Functional Modeling of Genes Upregulated in Chronic Myeloid Leukemia

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Abstract

Chronic myeloid leukemia (CML) is caused by the transformation of a primitive hematopoietic cell by the BCR/ABL1 fusion gene that is formed through the chromosomal translocation t(9;22). CML is currently successfully treated with tyrosine kinase inhibitors targeting the ABL1 kinase domain. However, the CML stem cells are insensitive to this drug and a large fraction of patients will have relapse following discontinuation of the drug. Thus, improved therapeutic strategies are needed towards the ultimate goal of curing CML. In Article I, the functional role of supressor of cytokine signaling 2 (SOCS2) in CML and in normal hematopoiesis was investigated. Mice deficient for the Socs2 gene displayed normal steady-state hematopoiesis and hematopoietic stem cell (HSC) function. Transduction of bone marrow (BM) cells from Socs2-deficient mice with BCR/ABL1 and subsequent transplantation resulted in a CML-like disease indistuingishable from the disease in control mice, suggesting that SOCS2 is dispensible for normal hematopoiesis and CML pathogenesis. In Article II, global gene expression analysis and subsequent flow cytometric analysis of normal and CML BM cells showed that interleukin-1 receptor acessory protein (IL1RAP) was highly upregulated on the cell surface of CML cells, allowing prospective separation of candidate CML stem cells from normal hematopoietic stem cells. The use of a polyclonal IL1RAP antibody enabled antibody-dependent cellular cytotoxicity (ADCC) of CD34+CD38- CML cells in vitro. In Article III, the function of IL1RAP in normal hematopoeisis and in CML was investigated. Mice lacking Il1rap displayed lower myeloid steady-state cell counts. Overexpression of IL1RAP in cord blood cells, followed by transplantation into immunodeficient mice, resulted in increased levels of myelopoeisis. Finally, CD34+CD38- CML cells were found to respond strongly to IL1B stimulation in vitro. Together, the results suggest that IL1RAP is a promising target for novel therapeutic approaches in CML.

Key words: Chronic myeloid leukemia, SOCS2, IL1RAP, BCR/ABL1, leukemia models

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"A model is a lie that helps you see the truth."

Howard Skipper

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Abbreviations

ALL	Acute lymphoblastic leukemia
AML	Acute myeloid leukemia
AP	Advanced phase
BM	Bone marrow
BP	Blast phase
CLL	Chronic lymphoblastic leukemia
CLP	Common lymphoid progenitor
CML	Chronic myeloid leukemia
CP	Chronic phase
CSC	Cancer stem cell
FTI	Farnesyl protein transferase inhibitor
GH	Growth hormone
GMP	Granulocyte macrophage progenitor
HSC	Hematopoietic stem cell
LIC	Leukemia-initiating cell
LMPP	Lymphoid multi-potential progenitor
LSC	Leukemic stem cell
MEP	Megakaryocyte erythrocyte progenitor

MPP	Multi-potent progenitor
PB	Pheripheral blood
Ph chromosome	Philadelphia chromosome
QPCR	Quantitative polymerase chain reaction
SCT	Stem cell transplantation
TKI	Tyrosine kinase inhibitor
WBC	White blood cell counts

Articles included in the thesis

This thesis is based on the following articles, which are referred to in the text by their Roman numerals (I–III).

I **Hansen N, Ågerstam H, Wahlestedt M, Landberg N, Askmyr M, Ehinger M, Rissler M, Lilljebjörn H, Johnels P, Ishiko J, Melo JV, Alexander WS, Bryder D, Järås M, Fioretos T.** SOCS2 is dispensable for BCR/ABL1-induced chronic myeloid leukemia-like disease and for normal hematopoietic stem cell function. *Leukemia* 2013;27(1):130-135.

II **Järås M, Johnels P, Hansen N, Ågerstam H, Tsapogas P, Rissler M, Lassen C, Olofsson T, Bjerrum OW, Richter J, Fioretos T.** Isolation and killing of candidate chronic myeloid leukemia stem cells by antibody targeting of IL-1 receptor accessory protein. *Proc Natl Acad Sci* 2010;107(37):16280–16285.

III **Hansen N, Reckzeh K, Ågerstam H, Askmyr M, Högberg C, Gordon S, Rissler M, Richter J, Järås M, Fioretos T.** IL1RAP promotes myelopoiesis and its upregulation on candidate CML stem cells is associated with increased IL1 sensitivity. *Manuscript.*

Other related publications

Askmyr M, Ågerstam H, Hansen N, Gordon S, Arvanitakis A, Rissler M, Juliusson G, Richter J, Järås M, Fioretos T. Selective killing of candidate AML stem cells by antibody targeting of IL1RAP. *Blood* 2013; 2;121(18):3709-3713.

Ågerstam H, Järås M, Andersson A, Johnels P, Hansen N, Lassen C, Rissler M, Gisselsson D, Olofsson T, Richter J, Fan X, Ehinger M, Fioretos T. Modeling the human 8p11-myeloproliferative syndrome in immunodeficient mice. *Blood* 2010;116(12): 2103-2111.

Preface

Chronic myeloid leukemia (CML) is a myeloproliferative disorder arising from a hematopoietic stem cell that has acquired the Philadelphia chromosome, which was discovered in 1960 as the first chromosomal abnormality consistently associated with a specific type of cancer. The fusion gene *BCR/ABL1* is transcribed from the breakpoint of the Philadelphia chromosome and 40 years after its discovery, a targeted therapy (imatinib), which inhibits the BCR/ABL1 fusion protein, was introduced in the clinic. Despite the remarkable success of imatinib and other tyrosine kinase inhibitors as therapy for CML, the CML stem cells are insensitive to the treatment and following drug discontinuation, a large proportion of the patients will experience relapse. Thus, an improved understanding of the disease mechanisms and new therapeutic strategies will be needed for complete cure of CML.

The aim of the work in this thesis was to study molecular mechanisms responsible for the emergence of CML. For this, we used various models of CML and normal hematopoiesis to assay the function of specific genes found to be upregulated in CML. This thesis is divided into three sections, the first of which provides a background to the subject by discussing normal hematopoiesis, CML, and the different experimental models available for the study of CML. The second part describes the studies performed during the work for the thesis and the conclusions we have drawn from them. The third and last part constitutes the articles that this thesis is based upon.

Lund

October, 2013

Summary in Swedish

Kroppens blodceller nybildas kontinuerligt ifrån blodstamceller (hematopoetiska stamceller) i benmärgen i en process som kallas hematopoes. Detta sker genom att stamcellerna ger upphov till både nya stamceller och till celler som mognar ut till olika typer av blodceller. Blodstamcellerna bildar en rad olika mogna celltyper med olika funktioner, så som syresättning av kroppens vävnader (röda blodkroppar, erytrocyter), förmåga att bekämpa infektioner (vita blodkroppar, leukocyter) och för att hejda blödningar (blodplättar, trombocyter). Cancer i den blodbildande benmärgen (leukemi) drabbar varje år ca 1200 personer i Sverige. Vid leukemi uppstår en okontrollerad delning av omogna blodceller, ofta i kombination med att dessa celler inte mognar ut eller dör undan på ett kontrollerat sätt. När detta sker uppstår brist på normala blodceller, vilket i sin tur bland annat kan leda till trötthet, blödningar, förstorad mjälte och ökad känslighet för infektioner. När celler delar sig organiseras allt DNA i 46 kromosomer och en typ av genetisk förändring som är vanlig i leukemier är så kallade translokationer, som uppstår när två kromosomer går sönder och felaktigt läker samman med varandra. I brytpunkterna mellan de olika kromosomdelarna, kan två olika gener sammanfogas och bilda en fusionsgen. En translokation mellan kromosomerna 9 och 22 kan skapa en fusionsgen där generna *BCR* och *ABL1* sammanfogas till *BCR/ABL1*, vilken cellen översätter till ett fusionsprotein som felaktigt aktiverar flera av cellens signalvägar. Om fusionsgenen *BCR/ABL1* uppstår i en blodstamcell kan den orsaka kronisk myeloisk leukemi (KML) som kännetecknas av en förhöjd produktion av en typ av vita blodkroppar benämnda ”myeloida celler”. Ungefär 80 personer om året insjuknar i KML i Sverige och behandlas med läkemedlet imatinib som blockerar BCR/ABL1. Imatinib har inneburit dramatiskt förbättrade utsikter för patienter med KML och framhålls idag som ett av de bästa exemplen där biologisk kunskap om cancerceller har möjliggjort utvecklingen av en målstyrd behandling. Behandlingen av KML är dock fortfarande förknippat med problem eftersom alla patienter inte svarar på behandlingen med imatinib. Dessutom krävs i de flesta fallen en livslång behandling eftersom en majoritet av patienterna återfaller i KML om medicineringen avbryts. Detta beror sannolikt på att leukemistamcellen i KML överlever behandlingen.

