# Molecular and functional studies of the BCR/ABL1 fusion gene

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# MOLECULAR AND FUNCTIONAL STUDIES OF THE *BCR/ABL1* FUSION GENE

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This thesis is dedicated to
all children and adults suffering from leukemia

to Matti and Tim
for endless love and support

# ORIGINAL ARTICLES

This thesis is based on the following publications and manuscripts, which will be referred to in the text by their Roman numerals:

**I.** Håkansson P, Lassen C, Olofsson T, Baldetorp B, Karlsson A, Gullberg U, and Fioretos T. Establishment and phenotypic characterization of human U937 cells with inducible P210 *BCR/ABL* expression reveals upregulation of CEACAM1 (CD66a). *Leukemia* 2004;18:538-547.

**II.** Håkansson P*, Segal D*, Lassen C, Gullberg U, Morse HC III, Fioretos T, and Meltzer PS. Identification of genes differentially regulated by the P210 *BCR/ABL1* fusion oncogene using cDNA microarrays. *Exp Hematol* 2004;32:476-482.

**III.** Håkansson P, Nilsson B, Andersson A, Lassen C, Gullberg U, and Fioretos T. Gene expression analysis of BCR/ABL1-dependent transcriptional response reveals enrichment for genes involved in negative feedback regulation. *Submitted*.

**IV.** Järås M*, Håkansson P*, Lassen C, Rissler M, Edén P, Bjerrum OW, Ågerstam H, Richter J, Fan X, and Fioretos T. Expression of P190 or P210 *BCR/ABL1* in cord blood $CD34^+$ cells leads to enhanced cell proliferation and differentiation towards the erythroid lineage. *Manuscript*.

* The first two authors contributed equally to these studies.

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# PREFACE

The Philadelphia chromosome (Ph) was discovered in 1960 by Nowell and Hungerford as the first consistent structural chromosome abnormality associated with leukemia. A decade later, the Ph chromosome was shown to originate from a reciprocal translocation between chromosomes 9 and 22. At the DNA level, the abnormality was shown in the early 1980s to involve the *BCR* and *ABL1* genes and to generate a *BCR/ABL1* fusion gene encoding a protein with increased tyrosine kinase activity. The *BCR/ABL1* fusion gene has since been studied extensively, and shown to induce expansion of the leukemic cell population by mediating growth-promoting and death-inhibiting signals, but the mechanisms by which *BCR/ABL1* elicits its transforming properties are unknown. The detailed molecular and functional characterization of the *BCR/ABL1* fusion gene recently allowed the development of inhibitors that target the tyrosine kinase activity of the fusion protein. However, mutations leading to drug resistance and persisting leukemic cells remain as challenging problems in the treatment of CML.

The general aim of this thesis has been to increase our understanding of *BCR/ABL1*-induced leukemogenesis by molecular and functional studies of *BCR/ABL1*-mediated signaling. The thesis is divided into three sections. The first is an introduction to Ph-positive leukemias and *BCR/ABL1*-mediated signaling, with the intention of putting the original articles into proper context. The second section outlines the specific aims, and summarizes the investigations on which this thesis is based. The third and final section contains the original articles on which the thesis is based.

Lund, August 2006

# ABBREVIATIONS

| | |
|---|---|
| ALL | acute lymphoblastic leukemia |
| AML | acute myeloid leukemia |
| AP | accelerated phase |
| BC | blast crisis |
| bcr | breakpoint cluster region |
| BM | bone marrow |
| CB | cord blood |
| CCR | complete cytogenetic remission |
| CLL | chronic lymphocytic leukemia |
| CLP | common lymphoid progenitor |
| CML | chronic myeloid leukemia |
| CMP | common myeloid progenitor |
| CMV | cytomegalovirus |
| CP | chronic phase |
| FDR | false discovery rate |
| GFP | green fluorescent protein |
| GMP | granulocyte/monocyte progenitor |
| HSC | hematopoietic stem cell |
| IRES | internal ribosomal entry site |
| LSC | leukemic stem cell |
| LTR | long terminal repeat |
| MEP | megakaryocyte/erythrocyte progenitor |
| MSCV | murine stem cell virus |
| NBT | nitro blue tetrazolium |
| PB | peripheral blood |
| Ph | Philadelphia chromosome |
| SAM | significance analysis of microarrays |
| SOCS | suppressor of cytokine signaling |
| WBC | white blood cell count |

# INTRODUCTION

## The Genetic Basis of Leukemia

Leukemia constitutes a heterogeneous group of malignant neoplasms of the blood-forming tissue. The word leukemia is Greek for "white blood", and refers to the unrestricted production of abnormal leukocytes. The imbalance of healthy and non-functional leukemic cells causes the symptoms of leukemia. The worldwide incidence of leukemia is approximately 5 cases per 100,000 persons and year, representing 3% of all cancers (Parkin et al. 1999). There are several types of leukemias that are traditionally classified according to how quickly they progress and which cells they affect. Acute leukemia is characterized by an accumulation of immature hematopoietic cells (blasts) in the bone marrow (BM) and peripheral blood (PB), whereas chronic leukemia typically displays a slow buildup of relatively mature blood cells. The acute and chronic leukemias may be further classified into lymphoid or myeloid leukemias according to the origin of the leukemic cells, where the main types are referred to as acute lymphoid leukemia (ALL), acute myeloid leukemia (AML), chronic lymphoid leukemia (CLL), and chronic myeloid leukemia (CML). In Sweden, approximately 250 and 450 new cases of AML and CLL, respectively, and 100 cases each of ALL and CML, are diagnosed every year (Socialstyrelsen 2006).

Leukemia is a clonal neoplastic disorder that originates in a single hematopoietic progenitor cell through acquired somatic genetic changes. At the chromosomal level, these genetic changes are visible either as balanced abnormalities (reciprocal translocations, inversions, and insertions) or unbalanced changes, including nonreciprocal translocations, deletions, numerical aberrations, and amplifications (Mitelman et al. 2006). Many of the cytogenetic abnormalities are intimately associated with a particular subtype of leukemia and provide clinically important diagnostic and prognostic information (Johansson et al. 2004; Mrozek et al. 2004). Thus, the current World Health Organization classification system now recognizes genetic changes as important factors for appropriate disease classification of hematological malignancies (Jaffe et al. 2001).

The most characteristic genetic changes in leukemia are the reciprocal chromosomal translocations that result in fusion genes. To date,

more than 250 different fusion genes have been described with at least two qualitatively different types of rearrangements being observed (Rabbitts and Stocks 2003; Mitelman et al. 2006). In a subset of malignancies of B- or T-cell origin, illigitimate recombinations result in the juxtaposition of a variety of structurally intact (onco)genes to regulatory control elements of the immunoglobulin loci or the T-cell receptor loci, resulting in deregulated expression (Rowley 2001; Rabbitts and Stocks 2003). The second and by far most common outcome of balanced translocations is the creation of fusion genes, resulting in the expression of chimeric fusion proteins. The most frequent targets of chromosomal translocations in acute leukemias are transcriptional control genes, whereas tyrosine kinase encoding genes are more common targets in chronic myeloproliferative disorders (Cross and Reiter 2002; Kelly and Gilliland 2002; Scandura et al. 2002; De Keersmaecker and Cools 2006).

The molecular and functional characterization of individual fusion genes has offered profound insights into leukemogenesis, and, more recently, has also enabled the development of targeted therapies of hematological malignancies (see below).

## Normal and Leukemic Hematopoiesis

Hematopoiesis is the dynamic process of blood cell formation and involves maintenance and proliferation of hematopoietic progenitor cells and their differentiation into mature blood cells. During adult life hematopoiesis occurs predominantly in the BM, where a small population of pluripotent hematopoietic stem cells (HSCs) resides and gives rise to all the different blood cells (Szilvassy 2003; Bonnet 2005). Normal PB is composed of three types of cells: erythrocytes, thrombocytes, and leukocytes. The most numerous of these cells are the erythrocytes (red blood cells), which transport oxygen to the tissues, and the thrombocytes (platelets), which are needed for blood coagulation and repair of damaged blood vessels. The leukocytes (white blood cells), which are important mediators of the innate and adaptive immune system, can be further divided into granulocytes and lymphocytes (Jandle 1996).

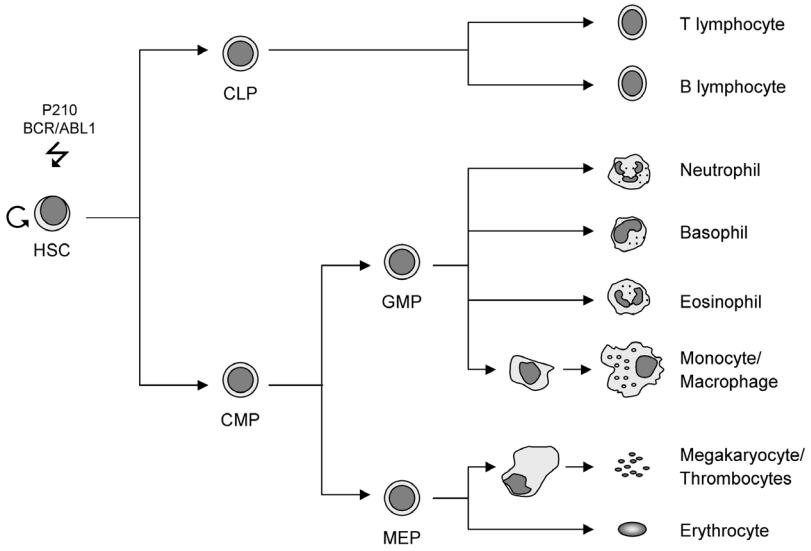


**Figure 1. Hematopoietic differentiation.** The pluripotent hematopoietic stem cell (HSC) has capacity of both self-renewal and differentiation. In the classical model of hematopoietic differentiation, the HSC first commits to either the lymphoid or myeloid branch through a common lymphoid progenitor (CLP) or a common myeloid progenitor (CMP), with the CMP further committing to either a granulocyte/monocyte progenitor (GMP) or a megakaryocyte/erythrocyte progenitor (MEP). Ultimately, the lymphoid branch gives rise to mature B- and T-lymphocytes. The GMP cells differentiate into neutrophils, basophils, eosinophils, and macrophages, whereas the MEP cells give rise to erythrocytes and thrombocytes. CML is characterized by presence of the P210 *BCR/ABL1* fusion gene, which is believed to arise in a pluripotent HSC.

Under normal conditions, most of the HSCs are in a quiescent or slow cycling state, but a small proportion remains active and continuously produces mature blood cells (Szilvassy 2003; Bonnet 2005). Lifelong hematopoiesis is maintained by the unlimited capability of HSCs to self-renew without differentiation, which maintains the HSC compartment at a steady state. The development of mature blood cells occurs in a hierarchical and linear manner that is commonly described as the progressive loss of self-renewing potential of HSCs, with gradual restrictions in cellular differentiation capacity. The classical and most accepted model of hematopoietic differentiation proposes that the HSCs first become commited to either the lymphoid or the myeloid branch through a common lymphoid progenitor (CLP) or a common myeloid progenitor (CMP) (Figure 1). The CMP further commits to either a granulocyte/monocyte progenitor (GMP) or

a megakaryocyte/erythrocyte progenitor (MEP). Ultimately, the lymphoid branch gives rise to mature B- and T-lymphocytes, whereas the myeloid branch differentiates into granulocytes (i.e. neutrophils, basophils, and eosinophils), macrophages, erythrocytes, and thrombocytes (Passegue et al. 2003; Hoang 2004). The decision between self-renewal and differentiation is strictly controlled by both intrinsic and extrinsic regulation exerted by transcription factors, cytokines, and the microenvironment in which the hematopoietic cells reside (Zhu and Emerson 2002; Hoang 2004; Kaushansky 2006).

The majority of the cells in the BM are blood cells at different stages of maturity and less than 0.1% of these cells correspond to an HSC (Szilvassy 2003). HSCs reside mainly within the BM, but can be mobilized into the PB by treatment with cytokines such as G-CSF, a method commonly used for stem cell transplantation. Umbilical cord blood (CB) is another source of primitive hematopoietic cells; however, use of it for transplantations is limited because of the low number of stem cells obtained from a single extraction (Szilvassy 2003). There is currently no single marker that can be used to distinguish the most primitive HSC population from other cells in the BM, but because CD34 is highly expressed on most human HSCs it has become widely used for identification of more primitive cells. CD34 is, however, a heterogeneously expressed cell-surface marker and is also found on more committed progenitor cells (Bonnet 2002; Szilvassy 2003).

