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DEPARTMENT OF CLINICAL GENETICS
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MOLECULAR AND FUNCTIONAL STUDIES OF *ABL1* AND *FGFR1* FUSION ONCOGENES IN MYELOPROLIFERATIVE NEOPLASMS

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Abstract

The tyrosine kinase encoding genes ABL1 and FGFR1 are involved in fusion genes underlying the myeloproliferative neoplasms chronic myeloid leukemia (CML) and the 8p11-myeloproliferative syndrome (EMS). CML and EMS are both myeloproliferative disorders with an initiating, relatively indolent, chronic phase that after some time progresses into acute myeloid or lymphoid leukemia. The mechanisms underlying disease progression are currently unknown, but additional genetic aberrations are commonly found in the progressed phase. The general aim of this thesis was to study BCR/ABL1 and FGFR1 fusion oncogenes in a relevant cellular context, that of primary human hematopoietic cells, in order to increase our understanding of disease mechanisms underlying the development of CML and EMS. In Article I, a secondary translocation between chromosomes 9 and 21, identified in the leukemic cells from a patient in the progressed phase of EMS, was investigated. The translocation was found to result in a truncated RUNX1 gene, suggesting that haploinsufficiency for RUNX1 could be a mechanism behind disease progression in EMS. It was also found that trisomy 21 is a common secondary change in EMS. In Article II, two variants of BCR/ABL1, P190 and P210, were retrovirally expressed in cord-blood derived human CD34-positive cells. Both variants induced erythroid expansion, increased proliferation, and similar gene expression profiles, indicating that P190 and P210 BCR/ABL1 have a similar mode of action. These results indirectly support the theory that the difference in disease manifestation between P190 and P210 BCR/ABL1 depends on separate cellular origins rather than intrinsic differences of the two fusion proteins. In Article III, retroviral expression of BCR/FGFR1 or ZMYM2/FGFR1 in human CD34-positive cells resulted in increased cellular proliferation and erythroid expansion, in similar to the effects caused by BCR/ABL1. Transplantation of BCR/FGFR1- and ZMYM2/FGFR1-expressing cells into immunodeficient mice resulted in engraftment of human cells in the mouse bone marrow. The human cells differentiated into a myeloid and erythroid direction. Both fusion genes induced similar EMS-like disorders in transplanted mice, with eosinophilia, splenomegaly, and accumulation of blasts. The established in-vivo model of EMS should constitute a valuable tool for obtaining further insights into FGFR1 fusion gene mediated leukemogenesis and for the development and evaluation of new treatment strategies in EMS.

Key words: 8p11-myeloproliferative syndrome, EMS, chronic myeloid leukemia, CML, BCR/ABL1, BCR/FGFR1, ZMYM2/FGFR1, immunodeficient mice

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ABBREVIATIONS

ALL	acute lymphoblastic leukemia
AML	acute myeloid leukemia
APL	acute promyelocytic leukemia
BM	bone marrow
CB	cord blood
CFA	colony forming assay
CLP	common lymphoid progenitor
CMP	common myeloid progenitor
CML	chronic myeloid leukemia
EMS	8p11-myeloproliferative syndrome
ET	essential thrombocytosis
FACS	flow cytometry
FISH	fluorescence in situ hybridization
GFP	green fluorescent protein
GMP	granulocyte/macrophage progenitor
HSC	hematopoietic stem cell
IRES	internal ribosome entry site
TKI	tyrosine kinase inhibitor
LSC	leukemia stem cell
MEP	megakaryocyte/erythrocyte progenitor
MSCV	murine stem cell virus
MPN	myeloproliferative neoplasm
PCR	polymerase chain reaction
Ph	Philadelphia chromosome
PMF	primary myelofibrosis
PV	polycythemia vera
SL-IC	SCID leukemia initiating cell
WHO	World Health Organization

ARTICLES INCLUDED IN THE THESIS

This thesis is based on the following articles, referred to in the text by their Roman numerals.

I. **Ågerstam H, Lilljebjörn H, Lassen C, Swedin A, Richter J, Vandenberghe P, Johansson B, and Fioretos T.** Fusion gene-mediated truncation of *RUNXI* as a potential mechanism underlying disease progression in the 8p11-myeloproliferative syndrome. *Genes Chromosomes Cancer* 2007;46:635-43.

II. **Järås M, Johnels P, Ågerstam H, Lassen C, Rissler M, Edén P, Cammenga J, Olofsson T, Bjerrum OW, Richter J, Fan X, and Fioretos T.** Expression of P190 and P210 *BCR/ABL1* in normal human $CD34^+$ cells induces similar gene expression profiles and results in a STAT5-dependent expansion of the erythroid lineage. *Experimental Hematology* 2009;37:367-75.

III. **Ågerstam H, Järås M, Andersson A, Johnels P, Hansen N, Lassen C, Rissler M, Gisselsson D, Olofsson T, Richter J, Fan X, Ehinger M, and Fioretos T.** Modeling the human 8p11-myeloproliferative syndrome in immunodeficient mice. *Blood* 2010;116:2103-11.

PREFACE

The discovery of the Philadelphia chromosome in leukemic cells from chronic myeloid leukemia (CML) patients in 1960 was the first time a consistent structural chromosomal abnormality was linked to a specific neoplastic disorder. In the early 1970's the Philadelphia chromosome was shown to arise as a result of a reciprocal translocation between chromosomes 9 and 22. Investigations at the DNA-level in the 1980's finally revealed that the t(9;22) resulted in a *BCR/ABL1* fusion gene. Since then, more than 350 recurrent fusion genes have been reported in neoplastic disorders. Fusion genes involving the *FGFR1* gene molecularly characterize the 8p11-myleoproliferative syndrome (EMS). Similar to CML, EMS is a chronic myeloproliferative disorder that eventually progresses into an acute phase that resembles acute leukemia. Albeit rare, EMS is more aggressive than CML, and there is currently no effective treatment. The only cure at present is allogenic bone marrow transplantation.