Den övergripande målsättningen med den här avhandlingen har varit att öka kunskapen om hur KML uppstår och hitta nya angreppspunkter för målstyrd terapi. För att göra detta har vi använt olika leukemimodeller som möjliggör detaljerade studier av sjukdomsbiologin vid KML. Avhandlingens första delarbete (Article I) syftade till att undersöka effekten av genen *SOCS2* som är uppreglerad hos KML-patienter. SOCS2-

proteinet fungerar som en broms i cellens signalsystem och har till uppgift att reglera svaret på specifika signalmolekyler. I delarbetet undersökte vi specifikt ifall SOCS2 även påverkade signaleringen som BCR/ABL1 startar i cellen. För att undersöka detta använde vi benmärgsceller från en mus som saknar *Socs2* genen (*Socs2* KO). Genom att transplantera dessa celler till normala möss, fann vi att även benmärgsstamceller utan SOCS2 ger upphov till alla de celltyper som normalt bildas i benmärgen. Dessa resultat tyder på att SOCS2 inte är viktigt för normal hematopoes eller att andra gener kan kompensera förlusten av SOCS2. Genom att uttrycka fusionsproteinet BCR/ABL1 i normala och i *Socs2* KO celler och sedan transplantera cellerna till normala möss, fann vi att mössen utvecklade samma KML-liknande sjukdomsbild även om de saknade SOCS2. Därför kunde vi dra slutsatsen att SOCS2 sannolikt inte har någon central betydelse för utvecklingen av KML.

I det andra delarbetet (Article II) var målet att hitta ett protein på cellytan som går att använda som markör för att skilja KML-stamceller från normala stamceller och potentiellt även som måltavla för behandling riktad mot KML-stamcellerna. För att göra det använde vi en metod (microarray) som gör det möjligt att jämföra uttrycket av ett stort antal gener, till att undersöka vilka gener som var högt uttryckta på cellytan av KML-celler jämfört med normala celler. I arbetet identifierade vi ett ökat uttryck av proteinet IL1RAP på cellytan av KML-celler jämfört med normala celler. IL1RAP är ett protein som utgör en viktig del av det komplex på cellytan som signalmolekylen IL1 binder till. Genom att använda antikroppar som binder till IL1RAP kunde vi även visa att denna cellytemarkör kan användas för att sortera ut och därmed särskilja KML-stamceller från normala stamceller. I arbetet kunde vi också visa att KML-stamceller selektivt kunde avdödas genom inbindning av en polyklonal antikropp riktad mot IL1RAP.

I det tredje delarbetet (Article III) undersökte vi vilken betydelse IL1RAP har för normal hematopoes och för celler som uttrycker BCR/ABL1. När vi undersökte blodet i möss som saknar *Il1rap*-genen (*Il1rap* KO möss), upptäckte vi lägre nivåer än normalt av myeloida celltyper i blodet. När vi sedan använde virus för att uttrycka IL1RAP i normala mänskliga celler som sedan transplanterades till en typ av möss med mycket försvagat immunförsvar (immundefekta möss), såg vi att fler av de omogna humana cellerna med IL1RAP utvecklades till myeloida typer av blodceller, än kontrollcellerna gjorde. Därav kunde vi dra slutsatsen att om omogna blodceller får förhöjt utryck av IL1RAP, kan detta påverka cellens förmåga att bli myeloida celler. Vi upptäckte även att de allra mest primitiva cellerna hos KML patienter, som har mycket högre nivåer av IL1RAP på cellytan än normala celler, reagerade kraftigt på signalmolekylen IL1 medan normala celler inte märkbart påverkades. Detta tyder på att de höga nivåerna av IL1RAP på KML-stamceller har betydelse för sjukdomsutvecklingen av KML genom att påverka hur cellen reagerar på IL1. Därför är det också sannolikt att behandlingsstrategier som blockerar IL1RAP kan komplettera imatinib i behandlingen av KML-patienter.

Sammanfattningsvis har studierna i den här avhandlingen bidragit till en ökad förståelse av de cellulära förändringarna som orsakar KML. Genom att undersöka två proteiner som är uppreglerade i KML-celler, har vi kunnat visa att ett den ena av dessa (IL1RAP) sannolikt har betydelse för utvecklingen av KML, genom att bl.a. öka leukemicellernas svar på signalmolekylen IL1. Därför kan IL1RAP vara ett lämpligt cellyteprotein att blockera vid utvecklingen av nya behandlingsmetoder i KML.

Introduction

Hematopoiesis

The gradual development of all mature blood cells from hematopoietic stem cells (HSCs) is called hematopoiesis and a trillion (1,000,000,000,000) new cells of different types migrate from the bone marrow (BM) into the peripheral blood (PB) every day (Ogawa 1993). During their lifetime, these cells are responsible for a wide variety of functions such as oxygen transport by erythrocytes, blood clotting after vessel damage by thrombocytes, production of antibodies by B-cells, killing of cells infected by virus or potentially damaged cells by T-cells and NK-cells, and innate immunity from macrophage/monocytes and granulocytes. In order to produce this variety of cells, the hematopoietic system is hierarchically organized with the self-renewing multi-potent HSCs at the top of the hierarchy. According to the classical model of hematopoiesis, HSCs differentiate into multi-potent progenitors (MPPs) that increase their proliferative rate while losing self-renewal capacity (Reya et al. 2001). More lineage-committed progenitors are gradually formed, and they have been defined based on the expression of different transcription factors and surface markers. These progenitors are generally named according to their potential to differentiate into different mature cell types, and some well-studied progenitor types are: common lymphoid progenitors (CLPs), which differentiate into B-cells, T-cells, and NK-cells; and granulocyte macrophage progenitors (GMPs). These give rise to innate immune cells. The megakaryocyte erythrocyte progenitors (MEPs) are precursors for thrombocytes and erythrocytes (Kondo et al. 1997; Akashi et al. 2000; Reya et al. 2001).

The differentiation pathways and surface markers needed to identify the various progenitor populations were first defined in the mouse, in the classical hematopoietic model described in Figure 1 (Kondo et al. 1997; Akashi et al. 2000; Reya et al. 2001). However, this model has been challenged after the identification of a lymphoid multi-potential progenitor (LMPP), which can give rise to the lymphoid, granulocytic, and monocytic lineages but not to erythroid cells (Adolfsson et al. 2005). This spurred further detailed investigations and proposal of alternative models for murine hematopoiesis (Giebel & Punzel 2008; Kawamoto et al. 2010). Human hematopoiesis was first believed to be consistent with the classical model. However, the discovery of a human cell population corresponding to the LMPP in the mouse prompted a revision of this model (Doulatov et al. 2010; Doulatov et al. 2012). Furthermore, it has recently been suggested

that the human hematopoietic model, established after the discovery of LMPPs, needs to be revised further, as LMPPs only give rise to neutrophilic granulocytes, while other types of granulocytes, macrophages, and erythrocytes develop from myeloid progenitors (Görgens et al. 2013).