The target cell for malignant transformation is mostly unknown, but cancer stem cells that are biologically distinct from the differentiated cells that characterize the disease have been demonstrated in both leukemias (Lapidot et al. 1994; Blair et al. 1997; Bonnet and Dick 1997; George et al. 2001; Jamieson et al. 2004) and other cancers (Al-Hajj et al. 2003; Singh et al. 2003). Because the leukemic stem cell (LSC) shares the capacity for unlimited self-renewal with normal HSCs, and also has some capacity to differentiate, it has been proposed that the initiating leukemic event occurs in an HSC, which—due to its lifelong persistence—has a high probability of accumulating mutations. Alternatively, the LSC may arise from a more committed progenitor that re-acquires the capability of self-renewal. There is currently support for both theories, indicating that the target cell may vary depending on the specific genetic aberration (Passegue et al. 2003; Bonnet 2005).

## Ph-Positive Leukemias and the *BCR/ABL1* Fusion Gene

### *The Philadelphia Chromosome*

The discovery of the Philadelphia (Ph) chromosome by Nowell and Hungerford in 1960 was the first description of a recurrent structural chromosomal abnormality associated with cancer, in this case CML (Nowell and Hungerford 1960). In 1973, Janet Rowley showed that the Ph chromosome results from a reciprocal translocation between the long arms of chromosomes 9 and 22 (Rowley 1973). Characterization of the breakpoints in the 1980s revealed that the translocation leads to fusion of the *BCR* gene at chromosome band 22q11 with the *ABL1* gene at 9q34, generating the *BCR/ABL1* fusion gene (Heisterkamp et al. 1985; Shtivelman et al. 1985). Molecular characterization of the chimeric protein later showed that the tyrosine kinase activity of BCR/ABL1 was indispensable for leukemic transformation in Ph-positive leukemias (Lugo et al. 1990). The leukemogenic effects of *BCR/ABL1* have been studied using different model systems, which have revealed that BCR/ABL1 affects several signal transduction pathways that influence proliferation, apoptosis, and adhesion of the leukemic cells (Deininger et al. 2000; Melo and Deininger 2004; Ren 2005). A major therapeutical breakthrough came with the development of imatinib, a drug that targets the tyrosine kinase activity of the BCR/ABL1 protein (Druker et al. 1996). Imatinib has now emerged as a front-line therapy in the treatment of CML patients, with increased survival advantages compared to previously available treatment regimens (O'Brien et al. 2003; Roy et al. 2006). However, some patients fail to respond to imatinib or develop drug resistance, and the reservoir of leukemic cells persists even in patients receiving treatment with more potent second-generation inhibitors (Copland et al. 2006). Hence, an increased understanding of how BCR/ABL1 mediates its leukemogenic effects will help us to identify complementary targets that can be used to eradicate the leukemic clone.

### *Clinical Characteristics of Ph-Positive Leukemias*

The Ph chromosome is found in 90–95% of patients with CML; the remaining cases have no cytogenetically visible Ph chromosome but are positive for the *BCR/ABL1* fusion, which then occurs either as a cryptic translocation or is masked within a complex karyotype (Jaffe et al. 2001).

CML accounts for about 15–20% of all leukemias in adults, with an incidence of 1–1.5 cases per 100,000 persons and year (Jaffe et al. 2001). The median age of presentation is around 53 years, but it does also occur at a low rate in children (D'Antonio 2005; Randolph 2005). Common symptoms at diagnosis include fatigue, weight loss, anemia, night sweats, and enlarged spleen—but many patients are asymptomatic and are diagnosed because of a high white blood cell count (WBC) in a routine blood test (D'Antonio 2005; Randolph 2005).

CML is a clonal myeloproliferative disorder characterized by an increased and premature release of primitive myeloid cells into the blood. Typically, the disease has a triphasic clinical course and is most often diagnosed during the initial, relatively indolent, chronic phase (CP), in which the expansion of mature myeloid cells leads to an increased number of white blood cells. Eventually the disease may progress into an accelerated phase (AP), showing an increased number of circulating blasts. After a short period of some months, an aggressive blast crisis (BC) resembling an acute leukemia of either myeloid origin (about two-thirds of cases) or lymphoid origin (about one-third of cases) develops (Randolph 2005). During BC the cells fail to mature and this phase is thus characterized by an accumulation of undifferentiated blasts and is often also associated with secondary genetic changes. The most common changes include trisomy 8, duplication of the Ph chromosome, and an isochromosome 17q (Johansson et al. 2002). If untreated, the BC finally leads to death in less than eight months (Randolph 2005).

The Ph chromosome is also found in 15–30% of adult ALL, and up to 5% of childhood ALL, and occasionally in newly diagnosed AML (Jaffe et al. 2001; Faderl et al. 2002; Kurzrock et al. 2003). Some cases of acute leukemia may represent an initial asymptomatic CML that has progressed to a later and acute stage of the disease. Ph-positive ALL represents the most common genetic abnormality in adult ALL, and its incidence increases with age (Faderl et al. 2002). Ph-positive ALL is characterized by clonal expansion and accumulation of immature lymphoid cells in the BM, blood, and lymphoid organs, and generally shows a higher WBC compared to other forms of ALL (Faderl et al. 2002; Kurzrock et al. 2003). The normal counterpart of the leukemic clone in Ph-positive ALL is pre-B cells (Faderl et al. 2002). Presence of the Ph chromosome in ALL is associated with a

poor prognosis when treated only with conventional chemotherapy (Radich 2001; Gleissner et al. 2002).

### *The Different BCR/ABL1 Fusion Genes*

The Ph chromosome is formed by fusion of the 3´ end of the *ABL1* gene at 9q34 to the 5´ end of the *BCR* gene at 22q11. The *ABL1* gene contains two alternative first exons followed by ten more, with the breakpoints occurring upstream of exon 2 (Deininger et al. 2000). Normally, the *ABL1* gene encodes a highly conserved and ubiquitously expressed non-receptor tyrosine kinase of 145 kDa, which is able to migrate between the nucleus and cytoplasm (Van Etten et al. 1989; Dhut et al. 1991; Taagepera et al. 1998). The ABL1 protein has been implicated in cell cycle regulation, in processes of stress response, and in signal transduction from growth factor receptors and integrins (Van Etten 1999; Saglio and Cilloni 2004). The *BCR* gene is also ubiquitously expressed and encodes a protein of 160 kDa with a serine/threonine kinase activity (Maru and Witte 1991). BCR has been suggested to be involved in the regulation of oxidative burst in neutrophils (Voncken et al. 1995b), to suppress RAS signaling (Radziwill et al. 2003), and to be important for cellular trafficking of growth factor receptors (Olabisi et al. 2006). The detailed normal cellular function of the BCR protein is, however, still largely unknown.

The *BCR* gene has 23 exons and the break usually occurs within one of three different breakpoint cluster regions (bcr), as indicated in Figure 2 (Deininger et al. 2000). In most cases of CML, as well as in 30–50% of the Ph-positive adult ALL cases (Jaffe et al. 2001; Faderl et al. 2002; Kurzrock et al. 2003), the break occurs within the major bcr (M-bcr) that spans exons 12–16, and results in either a fusion of *BCR* exons 13 or 14 with *ABL1* exon 2 (known as the b2a2 or b3a2 junctions, respectively). The resulting fusion protein is designated P210 BCR/ABL1 because it has a molecular weight of 210 kDa. If the break occurs within the minor bcr (m-bcr) instead, located in the 54 kb large intronic region between the alternative exon e2´ and exon 2, a smaller protein of 190 kDa is produced. This variant is called P190 BCR/ABL1 and is almost exclusively associated with Ph-positive ALL; it is the predominant form found in Ph-positive childhood ALL (Faderl et al. 2002; Kurzrock et al. 2003). The third bcr, the µ-bcr, is located downstream of exon 19 and the corresponding fusion protein is called P230 BCR/ABL1.

This variant is associated with a rare form of neutrophilic CML (Pane et al. 1996). Occasionally, some other fusion variants of *BCR/ABL1* are also detected (Melo and Deininger 2004).

Compared to the normal ABL1 protein, the BCR/ABL1 fusion protein is located in the cytoplasm and shows increased and constant tyrosine kinase activity (Lugo et al. 1990; Ilaria and Van Etten 1995). BCR/ABL1 contains several protein domains that allow interactions with adaptor molecules and mediate and regulate its function. The protein domains of the ABL1 part are identical in each of the P190, P210, and P230 BCR/ABL1 fusion variants, whereas the domains from the BCR segment differ (Figure 2). ABL1 contributes with the SRC homology domains SH2 and SH3, a tyrosine kinase domain, and also DNA- and actin-binding domains. The myristoylation site—encoded by the alternative exon 1b of *ABL1*—is not included in the BCR/ABL1 fusion protein and loss of this site has been suggested to favor an active conformation of ABL1 (Hantschel et al. 2003; Nagar et al. 2003). The SH2 and SH3 domains bind to tyrosine-phosphorylated proteins and proline rich residues, respectively, and are important domains for interaction with adaptor proteins such as CRK and CRKL (Feller et al. 1994b; Sattler et al. 1996). The tyrosine kinase activity of ABL1 is normally regulated by intramolecular interactions involving the SH2 and SH3 domains, which act to suppress the tyrosine kinase activity (Franz et al. 1989; Jackson and Baltimore 1989; Muller et al. 1993).

From the BCR part, the coiled-coil oligomerization domain and the serine/threonine kinase domain that harbors a tyrosine 177 site are present in all three different fusion proteins (Figure 2). The oligomerization domain is important for autophosphorylation of ABL1, leading to the increased tyrosine kinase activity of the BCR/ABL1 protein, and also promotes binding of BCR/ABL1 to actin (McWhirter et al. 1993; McWhirter and Wang 1993; Smith and Van Etten 2001; Zhang et al. 2001). The substrate of the serine/threonine kinase activity is unknown (Maru and Witte 1991), but residues overlapping this domain have been shown to be involved in several signal transduction pathways by binding to SH2 domains of other proteins. Phosphorylation of tyrosine 177 leads to recruitment and binding of the GRB2 adaptor protein (Pendergast et al. 1993), which is important for activation of the RAS pathway (Puil et al. 1994). Two additional domains of BCR include the Rho guanine nucleotide exchange factor (RhoGEF, also designated Dbl-like) and the pleckstrin homology (PH) domains, both of

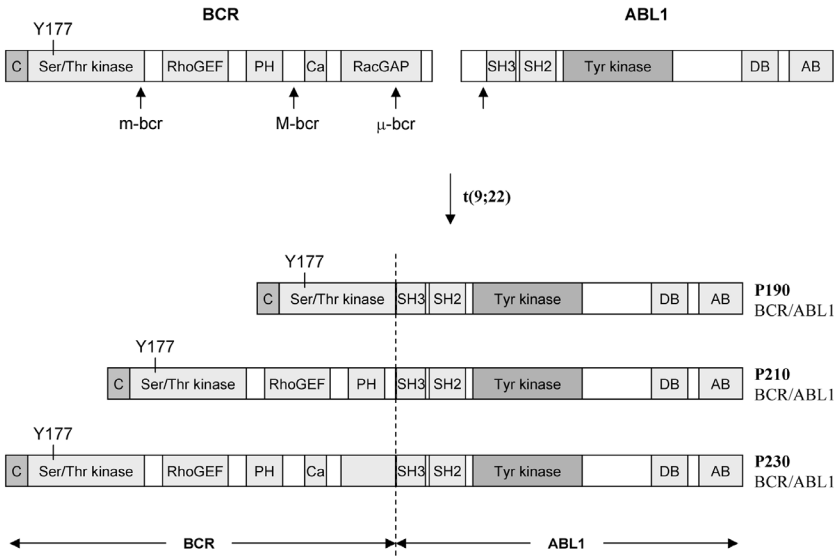


**Figure 2. Schematic representation of the BCR, ABL1, and BCR/ABL1 proteins.** The t(9;22) gives rise to mainly three different BCR/ABL1 fusion proteins: P190, P210, and P230 BCR/ABL1. The three fusion proteins consist of identical domains of the ABL1 part, but the contribution of the BCR protein differs. Three different BCR breakpoint regions have been identified: the major breakpoint cluster region (M-bcr), the minor breakpoint cluster region (m-bcr), and the micro breakpoint cluster region (μ-bcr). The P210 *BCR/ABL1* fusion variant is formed through a break within the M-bcr and is associated with CML and also a fraction of Ph-positive adult ALL. A break within the m-bcr is associated with formation of the P190 BCR/ABL1 fusion protein and is mainly seen in Ph-positive ALL. The P230 fusion variant is rare and results from a break in the μ-bcr. Domains/residues of the BCR protein: C, coil-coiled oligomerization domain; Ser/Thr kinase, serine/threonine kinase domain; Y177, tyrosine residue 177; RhoGEF, Rho guanine nucleotide exchange factor domain; PH, pleckstrin homology domain; Ca, calcium-dependent lipid-binding domain; RacGAP, RAC GTPase-activating protein domain. Domains of the ABL1 protein: SH2/SH3, SRC homology 2/3 domains; Tyr kinase, tyrosine kinase domain; DB, DNA-binding domain; AB, actin-binding domain. The figure is not drawn to scale.

which are present in P210 and P230 BCR/ABL1. The RhoGEF domain activates Rho GTPases by catalyzing the release of GDP and the binding of GTP (Denhardt 1996). Comparison of the P190 and P210 fusion proteins has suggested that differential activation of Rho proteins may play a role in the differences between P190 and P210 BCR/ABL1-induced leukemia (Harnois et al. 2003). PH domains have several putative functions, e.g. to mediate protein-lipid interactions (Lemmon 2004), but their role in BCR function is unknown. P230 BCR/ABL1 also harbors a calcium-dependent

lipid-binding domain, as well as a truncated RacGAP domain (Melo 1996; Advani and Pendergast 2002). The RacGAP domain of BCR has been shown to exhibit GTPase activity for RAC (Diekmann et al. 1991).

