of p96$^{ABL/BCR}$ to Ba/F3 cells positive for p185$^{BCR/ABL}$ led to an enhanced autophosphorylation of p185$^{BCR/ABL}$ on Y245 and Y177 sites (Figure 16A) with the more prominent effect on Y245.

The phosphorylation status of Bcr and Crkl, as substrates of p185$^{BCR/ABL}$ kinase, and activation of STAT5 and the MAP-kinase, Erk, was investigated by western blotting with specific Y-phosphorylation directed antibodies. Substrate phosphorylation was increased in the presence of p96$^{ABL/BCR}$, as shown by the increased phosphorylation of endogenous Bcr at Y177. However, no prominent change in the activation of Crkl was observed and the phosphorylation of STAT5 and Erk was slightly enhanced (Figure 16B).

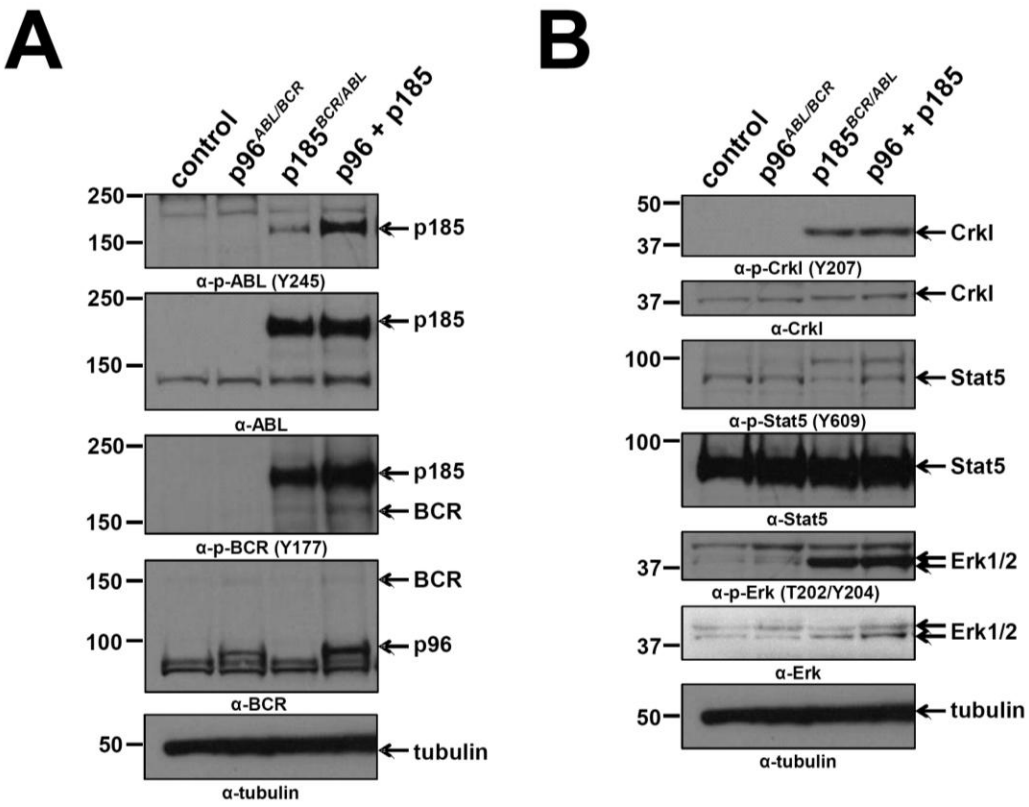

Figure 16. Effect of expression of p96$^{ABL/BCR}$ fusion protein in p185$^{BCR/ABL}$-positive Ba/F3 murine cells on the autophosphorylation of p185$^{BCR/ABL}$ and its downstream signaling pathways. (A) Ba/F3 murine cells were infected retrovirally with empty vector, p96$^{ABL/BCR}$, p185$^{BCR/ABL}$ or vector harboring both t(9;22) fusion proteins (p96-p2a-p185) and the autophosphorylation of p185$^{BCR/ABL}$ at Y245, Y177 and the substrate phosphorylation of BCR at Y177 was detected by western blotting with the indicated antibodies; **(B)** The effect of p96$^{ABL/BCR}$ on p185$^{BCR/ABL}$ substrate, Crkl, and its downstream signaling pathways, such as STAT5 and MAP-kinase pathways were investigated in the p185$^{BCR/ABL}$-positive Ba/F3 cells. This experiment was performed a total of three times with similar results. One representative experiment is presented.

3.2.5 The effect of ABL/BCR on the autophosphorylation of BCR/ABL is not limited to Ph$^+$ ALL, but also CML-associated t(9;22) fusion proteins

In contrast to Ph$^+$ ALL, where both t(9;22) fusion proteins are expressed, only 60% of CML cells contain both translocation products. In order to investigate if the CML associated ABL/BCR, p40$^{ABL/BCR}$, also enhances the phosphorylation of CML-related BCR/ABL, p210$^{BCR/ABL}$, Ba/F3 cells were retrovirally transduced with empty vector, p40$^{ABL/BCR}$, p96$^{ABL/BCR}$, p210$^{BCR/ABL}$ and the transgenes harboring the simultaneous expression of ABL/BCR fusion proteins and p210$^{BCR/ABL}$. The schematic representation of the used constructs is presented in Figure 17A. The phosphorylation status of p210$^{BCR/ABL}$ was detected using specific Y-phosphorylation directed antibodies.

As shown in Figure 17B, the autophosphorylation of p210$^{BCR/ABL}$ at Y245 and Y177 was enhanced in the presence of both ABL/BCR proteins.

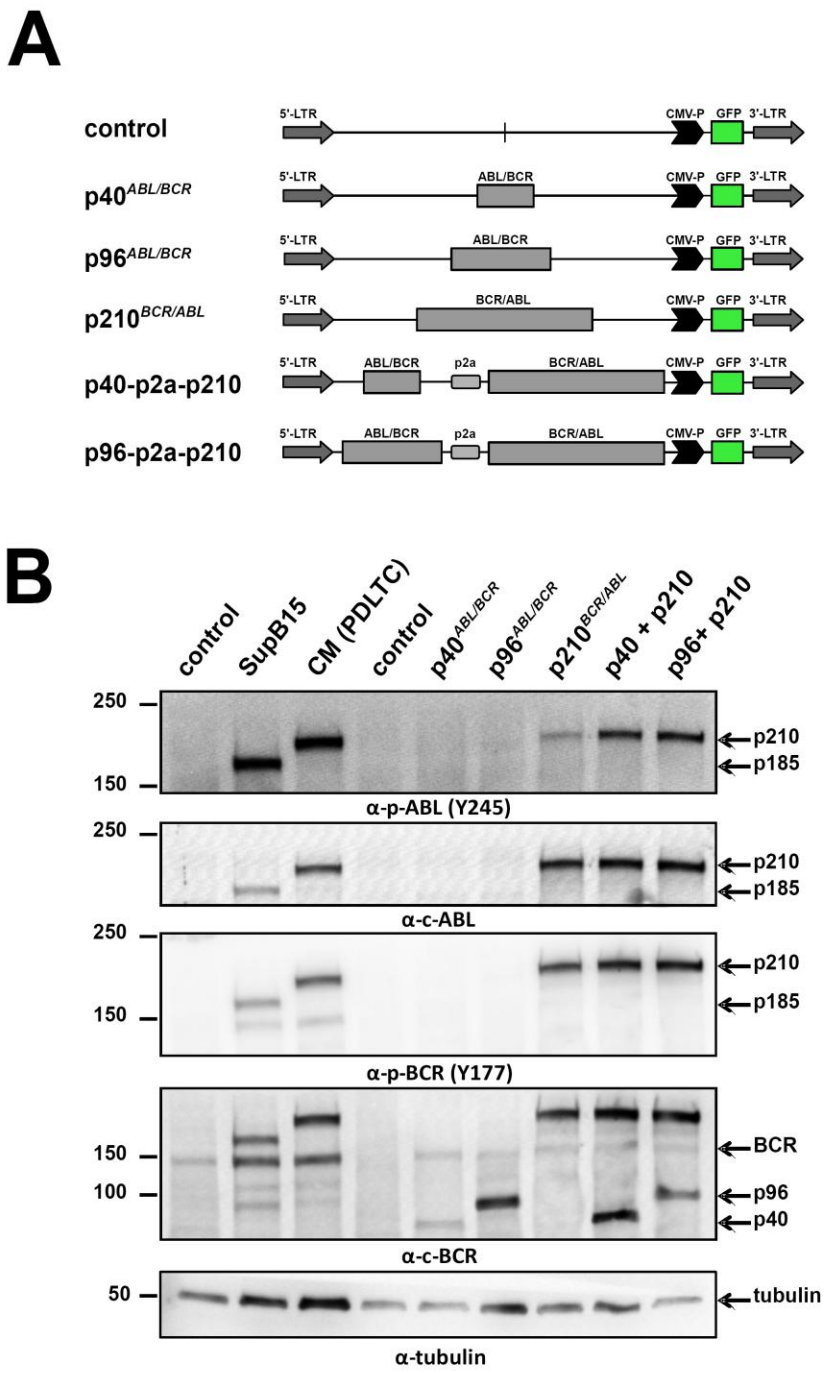

Figure 17. Effect of expression of p40$^{ABL/BCR}$ or p96$^{ABL/BCR}$ on the autophosphorylation of CML-associated BCR/ABL, p210$^{BCR/ABL}$. (A) Schematic representation of the used transgenes in this experiment; **(B)** Ba/F3 murine cells were transduced with the indicated retroviruses and the expression of transgenes and the phosphorylation status of p210$^{BCR/ABL}$ were analyzed by western blotting using the indicated antibodies. This experiment was performed a total of three times with similar results. One representative experiment is presented.

3.2.6 Down-regulation of p96$^{ABL/BCR}$ reduces the kinase activity of p185$^{BCR/ABL}$

Due to the fact that the enhanced phosphorylation of p185$^{BCR/ABL}$ in Ba/F3 cells was associated with a slight change in the activation of STAT5 and MAP-kinase pathways, SupB15 cell line was transduced lentivirally with shRNA against p96$^{ABL/BCR}$ (siR961-siR962) and the phosphorylation status of STAT5 and Erk upon down-regulation of p96$^{ABL/BCR}$ was investigated by western blotting. As shown in Figure 18, the phosphorylation of Erk is strongly abolished, when p96$^{ABL/BCR}$ expression was silenced due to the use of shRNA against p96$^{ABL/BCR}$. No effect on STAT5 activation could be observed.

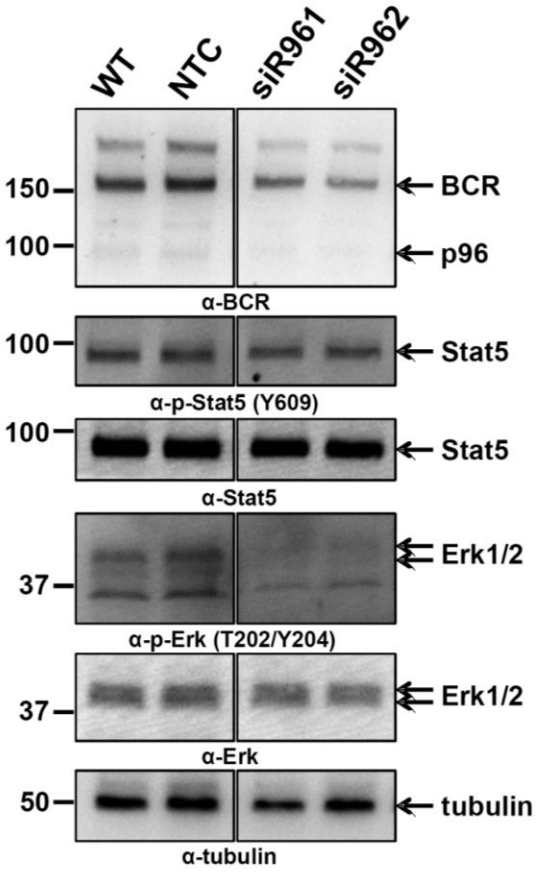

Figure 18. Effect of down-regulation of p96$^{ABL/BCR}$ on the p185$^{BCR/ABL}$-related signaling pathways. SupB15 cells were transduced with shRNA against p96$^{ABL/BCR}$ and the effect on STAT5 and MAP-Kinase pathway was detected using the indicated antibodies. Tubulin was taken as loading control.

In summary theses data indicate that knocking-down the p96$^{ABL/BCR}$ fusion protein using specific shRNAs reduces the kinase activity of p185$^{BCR/ABL}$, which was shown by the reduction in Erk kinase activity.

3.3 The t(9;22) fusion proteins are active at different level of mouse stem cell hierarchy

3.3.1 $p96^{ABL/BCR}$, but not $p185^{BCR/ABL}$, increases the replating efficiency of murine fetal liver HSCs

Since the majority of the leukemic stem cells are in non-proliferating status and anti-proliferative drugs are therefore not useful, it is of great importance to identify the effect of oncoproteins on the HSCs and figure out their target of transformation. In order to address the question whether the presence of $p96^{ABL/BCR}$ alters the transformation target of $p185^{BCR/ABL}$ and if there is an interplay between t(9;22) fusion proteins in different mouse HSC compartments, $Sca1^{+}/lin^{-}$ murine fetal liver cells, which were reported to be almost 7 times more enriched in long-term reconstituting HSCs than adult bone marrow cells (Morrison et al, 1995), were isolated and transduced retrovirally with empty vector, $p96^{ABL/BCR}$, $p185^{BCR/ABL}$ or $p96^{ABL/BCR}$-p2a-$p185^{BCR/ABL}$. The constructs used in this experiment are indicated in Figure 19A. As presented in Figure 19B, the expression of transgenes was controlled by FACS and the order of transduction efficiency was as following: empty vector > $p96^{ABL/BCR}$ > $p185^{BCR/ABL}$ > $p96^{ABL/BCR}$-p2a-$p185^{BCR/ABL}$, which is apparently due to the different sizes of the provirus in each construct. The acute promyelocytic leukemia (APL)-associated PML/RARα fusion protein was taken as positive control due to its well-known property in the maintenance of stem cell capacity via the activation of Wnt signaling (Muller-Tidow et al, 2004; Wang et al, 2010; Zheng et al, 2004).

As shown in Figure 20A, the replating efficiencies of the empty-vector and $p185^{BCR/ABL}$-transduced cells were limited to three cycles of replating. In contrast, $p96^{ABL/BCR}$, like PML/RARα, increased the replating efficiency over four cycles, with an increase in the number of CFUs in each round of replating. The simultaneous expression of $p185^{BCR/ABL}$ and $p96^{ABL/BCR}$ led to a higher number of colonies in comparison to $p185^{BCR/ABL}$, which was less than the capacity of $p96^{ABL/BCR}$-transduced cells (Figure 20A).