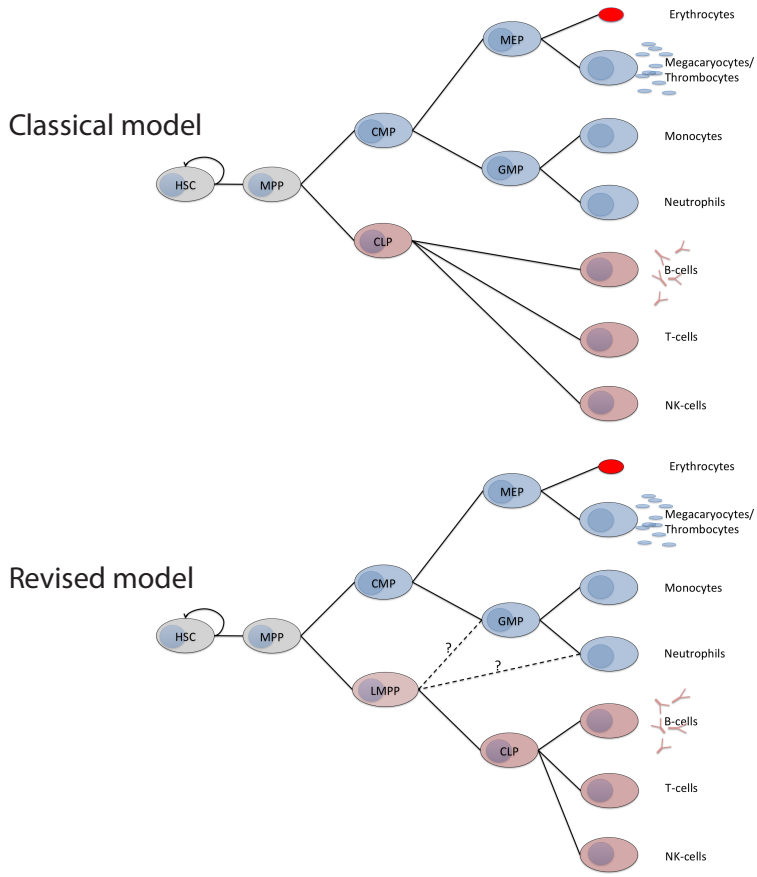

Figure 1. The classical model of hematopoiesis and a revised model with the recently described MLLPs and their possible differentiation pathways indicated. In the classical model of hematopoiesis, self-renewing hematopoietic stem cells (HSCs) differentiate into multi-potent progenitors MPPs, which lack self-renewal potential and produce common lymphoid progenitors (CLPs) and common myeloid progenitors (CMPs). The CLPs give rise to B-cells, T-cells, and NK-cells, while the CMPs give rise to megakaryocyte erythrocyte progenitors (MEPs) and granulocyte macrophage progenitors (GMPs). MEPs give rise to erythrocytes and megakaryocytes while monocytes and neutrophils are produced by GMPs. The discovery of lymphoid multi-potential progenitors (LMPPs) with lymphoid but also a limited myeloid lineage potential, caused a revision of the hematopoietic model. However, it is still not fully understood which subsets of myeloid cells LMPPs can give rise to and how these differentiation pathways are arranged.

A major limitation of the hematopoietic models is that they are constructed through functional evaluation of progenitor populations defined by expression of specific surface markers. However, these surface markers are not necessarily relevant for the cellular function of the progenitors and may not fully reflect gradual processes in the differentiating progenitor cells. Thus, the apparently homogeneous progenitor populations may be more heterogeneous than implied by the model and contain cells with slightly different properties and lineage potentials. Characterization of global molecular programs in order to better understand the differentiation process may be a feasible way to overcome this limitation, but the efforts so far have still been hampered by our inability to prospectively separate HSCs and progenitor cells without relying on individual surface markers and flow cytometry.

Cytokine signaling in hematopoiesis

Hematopoietic progenitor cells are guided in their decisions to proliferate, differentiate, and survive by external stimuli from cytokines. Each cytokine binds its specific receptor, which is expressed differentially among cell types, and can thereby activate downstream signaling pathways in a cell-specific manner. Despite numerous different receptors and several downstream pathways, cell signaling is, however, characterized by crosstalk between pathways, resulting in complex networks of intracellular signal transduction. A few pathways relevant to this thesis are outlined below.

KIT receptor

One of the best-known cytokines that regulate hematopoietic stem and progenitor cells is the stem cell factor (SCF), which signals through the KIT receptor (c-KIT) and can, by itself, regulate maintenance of HSCs, although a proportion of HSCs are generated and survive even in the absence of functional KIT (Li & Johnson 1994; Linnekin et al. 1995; Thoren et al. 2008). Stimulation of hematopoietic stem and progenitor cells with SCF is synergistic in combination with one of a number of other cytokines, for example interleukin-3 (IL3), granulocyte colony-stimulating factor (GCSF), and thrombopoietin (TPO) (Bernstein et al. 1991; Metcalf & Nicola 1991; Kobayashi et al. 1996), allowing *in vitro* expansion of progenitor cells and development of several myeloid cell types *in vivo* (Broudy 1997).

JAK/STAT

Janus kinase/signal transducer and activator of transcription (JAK/STAT) signaling, is another important pathway that can be activated by a number of different cytokines, among others interleukin-6 (IL6) and erythropoietin (EPO). The JAK/STAT pathway is normally activated by cytokines that bind and oligomerize cell-surface receptors, allowing them to bind and facilitate cross-phosphorylation of JAK proteins (Dorritie et al. 2013). Once JAK is activated, it phosphorylates STATs, which are then able to enter the nucleus

and initiate transcription of a number of target genes, including the suppressor of cytokine signaling (*SOCS*) family. The SOCS family act like classical feedback inhibitors of signaling (Krebs & Hilton 2001; Aaronson & Horvath 2002). The importance of JAK/STAT proteins can be exemplified by STAT3, which is activated by several cytokines including IL3 and GCSF (Duarte & Frank 2000). Furthermore, truncation of both *STAT5A* and *STAT5B* genes in mice results in lower B-cell counts, reduces the cellular response to erythropoietin, and may cause anemia (Teglund et al. 1998; Socolovsky et al. 1999; Sexl et al. 2000; Socolovsky et al. 2001), while complete deletion of the *STAT5* genes blocks development of several hematopoietic cell types and results in embryonic lethality (Hoelbl et al. 2006).

Interleukin-1 receptor

The Interleukin-1 receptor (IL1R)/Toll-like receptor (TLR) family includes both TLRs, which are mainly expressed on immune cells, and the IL1Rs, which have mainly been studied in the context of inflammation (O'Neill 2008). The two IL1 isoforms, IL1A/B, can bind IL1R type 1, which then forms a complex with the IL1R accessory protein (IL1RAP), resulting in recruitment of the adaptor protein MyD88 (Brikos et al. 2007). Following this, the proteins IRAK1 and IRAK4 bind to this complex and become phosphorylated, which in turn activates several downstream processes, leading to the activation of the nuclear factor kappa-light-chain-enhancer of activated B-cells 1 (NFKB or NF- κB) complex (O'Neill 2008). Although NFKB is a well-known transcription factor mainly studied in the context of immune response and T-cell development, only a few studies have been published on the role of NFKB signaling in hematopoiesis (Gerondakis et al. 2012). At the HSC level, NFKB has been suggested to regulate HSC self-renewal (Zhao et al. 2012a). Myelopoiesis may also be enhanced by NFKB through cooperation with C/EBPA (Friedman 2007).

Leukemia

Cancers, including leukemia, are caused by genetic aberrations that alter normal mechanisms regulating self-renewal, cellular proliferation, differentiation, and programmed cell death (apoptosis) (Hanahan & Weinberg 2011). The term leukemia refers to malignancies affecting immature hematopoietic cells, and it has traditionally been divided into acute and chronic leukemias, depending on various morphological and clinical characteristics. Acute leukemias are typically manifested by an accumulation of immature blast cells due to a block in differentiation, while chronic leukemias mainly show an increase in mature cells in PB and BM due to increased proliferation among the progenitors. Leukemias are subdivided further, based on which lineage(s) the cells differentiate into. Thus, patients with expansion of myeloid cells can be divided into having acute myeloid leukemia (AML) with immature myeloid blasts or chronic myeloid leukemia (CML) with an expansion of myeloid cells at all stages of maturation and, for the definition of CML, the presence of the *BCR/ABL1* fusion gene. The corresponding classification for lymphoid (B- and T-cell) leukemia is acute lymphoblastic leukemia (ALL) and chronic lymphoid leukemia (CLL). Currently, the classification of myeloid leukemias and neoplasms into clinically and scientifically relevant entities is based on genetic, morphological, cytochemical, and immuno-phenotypic characteristics with the genetic alterations playing an increasingly important role for the diagnosis of these malignancies (Vardiman et al. 2009). Myeloproliferative neoplasms and myelodysplastic syndromes are other important disease entities that mainly affect the myeloid lineage and that have the capacity to progress into AML (Vardiman et al. 2009).