### *Functional Differences Between the BCR/ABL1 Fusion Variants*

The three main translocation variants of *BCR/ABL1* (P190, P210, and P230) are primarily associated with different types of leukemias (Pane et al. 2002). Much work has been focused on trying to elucidate whether the intrinsic properties of these fusion proteins might explain their disease-specific preference, or if this can be explained by the fact that they originate in different cell types. Comparisons of the functional properties of the fusion proteins have revealed that P190 has a higher intrinsic tyrosine kinase activity than P210, and that P230 has the lowest kinase activity of the three (Lugo et al. 1990; Li et al. 1999). Transgenic mice expressing either P190 or P210 *BCR/ABL1* have been shown to develop distinct leukemic phenotypes, with relatively short and long latency periods associated with P190 and P210, respectively (Voncken et al. 1995a; Honda et al. 1998; Koschmieder et al. 2005). In contrast to P230 *BCR/ABL1*-expressing primary mouse bone marrow cells that remain dependent on growth factors for optimal growth in the absence of stroma, both the P190 and P210 *BCR/ABL1* variants induce independence from growth factors. All three variants, however, induce growth factor-independence in cytokine-dependent cell lines (Quackenbush et al. 2000). Although both P190 and P210 BCR/ABL1 have been shown to activate the JAK/STAT pathway by phosphorylating different STAT molecules, the P190 variant has—in contrast to P210—been reported to induce phosphorylation of STAT6 in Ba/F3 cells transfected with *BCR/ABL1* (Ilaria and Van Etten 1996). Notably, however, upon retroviral expression of the P190 and P210 *BCR/ABL1* variants in primary human hematopoietic cells, no differences in STAT phosphorylation were seen, with both fusion variants inducing STAT5 and STAT6 phosphorylation to similar extents (*Article IV*). Furthermore, P190 and P210 have been suggested to activate similar signal transduction pathways in hematopoietic cell lines, as demonstrated by phosphorylation of identical substrates (Okuda et al. 1996).

The various fusion proteins have been reported to drive expansion of different hematopoietic populations, where P190 efficiently induces

lymphoid expansion of primary mouse BM cells, whereas P210 and P230 expression result in myeloid cell expansion under the same conditions (Quackenbush et al. 2000). Furthermore, comparison of the leukemogenic activity of P210 BCR/ABL1 and an activated form of ABL1 revealed that ABL1 induces only lymphoid malignancies, whereas P210 BCR/ABL1 expression was found to result in a myeloproliferative disease (Gross et al. 1999). In contrast, other studies have demonstrated that P190 and P210 *BCR/ABL1* induce a similar leukemogenic phenotype in mouse B-lymphoid precursors, and also in mice reconstituted with BM cells expressing either of these variants. The P190 variant was, however, a more potent leukemic stimulus than P210 *BCR/ABL1* (McLaughlin et al. 1989; Kelliher et al. 1991). Furthermore, transplantation of mice with P190, P210, or P230 *BCR/ABL1*-expressing BM cells, was found to induce an identical CML-like myeloproliferative disorder with equal potency as that seen when BM cells from donors pretreated with 5-fluorouracil—which favors transduction of early progenitor/stem cells—were used (Li et al. 1999). Using BM from untreated donors, P190 was shown to induce lymphoid leukemia with shorter latency than that induced by P210 or P230 *BCR/ABL1* (Li et al. 1999). Thus, there are conflicting data suggesting either that the association of various BCR/ABL1 variants with different types of leukemias is related to their different intrinsic leukemogenic activities, or, alternatively, that the various *BCR/ABL1* translocations would arise in different hematopoietic cell populations. In this context, it is interesting to note that when P210 and P190 *BCR/ABL1* are expressed in human $CD34^+$-enriched umbilical CB cells, they both give rise to a similar (erythroid) cell expansion (*Article IV*).

### *Leukemic Cells in CML and Ph-Positive ALL*

In CML, aberrant cells mainly from the granulocytic lineage are released into the blood, but the Ph chromosome is also found in macrophages and cells from the erythroid, megakaryocytic, and B lymphoid lineages (Fialkow et al. 1978). This indicates that CML arises in a common blood-forming stem cell, however, the exact precursor cell remains to be identified. Because *BCR/ABL1* has also been detected in endothelial cells from CML patients, it has been suggested that CML may arise in a progenitor cell even earlier in the hierarchy than the HSC—possibly in a hemangioblast capable of generating both blood and endothelial cells (Gunsilius et al. 2000).

Although this should be confirmed in further studies, a later report has suggested that the *BCR/ABL1* translocation may originate in a more primitive cell than the HSC (Fang et al. 2005).

The mechanism behind myeloid expansion in CML is far from clear. It has been suggested that the population of *BCR/ABL1*-positive HSCs is renewed even more slowly than normal HSCs (Udomsakdi et al. 1992) and that myeloid expansion results from an increased self-renewal of the more differentiated progenitors (Jamieson et al. 2004). Although it is generally believed that CML arises in a pluripotent HSC (Figure 1), the cell of origin in Ph-positive ALL has been proposed to be a more committed lymphoid progenitor (Anastasi et al. 1996). The two largest studies investigating the origin of Ph-positive ALL, however, have reported conflicting results, the one demonstrating multilineage involvement suggesting a pluripotent stem cell origin (Schenk et al. 1998), and the other showing a lymphoid lineage restriction in all *de novo* Ph-positive ALL cases, indicating a lineage-committed origin (Pajor et al. 2000). In support of the latter, there has been a study of Ph-positive ALL in children suggesting that high-risk ALL in childhood originates in a B lineage-negative but lymphoid-restricted progenitor cell (Hotfilder et al. 2005). Interestingly, it has been reported that P210 *BCR/ABL1*-positive and P190 *BCR/ABL1*-positive ALLs have different cellular origins, with P210 originating in a pluripotent HSC and P190 in a committed B-cell progenitor (Castor et al. 2005). In indirect support of a separate cellular origin of P190 and P210 BCR/ABL1, expression of either of these two variants in primitive human $CD34^+$ cells was found to result in induction of very similar biological effects (*Article IV*).

### *Experimental Models of BCR/ABL1-Induced Leukemia*

Several experimental systems have been used to study how *BCR/ABL1* elicits its transforming activities. The different model systems include hematopoietic cell lines established mainly from patients in advanced stages of CML, *BCR/ABL1*-transduced cell lines, primary CML cells, and transgenic and retroviral mouse models (Deininger et al. 2000). Each of these systems has its individual advantages and disadvantages, but combined, they have contributed greatly to our current knowledge of *BCR/ABL1*-induced leukemogenesis. Retroviral transduction of primary mouse BM cells with *BCR/ABL1*—with subsequent transplantation into mice—reflects the clinical

features of Ph-positive leukemias well, and has been used to study the *in vivo* pathogenesis of CML (Wong and Witte 2001; Van Etten 2002; Ilaria 2004).

As for *BCR/ABL1*-transfected cell lines, most show stable expression of *BCR/ABL1*, and only a few cell lines of murine origin have been described in which this fusion gene can be conditionally expressed (Carlesso et al. 1994; Kabarowski et al. 1994; Cortez et al. 1997; Klucher et al. 1998; Pierce et al. 1998; *Article I*). Compared to stable transfection, conditional expression offers an advantage in that early effects of the introduced gene can be monitored in a controlled manner, and the risk of clonal selection is likely to be very low. Immortalized leukemic cell lines are relatively easy to work with and have thus become a widely used model system to study the basic biological features of Ph-positive leukemic cells (Drexler et al. 1999). These cells are, however, derived from cells arrested at a specific stage of hematopoietic differentiation, and have also evolved to tolerate and be dependent on the expression of the *BCR/ABL1* fusion gene. Although cell lines acquire additional genetic alterations during culture, it has been shown that immortalized hematopoietic cell lines with the same primary genetic change—including several Ph-positive cell lines—display a similar gene expression profile (Andersson et al. 2005), and that cell lines and clinical samples with the same aberration share a characteristic gene expression pattern (Fine et al. 2004; Andersson et al. 2005).

Retroviral transduction of *BCR/ABL1* into human $CD34^+$ progenitor cells has enabled the study of *BCR/ABL1*-induced transforming activities in cells resembling the ones in which leukemia originates, with the advantage that the transduced cells can be readily compared with normal hematopoietic progenitor cells (Zhao et al. 2001; Chalandon et al. 2002; Ramaraj et al. 2004; *Article IV*). Finally, primary CML cells from patients have also been widely used to study the effects of *BCR/ABL1*. However, their limited availability, poor growth, and tendency to mature *in vitro* restrict the use of primary cells from patients (Deininger et al. 2000). Moreover, when comparing the molecular and cellular phenotypes of primary CML cells with those of normal cells, it is important to use a clearly defined cell population such as selected $CD34^+$ cells.

During recent years it has become increasingly clear that the cellular context in which *BCR/ABL1* is expressed has a significant impact on the phenotype induced by the fusion gene (Deininger et al. 2000; Wong et al.

2003; Wetzel et al. 2005). Hence, care should always be taken before findings derived from, in particular cell lines and animal models, are extrapolated to primary CML cells and patients.

### *Biological Properties of BCR/ABL1-Mediated Transformation*

The constitutive tyrosine kinase activity of BCR/ABL1 is essential for its transforming properties (Lugo et al. 1990; Pear et al. 1998) and required both for the establishment and maintenance of Ph-positive leukemia. Different hypotheses have been postulated to explain the expansion of the leukemic clone—including activation of proliferation, reduction of apoptosis, and deregulation of cell adhesion—features also observed upon expression of *BCR/ABL1* in human hematopoietic progenitor cells (Zhao et al. 2001; Chalandon et al. 2002; Ramaraj et al. 2004). The proliferative advantage is believed to result from BCR/ABL1-mediated activation of a number of signal transduction pathways (Deininger et al. 2000; Melo and Deininger 2004; Ren 2005). Moreover, BCR/ABL1 has been shown to induce growth factor-independent proliferation in factor-dependent cell lines and primary CML cells, which may be at least partly due to autocrine production of IL3 and G-CSF (Hariharan et al. 1988; Sirard et al. 1994; Jiang et al. 1999).

Cell lines transfected with *BCR/ABL1* have been demonstrated to be less sensitive to apoptotic agents such as ionizing radiation and growth factor withdrawal (Bedi et al. 1994; McGahon et al. 1994; Cortez et al. 1995). The anti-apoptotic effect is, however, debatable because conflicting results have been reported in primary CML cells (Bedi et al. 1994; Amos et al. 1995; Albrecht et al. 1996). These contradictory findings have been suggested to be due to differences in the expression level of *BCR/ABL1* between primary CML cells and cell lines, where the latter have been reported to require high levels of *BCR/ABL1* expression to be able to be protected against apoptosis (Cambier et al. 1998; Issaad et al. 2000; Keeshan et al. 2001). A more recent study of primary CML progenitor cells has indicated that the dividing cells are sensitive, whereas the non-proliferating CML cells are resistant to apoptosis induced by, for example, imatinib and arsenic (Holtz et al. 2005).

In normal hematopoiesis, proliferation and differentiation are strongly regulated by cellular interactions with the BM stroma. Increased

proliferation and also premature release of immature myeloid cells at different stages of differentiation into the circulation have been suggested to result from reduced adhesion of CML progenitor cells to the stroma and extracellular matrix (Gordon et al. 1987; Verfaillie et al. 1992; Verfaillie et al. 1997). BCR/ABL1 has been proposed to perturb the receptor function of integrins by inducing a low-affinity state that reduces its adhesive properties (Verfaillie et al. 1997), and it appears that restoration of the normal integrin-dependent adhesion of CML progenitors is one of the mechanisms by which IFN-alpha treatment works (Bhatia et al. 1994). Altogether, accumulation of *BCR/ABL1*-positive cells may, besides its proposed effects on proliferation and apoptosis, also be due to reduced contact inhibition as a result of reduced interactions with the microenvironment.