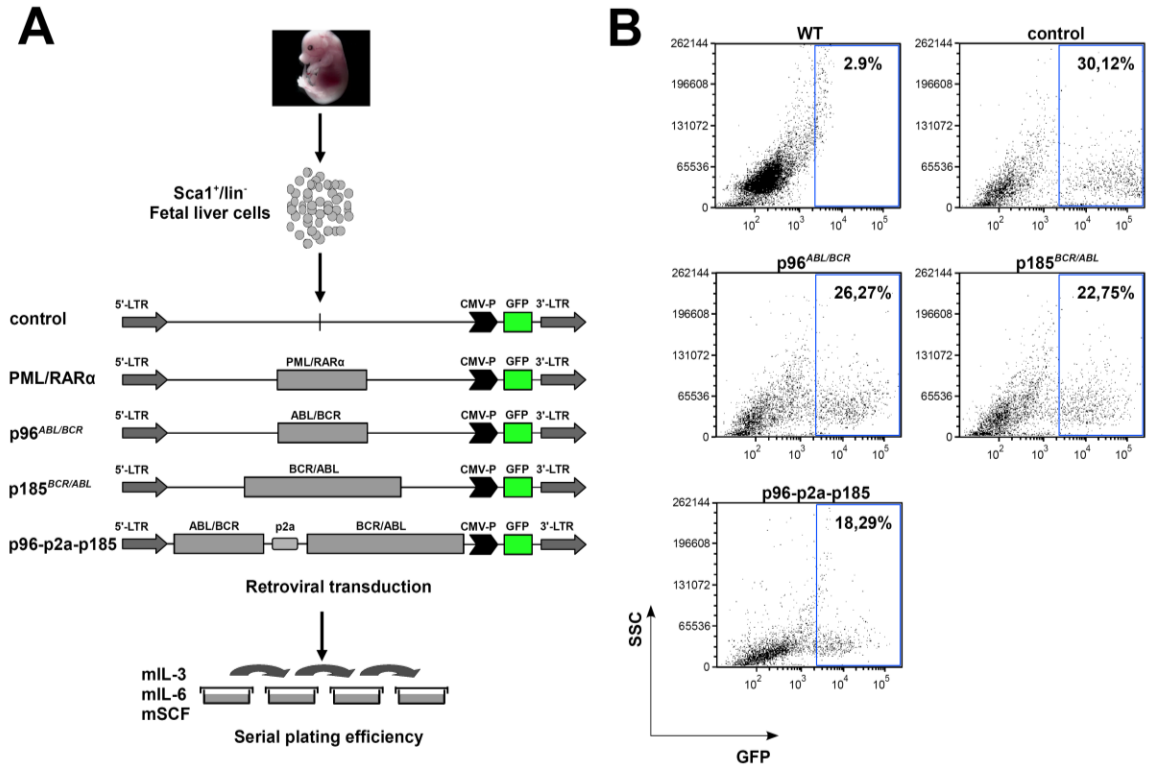

Figure 19. Effect of the t(9;22) fusion proteins on the biology of murine fetal liver HSCs. (A) Experimental strategy for studying the influence of the t(9;22) fusion proteins on the biology of murine fetal liver HSCs. Sca1^{+}/lin^{-} cells were immunomagenetically isolated from murine fetal liver, infected with the indicated retroviruses and plated in semi-solid medium supplemented with the indicated growth factors for determination of the serial replating potential; **(B)** The expression of transgenes was detected by FACS for the expression of GFP. The wild-type (WT) cells were taken as negative control for GFP expression.

The colony morphologies were classified as type A, B or C colonies according to the descriptions provided by Lavau and co-workers (Johnson et al, 2003; Lavau et al, 1997). The colony morphologies of p96$^{ABL/BCR}$ and PML/RARα were very similar and belonged to type A colonies, which are typical compact colonies (Figure 20B). Empty-vector control and p185$^{BCR/ABL}$-positive cells exhibited the type C morphology of diffuse colonies with mobile differentiating cells (Figure 20B). Interestingly, the expression of both t(9;22) fusion proteins supported the type B morphology, which seems to be a combination of type A and type C presented by a compact center surrounded by mobile cells.

Our observations in Sca1^{+}/lin^{-} murine fetal liver HSCs indicate that the presence of both t(9;22) fusion proteins impact the stem cell compartment distinctively to p96$^{ABL/BCR}$ or p185$^{BCR/ABL}$ alone. The presence of p96$^{ABL/BCR}$ confers stem cell capacity to p185$^{BCR/ABL}$ positive CFUs, which probably alters the transformation target of this oncoprotein. This may be of great importance in understanding the hematological features of Ph^{+} ALL and also CML-BC and in abrogating the leukemic stem cell in these malignancies.

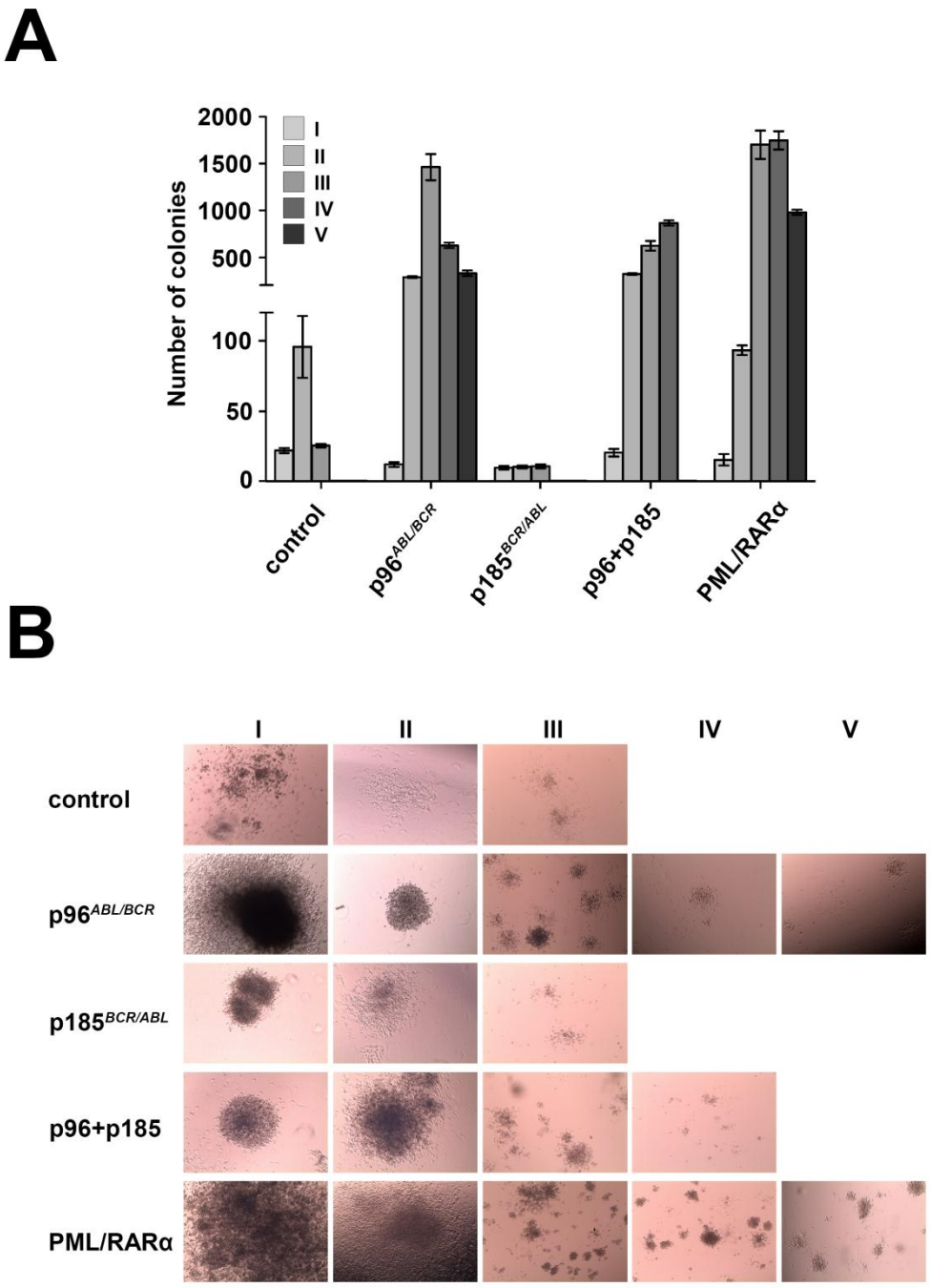

Figure 20. Effect of the t(9;22) fusion proteins on the biology of murine fetal liver HSCs. (A) Sca1^{+}/lin^{-} stem cells were transduced with the transgenes indicated in Figure 19A. empty vector and PML/RARα-transduced cells were used as negative and positive controls, respectively. 5000 cells/ml were seeded in semi-solid medium. At day 10, the colony numbers were enumerated, cells were harvested, analyzed, and 5000 cells/ml were replated again; **(B)** Colony morphology from the second plating were photographed using 20x magnification. Type A colonies (compact) were observed in cells transduced with p96$^{ABL/BCR}$ or PML/RARα, type B colonies (dense center surrounded by a halo of migrating cells) was noticed for p96-p2a-p185 transduced cells and type C colonies (diffuse colonies with mobile differentiating cells) were distinguished for empty vector or p185$^{BCR/ABL}$-positive cells.

3.3.2 The t(9:22) fusion proteins collaborate in the enhancement of self-renewal potential of early progenitors and short-term repopulating stem cells

The influence of ABL/BCR fusion proteins on hematopoietic stem cells was reported in previous studies (Zheng et al, 2009). These data revealed that ABL/BCR proteins block the myeloid differentiation and force the B-cell commitment in stem cells, which indicate that ABL/BCR fusion proteins target multipotent progenitors or even more immature HSCs. The *in vitro* results presented in part 3.3.1 confirmed these observations by the fact that $p96^{ABL/BCR}$ was able to confer/ or maintain multi-potential progenitor capacity of the HSCs in the semi-solid medium for more than 4 weeks. Knowing this, the effect of both t(9;22) fusion proteins on the self renewal capacity of early progenitors and short-term hematopoietic stem cells (ST-HSCs) was determined and attempted to address the question whether there is an hierarchical interplay between t(9;22) fusion proteins in the early stem cell compartment.

For this, Sca^{+}/Lin^{-} cells were isolated from murine bone marrow and retrovirally transduced with vectors harboring $p96^{ABL/BCR}$, $p185^{BCR/ABL}$ or $p96^{ABL/BCR}$-p2a-$p185^{BCR/ABL}$ as illustrated in Figure 21A. Cells transduced with empty vector were taken as negative control. The cells were kept 9 days in culture, in order to select for the stem cells affected by the used constructs and were subsequently transplanted into lethally (11Gy) irradiated recipients. The spleens were isolated after 12 days and the colony forming units-spleen (CFU-S) were counted (Figure 21A).

As shown in Figure 21B, $p96^{ABL/BCR}$ gave rise to CFU-S after 12 days. The $p185^{BCR/ABL}$ oncoprotein, like in CFU-assay, was unable to give rise to colony formation like empty vector. This was, however, completely modified when $p96^{ABL/BCR}$ was co-expressed simultaneously with $p185^{BCR/ABL}$. The size and number of colonies is an indication for the type and number of stem cells present, respectively (Till & McCulloch, 1961). As indicated in Figure 21B-C, CFU-S appeared in the presence of both t(9;22) transgenes showed enhanced number and also enlarged size.

Taken together, these data show that early progenitors and ST-HSCs expressing t(9;22)-associated fusion proteins maintain their stem cell capacity as demonstrated by their capacity to engraft *in vivo*.

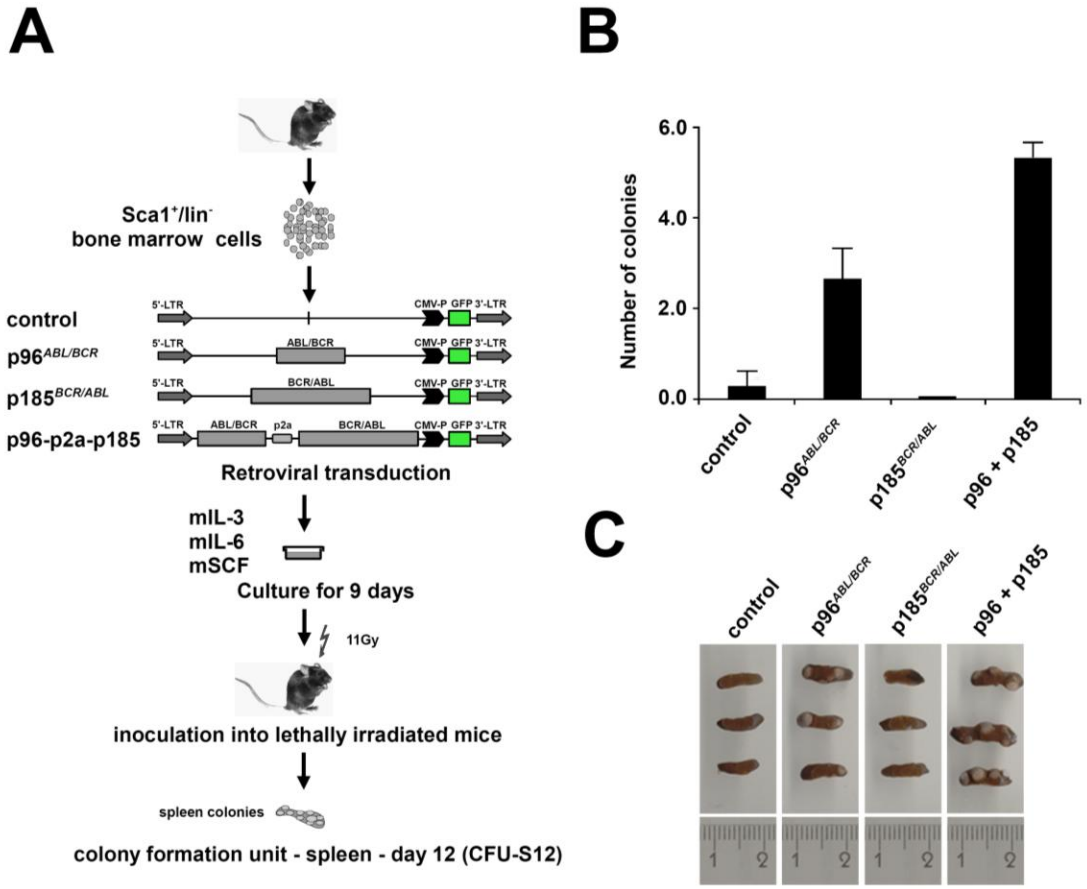

Figure 21. Stem cell cologenic potential of t(9;22) fusion proteins. (A) Experimental strategy for studying the influence of t(9;22) fusion proteins on the biology of murine early progenitors and ST-HSCs. $Sca1^{+}/lin^{-}$ bone marrow (BM) cells were infected with the indicated retroviruses and maintained for nine days in liquid culture supplemented with the indicated growth factors. 1 x 10^4 cells were inoculated into lethally (11Gy) irradiated recipients which were sacrificed at day 12 after transplantation; **(B)** Number of colonies on the spleens (n=3); **(C)** Obtained spleens after 12 days from all groups. The experiment was performed a total of three times with similar results. One representative experiment is given.

3.3.3 The ALL-associated $p96^{ABL/BCR}$ fusion protein seems to influences the fate of leukemia induced by $p185^{BCR/ABL}$ oncogene

One of the most sensitive, functional investigations of stem cell characteristics is due to their ability to reconstitute the leukemia *in vivo*. Earlier studies indicated the leukemogenic potential of ABL/BCR fusion proteins, since these fusion proteins were able to induce a myeloid leukemia-like phenotype in mice (Zheng et al, 2009). To address the question whether the presence of $p96^{ABL/BCR}$ influences the leukemia phenotype or initiation of $p185^{BCR/ABL}$-mediated leukemia, $Sca1^{+}$ murine HSCs were retrovirally transduced with $p96^{ABL/BCR}$, $p185^{BCR/ABL}$ or the transgene encoding both fusion proteins ($p96^{ABL/BCR}$-p2a-

p185$^{BCR/ABL}$) and inoculated into sub-lethally (4.5Gy) irradiated recipients. As controls, recipients inoculated with empty vector transduced Sca1^{+} cells were used (Figure 22A).