Leukemia stem cells

It is still debated from which cell types different leukemias arise and are maintained. Leukemic stem cells (LSCs) can be defined as cells that have the capacity to both self-renew and propagate cell populations expanded in the leukemia (Reya et al. 2001; Dick & Lapidot 2005). The discoveries leading to the current LSC concept were made by transplantation experiments where primary AML cells were transplanted into immunodeficeint mice (Lapidot et al. 1994; Bonnet & Dick 1997). Different AML cell populations were sorted and transplanted into non-obese diabetic/severe combined immunodeficiency disease (NOD/SCID) immune-incompetent mice to test whether only specific cell populations were able to propagate the leukemia. The most primitive CD34+CD38- fraction of cells was the only one that could reconstitute the leukemia, and it was proposed that the LSCs in AML were transformed normal primitive cells (Bonnet & Dick 1997). LSCs are not only found in AML, but they have also been found in various other leukemias and malignant disorders. In MDS with 5q deletions, the cell of

origin and the subsequent LSCs are found in the CD34+CD38- compartment (Nilsson et al. 2000). In B-ALL, various B-cell progenitor and blast populations, including the abnormal CD34+CD38-CD19+ population, have LSC activity (Cobaleda et al. 2000; Castor et al. 2005; le Viseur et al. 2008). The nature of the LSCs in AML was questioned a decade after their discovery, when Taussig et al. (2008) showed that the more mature CD34+CD38+ cell population in AML patients also contained LSCs. Furthermore, AML patients with NPM1 mutations even had their LSC activity in the CD34- cell population (Taussig et al. 2010). Although controversy remains, many of the diverse results could be explained by variations in the experimental systems used to study the LSCs; for example, treating recipient mice with immunosuppressing antibodies allows engraftment of CD38+ AML cells (Taussig et al. 2008). In the end, it is still uncertain which cells propagate AML, but there is a correlation between high engraftment potential and worse clinical outcome (van Rhenen et al. 2005; Pearce et al. 2006; Gentles et al. 2010; Eppert et al. 2011). Interestingly, NFKB is activated in primitive AML LSCs, and inhibition of this transcription pathway has led to successful eradication of CD34+CD38- cells in vitro (Guzman et al. 2001; Guzman et al. 2002; Guzman et al. 2005; Kuo et al. 2013).

When the LSC concept was first used to define the cells propagating a leukemia, they were termed LSCs, as they, like normal stem cells, have the potential for unlimited self-renewal and the ability to drive the leukemogenesis (Reya et al. 2001). The LSC concept does not, however, imply that the leukemic clone necessarily has to arise or share its surface marker profile with a normal stem cell. In fact, there are several examples where differentiated progenitors have been proven to regain self-renewal and become LSCs (Cozzio et al. 2003; Huntly et al. 2004; Krivtsov et al. 2006). Perhaps much confusion could have been avoided if another term, such as leukemia-propagating or leukemia-initiating cells (LICs), had been used instead of LSCs.

Chronic myeloid leukemia

In 1960, CML was the first cancer to be associated with a causative chromosomal aberration, when a minute chromosome named the Philadelphia chromosome (Ph chromosome), was discovered (Nowell & Hungerford 1960). The Ph chromosome is the result of a reciprocal translocation, t(9;22), which forms the *BCR/ABL1* fusion gene at its breakpoints (Rowley 1973; Heisterkamp et al. 1985; Shtivelman et al. 1985; Grosveld et al. 1986; Mes-Masson et al. 1986). Later mouse studies established that the expression of the *BCR/ABL1* fusion gene causes a CML-like disease in mice, and that the tyrosine kinase activity of BCR/ABL1 is indispensable (Daley et al. 1990; Lugo et al. 1990). Compared to AML, which is a heterogeneous disease caused by several different genetic aberrations, CML is always initiated by the *BCR/ABL1* fusion gene. Since its original discovery, CML and the disease-causing *BCR/ABL1* fusion gene have not only provided a framework for the study of leukemia, but have also provided an important basis for the study and dissection of several other tumor types. Several molecular and cellular characteristics of CML are discussed in the sections below.

Clinical characteristics of chronic myeloid leukemia and current treatment options

When diagnosed, CML often manifests in symptoms such as tiredness and clinical findings of an enlarged spleen accompanied by elevated white blood cell counts (WBC). However, today several cases are discovered accidentally from an elevated WBC count at a routine check-up performed for other medical reasons (Randolph 2005). The actual diagnosis of CML requires the presence of a t(9;22), or a confirmation that the *BCR/ABL1* fusion gene is present[1]. If left untreated, CML typically progresses through three phases. First, there is a chronic phase (CP), which manifests in an abnormal increase in mature granulocytes in the peripheral blood. After 3–5 years, the CP progresses into an accelerated phase (AP), with increasing numbers of immature myeloid progenitors. Finally, a blast phase (BP) (also called blast crisis or BC) will occur, with a block in differentiation that causes massive accumulation of immature blasts in both PB and BM (Sawyers 1999; Jabbour & Kantarjian 2012).

[1] According to the Swedish national treatment recommendations from 2010. http://www.sfhem.se/

Depending on the accumulation of either myeloid blasts (representing about 70% of all CML BP cases) or lymphoid blasts (30% of the cases), the BP is referred to as either 'myeloid BP' or 'lymphatic BP' (Randolph 2005; Jabbour & Kantarjian 2012). The blast accumulation, with its severe side effects, will be lethal within a few weeks if left untreated.

First-generation tyrosine kinase inhibitors

In 1996, a novel tyrosine kinase inhibitor (TKI) that blocks ABL1 activity was described (Druker et al. 1996), and it was later shown to be a very effective new treatment for CML (Druker et al. 2001). In long-term evaluations, this TKI called imatinib, has proved to effectively suppress the disease and induce remission in most patients (Druker et al. 2006). Since then, imatinib (or other TKIs) has become the recommended standard for the treatment of CP CML (Baccarani et al. 2013). The development of imatinib was made possible by a detailed characterization of the BCR/ABL1 fusion protein and the ability of different chemical compounds to block the ABL1 kinase activity. To date, imatinib most likely provides the best example of a truly targeted and successful therapy of any malignant disorder. Despite the remarkable success of imatinib, the majority of patients will experience relapse of the disease if the TKI treatment is discontinued, even after achieving undetectable levels of *BCR/ABL1* in BM and PB (Merante et al. 2005; Rousselot et al. 2007; Mahon et al. 2010). The most likely explanation for the recurrence of CML in patients stopping treatment with imatinib after achieving complete molecular remission, is a reservoir of residual CML stem cells that survive TKI treatment (Diamond & Melo 2011).

So far, it has not been possible to predict when the TKI therapy can be safely discontinued, but a fast decrease in *BCR/ABL1*-positive cells after initiation of therapy is believed to indicate a lower risk of relapse (Hughes et al. 2010). Other trials have indicated that in patients who achieve molecularly undetectable leukemia, which is defined as less than 0.0032% (4.5 log) *BCR/ABL1* transcripts per ABL1 transcript (as detected by quantitative polymerase chain reaction (QPCR)) (Baccarani et al. 2013), for a period of 2 years, between 50% and 60% will relapse after withdrawal of imatinib (Mahon et al. 2010; Ross et al. 2013). Thus, despite the revolutionary improvements in outcome following the introduction of TKIs in CML, additional therapeutic strategies are needed to meet the ultimate goal of a cure.

Second-generation tyrosine kinase inhibitors

Despite the success of imatinib in the treatment of CML, about 5% of patients in CP and virtually all of the responding patients in BP, will relapse with disease (Druker et al. 2006; Hochhaus et al. 2009). Relapses can have a number of explanations including upregulation of *BCR/ABL1*, low intracellular concentration of imatinib due to poor bioavailability or poor cellular uptake, other activated signaling pathways, or mutations at the active site of ABL1 (Diamond & Melo 2011). Imatinib binds competitively to the adenosine triphosphate (ATP) binding site on ABL1, thereby inhibiting the kinase activity of the BCR/ABL1 fusion protein (Schindler et al. 2000). Different point mutations at the active site of ABL1 can therefore prevent binding of imatinib, and are often found in relapse patients who have acquired resistance to therapy (Hochhaus et al. 2002). Today, several new small molecules have been developed with the aim of inhibiting ABL1, and they enable treatment of patients with ABL1 mutations. Using one of the second-generation TKIs (nilotinib or dasatinib) to treat relapsing patients, it is now possible to overcome most mutations in BCR/ABL1 except for T315I (Shah et al. 2004; Golemovic et al. 2005; Talpaz et al. 2006; Kantarjian et al. 2006). However, with the introduction of ponatinib, it has recently become possible to successfully treat also patients with the T315I mutation (O'Hare et al. 2009; Cortes et al. 2012).