### *Signaling Pathways Activated by BCR/ABL1*

The transforming potential of *BCR/ABL1* is likely to be mediated by a number of signal transduction pathways. Over the years, a large number of signal transduction pathways have been implicated in BCR/ABL1-mediated transformation, indicating that complex deregulatory mechanisms are involved in the leukemogenic events. Several different experimental systems have, however, been used for these investigations and some of the observed features are thus likely to be important only in the context of certain cells.

BCR/ABL1 contains several protein interaction domains/motifs that regulate and mediate its function by binding to and/or phosphorylating adaptor proteins, which activate downstream signaling pathways (Figure 3). Adaptor proteins that have been shown to interact with BCR/ABL1 include CRKL, CRK, GRB2, GRB4, GAB2, SHC, SOS, DOK, and CBL (Pendergast et al. 1993; Feller et al. 1994a; Matsuguchi et al. 1994; Oda et al. 1994; Puil et al. 1994; ten Hoeve et al. 1994; de Jong et al. 1995; Carpino et al. 1997; Coutinho et al. 2000; Sattler et al. 2002). The complex formed between BCR/ABL1 and the adaptor proteins results in activation of multiple signaling pathways, which in turn activate proteins and transcription factors such as RAS, PI3K, AKT, STAT5, NFκB, and MYC.

BCR/ABL1 activates the RAS signaling pathway by forming a complex with GRB2, which binds to the autophosphorylated tyrosine 177 site (Mandanas et al. 1993; Pendergast et al. 1993), and SOS, thereby leading to accumulation of the active GTP-bound form of RAS (Figure 3)

(Druker et al. 1992; Tauchi et al. 1994). Alternatively, RAS may be activated by the adaptor proteins SHC and CRKL. Following activation, RAS signals via MAP kinases (MAPKs) through a phosphorylation cascade and activates the ERK (Cortez et al. 1997) and the JNK/SAPK (Raitano et al. 1995) pathways, resulting in activation of transcription factors and stimulation of proliferation and differentiation. Inhibition of the RAS pathway has been shown to prevent the leukemogenic activity of BCR/ABL1 in fibroblasts and hematopoietic mouse cells (Sawyers et al. 1995).

Activation of the PI3 kinase (PI3K) pathway by BCR/ABL1 is thought to result from formation of a complex together with the CRKL, CRK, and CBL adaptor molecules (Sattler et al. 1996), which bind to the BCR/ABL1 fusion protein (Figure 3). The PI3K is composed of the regulatory p85 subunit and the catalytic p110 subunit, the first of which associates with BCR/ABL1 through interaction with the adaptor molecules CRKL and CBL (Sattler et al. 1996). Activation of the PI3K is required for transformation by BCR/ABL1, and downstream signaling through the AKT kinase appears to be important for the transforming event (Skorski et al. 1995; Skorski et al. 1997).

Another pathway thought to mediate growth-promoting signals in *BCR/ABL1*-positive cells is the JAK/STAT pathway (Figure 3). Normally, the JAKs are involved in signal transduction from tyrosine kinase-associated receptors and they phosphorylate and activate different STAT molecules, which then dimerize and are translocated to the nucleus where they activate gene transcription (Ivashkiv and Hu 2004). Mainly STAT5, but also STAT1 and STAT3, have been shown to become phosporylated in *BCR/ABL1*-transfected and leukemic cell lines (Carlesso et al. 1996; Ilaria and Van Etten 1996; Shuai et al. 1996; Chai et al. 1997; *Article I*). This activation has, however, been suggested to be independent of JAK signaling (Carlesso et al. 1996; Ilaria and Van Etten 1996). The JAK/STAT pathway has also been shown to be constitutively active in primary CML cells (Chai et al. 1997), and, after some years of controversy, STAT5 has recently been shown to be essential for BCR/ABL1-mediated transformation of primitive murine cells (Hoelbl et al. 2006). Thus, the available data suggest that JAK/STAT is an important pathway by which BCR/ABL1 mediates its transforming activities.

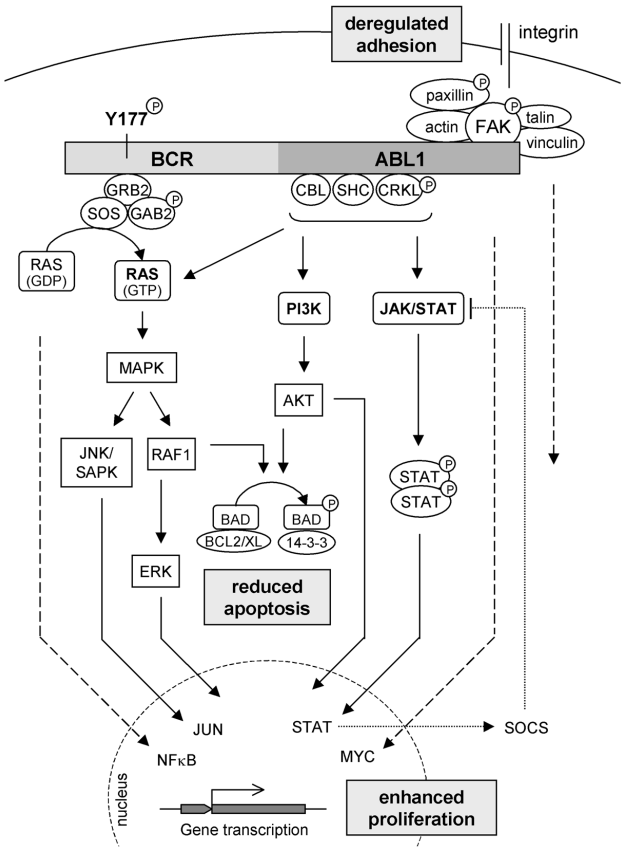


**Figure 3. Schematic overview of BCR/ABL1-mediated signaling.** BCR/ABL1 binds to and/or phosphorylates several adaptor proteins such as GRB2, CRKL, CBL, and SHC, resulting in activation of multiple downstream signaling pathways including RAS, PI3K, and JAK/STAT. BCR/ABL1 also activates a focal adhesion complex, including FAK and paxillin, that is associated with the integrin receptor. Ultimately, signaling through these pathways results in enhanced proliferation, reduced apoptosis, and deregulated adhesion.

The RAS, PI3K, and JAK/STAT signaling pathways may also contribute to a possible reduction of apoptosis in *BCR/ABL1*-positive cells. BCR/ABL1 has been shown to increase transcription of the anti-apoptotic BCL2 and BCLXL proteins by RAS/PI3K- and STAT5-mediated signaling, respectively (Sanchez-Garcia and Martin-Zanca 1997; Skorski et al. 1997; Horita et al. 2000). Normally, BCL2 and BCLXL are neutralized by the pro-apoptotic protein BAD, but in its phosphorylated form BAD is instead bound by to a 14-3-3 protein and can no longer inhibit the anti-apoptotic

activities. In *BCR/ABL1*-expressing cells, BAD has been suggested to become phosphorylated either by RAS-mediated activation of the RAF1 kinase, or by PI3K-mediated activation of AKT, which may be at least partly responsible for the increased survival of *BCR/ABL1*-expressing cells (Figure 3) (Neshat et al. 2000). Furthermore, BCR/ABL1 may inhibit apoptosis by downregulating *IRF8* (also designated ICSBP), thereby modulating the expression of *BCL2* and *BCLXL* (Gabriele et al. 1999; Burchert et al. 2004). Additionally, BCR/ABL1-mediated signaling may prevent accumulation of cytochrome c, resulting in suppressed activation of caspase 3, and hence suppressed apoptosis (Amarante-Mendes et al. 1998).

The integrins are a family of transmembrane receptors associated with the cytoskeleton through focal adhesions that connect them to cytoskeletal proteins, which is important for signal transduction between the cell and its environment (Ginsberg et al. 2005). The cytoplasmic location of the BCR/ABL1 protein permits interactions with the cytoskeleton by binding to focal adhesion kinase (FAK), paxillin, vinculin, F-actin, tensin, and talin (Figure 3) (McWhirter and Wang 1993; Gotoh et al. 1995; Salgia et al. 1995a; Salgia et al. 1995b). Phosphorylation and interaction of BCR/ABL1 with these cytoskeletal proteins may influence the function of integrins and mediate signal transduction, but there have been conflicting results reported regarding phosphorylation of FAK and paxillin in *BCR/ABL1*-transfected fibroblasts (Cheng et al. 2002). Primary CML cells have, however, been reported to show an enhanced association between the cytoskeleton and integrin receptors. This may influence the normal cellular interactions with the microenvironment, thereby affecting proliferation and normal contact inhibition (Bhatia et al. 1999).

Several transcription factors including MYC, JUN, and NFκB have been shown to become activated by BCR/ABL1 (Figure 3), but the mechanisms underlying this activation remain to be elucidated. Using a dominant-negative MYC mutant, it has been demonstrated that MYC is required for BCR/ABL1-mediated transformation of fibroblasts and primary mouse bone marrow cells (Sawyers et al. 1992). The activation of MYC appears to be dependent on the SH2 domain of BCR/ABL1 (Afar et al. 1994), and JAK2 has been suggested to be involved in the induction of MYC by BCR/ABL1 (Xie et al. 2002). JUN has been demonstrated to exhibit increased expression in *BCR/ABL1*-transfected murine and human cells, as well as in primary CML cells (Raitano et al. 1995; Burgess et al.

1998; *Article II*), and contributes to BCR/ABL1-mediated transformation (Raitano et al. 1995). The NFκB transcription factor is a key regulator of proliferation and apoptosis and is constitutively active in CML and Ph-positive ALL blasts (Reuther et al. 1998; Kirchner et al. 2003; Munzert et al. 2004). Activation of NFκB by BCR/ABL1 is dependent on the tyrosine kinase activity and is also partly dependent on the RAS signaling pathway (Reuther et al. 1998). Inhibition of the IKK complex that activates NFκB, abrogates proliferation of CML cell lines and primary CML cells (Cilloni et al. 2006).

In conclusion, BCR/ABL1 mediates its leukemogenic effects through activation and deregulation of several well-known signal transduction pathways. Although individual pathways have been demonstrated to be essential, they are likely to act in concert to induce transformation.

### *Transcriptional Effects Mediated by BCR/ABL1 Signaling*

Several of the different signal transduction pathways and transcription factors that have been found to become deregulated by BCR/ABL1 are likely to affect a number of downstream target genes. Identification of such genes, or perturbed transcriptional networks, may ultimately make it possible for us to identify, and hopefully target, novel pathways used by BCR/ABL1 to elicit its leukemogenic activity. Since the introduction of microarray technology in the 1990s, allowing gene expression profiling of several thousand genes at the same time, several studies investigating the influence of *BCR/ABL1* on the global gene expression pattern in different experimental systems have been reported.

One of the first microarray studies investigating the transcriptional effects of *BCR/ABL1* compared the gene expression profiles of mouse BM mononuclear cells retrovirally transduced with either P190 or P210 *BCR/ABL1* (Advani et al. 2004). This study revealed that P190 showed a significantly higher expression of IFN-inducible genes as compared to P210, which instead displayed a slightly increased expression of the IL3 receptor. An enrichment of IFN-alpha inducible genes was also found following inducible expression of P210 *BCR/ABL1* in human U937 cells (*Article II*). However, it is presently unclear whether the induction of IFN-responsive genes is part of the transformation process by BCR/ABL1, whether it is a negative feedback reaction against it, or whether it represents an entirely cell

context-dependent response. Global gene expression studies of Ph-positive cell lines following treatment with imatinib identified 142 genes that are dependent on the tyrosine kinase activity of BCR/ABL1 (*Article III*). Interestingly, BCR/ABL1 was found to positively regulate several genes involved in negative feedback regulation of known signaling pathways. Among these were three members of the suppressor of cytokine signaling (SOCS) family (Figure 3): *CISH*, *SOCS2*, and *SOCS3*. Aberrant expression of individual SOCS family members has previously been described to be associated with *BCR/ABL1* expression in transfected cell lines and primary CML cells (Tauchi et al. 2001; Schultheis et al. 2002; Liu et al. 2003; Radich et al. 2006), and thus seems to be a recurrent feature and an important response following BCR/ABL1 signaling. In addition, the latter study also found several genes to be negatively regulated by P210 BCR/ABL1, many of which are involved in metabolic pathways. In 32D mouse cells, P210 *BCR/ABL1* expression was also shown to deregulate the expression of several genes involved in carbohydrate metabolism (Hickey and Cotter 2006). In an attempt to investigate the transcriptional effects of BCR/ABL1 in a cellular context more closely resembling Ph-positive leukemias *in vivo*, human $CD34^+$ selected cells were retrovirally transduced with P210 and P190 *BCR/ABL1* (*Article IV*). Microarray analysis revealed no significant differences between the two fusion variants. When the gene expression profiles of both fusion genes were combined and compared with those of control cells, 222 potential downstream targets of P190/P210 BCR/ABL1 were identified (*Article IV*). The *SOCS2* gene was again identified as being upregulated and *BCR/ABL1* expression was found to activate genes involved in the MAPK and TGFB signaling pathways, for example, and to suppress the expression of genes involved in the immune response, cell communication, cell adhesion, and differentiation.