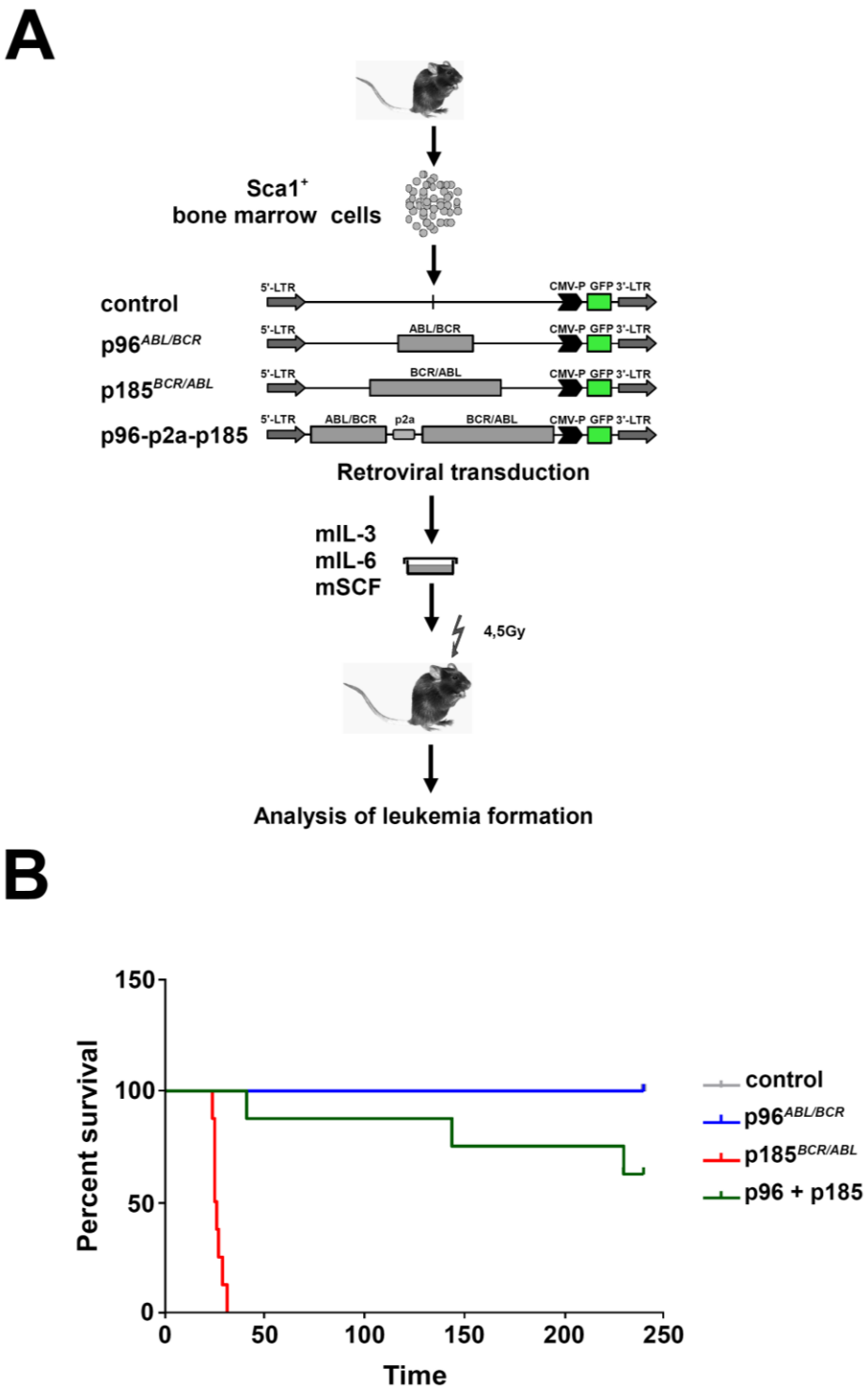

Figure 22. The leukemogenic potential of t(9;22) fusion proteins. (A) Experimental strategy to model leukemia induction by the reciprocal t(9;22) fusion proteins. Sca1^{+} bone marrow cells were infected with the indicated retroviruses and the same number of cells per group were inoculated into sub-lethally irradiated mice (n=8). Empty vector-transduced cells were used as control; **(B)** Survival curves present the frequency of recipients succumbing to disease after receiving the transduced cells.

Mice inoculated with $p185^{BCR/ABL}$-transduced cells developed rapidly myeloproliferative disease (MPD) leukemia phenotype, which was defined by splenomegaly (400-1200 mg spleen weight) (Figure 23A) and high numbers of Mac-1 (monocytes-macrophage) and Gr-1 (granulocytes) and a low number of B220 (mature B-cell) surface markers (Figure 23C). May-Gruenwald-Giemsa staining of BM and spleen cytospins present the type of the cells found in mice belonging to each of the groups. As indicated by arrows, mice inoculated with $p185^{BCR/ABL}$-positive cells showed the accumulation of myeloid lineage cells in both organs (Figure 23B). In contrast, the presence of both $p96^{ABL/BCR}$ and $p185^{ABL/BCR}$ induced a lymphoid-like leukemia phenotype, associated with moderate splenomegaly (90-300 mg spleen weight) (Figure 23A).

Further characterization of the bone marrow and spleen cells of these mice using FACS showed the presence of 82% and 64% of B220 expressing cells in the BM and spleen, respectively (Figure 23C). As indicated in Figure 23C, these mice had only 20% Mac-1 and Gr-1 positive cells. The presence of immature blast cells was observed using Gruenwald-Giemsa stained cytospins of BM and spleen cells (Figure 22B).

Collectively these data reveal that there is an interplay between ALL-associated t(9;22) fusion proteins in inducing an specific leukemia phenotype and that $p96^{ABL/BCR}$ might be one of the factors able to influence the fate of leukemia induced by $p185^{BCR/ABL}$ oncoprotein.

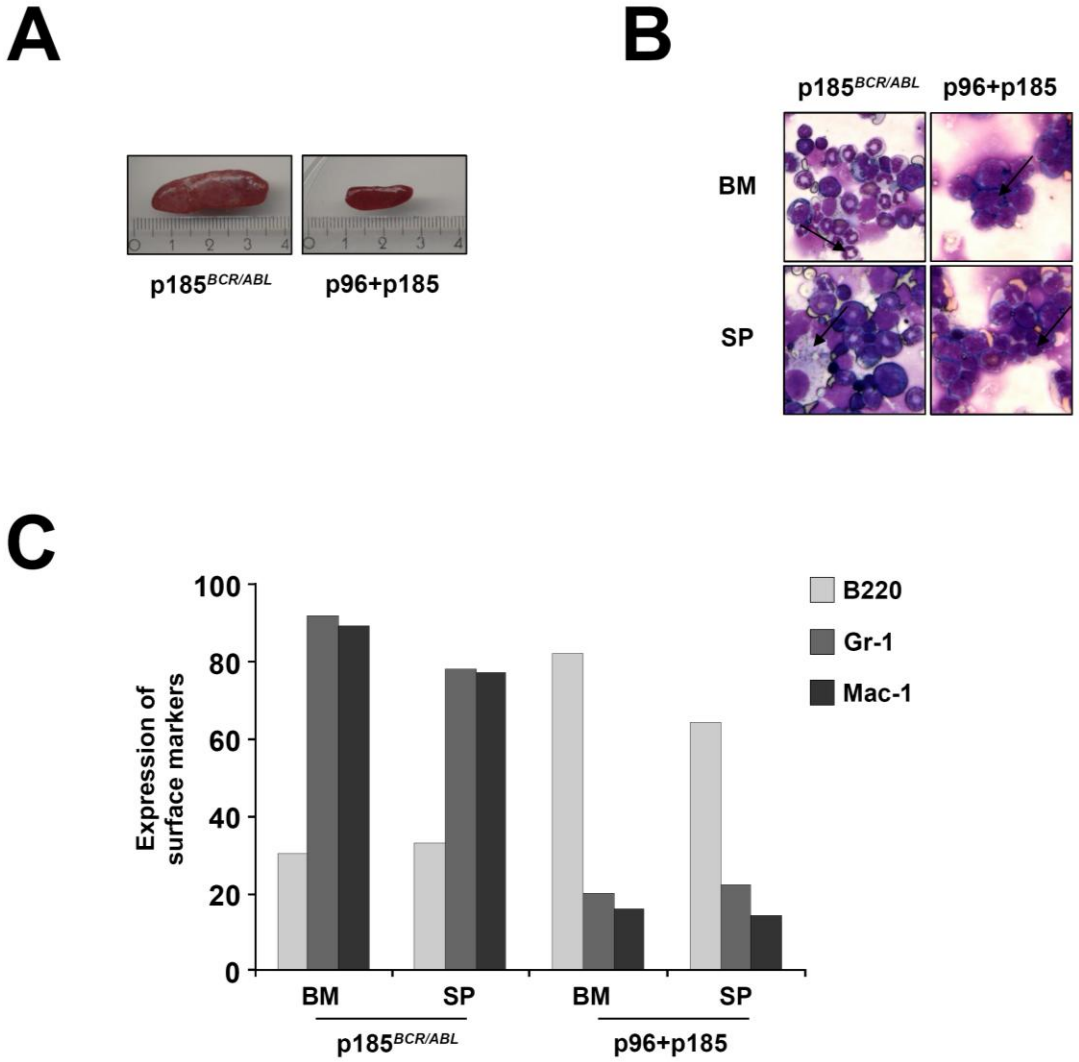

Figure 23. The leukemic phenotype of t(9;22) fusion proteins. (A) Sca1^{+} bone marrow cells were infected with empty vector or vectors harboring p96$^{ABL/BCR}$, p185$^{BCR/ABL}$ or p96-p2a-p185 and inoculated into sub-lethally irradiated mice. Relative splenomegaly of p185$^{BCR/ABL}$ and p96+p185-positive leukemia of one representative mouse in each group is presented; **(B)** May-Grünwald-Giemsa staining of cytospins from bone marrow (BM) and spleen (SP) of one representative mouse in each group. Arrows indicate cells of myeloid origin in p185$^{BCR/ABL}$-inoculated mice in comparison to blast cells observed in BM and SP of p96+p185 group; **(C)** Expression of differentiation-specific surface markers (Mac-1: monocytes- macrophage, Gr-1: granulocytes and B220: mature B-cells) of one representative mouse in p185$^{BCR/ABL}$ and p96+p185 groups.

3.3.4 The t(9;22) fusion proteins have distinct effect on the expression of cell cycle and apoptosis regulating genes

In order to explain the distinct effect of t(9;22) fusion proteins alone or in combination on the features related to mouse HSCs, the expression of different genes at RNA level was studied using micro array analysis. Therefore, RNA was isolated from the spleens obtained from the CFU-S12 experiment and gene expression profiles were performed using Affymetrix GeneChips (Santa Clara, USA). As shown in Figure 24, the gene expression

profile of spleens containing either p185$^{BCR/ABL}$ or both p96$^{ABL/BCR}$ and p185$^{BCR/ABL}$ transgenes showed that approximately all genes are expressed differentially when both t(9;22) fusion proteins are present in the mouse HSCs.

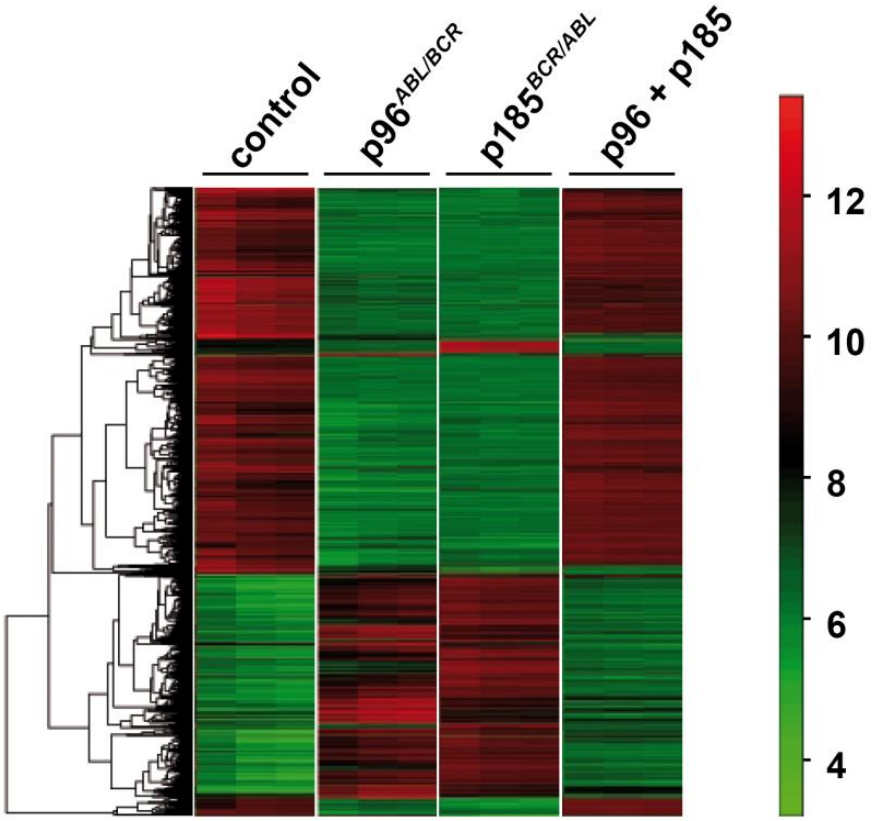

Figure 24. Gene expression profiles of spleens containing the t(9;22) fusion proteins. Total RNA was isolated from spleens obtained from the CFU-S12 *in vivo* experiment and gene expression profile was assessed using microarray analysis. Unsupervised clustering was done by taking genes with the highest standard deviation and sorted according to the similarity in expression level. The absolute expression is shown in this graph.

Due to the wide range of differentially expressed genes between p185 and p185+p96 samples, some cellular signaling pathways known to be important for the pathogenesis of BCR/ABL induced leukemia were taken and are shown in Figure 25. These pathways have at least 5 genes that are differentially regulated between p185 and p96+p185 groups and they mainly belong to the pathways related to cell cycle regulation, proliferation and apoptosis.

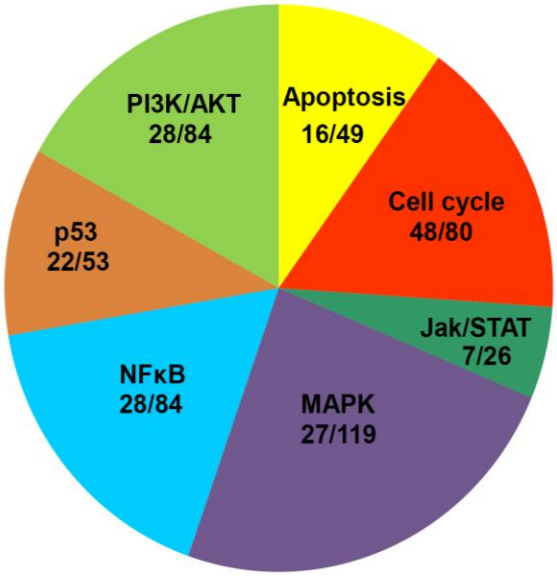

Figure 25. The differentially regulated signaling pathways in p185$^{BCR/ABL}$ versus p96+p185 group. Seven cellular pathways related to cell cycle regulation, proliferation and apoptosis are presented here. The numbers beyond each pathway indicate the number of differentially expressed genes between p185 and p96+p185 positive cells from the total number of the genes (related to each of the pathways) that were detected in the micro array analysis. As an example, 80 genes related to cell cycle regulation were detected in the micro array analysis and from this pool 48 genes were differentially regulated between p185 and p96+p185 groups.