Allogeneic stem cell transplantation

Before the introduction of TKI treatment in CML, allogeneic stem cell transplantation (SCT) remained the only curative treatment option (Sawyers 1999). As referred to earlier, this is likely to be still true in most cases, although a fraction of CML patients have been off drug treatment for several years without detectable BCR/ABL1 transcripts (Mahon et al. 2010). In patients diagnosed with BP CML or patients who develop resistance to second-generation TKIs, allogeneic SCT is still the recommended treatment of choice (Jain & van Besien 2011; Jabbour & Kantarjian 2012;). It is worth mentioning, however, that allogeneic SCT is associated with high treatment-related mortality and risk of graft-versus-host disease which, combined with the occurrence of relapse after transplant, results in a five-year survival ranging from 30% to 90%, depending on various risk factors (Jabbour et al. 2011; Kantarjian et al. 2012). Due to the risks associated with allogeneic SCT, and the challenge to find suitable HSC donors, it is still difficult to treat CML at advanced stages.

Blast phase and known secondary genetic changes

The exact mechanism and cellular effects leading to progression of CML into BP remain elusive, despite the fact that there have been several studies. Upregulation of *BCR/ABL1* has been suggested as one factor contributing to disease progression, and BCR/ABL1 is known to increase the rate of mutagenesis (Nowicki et al. 2004). As might be expected, CML cells in BC generally have a higher number of genetic aberrations than in CP (Johansson et al. 2002; Mullighan et al. 2008). As a consequence, several genes (e.g. *RUNX1*, *ASXL1*, *WT1*, *NRAS*, *KRAS*, and *TET2*) are deleted or mutated in a proportion of BP patients (Feinstein et al. 1991; Sill et al. 1995; Grossmann et al. 2011; Makishima et al. 2011; Zhao et al. 2012b). In addition, deletion of the transcription factors *IKZF1* and *CEBPA*, which are essential for differentiation into lymphoid and myeloid lineages, respectively, provide examples of genetic aberrations that may contribute to induction of the differentiation block required for lymphoid or myeloid blast formation (Wagner et al. 2006; Mullighan et al. 2008). Genetic events leading to gain of function of affected proteins may also contribute to progression of CML. For instance, formation of a secondary *NUP98/HOXA9* or *MSI2/HOXA9* fusion gene have been found important for the disease progression in CML (Yamamoto et al. 2000; Dash et al. 2002; Barbouti et al. 2003).

CML cells have an increased level of non-homologous end-joining, leading to increased susceptibility to chromosomal aberrations (Chakraborty et al. 2012). As a possible result, aberrations like +8, i(17q), and also an extra Philadelphia chromosome, are associated with progression to BP (Mitelman et al. 1976). In addition to the existence of an extra Philadelphia chromosome in patients with BP, increased *BCR/ABL1* expression in both the bulk leukemic cells and in cells with a GMP phenotype is associated with progression to BP (Gaiger et al. 1995; Jamieson et al. 2004). Cell line experiments have also shown that higher *BCR/ABL1* expression reduces cell adherence and increases transforming effects such as cytokine independence and migration (Barnes et al. 2005). These findings indicate that upregulation of *BCR/ABL1* may be involved in the transformation of CML to BP. Despite the finding of several genetic events that are associated with progression of CML to BP, this process is not yet fully understood, but the introduction of massive parallel sequencing will most likely give a more comprehensive view of the genetic landscape of CML in BP. Moreover, it may well be true that the most frequent cytogenetic changes observed in CML BP, i.e. trisomy 8 and isochromosome 17q, affect the expression levels of multiple genes that contribute to the progression of CML CP into BP.

CML stem cells

The first indication that the LSC in CML is created by transformation of a normal HSC was the finding that the *BCR/ABL1* gene is present not only in leukemic cells but also in multiple lineages of hematopoietic cells (Fialkow et al. 1977; Fialkow et al. 1978). Furthermore, experiments using transgenic mouse models closely mimic CML when *BCR/ABL1* is expressed specifically in HSCs and progenitor cells (Koschmieder et al. 2005). It is generally believed that the CML stem cells, which are insensitive to imatinib treatment, are the source of disease relapse in CML CP patients after withdrawal of imatinib. Several mechanisms for the insensitivity of CML stem cells to TKIs have been suggested, including high baseline expression of *BCR/ABL1*, protection of CML stem cells by the BM microenvironment, and insensitivity because the CML stem cells are kinase-independent, possibly due to cell cycle quiesence (Jiang et al. 2007; Corbin et al. 2011; B. Zhang et al. 2013). Recent studies using transgenic mice where *BCR/ABL1* expression was turned on and off after establishment of disease, strongly suggested that CML stem cells are not addicted to BCR/ABL1, as leukemia reappears after re-induction of the *BCR/ABL1* expression (Hamilton et al. 2012). Thus, it seems unlikely that TKIs by themselves would be able to provide an efficient cure for CML. In myeloid BP, CML stem cells appear to be present both in the CD34+CD38- population and in the GMP compartment (Jamieson et al. 2004), while in lymphoid BP, CML stem cells have been found even among CD34-CD38+ BM cells (Tanizaki et al. 2010).

Signaling pathways in chronic myeloid leukemia

Fusion of BCR to ABL1 results in a constitutive activation of the ABL1 tyrosine kinase activity and leads to phosphorylation and activation of several downstream signaling pathways. The network of signaling pathways activated by BCR/ABL1 is very complex, but a few main pathways that are very relevant to this thesis are described below and summarized in Figure 2.

RAS pathway

Autophosphorylation of the Tyr177 residue of BCR/ABL1 allows binding of the GRB2 adaptor protein (Pendergast et al. 1993), which enables subsequent binding of SOS and activation of the RAS/MAPK pathway (Druker et al. 1992; Tauchi et al. 1994; Cortez et al. 1997). Activated RAS can also induce signaling through the JUN amino-terminal kinase pathway (Raitano et al. 1995). RAS activity is essential in CML, and blocking of the RAS pathway reverses transformation by BCR/ABL1 (Sakai et al. 1994; Gishizky et al. 1995; Sawyers et al. 1995), Moreover, the cell transforming activity of RAS is dependent on a posttranslational modification, the addition of an isoprenoid group catalyzed by farnesyl protein transferase, which allows RAS to associate with the cell

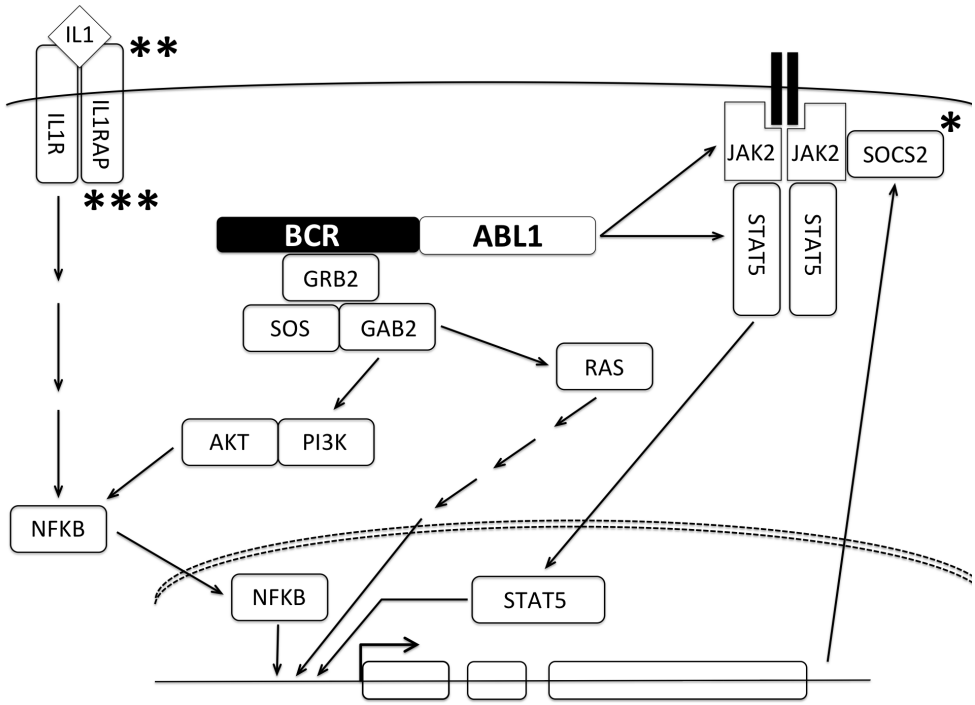

Figure 2. Schematic overview of signaling pathways activated by BCR/ABL1. The tyrosine kinase domain of ABL1 activates JAK2 and STAT5, both leading to translocation of phosphorylated STAT5 to the nucleus where it induces transcription of target genes. Phosphorylation of the Tyr177 in BCR/ABL1 allows binding of GRB2, which enables complex formation with SOS and GAB2 leading to activation of the RAS/MAPK pathway and transcriptional activation. GAB2 also activates PI3K, a signaling mediator with potential to cross-activate NFKB through AKT. The NFKB-complex is also activated downstream from the IL1R, which is dependent on IL1RAP for its activation. The focus of Article I, in which the role of SOCS2 was investigated in normal and malignant BCR/ABL1-induced hematopoiesis, is indicated with an asterix (*) in the figure. In Article II, IL1RAP was identified as upregulated on candidate CML stem cells, indicated by **. The effects of the signaling through IL1RAP were investigated in Article III, indicated by ***.