Relatively few microarray studies have been performed to investigate the transcriptional expression profiles of primary Ph-positive ALL and CML cells. Gene expression studies of Ph-positive ALL have revealed a more heterogeneous gene expression pattern than in other subgroups of ALLs harboring characteristic chromosomal aberrations (Ross et al. 2003; Fine et al. 2004; Haferlach et al. 2005). This finding has been suggested to reflect a less direct effect on the gene expression pattern by the P190 *BCR/ABL1* fusion gene than in cases harboring rearrangement of transcriptional control genes (Fine et al. 2004).

Gene expression studies in primary CML cells have concentrated on comparing normal cells with those obtained in CP (Bruchova et al. 2002; Li et al. 2002; Kaneta et al. 2003; Nowicki et al. 2003; Kronenwett et al. 2005), to identify gene signatures associated with disease progression by comparing cells obtained in CP with AP/BC cells (Ohmine et al. 2001; Radich et al. 2006; Zheng et al. 2006), or to identify molecular profiles at diagnosis that could predict the likelihood of treatment response or failure following administration of imatinib (Hofmann et al. 2002; Kaneta et al. 2002; Ohno and Nakamura 2003; McLean et al. 2004; Frank et al. 2006; Villuendas et al. 2006). One major drawback is that several of these studies have used a limited number of samples and/or heterogeneous, mainly mononuclear, cell populations (Bruchova et al. 2002; Li et al. 2002; Kaneta et al. 2003; Nowicki et al. 2003; Song et al. 2006), in which the influence of *BCR/ABL1* in a critical progenitor population may easily escape detection. A recent study compensated for this by subtracting the normal $CD34^+$ expression signature from the CML expression profile (Radich et al. 2006), and identified a set of 103 differentially expressed genes. At least four studies have used selected ($CD34^+$ or $AC133^+$) cells from CML patients (Ohmine et al. 2001; Kronenwett et al. 2005; Yong et al. 2006; Zheng et al. 2006), with two of them focusing on identification of genes involved in disease progression (Ohmine et al. 2001; Zheng et al. 2006), and one on prediction of an aggressive or indolent disease outcome from diagnostic CP CML samples (Yong et al. 2006). Kronenwett et al. (2005) used a restricted cDNA microarray of 1,185 defined genes to compare the expression profile of $CD34^+$ cells from five newly diagnosed CML patients in CP, and from ten healthy individuals. In total, 158 genes were differentially expressed and the authors speculated that the increased and reduced expression of GATA2 and CXCR4, respectively, would support the increased self-renewal and defective adhesive properties of the $CD34^+$-enriched progenitor cells seen in early phase CML.

Recently, Radich et al. (2006) reported an extensive gene expression study in which 3,500 genes were identified as being differentially expressed across the different disease phases of CML. A very similar expression profile was observed between AP and BC, suggesting that—at least at the transcriptional level—CML may be considered to be a two-step process rather than a three-step process. Of the genes involved in disease progression, *SOCS2* has been shown to have increased expression, whereas

members of the CEBP family that regulate myeloid differentiation show reduced expression in advanced phases relative to the CP of CML (Radich et al. 2006; Zheng et al. 2006).

Gene expression profiling has also been used to identify genes that can be used to predict the response and sensitivity of Ph-positive leukemias to imatinib treatment (Hofmann et al. 2002; Kaneta et al. 2002; Ohno and Nakamura 2003; McLean et al. 2004; Frank et al. 2006; Villuendas et al. 2006). Using unselected cells, these studies have reported that a number of genes can be used to distinguish between cytogenetically defined responders and non-responders to imatinib.

In conclusion, several global gene expression studies investigating the transcriptional effects of BCR/ABL1 in different experimental systems and in primary Ph-positive samples have been published. The use of different microarray platforms, experimental cellular systems, cell populations, and samples to which the *BCR/ABL1* expression pattern is compared makes it very difficult—and sometimes even impossible—to compare individual microarray investigations. In addition, an increased knowledge of transcriptional programs active in normal hemtopoiesis is needed to better interpret the aberrant transcriptional profiles of Ph-positive leukemia. Some recurrent features are, however, emerging; for example, the upregulation of SOCS family members by BCR/ABL1 seen in several studies. Experimental interference with such genes, for example by RNA interference-based strategies, will hopefully enable the development of novel targeted therapies.

### *Targeted Therapy Against BCR/ABL1-Positive Leukemias*

Imatinib mesylate (Gleevec/Glivec, formerly STI571; Novartis) is a highly selective and potent growth inhibitor of *BCR/ABL1*-expressing cells (Buchdunger et al. 1996; Druker et al. 1996; Deininger et al. 1997), and has emerged as the first-line therapy for CML. The imatinib molecule binds to the ATP-binding pocket of the ABL1 domain and stabilizes the inactive, non-ATP-binding conformation of BCR/ABL1 (Schindler et al. 2000; Nagar et al. 2002). This blocks the tyrosine kinase activity, and inhibits both BCR/ABL1-mediated autophosphorylation and substrate phosphorylation, resulting in abrogated downstream cell signaling and reduced proliferation of the *BCR/ABL1*-positive cells (Buchdunger et al. 1996; Druker et al. 1996;

Deininger et al. 1997; Gambacorti-Passerini et al. 1997). Although imatinib is highly specific for ABL1, the KIT, PDGFR, and ARG tyrosine kinase activities are also suppressed (Buchdunger et al. 1996; Druker et al. 1996; Carroll et al. 1997; Buchdunger et al. 2000), which has been suggested to be part of the inhibitory function of imatinib (Wong et al. 2004).

Imatinib is a well-tolerated drug with few side effects, but some influences on normal hematopoiesis have been reported (Appel et al. 2005). In the CP of CML, treatment with imatinib induces complete cytogenetic remission (CCR, i.e. no visible Ph chromosomes) in 70–80% of newly diagnosed patients (Kantarjian et al. 2003; O'Brien et al. 2003). With a median follow-up of 54 months, the overall relapse rate for imatinib-treated patients with newly diagnosed CML in the CP was found to be 16%. The relapse rates at 54 months for patients in CCR or with a 3-log reduction of *BCR/ABL1* transcripts were 7% and 3%, respectively (Druker 2006). Patients in more advanced stages of CML achieve CCR in about 5–20% of cases, but the response is generally not durable and relapse occurs relatively quickly (Ottmann et al. 2002; Sawyers et al. 2002; Talpaz et al. 2002).

Before the development of imatinib, CML was treated with IFN-alpha, which is normally involved in viral protection, inhibition of cell growth, and control of apoptosis (Stark et al. 1998; Platanias 2005). Treatment of CML with IFN-alpha suppresses the leukemic clone and prolongs survival (Talpaz 2001), but the mechanisms behind its effect are unknown. Compared to imatinib, treatment with IFN-alpha has major side effects and also gives a slower initial response (O'Brien et al. 2003). However, in some cases the effects of IFN-alpha have been reported to be durable even after interruption of treatment, which has not been observed for imatinib (Bonifazi et al. 2001; Cortes et al. 2004; Mauro et al. 2004). This discrepancy has been suggested to be due to targeting of different cell populations: IFN-alpha appears to be more toxic to primitive CML stem cells whereas imatinib mainly targets differentiated CML progenitors (Angstreich et al. 2005). In support of the latter idea, patients in imatinib-induced CCR have been shown to remain positive for leukemic progenitors (Bhatia et al. 2003; Chu et al. 2005). Furthermore, both *in vitro* studies and mathematical modeling of CML suggest that a small population of LSCs may survive treatment with imatinib, possibly by adopting a quiescent state (Holyoake et al. 1999; Graham et al. 2002; Michor et al. 2005).

Resistance to imatinib occurs frequently in patients with advanced CML or Ph-positive ALL, but is much rarer in CML patients treated with imatinib during their CP. The major molecular mechanism behind acquired resistance to imatinib is point mutation in the kinase domain of BCR/ABL1 that interferes with the binding of imatinib to ABL1 (Gambacorti-Passerini et al. 2003; Deininger 2005). This results in either no or incomplete inhibition of BCR/ABL1, and such mutations can be detected in 50–90% of relapsed patients (Deininger et al. 2005). Imatinib resistance has also been suggested to be conferred by increased expression of *BCR/ABL1*. Although amplification of the *BCR/ABL1* gene has been observed in some patients, overexpression may also result from other mechanisms (Gambacorti-Passerini et al. 2003; Deininger 2005).

Two newly developed inhibitors of the BCR/ABL1 kinase, dasatinib and nilotinib, have shown promising results in early-phase clinical trials involving patients with imatinib-resistant disease (Kantarjian et al. 2006; Talpaz et al. 2006). Dasatinib (BMS-354825; Bristol-Myers Squibb) has been shown to be a more potent inhibitor than imatinib, possibly due to its dual inhibitory effects on both ABL1- and SRC-family kinases. Dasatinib also binds to both the active and inactive conformations of the ABL1 kinase domain (Lombardo et al. 2004; Shah et al. 2004). Nilotinib (AMN107; Novartis Oncology), like imatinib, binds to the inactive conformation of ABL1, but is more potent and shows both higher binding affinity and selectivity for the ABL1 kinase (Weisberg et al. 2006). Although these two drugs have been shown to be effective against most imatinib-resistant mutations (Kantarjian et al. 2006; Talpaz et al. 2006), patients with imatinib-resistant acute Ph-positive leukemia still show a relatively short response to these drugs, and several kinase domain mutations that would confer resistance have been identified by *BCR/ABL1* mutagenesis (Burgess et al. 2005; Bradeen et al. 2006). Furthermore, although dasatinib has been shown to be more effective in targeting an earlier *BCR/ABL1*-positive progenitor population, quiescent primitive CML cells still remain viable (Copland et al. 2006).

In addition to tyrosine kinase inhibitors, compounds that target signaling pathways downstream of BCR/ABL1 have been developed. Inhibitors of farnesyl transferase, and cyclin-dependent kinases are currently undergoing clinical trials (Daley 2003; Deininger et al. 2005). The farnesyl transferase inhibitors work by abrogating RAS activation (Jabbour et al.

2004), and have been demonstrated to inhibit proliferation of BCR/ABL1-transformed cell lines and primary human CML cells, as well as to make imatinib-resistant cells sensitive to apoptosis (Peters et al. 2001; Hoover et al. 2002; Nakajima et al. 2003). Inhibitors that target the PI3K and MEK1 signaling pathways have also been reported to be effective compounds against imatinib-resistant cells (Klejman et al. 2002; Yu et al. 2002). Furthermore, BCR/ABL1 may be degraded by inhibition of HSP90 (Gorre and Sawyers 2002), which is required for stability of the fusion protein, or by histone deacetylase inhibitors which both promote degradation and reduce the expression level of BCR/ABL1 (Nimmanapalli et al. 2003). In conclusion, although novel compounds and tyrosine kinase inhibitors are under development, transplantation is likely to remain the only cure of Ph-positive leukemias until a new treatment (or combination of treatments) that efficiently targets and eliminates all leukemic cells has been developed.

# THE PRESENT STUDY

This section includes the specific aims of the thesis, a brief description of materials and methods, as well as the major results together with a short discussion of each article. At the end of this section a summary of the major conclusions is given together with some concluding remarks.

## Specific Aims of the Study

The general aim of this thesis has been to improve our understanding of *BCR/ABL1*-induced leukemogenesis through molecular and functional studies of BCR/ABL1-mediated signaling using different experimental systems. More specifically, the aims were:

- to establish a cell line allowing inducible expression of *BCR/ABL1*, and to characterize BCR/ABL1-mediated effects in this model (*Article I*),

- to investigate BCR/ABL1-mediated effects on the global gene expression pattern, using the *BCR/ABL1*-inducible cells established in the first study (*Article II*),

- to study BCR/ABL1-mediated effects on the global gene expression pattern, using blockage of the BCR/ABL1 tyrosine kinase activity in Ph-positive cell lines by imatinib (*Article III*), and

- to investigate the functional and molecular effects following retroviral transduction of *BCR/ABL1* into primitive human progenitor cells, and to compare the effects induced by the P190 and P210 *BCR/ABL1* variants (*Article IV*).

## Materials and Methods

For a more thorough description of how the individual studies were performed, the reader is referred to the original articles (*Articles I–IV*). In brief, the effect of *BCR/ABL1* on cellular proliferation and viability was studied in liquid cultures using trypan blue exclusion, assessment of apoptosis and differentiation was performed using Annexin V and nitro blue tetrazolium (NBT) reduction/May-Grünwald-Giemsa staining, respectively, FACS analysis was used to study the expression of cell-surface markers, and colony assays were performed to assess self-renewal and differentiation capacity. Microarray technology was used to investigate BCR/ABL1-mediated effects on the global gene expression pattern, and selected genes identified as being differentially expressed were studied further at the mRNA level using northern blot, RT-PCR, and real-time PCR analysis. Western blots were used to confirm and study BCR/ABL1-mediated effects at the protein level. Below follows a short summary of the experimental systems that were established, and a brief description of microarray technology.