The schematic representation of some of the genes related to these pathways is represented in Figure 27 to Figure 32, which was adapted from KEGG (Kyoto Encyclopedia of genes and genomes, Kanehisa laboratories) database. The red and green colors refer to the fold changes (FCs) observed between genes which were differentially regulated between p185 and p96+p185 groups. Red color represents changes with higher than +2 FCs and green color lower than -1 FCs. The colorless genes are not differentially regulated between these two groups.

In order to validate the data obtained from micro array analysis, real time-PCR was performed for three genes related to self renewal, proliferation and differentiation capacity of HSCs. As shown in Figure 26 the expression of p21 (CdKn1) as a target of p53 tumor suppressor gene was merely different between the samples and only a slight reduction in expression of this gene was detected in p96+p185 group; however a strong up-regulation of p53 and its target gene GADD45α (growth arrest and DNA damage inducible protein alpha) was observed. These results were in agreement with the obtained results from micro array analysis.

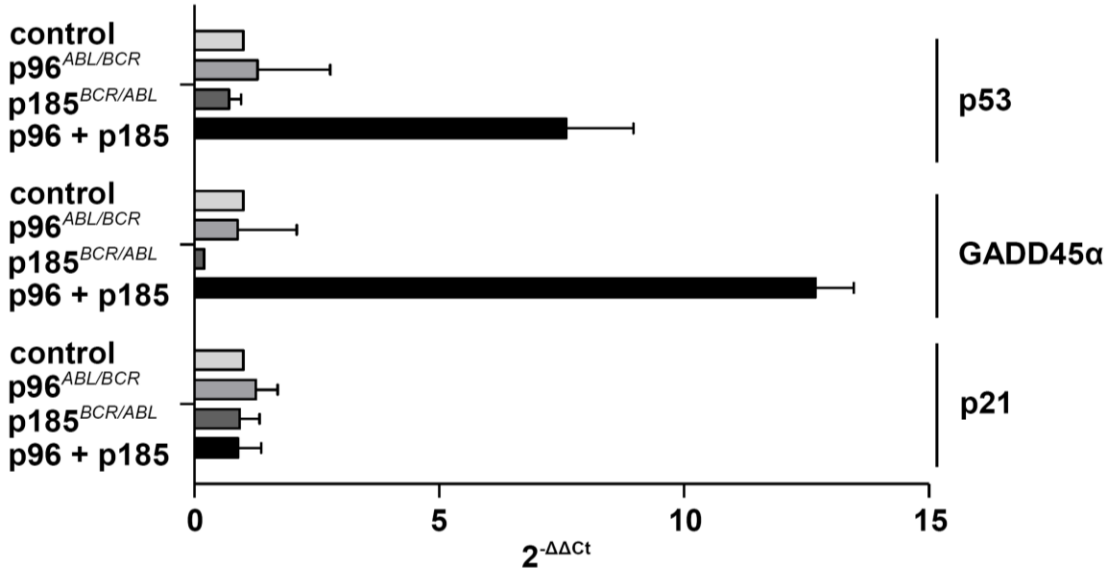

Figure 26. The real time- PCR validation of p53, GADD45α and p21 gene expression. Total RNA was isolated from spleens from the CFU-S12 *in vivo* experiment and after the cDNA was synthesized by reverse transcription PCR, the expression level of p53, p21 and GADD45α was quantified by Taqman real time-PCR. The relative concentration of each mRNA was normalized to the concentration of the housekeeping gene glyceraldehydes-3 phosphate dehydrogenase (GAPDH) and finally presented in $2^{-\Delta\Delta Ct}$. This experiment was performed in triplicates a total of two times with similar results. Mean of triplicates ± SD from one representative experiment is given.

These observations reveal that the expression of $p96^{ABL/BCR}$ alters the gene expression profile of $p185^{BCR/ABL}$ oncoprotein which was indicated by the up-regulation of p53 tumor suppressor gene and its target gene, GADD45α.

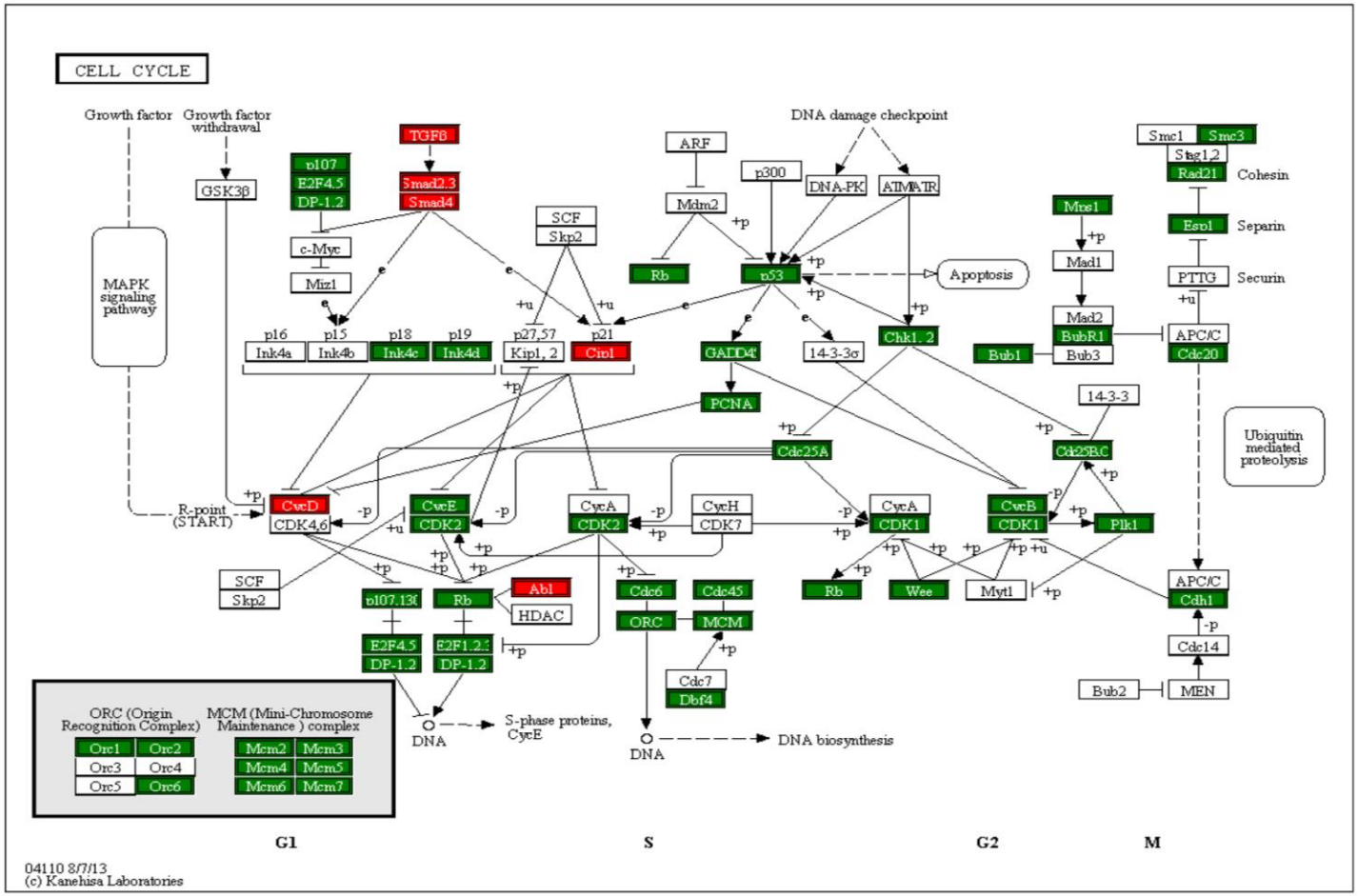

Figure 27. Schematic representation of genes related to cell cycle regulation. The cell cycle regulation genes and their interaction is presented in this picture. The colored (green and red) genes are differentially regulated between $p185^{BCR/ABL}$ versus p96+p185 groups. The green colored genes are down-regulated (fold changes < -1) in $p185^{BCR/ABL}$-containing spleens in comparison to spleens positive for both $p96^{ABL/BCR}$ and $p185^{BCR/ABL}$. The red colored genes are up-regulated (fold changes > +2) in $p185^{BCR/ABL}$ in comparison to p96+p185 group.

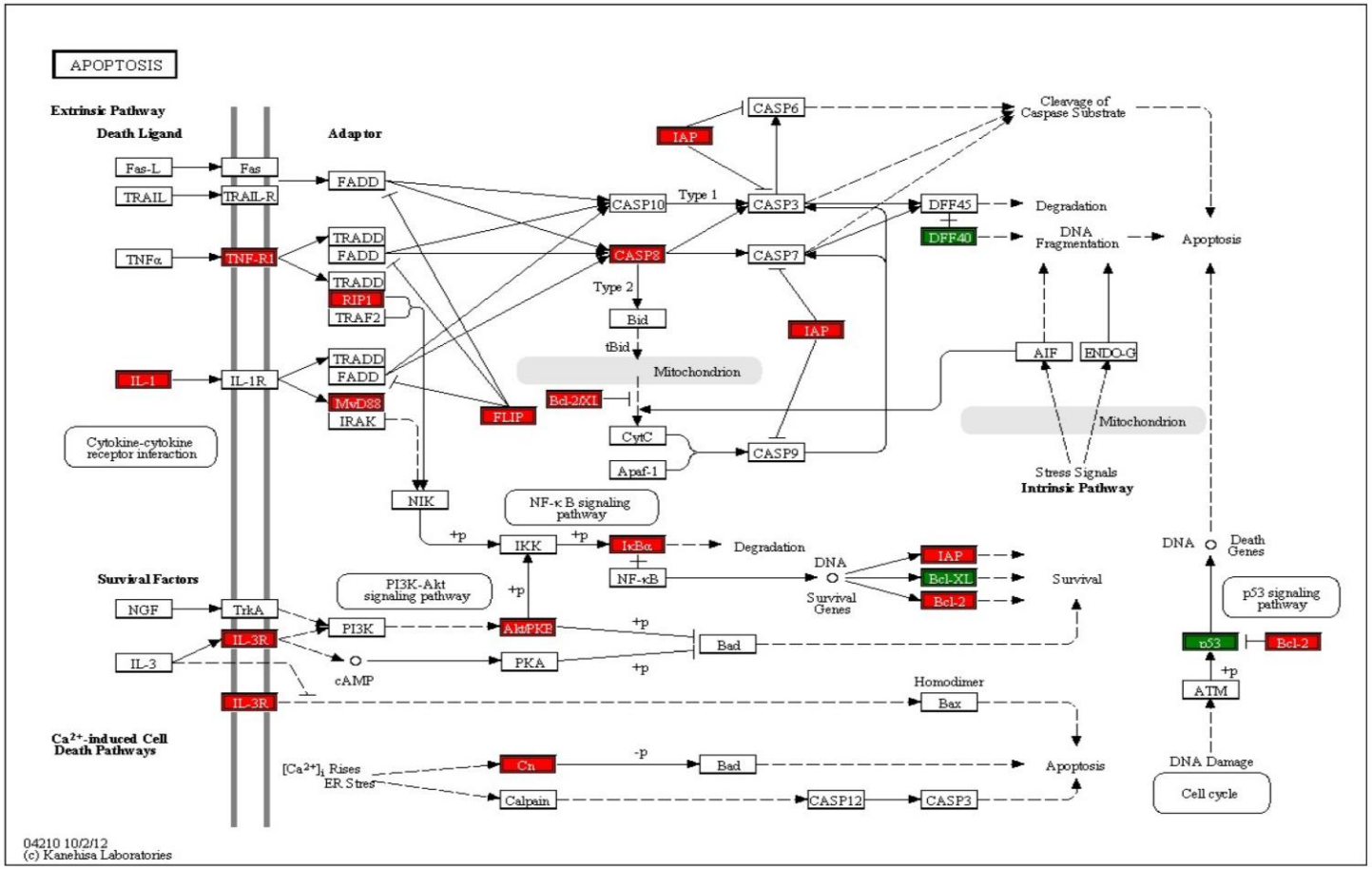

Figure 28. Schematic representation of genes related to apoptosis. The genes related to apoptosis signaling and their interaction is presented in this picture. The colored (green and red) genes are differentially regulated between $p185^{BCR/ABL}$ versus p96+p185 groups. The green colored genes are down-regulated (fold changes < -1) in $p185^{BCR/ABL}$-containing spleens in comparison to spleens positive for both $p96^{ABL/BCR}$ and $p185^{BCR/ABL}$. The red colored genes are up-regulated (fold changes > +2) in $p185^{BCR/ABL}$ in comparison to p96+p185 group.

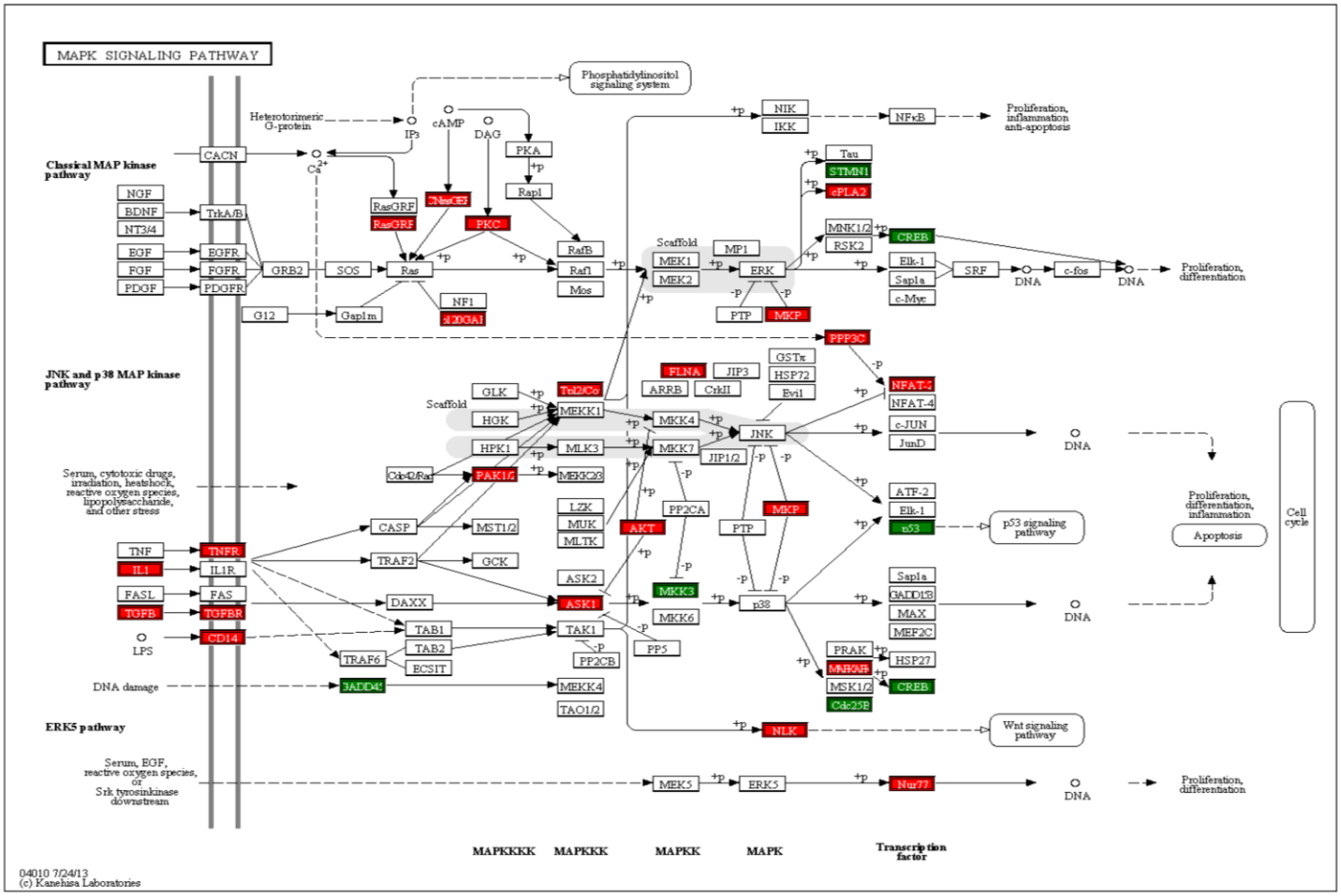

Figure 29. Schematic representation of genes related to MAP- kinase pathway. The genes related to MAP-kinase signaling and their interaction is presented in this picture. The colored (green and red) genes are differentially regulated between $p185^{BCR/ABL}$ versus p96+p185 groups. The green colored genes are down-regulated (fold changes < -1) in $p185^{BCR/ABL}$-containing spleens in comparison to the spleens positive for both $p96^{ABL/BCR}$ and $p185^{BCR/ABL}$. The red colored genes are up-regulated (fold changes > +2) in $p185^{BCR/ABL}$ in comparison to p96+p185 group.