membrane (Hancock et al. 1990; Gibbs et al. 1994). Thus, farnesyl protein transferase inhibitors (FTIs) were developed to inhibit both mutated RAS and normal RAS (Kato et al. 1992; Nagasu et al. 1995; Sepp-Lorenzino et al. 1995). It was, however, established later that RAS function could be partially maintained by alternative pathways and that FTIs had unspecific effects on a number of other farneslyated proteins (Downward 2003). Nevertheless, these compounds had some anti-tumorigenic activity, and FTIs have been tested in clinical trials as therapy for CML in CP, AP, and BP, but they only resulted in transient responses in a minority of patients tested (Cortes et al. 2003; Borthakur et al. 2006)

JAK2/STAT5 pathway

In CML, the JAK2/STAT5 pathway is directly activated by the BCR/ABL1 fusion protein, but the question of whether only STAT5 or also JAK2 is important for the BCR/ABL1-induced transformation is still being debated (Ilaria & Van Etten 1996; Carlesso et al. 1996; Shuai et al. 1996). However, STAT5 was believed to be dispensable in CML, given that *BCR/ABL1* transduction-transplantation experiments, using BM from mice with a truncated form of *STAT5*, was still able to induce a CML-like disease (Sexl et al. 2000). Although *STAT5* was truncated, these cells retained residual STAT5 activity and when the experiment was repeated with BM cells from mice lacking the complete *STAT5A* or *A/B* genes, BCR/ABL1 was shown to be dependent on STAT5 (Hoelbl et al. 2006; Ye et al. 2006; Hoelbl et al. 2010).

Normally, STAT5 is activated through phosphorylation of tyrosine residues by the upstream protein JAK2. Thus, it can be reasoned that because of a direct activation of STAT5 by BCR/ABL1 (Chai et al. 1997; Wilson-Rawls et al. 1997), JAK2 is redundant in CML. However, it has been argued that inhibition of JAK2 reduces the transforming effects of BCR/ABL1 and that JAK2 either cooperates with BCR/ABL1 to activate STAT5 or mediates cross-activation of other signaling pathways (Warsch et al. 2013). One of the main proofs supporting involvement of JAK2 in CML is that inhibition of JAK2 with a small-molecule inhibitor was shown to induce apoptosis in CML cells (Samanta et al. 2009). However, several of the inhibitors used in similar experiments were later shown to inhibit BCR/ABL1 as well (Hantschel et al. 2012). Hence, although this is still controversial, JAK2 now appears to be non-essential for the development of CML but has been suggested to be essential in mediating cytokine signaling to allow CML stem cells to survive TKI therapy (Warsch et al. 2013). In addition, it is quite possible that JAK2 has a different role in CML BP.

Another member of the JAK2/STAT5 pathway is the suppressor of cytokine signaling 2 (SOCS2), whose main function is feedback inhibition of cytokine signaling (Endo et al. 1997; Naka et al. 1997). *SOCS2* is upregulated upon STAT5 activation and although it is a feedback inhibitor, high levels of SOCS2 have been reported to stimulate signaling, possibly by inhibiting other inhibitors (Tannahill et al. 2005; Piessevaux et al. 2006). We and other investigators have previously found that *SOCS2* expression is upregulated in CML (Schultheis et al. 2002; Håkansson et al. 2008). In Article 1, we used a *Socs2*-deficient mouse strain to evaluate whether SOCS2 is a critical regulator of BCR/ABL1 signaling and important for the pathogenesis of CML. We showed that cells from *Socs2*-deficient mice develop leukemia indistinguishable from wild-type cells upon transplantation after transduction with *BCR/ABL1*. Our results indicate that SOCS2 is not critical for the induction of CML and STAT5 phosphorylation by BCR/ABL1. In myeloproliferative neoplasms, STAT5 phosphorylation is often achieved by mutations activating JAK2, so SOCS2 may be of more importance in that setting (Quentmeier et al. 2008).

NFKB pathway

BCR/ABL1 activates NFKB in a tyrosine kinase- dependent fashion (Hamdane et al. 1997; Kirchner et al. 2003). However, it has also been suggested that RAS activation is involved in transactivation of NFKB (Reuther et al. 1998). Thus, it is still possible that multiple co-operating pathways contribute to the activation of NFKB in CML. NFKB is known to enhance resistance to apoptosis and to increase proliferation in some forms of cancer, and it is known to be activated in both CML and AML (Hamdane et al. 1997; Reuther et al. 1998; Guzman et al. 2001; Karin et al. 2002; Kirchner et al. 2003), possibly through crosstalk from the PI3K/AKT signaling pathway (Zhu et al. 2011). Furthermore, inhibition of the NFKB complex by blocking of various proteins in the signaling pathway suggests that NFKB may be a possible therapeutic target in CML (Cilloni et al. 2006; Lounnas et al. 2009; Lu et al. 2010; Wei et al. 2013). IL1 is one cytokine known to activate NFKB in several types of immune cells (Fitzgerald & O'Neill 2000). In addition, a study of microenviromental effects in CML showed that IL1 and GCSF induce more proliferation of primitive CD34+CD38- CML cells than of corresponding normal cells (Zhang et al. 2012). Furthermore, altered cytokine expression in CML cells with an increase in IL1A, GCSF, and other cytokines provides a selective advantage for the CML stem cells (Zhang et al. 2012). The intracellular mechanisms underlying the growth advantage of primitive CML cells over normal HSCs after IL1 stimulation are unclear, but they could be an effect of additional NFKB activation. In Article II, we found that expression of the IL1 receptor accessory protein (IL1RAP) was upregulated in primitive CD34+CD38- CML cells compared to the corresponding normal cells. IL1RAP is an essential component of the IL1 signaling pathway which (among other effects) results in NFKB activation (Cullinan et al. 1998). Thus, in Article III, we continued to investigate whether IL1RAP has a role in hematopoiesis and whether upregulation of *IL1RAP* is sufficient to induce features of a myeloproliferative disease. We demonstrated that *Il1rap*-deficient mice have a reduction in myeloid cells in PB. We also showed that CML CD34+CD38- BM cells, the population with the highest *IL1RAP* expression compared to corresponding normal cells, are hypersensitive to IL1B, while IL1B has limited effect on more mature CD34+ cells. It may be speculated that since IL1RAP is essential for activation of the IL1R, the higher levels of IL1RAP on the cell surface of CD34+CD38- CML cells may help to sensitize the receptor complex and downstream signaling pathways to activation, leading to a CML-specific cell expansion.

Experimental models of chronic myeloid leukemia

Cell culture

Immortalized leukemic cell lines are widely used in research and offer a system that is easy to work with, is renewable, and has the benefit that experiments can be carried out at multiple sites using the same cell model. Several CML cell lines are available, all of which were established from PB or BM of patients in advanced BP, but there are no cell lines that faithfully represent CML CP. Despite the convenience of using immortalized cell lines, they have several drawbacks that limit their use. Cell lines have usually been cultured extensively, leading to the acquisition and selection of new genetic alterations and to genetic drift, when the cells adapt to *in vitro* culture (Drexler et al. 1999; Dirks et al. 2010).

To overcome the drawbacks of immortalized cell lines, retrovirally transduced primary cells are often used when studying the cellular and molecular effects of BCR/ABL1 expression. These cell cultures are believed to reflect leukemic cells more closely, as they have not been immortalized and retain much of their intrinsic differentiation potential and cytokine dependency. Compared to *in vitro* culture of primary leukemic cells, retrovirally transduced cells have both benefits and drawbacks. While retroviral transduction is often a way of ensuring access to sufficient numbers of cells for a successful study (especially when rare genetic aberrations are studied), primary leukemia cells often contain additional genetic and epigenetic changes that are difficult to mimic in transduced cells.