### *Experimental Model Systems*

In this thesis, three different experimental models were established and used to study functional and transcriptional effects of BCR/ABL1-mediated signaling. First, Clontech's Tet-On Gene Expression System was used to prepare human U937 cells with inducible *BCR/ABL1* expression. The U937 cell line was chosen because it is a well-characterized leukemic cell line that can be induced to differentiate using various compounds (Sundström and Nilsson 1976; Olsson and Breitman 1982; Olsson et al. 1983), thus allowing evaluation of BCR/ABL1-mediated effects on cellular differentiation. The model system was established by electroporation of U937 cells containing the pTET-On regulatory plasmid with a vector carrying P210 *BCR/ABL1* under the control of a tetracycline-inducible cytomegalovirus (CMV) promoter lacking the strong enhancer elements. The regulatory plasmid also conferred resistance to geneticin, which permitted selection and establishment of stable clones. For the phenotypic characterization of the established cells (*Article I*), the tetracycline analog doxycycline was used to induce *BCR/ABL1* expression at different time intervals between 30 min and 72 h.

The transcriptional effects were studied 12, 24, 36, and 48 h after *BCR/ABL1* induction and compared to the expression profiles of uninduced cells and of control (mock) clones obtained from transfections with an empty vector (Figure 4A) (*Article II*).

In a second experimental system, Ph-positive cell lines were used to study BCR/ABL1-mediated transcriptional effects (*Article III*). Five Ph-positive cell lines (K562, KU812, JK-1, Meg-01, and LAMA-84), derived from CML cells in BC, were selected. For the microarray analysis, all cells were treated with imatinib mesylate for 3 and 12 h, after which cells were collected and total RNA extracted (Figure 4B). Following expression profiling, a mean expression value based on the two time points was calculated. Because imatinib is a specific abrogator of BCR/ABL1-mediated signaling, the genes that become downregulated upon imatinib treatment were considered to be positively regulated by BCR/ABL1, whereas the upregulated genes were considered to be negatively regulated. Control experiments included untreated cells and Ph-negative cell lines treated with imatinib.

The third model system consisted of human hematopoietic progenitor cells transduced with virus containing a murine stem cell virus (MSCV)-based retroviral vector (*Article IV*). The bicistronic vector contained either P190 or P210 *BCR/ABL1* cDNA, and a separate internal ribosomal entry site (IRES) element linked to green fluorescent protein (GFP). The expression of *BCR/ABL1* and GFP was driven by the constitutively active viral long terminal repeat (LTR) promoter. Before transduction, the $CD34^+$ umbilical CB cells were stimulated with early acting cytokines for 48 h to induce active cell cycling, which is needed for integration of the gene carried by the virus into the host genome. The cellular effects of BCR/ABL1, including proliferation, differentiation, and colony-forming capacity, were monitored over several days in culture. In contrast, the transcriptional response was examined at earlier time points, i.e. 2, 3, and 4 days after transduction, to monitor more immediate transcriptional effects of BCR/ABL1 (Figure 4C). The results were compared with those in control (MIG) cells obtained from retroviral transduction of a vector encoding GFP only.

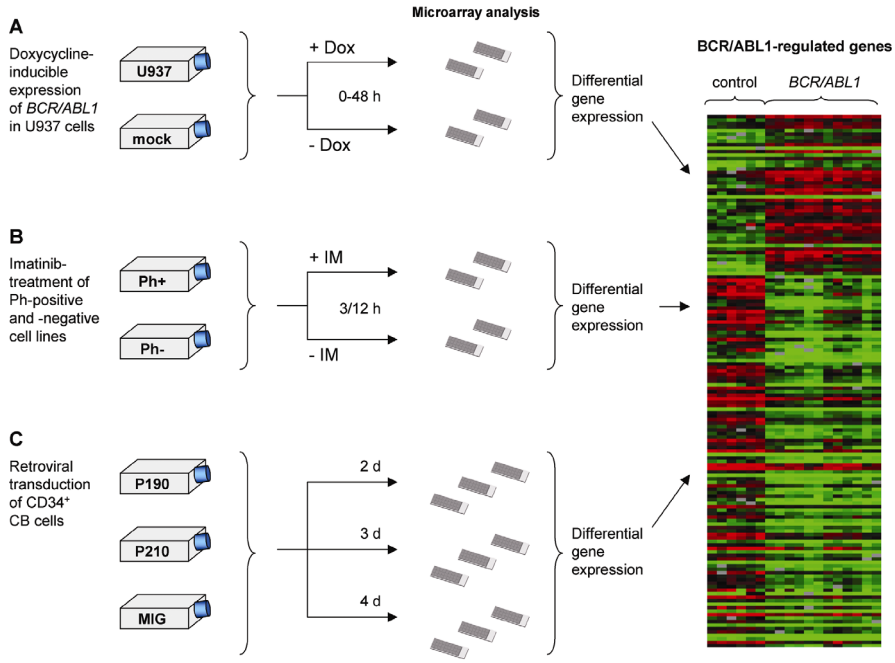


**Figure 4. Experimental models used to identify BCR/ABL1-regulated genes.** The figure is a schematic representation of the different experimental set-ups used to identify differentially expressed genes by microarray analysis. (**A**) Doxycycline (Dox)-inducible expression of *BCR/ABL1* in U937 cells. The transcriptional response was studied at six different time points between 0–48 h after doxycycline-induced expression of *BCR/ABL1*, and compared to the expression profiles of uninduced cells and control mock cells. (**B**) Imatinib (IM)-treatment of Ph-positive and Ph-negative cell lines. To extract BCR/ABL1-regulated genes, expression profiling were performed 3 and 12 h after blockage of the tyrosine kinase activity of BCR/ABL1 in five Ph-positive cell lines. The gene expression profiles were compared to the profiles of untreated Ph-positive cells and to the expression profiles of five untreated and imatinib-treated Ph-negative cell lines. (**C**) Retroviral transduction of human $CD34^+$ CB cells. The transcriptional effects of P190 and P210 *BCR/ABL1* expression were studied 2, 3, and 4 days after transduction of $CD34^+$ CB cells and compared with the expression profiles of control MIG cells.

## *Gene Expression Profiling Using Microarrays*

Gene expression analysis using microarrays was used to screen for novel downstream target genes of BCR/ABL1. Microarray technology permits parallel analysis of the expression of thousands of genes at a fixed time point (Duggan et al. 1999), and relies on the inherent ability of single-stranded DNA to base pair with a complementary sequence (Southern et al. 1999). Briefly, two different fluorescent dyes were used to label the test and

reference samples, which were then allowed to hybridize to known single-stranded cDNA or oligonucleotide probes immobilized on a glass slide (Figure 5). Because the hybridization occurs competitively, the amount of bound target is proportional to the level of expression (Duggan et al. 1999; Southern et al. 1999). Following hybridization and washing, the slides were scanned to measure the emission of the two dyes and the final outcome was presented as a color image of the spots with red and green representing up- and downregulation of the corresponding gene in the test sample relative to the reference. The expression of each gene was then quantified as the $\log_2$-ratio between the intensity values of the test and reference samples.

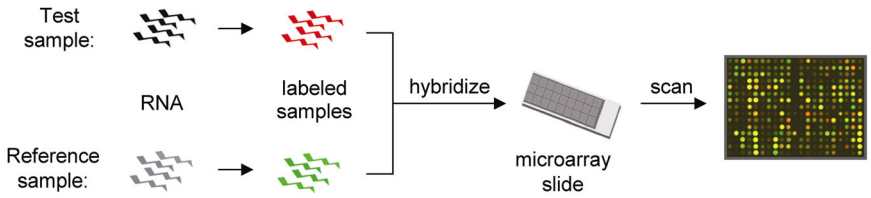


**Figure 5. Brief overview of microarray technology.** Total RNA extracted from test and reference samples are independently labeled with two different fluorescent dyes. The labeled samples are subsequently mixed and hybridized to a microarray slide. Following washing, the slides are scanned to quantify the emission of the two dyes, finally providing a color image with red and green representing up- and downregulation of the corresponding gene in the test relative to the reference sample. Yellow spots designate genes with an equal expression level in the test and reference samples.

In *Articles II* and *III*, cDNA microarrays containing 6,450 and 25,000 cDNAs, respectively, were used. Total RNA from the test samples was extracted, purified, and reversed transcribed into cDNA. The reference sample used in *Article II* was prepared from uninduced cells corresponding to the test sample, whereas *Article III* made use of a common human reference RNA. For the microarray analysis in *Article IV*, oligonucleotide slides of about 27,000 oligos were used. Because of the small amount of total RNA available for this investigation, both the test sample and human reference RNA were amplified using one round of amplification. To extract genes differentially expressed as a result of the *BCR/ABL1* fusion gene, the second study used several selection criteria based on ratio and spot intensity (*Article II*). In the third study, a mean difference statistic was used to quantify the response to imatinib, and a significance threshold of 5% false

discovery rate (FDR) was used (*Article III*). In the final microarray study (*Article IV*), the differentially expressed genes were extracted using the significance analysis of microarrays (SAM) method, which ascribes a score to each gene based on the change in expression level and the standard deviation of repeated measurements (Tusher et al. 2001). In the latter study, an FDR of 0% was used.

## Results and Discussion

Below follows a brief summary and discussion of the results obtained in the present study. For a more comprehensive description and discussion of the individual results, the reader is referred to the original articles in the final section.

### *Article I*

**Establishment and phenotypic characterization of human U937 cells with inducible P210 *BCR/ABL* expression reveals upregulation of CEACAM1 (CD66a)**

In this study U937 cells were established, allowing inducible expression of the P210 *BCR/ABL1* fusion gene. Two *BCR/ABL1*-inducible clones, c6 and e9, were obtained from independent transfections of U937 cells with a vector expressing the P210 *BCR/ABL1* gene under the control of a tetracycline-inducible promoter. In their uninduced state, no BCR/ABL1 protein could be detected in the c6 clone, whereas the e9 clone showed a weak leakage. Upon doxycycline treatment, both clones showed a rapid induction of BCR/ABL1 protein expression, but the c6 clone was able to induce a higher level of expression.

*BCR/ABL1* expression resulted in an early phosphorylation of STAT5A/B and a later and less pronounced phosphorylation of STAT1 and STAT3, as determined by western blot analysis. Activation of the JAK/STAT pathway, involving activation of mainly STAT5, has been shown in most *BCR/ABL1*-positive cell lines and primary CML cells (Carlesso et al. 1996; Ilaria and Van Etten 1996; Shuai et al. 1996; Chai et al. 1997). Furthermore, STAT5 was recently demonstrated to be essential for P210 *BCR/ABL1*-induced transformation of primitive murine cells (Hoelbl et al. 2006).

Phenotypic characterization of the *BCR/ABL1*-inducible clones, using different techniques such as the NBT reduction test and FACS analysis, revealed that *BCR/ABL1* expression induced a slight decrease in proliferation and viability without having a marked effect on cellular differentiation, cell cycle distribution, or the rate of apoptosis. Thus, BCR/ABL1 does not appear to confer a proliferative advantage under these conditions. Although BCR/ABL1 is generally considered to confer a proliferative advantage and/or increased survival, *in vivo* studies have not been able to confirm that CML cells proliferate more rapidly than their normal counterparts (Stryckmans et al. 1977; Thiele et al. 1997); nor has it been possibly to demonstrate an anti-apoptotic effect in primary CML cells (Amos et al. 1995; Albrecht et al. 1996).

Increased cell-surface expression of the CEACAM1 (CD66a) molecule was observed following induction of *BCR/ABL1*, which was in accordance with increased levels of transcription and translation. Using semiquantitative RT-PCR analysis, the increased transcription was found to be reversible upon treatment of c6 cells with imatinib. This was also true of K562 cells, showing that *BCR/ABL1* also regulates *CEACAM1* expression in another cellular context. The tyrosine kinase activity associated with CEACAM1 has been suggested to be involved in important cellular processes such as signal transduction, cell adhesion, proliferation, apoptosis, and immune responses (Hammarström 1999; Gray-Owen and Blumberg 2006). The increased expression of this protein could thus play an important role in the leukemogenic process. In this respect, it is interesting to note that members of the CEA family have been shown to be aberrantly expressed in Ph-positive ALL (Mori et al. 1995; Sugita et al. 1999; Carrasco et al. 2000). However, in normal hematopoiesis, the expression of CEACAM1 and some of its family members has been shown to change during differentiation along the myelocytic lineage (Grunert et al. 1998), and it is quite possible that the increased expression of CEACAM1 reflects an indirect upregulation by BCR/ABL1 caused by a small shift in the differentiation of U937 cells. This hypothesis is supported by studies showing that CEACAM1 is upregulated following retinoic acid-induced differentiation of U937 cells (Botling et al. 1995).