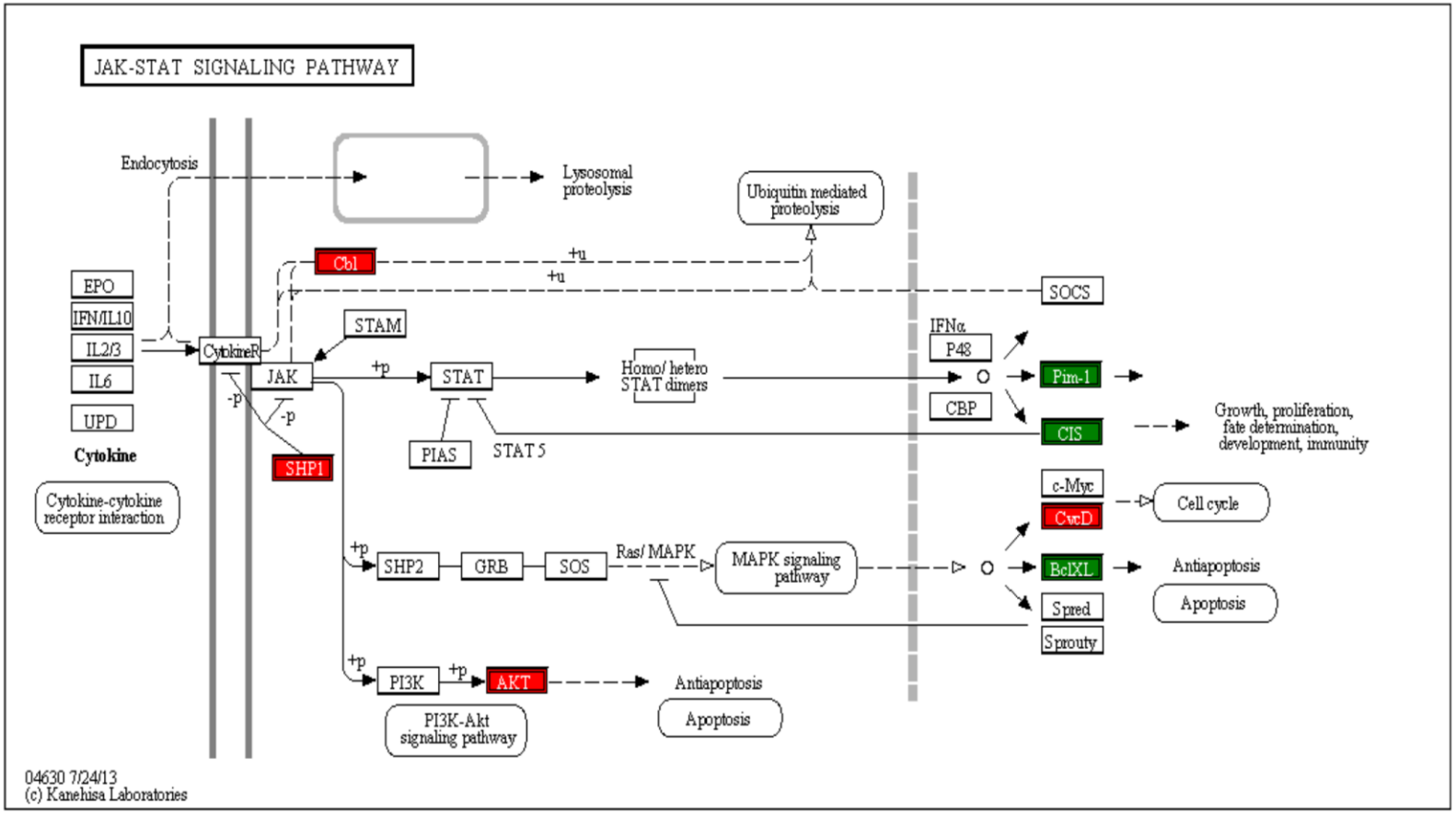

Figure 30. Schematic representation of genes related to JAK-STAT signaling pathway. The genes related to JAK-STAT signaling and their interaction is presented in this picture. The colored (green and red) genes are differentially regulated between $p185^{BCR/ABL}$ versus p96+p185 groups. The green colored genes are down-regulated (fold changes < -1) in $p185^{BCR/ABL}$-containing spleens in comparison to the spleens positive for both $p96^{ABL/BCR}$ and $p185^{BCR/ABL}$. The red colored genes are up-regulated (fold changes > +2) in $p185^{BCR/ABL}$ in comparison to p96+p185 group.

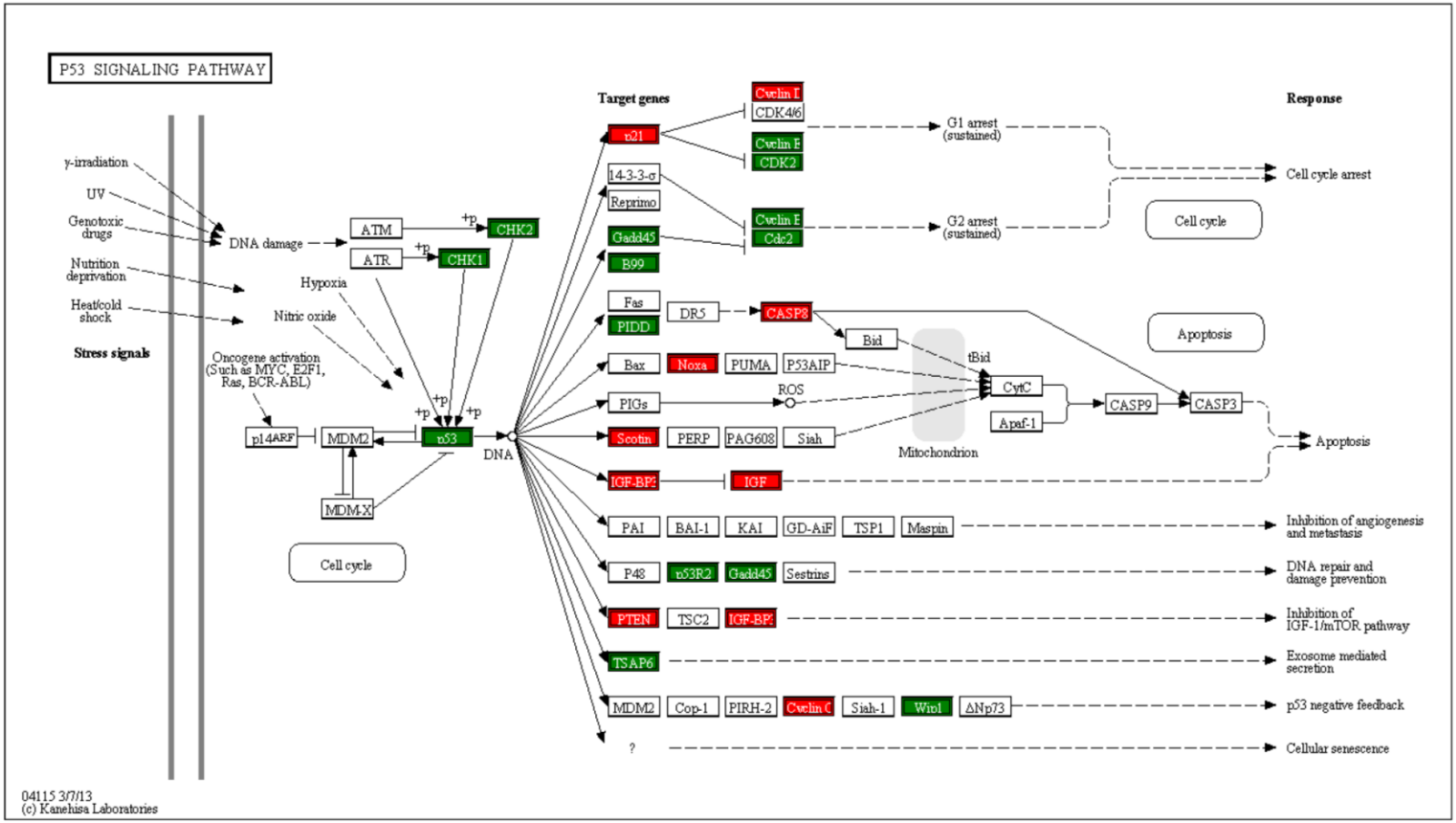

Figure 31. Schematic representation of genes related to p53 pathway. The genes related to p53 pathway and their interaction is presented in this picture. The colored (green and red) genes are differentially regulated between $p185^{BCR/ABL}$ versus p96+p185 groups. The green colored genes are down-regulated (fold changes < -1) in $p185^{BCR/ABL}$-containing spleens in comparison to the spleen which contained both $p96^{ABL/BCR}$ and $p185^{BCR/ABL}$. The red colored genes are up-regulated (fold changes > +2) in $p185^{BCR/ABL}$ in comparison to p96+p185 group.

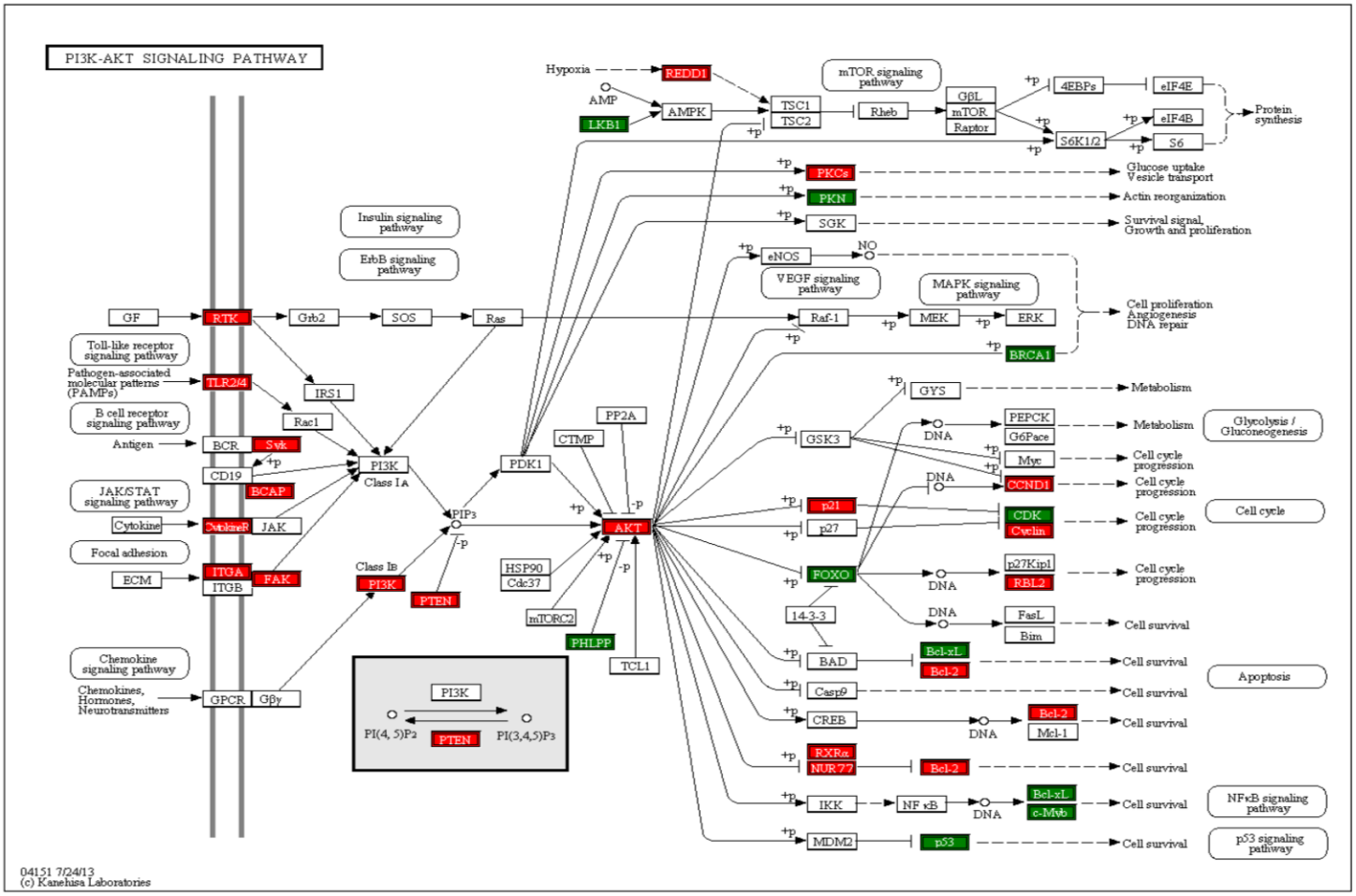

Figure 32. Schematic representation of genes related to PI3K/AKT pathway. The genes related to PI3K/AKT pathway and their interaction is presented in this picture. The colored (green and red) genes are differentially regulated between $p185^{BCR/ABL}$ versus p96+p185 group. The green colored genes are down-regulated (fold changes < -1) in $p185^{BCR/ABL}$- containing spleens in comparison to the spleens which contained both $p96^{ABL/BCR}$ and $p185^{BCR/ABL}$. The red colored genes are up-regulated (fold changes > +2) in $p185^{BCR/ABL}$ in comparison to p96+p185 group.

4 DISCUSSION

The discovery of BCR/ABL fusion tyrosine kinase as the causative agent of more than 95% of CML and 20-30% of ALL led to the development of ATP competitive tyrosine kinase inhibitor imatinib and other drugs in this class such as nilotinib, dasatinib, bosutinib and ponatinib (Druker, 2008; Faderl et al, 1998; Faderl et al, 1999; Hantschel et al, 2012; Lugo et al, 1990) . The majority of CML patients under TKI therapy achieve complete cytogenic remission (Hochhaus et al, 2008), emphasizing the important role of BCR/ABL kinase activity in the induction of the disease. The application of TKIs is however not always successful due to the occurrence of point mutations in BCR/ABL, which reduce the sensitivity to TKIs leading to relapse (Apperley, 2007; O'Hare et al, 2010). Not only mutation-caused relapse, but also the appearance of non mutational resistance represents clinical complications (Apperley, 2007). The fact that one third of the CML patients progressing into a CML-BC develop an ALL-like disease phenotype, which similar to ALL is accompanied by poor prognosis, raise the question whether BCR/ABL collaborates with other factors and if this interplay modulates the biology of the disease. Since the t(9;22) is a reciprocal translocation, one of the most obvious factors, the reciprocal ABL/BCR fusion proteins, did not obtain much attention, since no relation between the presence of the transcript and clinical features of the disease like prognosis or therapy response was observed (Melo et al, 1996). The lack of correlation between ABL/BCR expression and the response of patients to IFNα was, however, exclusively based on the observation in CML patients and therefore the role of ABL/BCR protein in the pathogenesis of Ph^+ ALL, where the transcript is present in 100% of the examined cases and is translated into a functional protein (Melo et al, 1993b; Zheng et al, 2009), is still unknown.