Mouse models

Instead of culturing *BCR/ABL1*-transduced cells *in vitro*, it is possible to transplant transduced mouse cells into mice of the same genetic background in order to study the cellular functions *in vivo*. Daley et al. (1990) established a mouse model of CML by transducing mouse BM cells with a retrovirus expressing *BCR/ABL1* and transplanting cells into irradiated recipients. These mice developed several different hematopoietic malignancies, including macrophage tumors and lymphoid leukemia. Subsequently, the transduction and transplantation model was gradually improved and better characterized to give a disease more similar to CML (Zhang & Ren 1998; Li et al. 1999). These models have been used successfully combined with KO strains, to investigate the role of specific genes in CML, such as *Stat5* and *Alox5* (Hoelbl et al. 2006; Ye et al. 2006; Chen et al. 2009). However, the transduction-transplantation model of CML still has a number of limitations. The retroviral constructs target not only HSCs but also progenitor cells, leading to massive progenitor proliferation that complicates the evaluation of experiments. The irradiation of recipient mice and the variation in *BCR/ABL1* expression levels may also increase the variability of the experiments. In addition, the retroviral models typically show a very aggressive disease, with death occurring around three weeks

after transplantation. Thus, studies investigating the contributions by various 'associated genes' such as *SOCS2* (Article 1) may not necessarily exclude the possibility that these genes play a role in the pathogenesis of CML, as, due to its limitations, this mouse model may fail to account for the function of that specific gene. An alternative way to model CML that has been widely used is to generate transgenic *BCR/ABL1* mice (Koschmieder & Schemionek 2011). Currently, the most elegant one is transgenic mice that express *BCR/ABL1* from an HSC-specific tetracycline-inducible promoter (Huettner et al. 2003; Koschmieder et al. 2005). This approach makes it possible to both induce and to reverse a CML-like disease by removal or re-administration of tetracycline to the drinking water of the mice, thereby allowing detailed *in vivo* studies of CML pathogenesis (Zhang et al. 2012). To date, this transgenic model is probably the most elegant and pure murine model of CML.

Immunodeficient mouse models

Some immunodeficeint recipient mice, such as NOD/SCID mice, have impaired T-cell and B-cell development and can therefore be engrafted by human cells (Prochazka et al. 1992; Lowry et al. 1996). Xenotransplantation of human cells into these mice have been of great importance for the functional characterization of normal human hematopoiesis and HSCs (Lowry et al. 1996; Doulatov et al. 2012). However, not only can normal hematopoietic cells be transplanted into NOD/SCID mice, but CML cells also give long-term engraftment (Lewis et al. 1998, Wang et al. 1998). Although they can be robustly engrafted by *BCR/ABL1*-positive cells, none of the transplanted mice were reported to have succumbed from development of a disease similar to CML.

Immunodeficient mouse models for xenotransplantation of human cells are still being improved to allow higher engraftment levels and to better support differentiation of cells into lineages. The NOD/SCID model has been improved by deletion of the IL2R gamma chain, which leads to lack of functional NK-cells in these mice, commonly referred to as NSG (Shultz et al. 2005). In Article III of this thesis, we used NSG mice to study the effect of IL1RAP overexpression in human HSCs and progenitor cells. Clearance of human donor cells by recipient immune cells is, however, not the only limiting factor for xenotransplantation efficiency. Lack of cross-reactivity of some niche factors and cytokines is also likely to limit the development of leukemic cells after xenotransplantation. Mice transgenically expressing human SCF, granulocyte macrophage colony-stimulating factor (GM-CSF), and IL3 have increased development of both normal human hematopoietic cells and leukemic cells after transplantation (Feuring-Buske et al. 2003; Nicolini et al. 2004).

Recently, Wunderlich et al. (2010) crossed these mice with NSG mice to generate an NSG strain expressing human SCF, granulocyte macrophage colony-stimulating factor (GM-CSF), and IL3 (referred to as NSGS mice), which even better supports the engraftment of human leukemic cells. As it has already been reported that CML cells transplanted into NOD/SCID mice achieve higher engraftment after injections of human SCF into the recipients (Lewis et al. 1998), it is quite possible that NSGS mice are more permissive in allowing engraftment of primary CML cells.

The present investigation

This section describes the aims of the present study and the articles included, a summary and discussion of the main findings, and the conclusions arrived at. The experimental procedures are based on well-known methods and will not be discussed in this chapter. Instead, the reader is referred to the materials and methods sections of the original articles (I–III), which are presented in the third section of this thesis.

Aims of the study

The overall aim of this study was to identify genes upregulated by the BCR/ABL1 fusion protein and to use *in vitro* and *in vivo* models to study their function in normal and malignant hematopoiesis. More specifically, the aims were:

- To determine the function of SOCS2 in normal hematopoiesis and its role in BCR/ABL1-induced cell signaling (Article I).
- To identify a cell-surface marker for primitive CML cells, allowing prospective isolation of candidate CML stem cells (Article II).
- To study the function of IL1RAP in normal hematopoiesis and the consequences of upregulation of *IL1RAP* in malignant hematopoiesis (Article III).

Results and discussion

Article I

SOCS2 is dispensable for BCR/ABL1-induced chronic myeloid leukemia-like disease and for normal hematopoietic stem cell function.

Signaling cascades activated by BCR/ABL1 elicit transcriptional gene expression signatures that are thought to contribute to CML pathogenesis. Thus, not only may the primary downstream mediators of BCR/ABL1 be important for the establishment of CML, but also genes becoming upregulated as a result of signaling by BCR/ABL1. The STAT5 signaling pathway is one important mediator of BCR/ABL1-induced transformation. Activation of STAT5 is well known to induce expression of its feedback inhibitor SOCS2, which had already been found to be highly expressed in primary BM cells from patients with CML. However, at the outset of this study it had not been established whether SOCS2 is involved in the pathogenesis of CML or whether it is important for normal HSC function. In Article I, we demonstrated that although *Socs2* was found to be preferentially expressed in long-term HSCs, *Socs2*-deficient HSCs were indistinguishable from wild-type HSCs when challenged in competitive BM transplantation experiments. Furthermore, by using a retroviral BCR/ABL1-induced mouse model of CML, we showed that SOCS2 is dispensable for the induction and propagation of the disease. The phosphorylation of STAT5 following *BCR/ABL1* transduction was unaffected by SOCS2, suggesting that the SOCS2-mediated feedback regulation of the JAK/STAT pathway is deficient in BCR/ABL1-induced CML.

Article II

Isolation and killing of candidate chronic myeloid leukemia stem cells by antibody targeting of IL-1 receptor accessory protein.

Therapeutic strategies aimed at cure of CML will most likely require full eradication of the CML stem cells. In an attempt to find cell-surface markers useful for identification and isolation of CML stem cells, we performed global gene expression analysis both on primary CD34+ CML cells and umbilical cord blood (CB) cells transduced with BCR/ABL1. The IL1R1 co-receptor IL1RAP was found to be selectively expressed on CML cells and to become upregulated following expression of BCR/ABL in CB cells. To determine whether IL1RAP expression would allow a prospective isolation of Ph-positive and normal residual Ph-negative cells within the CML CD34+CD38- cell compartment, we established a new protocol for FISH analysis on small numbers of sorted cells.

Interestingly, we found that the CML CD34+CD38−IL1RAP+ cells were preferentially Ph-positive, whereas CML CD34+CD38−IL1RAP− cells were almost exclusively normal. We hypothesized that, being exposed on the cell surface of candidate CML stem cells, IL1RAP could provide a target for a novel antibody-based therapy of CML. After generating a polyclonal anti-IL1RAP antibody, we found that IL1RAP could be used to induce antibody-dependent cell-mediated cytotoxicity (ADCC) of CD34+CD38− CML cells. In summary, this study identified IL1RAP as the first specific marker for candidate CML stem cells and also demonstrated that targeting of IL1RAP by an antibody-based approach may open up new avenues in the treatment of CML.

Article III

IL1RAP promotes myelopoiesis and its upregulation on candidate CML stem cells is associated with increased IL1 sensitivity.