Thus, further studies are needed before a firm conclusion can be drawn about the mechanism behind the observed upregulation of CEACAM1 by BCR/ABL1 and its possible role in leukemogenesis. Even

so, the establishment of U937 cells with conditional expression of *BCR/ABL1* in the present study provides a valuable model for studying BCR/ABL1-mediated cellular effects, in particular early effects elicited by this fusion gene.

### *Article II*

### Identification of genes differentially regulated by the P210 *BCR/ABL1* fusion oncogene using cDNA microarrays

BCR/ABL1 activates a wide range of signal transduction pathways, but little is currently known about the effects on downstream target genes. In this study, cDNA microarrays were used to study the expression pattern of 6,450 genes in the *BCR/ABL1*-inducible U937 model system established and characterized during the first study (*Article I*).

A time-course experiment in which RNA was extracted 0, 12, 24, 36, and 48 h after induction of P210 *BCR/ABL1* and analyzed using cDNA microarrays, revealed approximately 60 genes to become upregulated upon *BCR/ABL1* expression. Five of these genes were validated by northern blot analysis and confirmed the previously reported upregulation of the *PIM1* and *JUN* oncogenes by *BCR/ABL1* (Burgess et al. 1998; Nosaka and Kitamura 2002). The most striking feature of the microarray analysis was that approximately one-third of the genes were found to be IFN-responsive, including *OAS1*, *IFIT1*, and *IFI16*, as well as the transcription factor genes *ISGF3G* and *STAT1*, with the latter two also activating other IFN-responsive genes (Lau and Horvath 2002; Platanias 2005). In addition, seven other genes were found to be IFN-responsive as determined by IFN-alpha treatment of the control cells, which also induced phosphorylation of STAT1, STAT3, and STAT5. Normally, IFNs are involved in protection against viruses, and also inhibit cell growth and modulate differentiation (Platanias 2005). Most likely, the observed effect did not result from an autocrine action of BCR/ABL1-induced IFNs because no IFNs could be identified in the culture media using ELISA; nor did conditioned media from *BCR/ABL1*-expressing cells induce expression of IFN-responsive genes when added to control U937 cells.

In principle, there are a number of possible mechanisms to explain the observed induction of IFN-responsive genes. Both IFN and BCR/ABL1 have been shown—by us and others—to use similar signaling pathways,

including phosphorylation of STAT molecules and of adaptor proteins such as CRKL and CBL (Voutsadakis 2000; Platanias 2005). This may result in the activation of a common subset of genes, and could provide a mechanism by which the observed clinical effects of treatment with IFN-alpha work in CML. Thus, the growth-inhibiting effects of IFN-alpha in CML may be the result of its interference with—and/or competition for—common substrates used by BCR/ABL1 to elicit its transforming properties. Alternatively, *BCR/ABL1* expression might activate cellular defense mechanisms or negative feedback mechanisms, of which IFN-inducible genes are a known part (Stark et al. 1998; Platanias 2005). In support of this, the *CISH* gene, a member of the SOCS family, was found to be upregulated by BCR/ABL1 in this study. The CISH protein has previously been reported to diminish transformation of *BCR/ABL1*-expressing cells (Tauchi et al. 2001). Interestingly, a study of purified progenitor cells from CML patients found several IFN-responsive genes to be upregulated during the CP, while there was marked downregulation with disease progression (Ohmine et al. 2001). Finally, it is also possible that the observed activation of IFN-inducible genes may be entirely cell-context dependent, i.e. specific for U937 cells.

BCR/ABL1 was also found to upregulate the expression of several genes with common functional properties. The expression profile included genes encoding transcription factors, kinases, and signal transduction molecules, and also genes regulating cell growth, differentiation, and apoptosis, for example—features previously implicated in BCR/ABL1-mediated transformation (Deininger et al. 2000; Melo and Deininger 2004; Ren 2005). Thus, in this article a number of genes were identified that may provide insights into early transcriptional alterations (< 48 h) following induction of *BCR/ABL1*.

### *Article III*

### Gene expression analysis of BCR/ABL1-dependent transcriptional response reveals enrichment for genes involved in negative feedback regulation

In this study, we used global gene expression profiling of imatinib-treated Ph-positive cell lines to identify genes that are dependent on *BCR/ABL1* expression. A particular advantage of this experimental set-up is that

problems associated with non-physiological and forced expression of the *BCR/ABL1* fusion gene to a large extent are circumvented.

Following treatment with imatinib, the five Ph-positive cell lines (but not control cell lines) showed reduced growth and viability, as well as reduced phosphorylation of BCR/ABL1 and STAT5, thus confirming the selective effect of imatinib in Ph-positive cell lines. cDNA microarray analysis was used to study the transcriptional response 3 and 12 h after blockage with imatinib, i.e. at time points before any effects on proliferation or viability could be detected. In total, 142 genes were identified as potential targets of BCR/ABL1-mediated signaling. Half of these genes were considered to be positively regulated by BCR/ABL1 and they included genes mainly involved in signal transduction via, for example, the JAK/STAT, MAPK, and TGFB signaling pathways. BCR/ABL1 was also found to affect genes involved in several metabolic pathways, which most likely reflects a restoration of the increased metabolic rate characterizing *BCR/ABL1*-transformed cells.

Interestingly, BCR/ABL1 was found to positively regulate several genes involved in negative feedback regulation of known signaling pathways. Among these were three members of the SOCS family, *CISH*, *SOCS2*, and *SOCS3*, of which *CISH* was also found to become activated upon *BCR/ABL1* expression in *Article II*. Normally, the SOCS family controls the strength and duration of cytokine-mediated signaling, particularly via the JAK/STAT pathway (Krebs and Hilton 2001; Naka et al. 2005). Based on the deregulated gene expression pattern, this study suggests that BCR/ABL1-mediated signaling activates negative feedback regulatory systems that might initially act to limit the tumor-promoting effects of this fusion gene. Aberrant expression of individual SOCS family members has previously been associated with BCR/ABL1 and CML (Tauchi et al. 2001; Schultheis et al. 2002; Liu et al. 2003; Radich et al. 2006), where, for example, *SOCS2* has been shown to be overexpressed in advanced stages of the disease (Schultheis et al. 2002; Radich et al. 2006) and *CISH* has been reported to suppress transformation of *BCR/ABL1*-transfected cell lines (Tauchi et al. 2001). BCR/ABL1 was also found to activate *PIM1*, a gene previously suggested to be involved in negative feedback regulation of STAT5 (Paukku and Silvennoinen 2004), and also *TNFAIP3* and *DUSP6*, which have been implicated in negative feedback regulation of NFκB activation and the ERK/MAPK pathway, respectively (Beyaert et al. 2000;

Platanias 2003). In summary, this article describes both new and previously known targets of BCR/ABL1-mediated signaling, and provides additional insights into the complex downstream effects of this fusion protein.

### *Article IV*

### Expression of P190 or P210 *BCR/ABL1* in cord blood CD34$^+$ cells leads to enhanced cell proliferation and differentiation towards the erythroid lineage

The two *BCR/ABL1* variants P190 and P210 are clinically associated with distinct types of leukemias in which different hematopoietic subpopulations are expanded, but only a few studies comparing the molecular effects of P190 and P210 BCR/ABL1-mediated signaling have been published (Ilaria and Van Etten 1996; Okuda et al. 1996; Advani et al. 2004). Moreover, most studies on BCR/ABL1-mediated signaling have been performed in cell lines or transgenic animal models. Thus, the functional and transcriptional effects of retroviral transduction of P190 and P210 *BCR/ABL1* into primitive human hematopoietic progenitor cells were investigated in this study.

A retroviral vector system was created to express either P190 or P210 *BCR/ABL1* in human umbilical CB CD34$^+$ cells. The results showed that expression of both P190 and P210 *BCR/ABL1* resulted in phosphorylation of STAT5A/B, and rapidly induced a substantially increased cell proliferation accompanied by differentiation towards the erythroid lineage. Although contradictory findings have been published (Zhao et al. 2001; Ramaraj et al. 2004), P210 BCR/ABL1 has previously been described to induce an erythroid phenotype (Chalandon et al. 2002). Given the requirement of STAT5 for BCR/ABL1-mediated leukemogenesis (Hoelbl et al. 2006) and the important role of STAT5A in the regulation of erythroid hematopoiesis (Schuringa et al. 2004), we speculate that STAT5 may be important for the observed differentiation towards the erythroid lineage.

Global gene expression profiling of P190 and P210 *BCR/ABL1*-transduced cells using oligonucleotide microarrays failed to identify any significant differences between the two fusion variants. Combining the gene expression profiles of both fusion genes and comparing them with MIG transduced cells allowed identification of 222 genes as potential targets of P190/P210 BCR/ABL1. In this study, *BCR/ABL1* expression was found to activate genes involved in, for example, the MAPK, and TGFB signaling

pathways, and to suppress the expression of genes involved in the immune response, cell communication, cell adhesion, and differentiation. In accordance with the erythroid lineage phenotype, both P190 and P210 BCR/ABL1 were found to suppress *CEBPA* and *CEBPD*, which are important for myeloid differentiation and which have also been reported to suppress leukemogenesis (Perrotti et al. 2002; Gery et al. 2005; Rosmarin et al. 2005; Ferrari-Amorotti et al. 2006). Furthermore, as in *Article III*, *SOCS2* was again found to be activated by BCR/ABL1.

In conclusion, expression of P190 and P210 *BCR/ABL1* had no detectable functional differences, and only subtle transcriptional differences, when expressed in the same cellular context. These findings also indirectly support a separate cellular origin of P190 and P210 BCR/ABL1-induced leukemias.

# CONCLUSIONS

The main findings of the present thesis can be summarized as follows:

### *Article I*

- Conditional expression of P210 *BCR/ABL1* in the myeloid U937 cell line results in activation of the JAK/STAT pathway, but does not confer a proliferative advantage or any marked effects on differentiation and apoptosis.

- Expression of P210 *BCR/ABL1* in U937 cells induces a reversible expression of the cell-surface marker CEACAM1, which has been implicated in cell adhesion and signal transduction, and may thus be important in the leukemogenic process.

- A widely requested U937 cell model with inducible expression of P210 *BCR/ABL1* was established.

### *Article II*

- Gene expression profiling of U937 cells with inducible P210 *BCR/ABL1* expression, identified upregulation of 61 potential target genes of BCR/ABL1-mediated signaling.

- BCR/ABL1 induces expression of a large number of IFN-responsive genes, which may indicate an overlap with IFN-induced signal transduction pathways, or activation of cellular defense or negative feedback mechanisms.

- *BCR/ABL1* expression affects genes encoding transcription factors, kinases, and signal transduction molecules, as well as genes regulating cell growth, differentiation, apoptosis, and cell adhesion.

### *Article III*

- Blockage of the P210 BCR/ABL1 tyrosine kinase activity in Ph-positive cell lines allowed identification of 142 genes as potential targets of BCR/ABL1-mediated signaling using global gene expression profiling.

- *BCR/ABL1* expression affects mainly genes involved in signal transduction, e.g. the JAK/STAT, MAPK, TGFB, and insulin signaling pathways, and in regulation of metabolism.

- BCR/ABL1 activates several genes involved in negative feedback regulation of important cell signaling pathways, which may possibly act to suppress tumor-promoting effects elicited by BCR/ABL1.

### *Article IV*

- Expression of both P190 and P210 *BCR/ABL1* in retrovirally transduced primitive human cells results in increased proliferative capacity and differentiation towards the erythroid lineage.

- Global gene expression profiling of P190 or P210 *BCR/ABL1* expression in primitive human cells revealed no major differences in the transcriptional response between the two fusion genes, but allowed identification of 222 genes as potential targets of both P190 and P210 BCR/ABL1-mediated signaling.

- Expression of P190 or P210 *BCR/ABL1* in primitive human cells induces very similar biological effects, which indirectly supports the hypothesis of a separate cellular origin for these fusion genes to explain their association with different leukemias.

# CONCLUDING REMARKS

More than four decades have passed since the original discovery of the Ph chromosome and our knowledge about this specific translocation has increased rapidly over recent years. It has been established that the Ph chromosome results in the formation of mainly three different *BCR/ABL1* fusion genes. The transforming properties of *BCR/ABL1* have been elegantly demonstrated in retroviral and transgenic animal models, and much work has been focused on trying to understand how BCR/ABL1 elicits its transforming properties. Without a doubt, it has emerged that there is a very complex biochemical basis for BCR/ABL1-mediated signaling with a number of intracellular pathways/proteins becoming activated or phosphorylated by the fusion protein. Most of this knowledge has, however, been gained by analyses of individual adaptor proteins and signal transduction molecules rather than by using more systematic approaches to address BCR/ABL1-mediated signaling.