Due to the fact that ABL/BCR proteins are BCR mutants lacking important functional domains in BCR, investigations were performed to figure out the oncogenic potential of ABL/BCR fusion proteins. These investigations led to the finding that both ABL/BCR fusion proteins lost fundamental functional features of BCR regarding the regulation of small Rho-like GTPases with negative consequences on cell motility, in particular on the capacity to adhere to endothelial cells (Zheng et al, 2006). Deregulated cell motility leads to an abnormal mobilization of early leukemic precursors into the peripheral blood and leukemic infiltration of extra-medullar tissues. In addition to this an abnormal capacity to

pass through the blood-brain barrier with consequent meningeal leukemia, as frequently seen in Ph^+ ALL, is the consequence of deregulated cell motility (Pfeifer et al, 2003). The leukemogenic potential of both ABL/BCR fusion proteins was shown recently in a syngenic mouse model and was supposed to be due to their effect on stem cell capacity of mouse HSCs (Zheng et al, 2009). Both CML and Ph^+ ALL are stem cell diseases (Faderl et al, 1999; Pui et al, 2004). The failure of TKIs to eradicate the leukemic stem cell due to their quiescent attribute (Graham et al, 2002), rekindled the interest in understanding the signaling pathways that mediate the leukemic phenotype and the survival of the leukemic stem cell.

In order to figure out the role of $p96^{ABL/BCR}$ on the leukemogenic potential of BCR/ABL and to determine whether there is an interplay between these proteins in Ph^+ ALL two aspects were investigated in this study: (i) As $p96^{ABL/BCR}$ is expressed in all of Ph^+ ALL patients what is the role of this fusion protein in the pathogenesis of Ph^+ ALL and whether the ubiquitous expression of this protein is due to its effect on the transformation potential of $p185^{BCR/ABL}$; (ii) The fact that in contrast to the $p185^{BCR/ABL}$, $p96^{ABL/BCR}$ allows for both myeloid and lymphatic commitment of murine HSCs reveals that this fusion protein is active in more immature HSC compartment. Therefore it would be of great importance to determine whether the presence of $p96^{ABL/BCR}$ alters the target of transformation by $p185^{BCR/ABL}$ oncoprotein. In addition, this study attempted to determine if the Ph^+ ALL-associated t(9;22) fusion proteins are distributed hierarchically in the regulation of the leukemic stem cell compartment and if there is an interplay between these proteins at the stem cell level. This would be of high significance for the design of new therapy approaches dealing with eradication of leukemic stem cell.

Our data obtained in this study suggest that ABL/BCR contribute to the maintenance of the Ph^+ ALL. This is supported by the fact that the shRNA-mediated targeting of $p96^{ABL/BCR}$ strongly reduced the proliferation of not only an ALL cell line, but also that of PD-LTCs, which was accompanied by induction of apoptosis. The double strand shRNA used for silencing $p96^{ABL/BCR}$ expression binds to the 3' UTR of BCR at the transcription level and interferes with the transcription machinery and eventually translation of the target gene (Elbashir et al, 2001; Hutvagner & Zamore, 2002). Due to the binding site of this shRNA to the 3' UTR sequence of BCR, the expression level of endogenous BCR was also modified in this model. An exclusive targeting of ABL/BCR is technically not possible,

since the only exclusive sequence at the breakpoint is not accessible for the design of potentially efficient shRNA. However the comparison between the down-regulation level of endogenous BCR and that of $p96^{ABL/BCR}$ indicated a minimal reduction in expression of BCR protein in comparison to that of $p96^{ABL/BCR}$. In addition to this, down-regulation of endogenous BCR in a Ph^- ALL cells (HP) using shRNA had no effect on the proliferation capacity of these cells. In line with this observation down-regulating BCR in a TEL/ABL-positive cell had no prominent impact on the proliferation potential of the cells. The BCR/ABL and TEL/ABL fusion proteins were reported to activate similar signaling pathways (Baeumler et al, 2008; Okuda et al, 1996). These findings exclude a role of BCR in the proliferation capacity of the cells. Furthermore several studies reported that the expression of BCR had either no effect or negative effect on the activation and oncogenic potential of BCR/ABL. BCR/ABL was reported to phosphorylate BCR on tyrosine residues and by this inhibits the serin phosphorylation and subsequently reducing the activity of BCR. A phosphoserin form of BCR inhibits the oncogenic effects of BCR/ABL and that of ABL (Lin et al, 2001; Liu et al, 1996a; Liu et al, 1996b; Perazzona et al, 2008). In addition no effect could be observed on the pathogenesis of BCR/ABL-mediated disease in $BCR^{-/-}$ mice (Voncken et al, 1998). A recent study related to the role of BCR in the pathogenesis of BCR/ABL indicated that down-regulation of BCR in murine cells (Ba/F3 cell line) had no effect on the proliferation of BCR/ABL-positive cells (Mian et al, unpublished data). Collectively it can be concluded that the observed effect on the proliferation capacity of Ph^+ ALL cell line or PD-LTCs was exclusively due to the down-regulation of $p96^{ABL/BCR}$ and not that of endogenous BCR. The role of $p96^{ABL/BCR}$ in the proliferation capacity of the Ph^+ ALL cells suggests that the inhibition of this protein could serve as a potential target regarding the treatment of Ph^+ ALL.

The cell growth arrest and induction of apoptosis together with an increased response to BCR/ABL kinase inhibition upon the down-regulation of $p96^{ABL/BCR}$ strongly suggest a functional interplay between the t(9;22) reciprocal products in Ph^+ ALL. The existence of a correlation between the function of $p185^{BCR/ABL}$ and $p96^{ABL/BCR}$ was further confirmed by the findings that the $p96^{ABL/BCR}$ enhanced the transforming kinase activity of $p185^{BCR/ABL}$ leading to a higher proliferation rate of factor-dependent Ba/F3 murine cells. In addition to this, the co-expression of these proteins in Rat-1 fibroblast increased the colony formation in the classical transformation assays and led to an increased proliferation of $CD34^+CD38^-$ human cells by reduction of apoptosis rate. The latest are reported to present early stem

cells (Passegue et al, 2003). The observed effect of simultaneous expression of t(9;22) on the proliferation and apoptosis of $CD34^{+}CD38^{-}$ primary human cells is of great importance, since the overexpression of BCR/ABL in human cells attempting to develop human CML models was unsuccessful and did not result in hematologic disease (Chalandon et al, 2005), which was in contrast to murine bone marrow transduced with BCR/ABL, as in these cells BCR/ABL was sufficient to induce transplantable leukemias in almost all recipient mice. This indicates that in human cells BCR/ABL needs additional factors for its complete transformation potential. The collaboration of Bmi1 protein with BCR/ABL in leukemic transformation of human $CD34^{+}$ cells was recently reported and this protein was suggested to be involved in the progression of the CML disease (Rizo et al). According to these findings and the observations regarding the proliferation and apoptosis capacity of $CD34^{+}CD38^{-}$ primary human cells in this study, $p96^{ABL/BCR}$ could possibly represent an important collaborator of BCR/ABL oncoprotein in these cells.

The functional interplay between the t(9;22) fusion proteins seems to be based on an enhanced kinase activity of BCR/ABL in the presence of ABL/BCR leading to an increased activation of Erk kinase, which may contribute to an increased transformation potential of BCR/ABL, since Erk1/2 were reported to stimulate DNA synthesis and stimulates cell proliferation (Graves et al, 2000; Treinies et al, 1999). The MAP-kinase pathway regulates important cellular processes including gene expression, cell proliferation, cell survival and death and cell motility and activates the expression of its downstream target genes (Johnson & Lapadat, 2002); therefore it still has to be elucidated whether the activation of MAP-kinase pathway in the presence of ABL/BCR alters the phosphorylation of BCR/ABL by factors upstream of this protein.

The difference between CML-associated ABL/BCR and the ALL-associated fusion protein is the existence of a Rho-GEF domain in $p96^{ABL/BCR}$. This domain is important for the activation of Rho family of small GTPases. Rho GTPases are Ras-like molecules that integrate signal transduction pathways linking multiple cell-surface receptors to a variety of intracellular signaling proteins (Bar-Sagi & Hall, 2000; Vojtek & Der, 1998). The GTP-bound Rho GTPases interact with a variety of effectors to control actin cytoskeleton reorganization, cell shape, and cell polarity (Jaffe & Hall, 2005). The deletion of Rho-GEF domain in $p40^{ABL/BCR}$ had no effect on the transformation potential of the CML-associated $p210^{BCR/ABL}$, since this protein, similar to $p96^{ABL/BCR}$, was able to enhance the activation of

$p210^{BCR/ABL}$. Earlier reports demonstrated that Rac-GTPases are the key regulator of $p210^{BCR/ABL}$-dependent leukemogenesis (Thomas et al, 2008). The activation of Rac by ABL/BCR proteins was reported in earlier studies (Zheng et al, 2006). Whether the activation of Rac is the reason behind the enhanced phosphorylation of BCR/ABL, which probably leads to an enhanced proliferation of the cells, is still unclear.

Taken together, these data suggest a role for ABL/BCR in the maintenance and probably non-mutational resistance of Ph^+ ALL. The functional interplay between BCR/ABL and ABL/BCR may promote the disease in the absence of BCR/ABL inhibition and the functional independence of ABL/BCR as a self-standing leukemogenic factor may contribute to the maintenance of the disease upon efficient BCR/ABL inhibition and therefore contribute to the only transient response of Ph^+ ALL to TKI treatment. The mechanism behind this effect should be the focus of future work, due to its importance in gaining better understanding of the biology and pathogenesis of Ph^+ ALL. Gene expression analysis may present an appropriate technique to disclose the genes involved in this process. Such recognition might lead to targeting multiple signaling pathways in the leukemic cells and could prevent or overcome the acquired TKI resistance and lead to complete remission.

Leukemia is characterized by block of differentiation, accelerated cell cycle progression and increased self-renewal. The self-renewal capability is not only important for normal hematopoiesis but also crucial for the maintenance of leukemia (Reya et al, 2001). Compared with the widely investigated block of differentiation, there is limited data on the role of aberrantly regulated self-renewal of HSC and progenitor cells in leukemogenesis. According to the theory of tumor stem cells, it is the leukemic stem cells, which maintain the growth of a tumor and is thought to be the origin of relapse (Reya et al, 2001). Unlimited self-renewal capacity of stem cells is an absolute prerequisite for any malignancy, and is the ultimate decider of the continuous growth and metastasis of tumors (Huntly et al, 2004). Acute leukemia has been viewed as collaboration between two types of oncogenic proteins. In this picture, class I oncoproteins confer a proliferative and/or survival advantage, whereas class II oncoproteins impair differentiation (Gilliland, 2001). Both together cause the acute leukemic phenotype of enhanced proliferation and survival with impaired differentiation. Earlier studies revealed that in CML and Ph^+ ALL, translocation products $p210^{BCR/ABL}$ and $p185^{BCR/ABL}$ had no effect on the self-renewal of

HSCs/progenitors although they both induce proliferation of blast cells and provide a survival advantage due to the constitutive activation of the Abl kinase (Huntly et al, 2004). Results obtained in the present study confirmed the previous observations since $p185^{BCR/ABL}$ was unable to transform the $Sca1^{+}/Lin^{-}$ cells in the CFU or CFU-S12 stem cell assays. In contrast to BCR/ABL proteins, the reciprocal fusion products $p40^{ABL/BCR}$ and $p96^{ABL/BCR}$ were able to increase the replating efficiency and stem cell re-population potential of $Sca1^{+}/lin^{-}$ HSCs (Zheng et al, 2009). The functional interplay between BCR/ABL and ABL/BCR shown in the present study by the enhanced CFU number and the elevated replating capacity of the CFUs and also the increased CFU-S number in the presence of both t(9;22) fusion proteins confirmed these results. Taken these observations together one can assume that there is a functional hierarchy between $p185^{BCR/ABL}$ and $p96^{ABL/BCR}$ in HSC compartment. Due to the fact that the $p185^{BCR/ABL}$ protein did not confer replating capacity to the Sca^{+}/Lin^{-} HSCs, or the capacity to develop colonies in the spleen, the activation of this oncoproteins seems to be limited at the lineage restricted/ mature progenitor cells, which are rapidly exhausted in their progeny capacity in CFU and CFU-S12 assays. Earlier studies reported that the CFU-S derived at day 12 originates from primitive MPPs and at day 8 from relatively late myeloerythroid-committed progenitors (MEPs) (Na Nakorn et al, 2002). Whether $p185^{BCR/ABL}$ gave rise to colonies which appeared early in the spleen but disappeared until day 12 and therefore were not detectable, has still to be elucidated in order to refer the targeted population by BCR/ABL. In contrast to BCR/ABL, ABL/BCR seems to be active at earlier stage of differentiation, since its effect on stem cell capacity was comparable with the AML-associated fusion protein PML/RARα, whose activation was reported to enhance the self-renewal capacity of primitive HSCs via Wnt signaling (Muller-Tidow et al, 2004; Reya et al, 2003; Zheng et al, 2004). Gene expression analysis performed in this study revealed the enhanced expression of Wnt signaling-related transcription factors TCF/LEF1 in the presence of $p96^{ABL/BCR}$. The downstream signaling through TCF/LEF1 transcription factors induces proliferation and cellular transformation (Polakis, 2000) and might be the reason for the enhanced colony numbers, as an indication for the proliferation capacity of the cells, in each replating round.

Collectively these data indicate that each of Ph^{+} ALL-related t(9; 22) fusion proteins have their own target of transformation in the HSC compartment and this is probably organized in a hierarchical manner. The $p96^{ABL/BCR}$ confers its leukemogenic potential in immature HSC components that are able to enhance the cologenic potential of HSCs. In contrast,

$p185^{BCR/ABL}$ targets the more mature/committed progenitor cells and is unable to transform HSCs in LT-stem cell, ST-stem cell or progenitor-assays such as CRA (Zheng et al, 2009), CFU-S12 or CFU assays (present study). The observed phenotype alteration of the CML-like disease induced by $p185^{BCR/ABL}$ to an ALL-like disease in the presence of $p96^{ABL/BCR}$ further confirms the hypothesis of existence of an interplay between the t(9;22) fusion proteins at the stem cell level, since the capacity of $p185^{BCR/ABL}$ oncogene in transforming the cells able to give rise to myeloid-leukemia like disease in mice was abrogated in the presence of $p96^{ABL/BCR}$. Based on the observation that the ABL part in the chimeric protein is constant, while the BCR portion varies, it can be concluded that ABL is likely to carry the transforming principle, whereas different sizes of the BCR sequence may determine the phenotype of the disease (Deininger et al, 2000).