In addition to several progenitor populations, IL1RAP is expressed on candidate CML stem cells and other myeloid malignancies (Article II; Barreyro et al. 2012; Askmyr et al. 2013). IL1RAP is an essential co-receptor for IL1R1 and IL33R (ST2), mediating signals from IL1 and IL33, respectively. However, little is known about the cellular consequences of IL1RAP-mediated signaling in these cell types. In Article III, we found that *Il1rap*-deficient mice have reduced myeloid cell counts in peripheral blood at steady state, but that IL1rap is dispensable for normal hematopoietic stem cell function, as assessed by competitive SCTs. In xenotransplantation studies, enforced expression of IL1RAP in CB CD34+ cells resulted in a myeloid lineage skewing with expansion of a $CD33^{low}$CD15+ cell population. We also determined whether CB cells with enforced IL1RAP expression and primary CML cells showed a different sensitivity for cytokines signaling through receptors known to associate with IL1RAP in other cell types. Whereas IL1B did not support *in vitro* proliferation of normal CB $CD34^{+}$ cells, not even upon enforced IL1RAP expression, primary CML CD34+CD38- cells expanded significantly in response to IL1B stimulation. Furthermore, we found that CD34+ CML cells under stimulation by SCF expanded more than the primitive CD34+CD38- population. This is in accordance with a recent report showing differential sensitivity of CML cells to SCF (Corbin et al. 2013). In summary, these results suggest that upregulation of IL1RAP may contribute to the pathogenesis of myeloid neoplasms by increasing the sensitivity to IL1 signaling and promoting myelopoeisis.

Conclusions

The main findings of this thesis are presented below:

Article I

- SOCS2 is not required for normal hematopoiesis and HSC function.
- SOCS2 is not critical for BCR/ABL1 to initiate disease.
- The direct activation of STAT5 by BCR/ABL1 probably by-passes the SOCS2-mediated feedback inhibition of the JAK2/STAT5 pathway.

Article II

- IL1RAP is a cell-surface marker for candidate CML stem cells and can be used to prospectively isolate such cells.
- Antibodies to IL1RAP can be used to induce ADCC in candidate CD34+CD38- CML stem cells, while sparing the corresponding normal cells.

Article III

- IL1RAP is dispensable for HSCs but promotes the myeloid lineage *in vivo*.
- Forced IL1RAP expression is insufficient to increase the sensitivity of CB cells to IL1B, while primitive CML cells are hypersensitive and expand massively *in vitro* upon IL1B stimulation.

Concluding remarks and future perspectives

Cancer is caused by a variety of genetic aberrations, resulting in loss or abnormal activation of protein functions. Recent technological advancements have facilitated genetic studies, with the sequencing of the human genome as an important landmark (Lander et al. 2001; Venter et al. 2001). Since then, sequencing technology has improved and has reduced costs further. Currently, massive parallel sequencing technology provides large amounts of data describing the genetic landscape of cancer and leukemia (Cancer Genome Atlas Research Network 2013a; Cancer Genome Atlas Research Network 2013b). The human genome project had great hopes of giving benefit to patients in the clinic, but it has proven to be more difficult than expected to understand the functional relevance, at the cellular and tissue levels, of the genetic findings. Several new functional methods such as shRNA technology and refined mouse models are being developed to facilitate experiments to understand the functional effects of the genetic findings.

The aim of this thesis was to improve our understanding of the functional effects of the upregulation of *SOCS2* and *IL1RAP* seen in CML cells. SOCS2 is known to be involved in intracellular regulation of cytokine signaling, but as described in this thesis, SOCS2 is dispensable for BCR/ABL1 inducing disease *in vivo*, as assessed in mice receiving transplants with BCR/ABL1-transduced mouse BM cells. As referred to earlier, it is worth noting that this retroviral CML model has several limitations; for example, the disease observed is very aggressive with a short latency period, and as such it is difficult to entirely rule out a role of SOCS2 in the pathogenesis of CML. A relevant follow-up study would be to cross *Socs2*-deficient mice with transgenic mice expressing *BCR/ABL1* from an HSC-specific inducible promoter (Koschmieder et al. 2005). Interestingly, there have been several publications indicating that SOCS2 is important in other tumor types, including prostate cancer, and in MPDs with JAK2 mutation (Quentmeier et al. 2008; Iglesias-Gato et al. 2013).

The current CML therapy is based on inhibition of BCR/ABL1 or stem cell transplantation. However, as LSCs appear to be independent of the BCR/ABL1 tyrosine kinase activity, it is questionable whether ABL1 inhibitors would have the potential to be highly curative in CML. In addition, TKIs have proven to be ineffective in advanced stages of the disease (Pellicano et al. 2011). One way to overcome the TKI insensitivity is to find alternative targets in the CML cells, such as proteins with a function that becomes

critical after transformation by BCR/ABL1. One example of such a target in CML is RAD52, which is involved in the repair of double-strand breaks (Cramer-Morales et al. 2013). TKI treatment of CML cells has also been shown to induce autophagy, a process that leads to TKI resistance (Mishima et al. 2008; Kamitsuji et al. 2008). Inhibition of autophagy increases leukemic cell death and significantly increases the elimination of candidate CML stem cells in combination with a TKI (Mishima et al. 2008; Bellodi et al. 2009). Thus, autophagy may be a promising novel therapeutic target in CML.

The *IL1RAP* protein is an alternative target in CML that was discovered in a quest to find a cell-surface marker specific for CML stem cells (Article II). Cell-surface proteins can be used both to identify leukemic cells and as targets for different antibody-based therapies (Morris & Waldmann 2009). One alternative to the ADCC-based approach using NK-cells in Article II is the cancer vaccine concept, where synthetic peptides from leukemia-specific proteins induce T-cell-mediated cell killing (Dao & Scheinberg 2008). Based on this concept, there have been several trials using various BCR/ABL1 fusion peptides as vaccines in CML treatment (Maslak et al. 2008; Casnici et al. 2011). Peptides of WT1 are another target of vaccines tested for AML, but they have recently also been used for antibodies targeting the peptide when presented by human leukocyte antigen (HLA) in Ph chromosome-positive ALL (Maslak et al. 2010; Dao et al. 2013). These approaches might prove to be feasible ways to achieve an improved therapy of CML and Ph chromosome-positive ALL. Most likely, however, targeting of cell-surface proteins will turn out to be an easier and more effective approach, as exemplified by the successful therapy of B-cell malignancies using anti-CD20 antibodies (e.g. Rituximab) (Barth et al. 2012).

In Article III, we studied the function of IL1RAP and found that mice deficient for *Il1rap* had fewer myeloid cells in PB and that forced expression of *IL1RAP* in human CB cells resulted in myeloid skewing after transplantation into NSG mice. These findings indicate that IL1RAP has a functional role in promoting myelopoiesis by enabling cytokine signaling in normal hematopoiesis. It should, however, be noted that *in vitro* cultures of cells with forced expression of IL1RAP did not yield higher cell numbers after cytokine stimulation with IL1RAP-dependent cytokines. Thus, the exact mechanism of IL1RAP function in normal hematopoiesis is not yet understood, and should be further investigated. In contrast, primitive CML CD34+CD38- cells with increased expression of IL1RAP expanded significantly in response to IL1B stimulation (Article III; Zhang et al. 2012). This effect of IL1 in the primitive CML cell population supports the idea that extrinsic microenviromental regulation, and in particular IL1, may be of importance to the LSCs. IL1 signals through the IL1R1/IL1RAP complex and is known to result in NFKB activation (Cullinan et al. 1998). It is also known that NFKB activation increases the self-renewal of normal HSCs (Zhao et al. 2012a), which may indicate that upregulation of IL1RAP combined with expression of IL1R leads to increased self-renewal of the LSCs. However, our results with cell expansions after liquid cultures suggest more of a proliferative expansion of the CD34+CD38- CML fraction. A detailed

analysis of the consequences of IL1 signaling, regarding both gene expression response and in terms of cellular response of primitive CML cells, will therefore be needed to clarify how primitive CML cells respond to IL1. It may very well be that an IL1RAP-based therapy would both inhibit IL1 signaling in the CML cells and target them for immune-mediated killing in order to be effective.

Despite the fact that CML is always initiated by the formation of the *BCR/ABL1* fusion gene, CML cells may evolve into separate heterogeneous sub-clones during disease progression, which is indicated by the variation in mutations and genetic aberrations found in CML cells (Grossmann et al. 2011; Makishima et al. 2011). Thus, it may be not be enough to use only one therapeutic approach, but combinational therapy may be needed in order to find cures for CML patients in the future. Given the heterogeneity of most cancers, it may be that therapy could be based on the presence of specific target molecules rather than the type of cancer. For example, IL1RAP is expressed in CML, and also in a proportion of AML patients, and antibodies against IL1RAP are also capable of inducing *in vitro* ADCC in cells from these patients (Barreyro et al. 2012; Askmyr et al. 2013). Perhaps a large panel of immune-based therapy targets, like anti-IL1RAP agents, cancer vaccines, or therapies inhibiting the key driver mutations (like imatinib) will eventually become the basis of cancer therapy, where treatment is selected by genome-wide analysis to identify target proteins, enabeling individually selected drug combinations irrespective of the tumor type.

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