A major aim of the present study was to identify BCR/ABL1-regulated genes. To this end, we developed three different experimental systems and used global gene expression analysis to identify such genes systematically. A recurrent and interesting finding in the three experimental systems was the deregulated expression of different SOCS family members, but otherwise there were only a few genes that overlapped in the different studies. While this can be at least partly explained by the different platforms and statistical methods used for gene extraction, it is more likely to be a result of the "different inherent properties" of the established experimental systems. The *BCR/ABL1*-inducible U937 cell line model is likely to help unravel the immediate transcriptional effects of BCR/ABL1-mediated signaling, thus giving us insight into early transcriptional effects elicited by BCR/ABL1 in cells never exposed to this fusion protein before. In contrast, the genes identified after blockage of the tyrosine kinase activity in immortalized Ph-positive cell lines are likely to reflect BCR/ABL1-mediated transcriptional effects in cells that have evolved to tolerate—and be dependent on—constitutive expression of *BCR/ABL1*.

The experimental system that perhaps most closely resembles cells in which Ph-postive leukemias originate is the model in which human $CD34^+$ CB cells are transduced with retroviruses encoding either P190 or

P210 *BCR/ABL1*. Apart from showing that both fusion gene variants induced a similar phenotypic and transcriptional response, providing circumstantial evidence for a "separate cellular origin" to explain their association with different types of leukemia, we identified 222 genes as potential BCR/ABL1 targets. *BCR/ABL1* expression was found to affect genes involved in, for example, the MAPK and TGFB signaling pathways, and in the immune response, cell communication, cell adhesion, and differentiation—i.e. cellular processes that are consistent with our current understanding of CML biology. Results from an ongoing gene expression study of $CD34^+$ selected primary CML CP cells in our laboratory support the relevance of the latter model. As outlined in *Article IV*, there was a 6-fold overabundance of differentially expressed genes between the two studies than would be expected by chance, suggesting that the retroviral expression of *BCR/ABL1* in human $CD34^+$ CB cells at least partly reflect aberrant transcriptional patterns present in primary CML cells.

In conclusion, the present study has identified a number of pathways/individual genes that become deregulated by BCR/ABL1 in different experimental systems. Further experimental studies, for example by RNA interference-based strategies, should help to elucidate the role of some of the identified genes in BCR/ABL1-mediated transformation. In addition, an increased knowledge of transcriptional programs underlying normal hematopoiesis is needed before the aberrant transcriptional profiles of *BCR/ABL1*-expressing cells can be put into a biologically meaningful context. Such efforts should provide additional pathogenetic insights into BCR/ABL1-mediated leukemogenesis and, hopefully, enable the development of novel targeted therapies that can be used either alone or as a complement to already available tyrosine kinase inhibitors.

# SVENSK SAMMANFATTNING
# (Summary in Swedish)

## Molekylärgenetiska och funktionella studier av *BCR/ABL1* fusionsgenen

Det finns tre sorters blodkroppar, röda, vita och trombocyter, som alla bildas från gemensamma stamceller i benmärgen. När stamcellerna delar sig bildas det antingen nya stamceller eller förstadier till blodkroppar som efter ytterligare delningar blir till mogna blodkroppar. Ibland uppkommer särskilda genetiska förändringar i något av dessa förstadier eller stamceller, vilket omvandlar normala celler till cancerceller. Vid leukemi trängs den normala blodbildningen undan av en kraftig expansion av dessa leukemiceller vilket orsakar brist på normala mogna blodkroppar samtidigt som en ökad mängd omogna vita blodkroppar släpps ut i blodet.

I Sverige insjuknar ungefär 1000 personer årligen i en akut eller kronisk leukemi, varav ca 100 fall utgörs av kronisk myeloisk leukemi (KML) (http://cancerfonden.se). Medan de akuta leukemierna ses hos både barn och vuxna förekommer kronisk leukemi främst bland vuxna. KML karaktäriseras av olika faser där den första kroniska fasen är ganska lätt att kontrollera, medan den slutliga blastfasen istället liknar en akut leukemi. I majoriteten av alla fall av KML uppkommer ytterligare genetiska avvikelser under sjukdomsutvecklingen, vilket man tror kan ha betydelse för leukemins framskridande.

De allra flesta fall av KML, samt 15-20% av akut lymfatisk leukemi (ALL), kännetecknas av en specifik kromosomavvikelse, Philadelphia (Ph) kromosomen. Denna specifika avvikelse uppkommer genom en translokation (utbyte av genetiskt material) mellan kromosomerna 9 och 22. Resultatet blir att tyrosinkinasgenen *ABL1* på kromosom 9 sätts ihop med *BCR* genen på kromosom 22, vilket leder till att antingen ett P190 eller P210 BCR/ABL1 fusionsprotein med förhöjd tyrosinkinasaktivitet bildas. Medan P190 BCR/ABL1 varianten främst är förknippad med ALL, ses P210 i både KML och i 30-50% av patienterna med Ph-positiv ALL. Under senare år har det utvecklats lovande läkemedel som syftar till att hämma aktiviteten av BCR/ABL1 proteinet i cellen. Tyvärr utgör dessa mediciner ingen bot mot

sjukdomen och många patienter utvecklar så småningom resistens mot dem vilket gör att leukemin återkommer.

För att förstå och så småningom bota denna sjukdom är det viktigt att känna till hur BCR/ABL1 proteinet signalerar och påverkar omvandlingen till leukemiceller. Senare års forskning har inriktat sig på att försöka förstå hur BCR/ABL1 ger upphov till leukemi och varför P190 och P210 BCR/ABL1 företrädesvis är associerade med olika typer av leukemier. Studier har visat att både P190 och P210 uppvisar transformerande egenskaper i olika experimentella system och ett flertal proteiner och signalvägar, viktiga för bl.a. reglering av celltillväxt och programmerad celldöd, har visats interagera med eller aktiveras av BCR/ABL1. Detta gör det sannolikt att *BCR/ABL1* uttrycket leder till aktivering eller nedreglering av ett flertal målgener. Mycket lite är emellertid känt om vilka dessa gener är.

Systematiska studier av de genetiska program och signalvägar som påverkas av BCR/ABL1 har saknats. I denna avhandling har olika experimentella system utvecklats för att studera hur signalering via P190 och P210 BCR/ABL1 proteinerna påverkar cellerna. Genom att använda olika cellbiologiska och molekylärgenetiska metoder har de fenotypiska och transkriptionella effekterna av BCR/ABL1 aktivering undersökts. De erhållna resultaten utgör ett bidrag till vår kunskap om de cellulära effekter och förändringar på gennivå som styrs av *BCR/ABL1* fusionen, vilket på sikt skulle kunna få betydelse för utvecklingen av nya behandlingsstrategier. Nedan följer en kort summering av resultaten från de publikationer och delarbeten som ingår i denna avhandling.

## *Artikel I*

### Etablering och karaktärisering av en human cellinje med inducerbart uttryck av *BCR/ABL1* genen

En stor del av dagens kunskap om hur BCR/ABL1 påverkar celler kommer från studier utförda på humana cellinjer eller cellinjer från möss som visar ett stabilt uttryck av denna fusionsgen. I detta delarbetet etablerades en human cellinje i vilken uttrycket av *BCR/ABL1* kan slås på genom att tillsätta doxycyklin till odlingsmediet. Fördelarna med detta system är att det blir möjligt att undersöka tidiga och omedelbara effekter av BCR/ABL1 signalering samtidigt som risken för klonal selektion, dvs. att enbart studera en grupp celler som erhållit någon tillväxtfördel, minskar. *BCR/ABL1* genen

fördes in i U937 celler som är en välstuderad leukemisk cellinje som kan fås att differentiera och mogna ut längs den myeloida vägen i hematopoesen (blodbildningen). Vi kunde visa att ett tidigt uttryck av BCR/ABL1 leder till fosforylering/aktivering av i huvudsak STAT5, men även av STAT1 och STAT3. Dessa molekyler är del av ett viktigt signalsystem som bl.a. tros påverka cellers tillväxt och överlevnad. Aktivering av STAT5 via BCR/ABL1 har även visats vara viktig/nödvändig för fusionsgenens möjligheter att förändra cellen. Uttryck av *BCR/ABL1* genen i detta modellsystem gav en något minskad cellexpansion, dvs. fusionsgenen tillför ingen tillväxtfördel vid dessa betingelser, medan ingen synlig påverkan av förmågan att mogna ut eller begå programmerad celldöd (apoptos) kunde ses. Slutligen visades även en reversibel ökning av cellytemarkören CEACAM1, vilket bekräftades i ett oberoende modellsystem. Medlemmar i samma familj som CEACAM1 molekylen har tidigare visats ha ett förändrat uttrycksmönster i Ph-positiv akut leukemi.

### *Artikel II*

#### Identifiering av gener med förändrad uttrycksnivå till följd av tidig påverkan av BCR/ABL1 signalering

I detta delarbetet användes det *BCR/ABL1* inducerbara U937 modellsystemet för att studera uttrycket/aktiviteten av ett mycket stort antal olika gener med hjälp av den s.k. microarray tekniken. Uttryck av *BCR/ABL1* fusionsgenen ledde till ökat uttryck av ca 60 olika gener, däribland gener inblandade i olika signalvägar samt gener med betydelse för tillväxt, utmognad och celldöd. Intressant nog utgjordes ungefär en tredjedel av de aktiverade generna av s.k. interferon-inducerade gener. Normalt aktiveras ett flertal signalvägar och gener av interferoner som bl.a. har betydelse för cellens försvar mot virus och även verkar tillväxthämmande. Flera olika tolkningar av dessa resultat är möjliga. Det är t.ex. tänkbart att BCR/ABL1 och interferon använder sig av delvis samma signalvägar, vilket resulterar i aktivering av samma gener. En annan möjlighet är att de interferon-inducerade generna speglar ett tidigt försvar mot *BCR/ABL1* genens påverkan i cellen.

## *Artikel III*

### Identifiering av gener med förändrad uttrycksnivå till följd av avstängd BCR/ABL1 signalering i Ph-positiva cellinjer

I det här delarbetet använde vi oss av Ph-positiva cellinjer för att studera hur BCR/ABL1 signalering påverkar genuttrycksmönstret i cellen. Detta delarbete utgår ifrån att en blockering av BCR/ABL1 aktiviteten i celler som anpassat sig till att leva med denna avvikelse bör vara fördelaktigare än att tillföra ett onormalt uttryck av fusionsgenen. Genom att behandla fem Ph-positiva cellinjer med imatinib som specifikt hämmar tyrosinkinasaktiviteten hos BCR/ABL1 proteinet, studerade vi hur genuttrycket i dessa celler förändrades jämfört med samma behandling av Ph-negativa cellinjer. Totalt identifierades ca 140 gener som beroende av BCR/ABL1 aktiviteten. Dessa gener var framförallt associerade med olika signalvägar (JAK/STAT, MAPK och TGFB) och reglering av cellens metabolism (ämnesomsättning). BCR/ABL1 befanns också öka aktiveringen av ett flertal gener involverade i s.k. ”negative feedback reglering” av viktiga signalvägar i cellen, vilken skulle kunna tyda på en funktion hos cellerna för att undertrycka fusionsproteinets påverkan.

## *Artikel IV*

### Uttryck av två olika *BCR/ABL1* varianter i humana stamceller leder till ökad cellexpansion och utmognad längs den erytroida linjen

De två olika varianter av *BCR/ABL1* som kallas P190 och P210 är huvudsakligen ansvariga för uppkomsten av olika typer av leukemier. Den mesta kunskapen om hur *BCR/ABL1* omvandlar normala celler till leukemiceller kommer från studier av P210 *BCR/ABL1* varianten i olika cellinjer eller djurmodeller. I detta delarbetet användes virus för att föra in de två olika fusionsgenerna i normala humana stamceller tagna från navelsträngsblod. Resultaten visar att såväl P190 som P210 *BCR/ABL1* uttrycket leder till fosforylering/aktivering av STAT5 samt en kraftig expansion av cellerna som efter några dagar åtföljs av en omogen utmognad längs den erytroida vägen som normalt ger upphov till röda blodkroppar. Vi visar också att de två varianterna av fusionsproteinet ger en liknande effekt på genuttrycksmönstret i cellen och 222 gener identifierades som påverkade av tidig BCR/ABL1 signalering. De sammantagna fynden talar för att de två fusions-

proteinerna inte skiljer sig åt beträffande sin biologiska påverkan när de uttrycks i samma cellpopulation. Detta ger ett indirekt stöd åt de resultat från tidigare studier som tyder på att P190 och P210 BCR/ABL1 avvikelserna måste uppkomma i olika cellpopulationer under blodcellsbildningen för att kunna ge upphov till olika sorters leukemi.

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