The distinct effect of BCR/ABL and ABL/BCR alone or in combination on different HSC compartments might influence the hematopoietic homeostasis by altering the regulation cell cycle, senescence and apoptosis. The results obtained from gene expression analysis performed in this study provided further evidence for this phenomenon, since only the presence of both t(9;22) fusion proteins led to increased expression of *Tp53* (p53) tumor suppressor gene. An activated p53 triggers a multifaceted transcriptional program, initiates cell cycle arrest, senescence, or apoptotic pathways, which depends on the cell type and damage signal (Vousden & Prives, 2009). As presented in Figure 33, the expression level and activity of p53 changes during differentiation of HSCs into lineage components with the aim of maintaining the homeostasis of hematopoiesis (Pant et al, 2012). During steady state, basal p53 activity regulates HSC quiescence and self-renewal. Lack of p53 activity promotes HSC proliferation, which eventually leads to accumulation of DNA damage and tumor development. Excessive p53 activation, on the other hand, adversely affects cellularity of key organs and promotes loss of hematopoietic progenitors (Pant et al, 2012). The expression of this protein seems to be the most in immature stem cell, with the aim of keeping these cells in quiescent in order to preserve the lifelong pool of HSCs and the reduction in p53 expression is accompanied by elevated proliferation capacity (Akala et al, 2008; Liu et al, 2009; TeKippe et al, 2003). The complex role of p53 is however indicated by the fact, that this protein has distinct effects on different HSC compartments regarding DNA damage (DD) response, which indicates distinct roles for this protein in normal or malignant hematopoiesis. The activation of p53 in the absence of induction of apoptosis in response to DD was shown to occur in the short term stem cell compartment. In LT-HSCs

no p53 activation and no apoptosis was observed in response to DD, which was altered to p53 activation and occurrence of apoptosis in CMPs and MPPs. This suggests for the existence of different DD responses in stem cell/progenitor populations with distinctive self renewal and differentiation properties (Insinga et al, 2012).

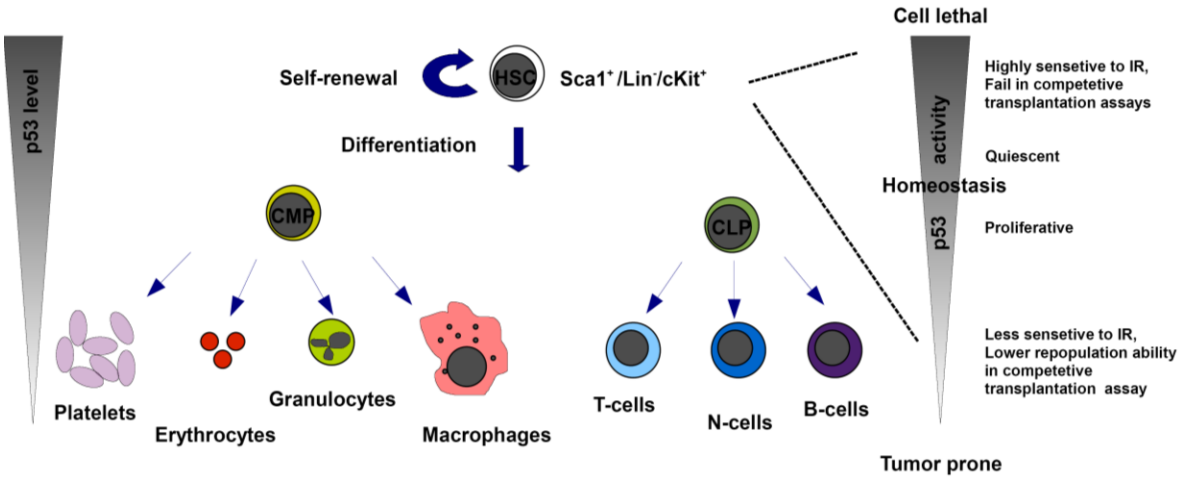

Figure 33. Role of p53 in HSCs. A simplifies model depicting changes in p53 level during differentiation of HSCs into the lineage components (left). The p53 activity levels influence HSC functionality (right) (Pant et al, 2012).

Collectively one can assume that according to the maturity level of the targeted HSC by t(9;22) fusion proteins, a distinct effect on p53 expression could be triggered, which influences the HSC functionality. Whether up-regulation of p53 expression is the response of particular stem cell compartment to the simultaneous expression of $p96^{ABL/BCR}$ and $p185^{BCR/ABL}$, as oncogenic events, with the aim to suppress the proliferation of the cells and induce senescence as a tumor suppression mechanism, or, the expression of this gene is controlled by the oncogenes independently of the differentiation level of the cell, is still unclear. The third explanation for the elevated expression of p53 in the presence of both $p185^{BCR/ABL}$ and $p96^{ABL/BCR}$ might be independent of the presence of these oncogenes but rather the differentiation level of the cell targeted by the t(9;22) fusion proteins. The distinct effect of $p185^{BCR/ABL}$ and $p96^{ABL/BCR}$ in CFU-S12 assay regarding the cologenic potential of these fusion proteins reveals the distinct effect of these oncogenes on stem cell compartment and as discussed before is an indication for the distribution of these oncogenes in a hierarchical manner. This represents the urgent for the recognition of the target cell for transformation by t(9;22) oncoproteins alone or in combination. The effect of the t(9;22) oncoproteins on the expression of p53 gene regarding the level of

differentiation and proliferation capacity of the transformed cell remains to be elucidated in the future works. This could provide insights into the leukemogenic potential of t(9;22) fusion proteins at different stem cell levels and might disclose whether the maintenance of leukemia, even upon efficient BCR/ABL inhibition, is due to the presence of ABL/BCR due to the change in the transformation target, which is independent of BCR/ABL oncogenic activity.

The up-regulation of GADD45α, as a downstream target of p53 in the presence of $p185^{BCR/ABL}$ and $p96^{ABL/BCR}$ fusion proteins supports the prominent role of p53. The GADD45 proteins are implicated in cell cycle arrest (Wang et al, 1999), DNA repair (Smith et al, 1994), cell survival and apoptosis (Gupta et al, 2005; Harkin et al, 1999). The dual role of GADD45 proteins as cell survival mediator but also proapoptotic agents might be dependent on the stress stimulus encountered, cell type, and interaction with other proteins that modulate gadd45 function and ultimately determine whether the outcome will be DNA repair and cell survival, or apoptotic cell death (Liebermann & Hoffman, 2007). The expression of p21, another target of p53, which was reported to be implicated in the in the regulation of hematopoietic stem cell quiescence, was down-regulated in the presence of both t(9;22) oncoproteins. Given the fact that p21 is the attenuator of BCR/ABL mediated cell proliferation (Forster et al, 2008), the down-regulation of this protein in the presence of $p96^{ABL/BCR}$ together with the activation of GADD45α may contribute to the enhanced transformation potential of $p185^{BCR/ABL}$ in the presence of $p96^{ABL/BCR}$. Hence, one can assume that the presence of $p96^{ABL/BCR}$ in immature HSCs leads to release the cells from quiescent and enhance their proliferation capacity.

Taken together, this study gives the first indications for the existence of a collaboration between the Ph^+ ALL-related t(9;22) fusion proteins ($p96^{ABL/BCR}$ and $p185^{BCR/ABL}$) in the determination of Ph^+ ALL pathogenesis and the regulation of HSC compartment. The $p96^{ABL/BCR}$ fusion protein target immature HSC compartment for its oncogenic transformation, where is acts as an attenuator of cell cycle arrest and quiescence. In these cells $p185^{BCR/ABL}$ seems to confer survival advantage to the cells, which was shown in the enhanced cologenic potential of murine primary cells in the presence of $p96^{ABL/BCR}$ and $p185^{BCR/ABL}$ in comparison to $p96^{ABL/BCR}$ or $p185^{BCR/ABL}$ alone. The role of this protein in more committed HSCs/progenitors seems to be limited and taken over by $p185^{BCR/ABL}$ protein. The growing evidence that the chronic phase CML stem cells may be less sensitive

to BCR/ABL inhibitor than the bulk of the CML cells (Graham et al, 2002), emphasis the important role of the interplay between BCR/ABL and ABL/BCR at the stem level and suggests the necessity for further work in characterizing the mechanism behind this interplay. Factors such as $p96^{ABL/BCR}$ itself or p53, whose expression was strongly up-regulated in the presence of both t(9;22) fusion proteins could serve as appropriate targets in the eradication of leukemic stem cell. Such strategies may lead to the complete eradication of the LSCs and prevent the course of remission.

5 SUMMARY

The hallmark of Philadelphia-positive (Ph^+) leukemia, chronic myeloid leukemia (CML) and Ph^+ acute lymphatic leukemia (Ph^+ ALL) is the BCR/ABL kinase activity, which is successfully targeted by selective ATP-competitors. However, inhibition of BCR/ABL is unable to eradicate Ph^+ leukemic stem cell (LSC), which if untreated leads to relapse. Thus at the stem cell level the Ph^+ leukemia may be independent of the aberrant kinase activity of BCR/ABL. The Philadelphia chromosome is the cytogenetic correlate of the t(9;22), a reciprocal translocation. In fact the transcript of the reciprocal translocation product, ABL/BCR on der 9, which can be detected in 65% of CML and 100% of Ph^+ ALL cases, is translated into a functional protein. The biological role of ABL/BCR fusion proteins was reported recently and these proteins were shown to have oncogenic potential and the capacity to influence the lineage commitment of hematopoietic stem cells (HSCs). Aim of this study was to further disclose the role of the Ph^+ ALL associated $p96^{ABL/BCR}$ in the pathogenesis of this malignancy and to investigate whether there is an interplay between t(9;22) associated fusion proteins, $p96^{ABL/BCR}$ and $p185^{BCR/ABL}$, and if this interplay influences the pathogenesis of Ph^+ ALL. Down-regulation of $p96^{ABL/BCR}$ using specific shRNA reduced the growth and proliferation potential of Ph^+ ALL cell line and patient-derived long-term cultures (PD-LTCs), which was accompanied by induction of apoptosis. This indicates the important role of $p96^{ABL/BCR}$ in survival mechanisms of the Ph^+ ALL cells. These results raise the question whether the down-regulation of $p96^{ABL/BCR}$ sensitizes the $p185^{BCR/ABL}$-positive cells to ABL inhibitors, such as Imatinib. In order to investigate this, the expression ABL/BCR fusion gene was targeted using shRNA in Ph^+ ALL PD-LTCs and the cells were treated with ABL kinase and also allosteric inhibitor. The simultaneous targeting of $p96^{ABL/BCR}$ and the application of ABL inhibitors sensitized the cells to the ABL inhibitors, providing evidence that $p96^{ABL/BCR}$ could be an appropriate target in Ph^+ ALL, especially in resistance caused due to non-mutational reasons. Since the shRNA used in these experiments also down-regulated the expression of endogenous BCR and in order to demonstrate that the down-regulation of $p96^{ABL/BCR}$ and not that of BCR is interfering with the proliferation properties of the Ph^+ ALL cells, a TEL/ABL-positive PD-LTC was selected and the expression of BCR was targeted using shRNA. The down-regulation of BCR had no significant effect on the proliferation potential of these cells indicating that the effect of shRNA on the proliferation of Ph^+ ALL cells was solely du the

down-regulation of $p96^{ABL/BCR}$. Expression of both t(9;22) fusion proteins in Ba/F3 pro-lymphocytic murine cells, Rat-1 fibroblasts and $CD34^{+}CD38^{-}$ primary human cells enhanced the transformation capacity of $p185^{BCR/ABL}$ in untransformed fibroblasts and increased the proliferation of Ba/F3 and $CD34^{+}CD38^{-}$ cells, which was, as shown in Ba/F3 cells, accompanied by an enhanced phosphorylation of $p185^{BCR/ABL}$ on Y177 and Y245. The phosphorylated Y177 connects BCR/ABL with the MAP-kinase pathway; hence the phosphorylation of the MAP- kinase, Erk1/2 was investigated in the Ba/F3 cells. The co-expression of $p96^{ABL/BCR}$ and $p185^{BCR/ABL}$ in Ba/F3 cells led to an increased activation of MAP-kinase pathway in comparison to the cells transduced with $p96^{ABL/BCR}$ or $p185^{BCR/ABL}$ alone. Since the co-expression of CML-related ABL/BCR ($p40^{ABL/BCR}$) with $p210^{BCR/ABL}$ also showed similar results on the phosphorylation of $p210^{BCR/ABL}$, it still remained unclear if ABL/BCR is one of the co-factors, which determines the progression of CP-CML to the blast crisis.

Previous studies demonstrated the effect of ABL/BCR on the lineage commitment of BCR/ABL-positive murine stem cells. In order to determine whether the effect on the lineage commitment of the stem cells impact the cells that are targeted and transformed by BCR/ABL, *in vitro* and *in vivo* stem cell surveys were performed in murine hematopoietic stem cells. The *in vitro* and *in vivo* stem cell studies, such as colony forming unit assay (CFU), colony forming unit spleen- day 12 (CFU-S12) and transduction-transplantation experiments revealed a hierarchical organization of the t(9;22) fusion proteins in mouse hematopoietic stem cell. The $p185^{BCR/ABL}$ oncoprotein was not able to transform the Sca^{+}/Lin^{-} stem cells, which are assumed to be "early" stem- and progenitor cells. In contrast, $p96^{ABL/BCR}$ was able to transform these cells conferring the cells the capacity to proliferate and differentiate in semi-solid medium for more than five weeks. Also the production of visible colonies in the spleens was an indication for the ability of $p96^{ABL/BCR}$ to transform cells able to give rise to multipotent progenitor cells. In the presence of both t(9;22) translocation products the target cells of both fusion proteins seemed to be altered, since the appearance and number of colonies in both CFU and CFU-S12 assays were distinctly changed, which could be observed by enhance colony formation in spleen in CFU-S12 assay and distinct colony morphology in CFU assay in the presence of both t(9;22) translocation products. This is of highly clinical interest, since targeting the malignant stem cell in CML is one of the greatest challenges to date.

In agreement with the fact, that t(9;22) fusion proteins exhibit a hierarchical organization in mouse stem cells, the present study shows that the introduction of both $p185^{BCR/ABL}$ and $p96^{ABL/BCR}$ to murine HSCs ($Sca1^+$ cells) induced an ALL-like disease in mice which was characterized by the presence of a high number of lymphatic blasts in the bone marrow and spleen. The $p185^{BCR/ABL}$ fusion protein induced a myeloid leukemia-like disease in mice. Taken together the stem cell studies indicate that $p96^{ABL/BCR}$ and $p185^{BCR/ABL}$ have distinct transformation targets in the mouse hematopoietic stem cell and that there is interplay between these proteins at stem cell level, which can be seen as a collaboration changing the outcome of stem cell proliferation and probably differentiation capacity.

In order to further disclose the mechanism by which $p96^{ABL/BCR}$ enhances the transformation potential of $p185^{BCR/ABL}$ and to understand the interplay between these proteins at the stem cell level, gene expression analysis was performed using microarray studies. The obtained results indicated that the expression profile of $p185^{BCR/ABL}$ is completely altered when $p96^{ABL/BCR}$ is expressed simultaneously in the mouse HSCs. The expression level of genes related to cell cycle, apoptosis and proliferation of stem cell like p53 and its target gene GADD45α was enhanced in the presence of both transgenes in comparison to $p96^{ABL/BCR}$ or $p185^{BCR/ABL}$ alone. Since recent studies showed that genes involved in cell cycle and apoptosis are involved in the determination of stem cell capacity and self-renewal potential, it can be concluded that the enhanced expression of genes like GADD45α in the presence of $p96^{ABL/BCR}$ probably leads to release from quiescent and enhanced proliferation of the stem cells. The reduction of p21 expression in the presence of both transgenes confirmed this hypothesis, since this protein is known to maintain the stem cells in quiescence.

In summary, our observations in this study reveal an important role for $p96^{ABL/BCR}$ in the determination of the leukemogenesis potential of $p185^{BCR/ABL}$ and the therapy resistance of Ph^+ ALL.

6 ZUSAMMENFASSUNG

Bei ca. 95% der chronisch myeloischen Leukämie (CML) und 20-30% der akuten lymphatischen Leukämie (ALL) des Erwachsenen liegt eine reziproke Chromosomentranslokation t(9;22)(q34;q11) vor, in deren Rahmen das BCR (Breakpoint Cluster Region) Gen auf Chromosom 22 mit dem ABL (Abelson-Leukämie-Virus) Gen auf Chromosom 9 fusioniert. Auf Chromosom 22 gibt es zwei verschiedene Bruchpunkte, die somit zur Bildung von unterschiedlichen Fusionsgenen führen. Bei der CML findet man den sogenannten „großen" Bruchpunkt (M-bcr), während bei der Ph+ ALL der sogenannte „kleine" Bruchpunkt (m-bcr) vorkommt. Das hybride Fusionsgen auf Chromosom 22q+ (Philadelphia-Chromosom) kodiert für das jeweilige BCR/ABL Protein, während das Fusionsgen auf Chromosom 9q+ für das reziproke ABL/BCR Protein kodiert. Das ABL-Protein ist eine Nicht-Rezeptor Tyrosinkinase, die eine wichtige Rolle in der Signaltransduktion und der Regulation des Zellwachstums spielt. Im BCR/ABL Fusionsprotein wird die Kinase-Aktivität von ABL, die im Normalfall streng reguliert ist, durch die Fusion mit BCR konstitutiv aktiv. Dadurch kommt es zur Deregulierung intrazellulärer Signalwege, welche die maligne Transformation hämatopoetischer Zellen verursacht. Eine zielgerichtete Inhibierung von BCR/ABL mittels ABL-Kinase-Inhibitoren induziert Apoptose in BCR/ABL transformierten Zellen, was eine komplette Remission im größten Teil Ph^+ Leukämie Patienten zur Folge hat. Allerdings ist die Inhibierung des BCR/ABL Proteins nicht in der Lage die Ph^+ Stammzelle zu eradizieren, was darauf hindeutet, dass Ph^+ leukämische Stammzellen unabhängig von der aberranten BCR/ABL Kinase sind. Da die t(9;22) eine reziprokale Translokation ist, wird bei der Erzeugung von BCR/ABL ein anderes Fusionsgen an dem Chromosom 9 generiert, welches ABL/BCR genannt wird und sowohl in ALL als auch in CML Fällen nachgewiesen werden kann. Es wurde bereits gezeigt, dass das ALL-assoziierte ABL/BCR Protein ($p96^{ABL/BCR}$) bei 100% der Ph^+ ALL Patienten vorhanden ist. Dies ist hingegen nicht der Fall für das CML-assoziierte Protein ($p40^{ABL/BCR}$), welches nur bei 60% der Patienten festgestellt werden kann. Im Rahmen einer vorherigen Studie wurden diese Angaben bestätigt und es wurde gezeigt, dass in Ph^+ ALL Zelllinien und Patientenzellen das ABL/BCR Gen nicht nur an der Transkriptionsebene, sondern auch als Protein nachgewiesen werden kann. Weiterhin konnte gezeigt werden, dass die Expression beider ABL/BCR Fusionsproteine zur Induktion einer Leukämie-ähnlichen Krankheit in der Maus führt. Da das $p96^{ABL/BCR}$ in

100% aller Ph^+ ALL Fälle detektiert wurde, hat sich diese Arbeit ausschließlich mit dem Ph^+ ALL-assoziierten Protein $p96^{ABL/BCR}$ befasst. Um die Bedeutung des $p96^{ABL/BCR}$ Proteins in der Leukämogenese der Ph^+ ALL aufzuklären, wurde dieses Protein in einer Ph^+ ALL Zelllinie mittels shRNA herunterreguliert und die Wachstumskapazität der Zellen untersucht. Hiermit konnte gezeigt werden, dass (i) die Herunterregulierung des $p96^{ABL/BCR}$ Proteins in der Ph^+ ALL zur Inhibierung der Wachstumskapazität der Zellen führt; (ii) die reduzierte Wachstumskapazität der Zellen von einer Apoptoseinduktion begleitet ist; (iii) die gleichzeitige Behandlung der Zellen mit shRNA gegen $p96^{ABL/BCR}$ und ABL Kinase-Inhibitoren zur Sensibilisierung der Zellen gegen die ABL Kinase-Inhibitoren führt; (iv) der festgestellte Effekt an der Wachstumskapazität der Ph^+ ALL Zellen lediglich auf die Herunterregulierung des ABL/BCR Proteins und nicht auf das endogene BCR Protein zurückzuführen war. Die oben erwähnten Ergebnisse deuten drauf hin, dass das $p96^{ABL/BCR}$ Protein einen wirkungsvollen therapeutischen Ansatz zur Behandlung der Ph^+ ALL präsentieren könnte. Dies ist von großer Bedeutung, da die Inhibierung von $p185^{BCR/ABL}$ zur mutationsbedingten oder aber auch zur nicht mutationsbedingten Resistenz der Zellen gegenüber der ABL Kinase Inhibitoren führen kann.

Um den möglichen Einfluss von $p96^{ABL/BCR}$ auf das Transformationspotenzial des $p185^{BCR/ABL}$ Onkogens zu überprüfen, wurden zwei weitere Zelllinien verwendet: Ba/F3 Zellen (Mauspromyelozyten) und Rat-1 Fibroblastenzellen. Die erhaltenen Ergebnisse für Ba/F3 und Rat-1 Zellen zeigen, dass (i) $p96^{ABL/BCR}$ in der Lage ist, das Transformationspotenzial des $p185^{BCR/ABL}$ Proteins und die Wachstumsrate dieser Zellen zu erhöhen; (ii) die Expression des $p96^{ABL/BCR}$ Proteins zur Steigerung der Phosphorylierung der $p185^{BCR/ABL}$ Kinase an Y245 und Y177 führt; (iii) die gesteigerte Phosphorylierung von $p185^{BCR/ABL}$ zu einem Anstieg der MAP-Kinase Signalkaskade führt, deren Aktivierung mehrere zelluläre Prozesse wie Proliferation, Apoptose und die Expression unterschiedlicher Gene reguliert; (iv) die Präsenz des $p96^{ABL/BCR}$ Proteins keinen Effekt auf die Aktivierung von Crkl oder STAT5 durch $p185^{BCR/ABL}$ hatte; (v) die gesteigerte Phosphorylierung von BCR/ABL nicht spezifisch für $p185^{BCR/ABL}$ war, sondern auch CML-assoziiertes $p210^{BCR/ABL}$ eine erhöhte Phosphorylierung an Y245 zeigte. Die Expression des $p96^{ABL/BCR}$ Proteins in $CD34^+CD38^-$ humanen Stammzellen hat gezeigt, dass das Zusammenspiel zwischen diesem Protein und $p185^{BCR/ABL}$ zur einer gesteigerten

Proliferation und Inhibierung der Apoptose führt, da keins der Proteine alleine in der Lage war die Wachstumskapazität dieser Zellen zu erhöhen.

In einer bereits vorliegenden Studie wurde gezeigt, dass die ABL/BCR Fusionsproteine, insbesondere $p96^{ABL/BCR}$, im Gegensatz zu BCR/ABL ein großes Stammzellpotential aufweisen. $P96^{ABL/BCR}$ war in der Lage die Aktivierung des Wnt Signalwegs und die Expression von HoxB4 zu erhöhen. Dies führte zu einer erhöhten Replating-Kapazität der Zellen und war in Übereinstimmung mit den erhaltenen Daten dieser Studie, da im Rahmen dieser Studie gezeigt werden konnte, dass die Replating-Kapazität der $p96^{ABL/BCR}$-positiven Zellen über 5 Wochen andauerte und einen sehr ähnlichen Effekt wie das Fusionsprotein PML/RARα auf die Proliferation und das Selbsterneuerungspotential der Stammzellen aufwies. In der *in vitro* Stammzell-Untersuchung in der Methylzellulose konnte gezeigt werden, dass (i) die Stammzellen positiv für das $p185^{BCR/ABL}$ Transgen keine erhöhte Stammzell- oder Proliferationskapazität aufweisen, wohingegen die gleichzeitige Expression des $p96^{ABL/BCR}$ Proteins und des BCR/ABL Proteins in der Lage war die Stammzellkapazität der Zellen zu erhöhen; (ii) das Zusammenspiel der $p96^{ABL/BCR}$ und $p185^{BCR/ABL}$ Proteine zur einer Veränderung der bei $p185^{BCR/ABL}$ entstehenden Kolonie-Morphologie führte. Auch die CFU-S12 *in vivo* Untersuchung in der letal bestrahlten Maus deutet auf ein Zusammenspiel zwischen $p96^{ABL/BCR}$ und $p185^{BCR/ABL}$ in der Stammzellebene hin. Unseren Ergebnissen zufolge ist das $p185^{BCR/ABL}$ nicht zur Kolonienbildung in der Milz fähig, während $p96^{ABL/BCR}$ in der Lage ist, Kolonien zu bilden. Die Anwesenheit beider Proteine führt zu einer gesteigerte Kolonienbildung in der Milz. Die erlangten Ergebnisse der *in vitro* und *in vivo* Kolonien-Experimente lassen von der Hypothese ausgehen, dass $p96^{ABL/BCR}$ auf Stammzellebene für die Erhaltung der Leukämie verantwortlich ist, während $p185^{BCR/ABL}$ erst in reiferen Vorläuferzellen und in den Blasten seine onkogene Funktion ausübt. Da das Transduktions-Transplantationsmausmodell bereits zeigen konnte, dass beide ABL/BCR Fusionsproteine (p40 und p96) ein eigenes leukämogenisches Potential besitzen und Leukämie im Mausmodel induzieren, stellte sich die Frage, ob das Zusammenspiel zwischen $p96^{ABL/BCR}$ und $p185^{BCR/ABL}$ den Phänotyp und den Fortschritt der Leukämie beeinflussen kann. In dieser Arbeit konnte gezeigt werden, dass (i) BCR/ABL eine CML-ähnliche Krankheit in der Maus auslösen konnte, die einen sehr aggressiven Verlauf besitzt; (ii) die gleichzeitige Expression beider Fusionsproteine allerdings zu einer Veränderung des Krankheitsphänotyps führte und Fälle von ALL gezeigt werden konnten;

(iii) der Verlauf und die Latenzzeit dieser ALL-ähnlichen Leukämie im Vergleich zu der von $p185^{BCR/ABL}$ induzierten Krankheit verlangsamt wurde, obwohl das Knochenmark und die Milz zum größten Teil leukämische Blastenzellen der lymphatischen Ausrichtung aufwiesen.

Anhand der erhaltenen Ergebnissen dieser Arbeit konnte eindeutig gezeigt werden, dass die Präsenz des $p96^{ABL/BCR}$ Proteins in Ph^+ ALL oder in den Stammzellen eine wichtige Rolle in der Leukämogenese des $p185^{BCR/ABL}$ Proteins spielt. Dieses Protein ist in der Lage das Transformationspotential des $p185^{BCR/ABL}$ Proteins zu steigern und diesem Protein die Fähigkeit zu verleihen die Proliferation und möglicherweise das Differenzierungspotential der Stammzellen zu beeinflussen. Diese Erkenntnisse sind von sehr großer Bedeutung für neuartige molekulare Therapieansätze, da die Inhibierung des BCR/ABL Onkogens in den Stammzellen nicht erfolgreich ist und die Weiterentwicklung der Krankheit zur Folge hat.

Die Durchführung der Genexpressionanalyse mittels Microarray hat gezeigt, dass sich die Genexpression in Gegenwart von beiden t(9;22) Translokationsprodukten, $p96^{ABL/BCR}$ und $p185^{BCR/ABL}$, im Vergleich zur Anwesenheit der einzelnen Fusionsproteine unterscheidet. Diese Analyse hat ergeben, dass die Expression des p53 Gens und dessen Targets GADD45α durch den Einfluss von ABL/BCR in BCR/ABL positiven Proben signifikant steigt. Da die Hochregulierung der dem Zellzyklus zugehörigen Gene einen Einfluss auf das Selbsterneuerungspotenzial, die Vermehrung und die Ausdifferenzierung der Stammzellen hat, deuten unsere Ergebnisse drauf hin, dass die Hochregulierung der p53 und GADD45α Gene vermutlich zur Aufhebung der Stilllegung (quiescent) und einer erhöhten Proliferation der unreifen Stammzellen führt. Diese Veränderung des Stammzellenbildes ist vermutlich der Auslöser für eine gesteigerte Kolonienbildung in Methylzellulose und Milz sowie für eine Veränderung des von $p185^{BCR/ABL}$ vermittelten Leukämie-Phänotyps. Da die Aktivierung von p21 eine Stilllegung (quiescent) der Stammzellen induziert, deutet die herunterregulierte Expression von p21 (Cdkn1) in der Anwesenheit beider Translokationsprodukte auf einen aktiven Zellzyklus der Stammzellen hin.

Zusammenfassend bietet diese Studie erste Beweise, dass Ph^+ ALL eine Krankheit mit zwei potentiellen Auslösern ist, $p96^{ABL/BCR}$ und $p185^{BCR/ABL}$. Die Erscheinung des $p96^{ABL/BCR}$ Onkogens ist für die Entstehung der Krankheit unerlässlich. Die Anwesenheit der Reziproke des $p185^{BCR/ABL}$ führt allerdings zu einer erhöhten Aktivierung dieses

Proteins und einer Hochregulierung des MAP-Kinasewegs, die vermutlich zu dem gesteigerten Vermehrungspotential der Zellen führt. Die erhöhte Expression anderer Gene, wie p53 und GADD45α und die Herunterregulierung des p21 Gens in der Präsenz von $p185^{BCR/ABL}$ führt zur Aktivierung des Zellzyklus. Durch die Veränderung des Vermehrung und Differenzierungspotentials der $p185^{BCR/ABL}$ positiven Stammzellen wird die Ausrichtung der Leukämie beeinflusst. Desweiteren zeigen die vorliegenden Daten, dass die An- und Abwesenheit des ABL/BCR Fusionsproteins eine mögliche Erklärung für schlechte klinische Prognosen der Patienten mit BCR/ABL-positiven Blasten darstellt, da die Präsenz dieser Proteine die Aktivität des BCR/ABL Proteins erhöht. Die Inhibierung dieses Protein in einer Kombination mit BCR/ABL Kinase Inhibitoren könnte daher eine Verbesserung des Therapieansatzes ermöglichen.

7 EHRENWÖRTLICHE ERKLÄRUNG

EHRENWÖRTLICHE ERKLÄRUNG

Ich erkläre hiermit ehrenwörtlich, dass ich die dem Fachbereich Biochemie, Chemie und Pharmazie zur Promotionsprüfung eingereichte Arbeit mit dem Titel

''The role of ABL/BCR in the leukemogenic potential of BCR/ABL in Philadelphia chromosome positive leukemia''

im Zentrum der Inneren Medizin, Medizinische Klinik III, Abteilung Hämatologie des Universitätsklinikums Frankfurt bei Prof. Dr. Hubert Serve und unter Leitung von Prof. Dr. Rolf Marschalek mit Unterstützung von PD. Dr. Martin Ruthardt ohne sonstige Hilfe selbst durchgeführt und bei der Abfassung der Arbeit keine anderen als die in der Dissertation angeführten Hilfsmittel benutzt habe.

Ich habe bisher an keiner in- oder ausländischen Biologischen Fakultät ein Gesuch um Zulassung zur Promotion eingereicht noch die vorliegende Arbeit als Dissertation vorgelegt.

Frankfurt, den 10.07.2014

Anahita Rafiei (M.Sc.)

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