The general aim of this thesis was to increase our understanding of disease mechanisms in CML and EMS by molecular and functional studies of *BCR/ABL1* and *FGFR1* fusion oncogenes in a relevant cellular context; that of normal human hematopoietic cells. Furthermore, the aim was to establish and to characterize a model of human EMS in immunodeficient mice. This model of human EMS should constitute a valuable tool for future studies of this disorder, and provides a first line evaluation model for new treatment strategies of EMS.

This thesis is divided into three sections. The first provides an introduction to hematopoiesis and myeloproliferative disorders in general, with an emphasis on *FGFR1* fusion genes. The second is a description of the investigations that were performed during the work of this thesis. The third and final section constitutes the articles upon which this thesis is based.

Lund, September 2010

INTRODUCTION

Normal hematopoiesis and hematopoietic stem cells

There are many types of cells in the blood, each with their specific function. Erythrocytes transport oxygen to cells in the body, thrombocytes are necessary for the induction of blood clotting, and granulocytes and lymphocytic cells are involved in the immunological defense against viruses, bacteria and parasites. All types of blood cells develop from hematopoietic stem cells (HSCs), during a process called hematopoiesis. During most of fetal life, the liver is the main organ for production of blood cells. In children from about six months of age and through the rest of life the formation of new blood cells take place in the bone marrow (BM). The classical model of hematopoiesis is strictly hierarchical, and depending on the absence or presence of different external stimuli in combination with intrinsic signals, cells are gradually committed to certain differentiation paths (Figure 1) (Kondo et al. 1997; Akashi et al. 2000).

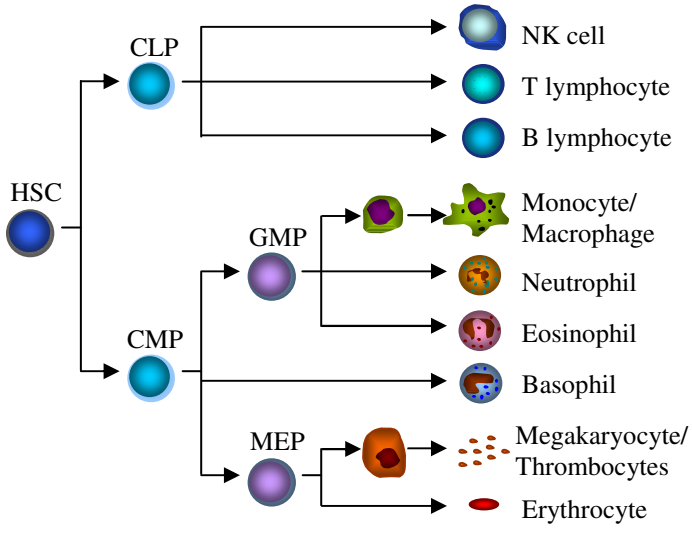

Figure 1. The classical model of hematopoiesis. The hematopoietic stem cell (HSC) can commit into a common lymphoid progenitor (CLP) which can differentiate into B cells, T cells, or NK cells, or a common myeloid progenitor (CMP). The CMP can commit to a granulocyte-monocyte progenitor (GMP) with subsequent differentiation into macrophages, or granulocytes of neutrophilic or eosinophilic type. The other alternative for CMP is megakaryocyte-erythrocyte progenitor (MEP), giving rise to thrombocytes and erythrocytes.

In the classical model of hematopoiesis the separation of the lymphoid system from the myeloid system is early and distinct, and accordingly the lymphoid progenitors have lost all myeloid potential. Contradictory to the classical model, several investigations have, however, shown that early lymphoid B and T cells under certain conditions can differentiate into myeloid cells (Schaniel et al. 2002; Bell and Bhandoola 2008; Wada et al. 2008). In response to the new findings, several alternative models of hematopoiesis have been proposed (Adolfsson et al. 2005; Kawamoto 2006; Ye and Graf 2007; Ceredig et al. 2009). These models commonly suggest that the segregation of lymphoid and myeloid differentiation pathways occurs later than in the classical model, and that myeloid potential is retained in lymphoid progenitors. Another common feature is that the commitment into erythroid and megakaryocytic cells may be an early branching point. Recent studies of human progenitor cells support the idea of a classical common myeloid progenitor (CMP) without lymphoid potential, but in line with the alternative models, lymphoid progenitors have retained certain myeloid lineage potential (Doulatov et al. 2010). Clearly, the process of normal hematopoiesis is not yet fully understood, and many questions remains to be answered in future investigations.

Irrespective of how blood cell differentiation is carried out, it begins with the HSC. The concept of HSC was demonstrated in the beginning of the 1960's (Till and McCulloch 1961; Siminovitch et al. 1963), and HSCs are defined as cells that 1) have the capacity for self renewal, which means that upon cell division at least one daughter cell can retain HSC properties, and 2) are capable of multilineage differentiation into all types of blood cells (Till and McCulloch 1961; Siminovitch et al. 1963; Weissman 2000). Accordingly, at least theoretically, a single HSC can engraft an ablated host and reconstitute the entire hematopoietic system. A prerequisite for sustained engraftment is that the cell is a long-term HSC which has the capacity for self-renewal for the life of the host, in contrast to the short-term HSC, which has retained self renewal capacity for only approximately eight weeks (Morrison and Weissman 1994). Despite having extensive proliferative capacity, HSCs are normally in a quiescent state (Fleming et al. 1993). During the differentiation into progenitor cells the ability for self renewal is lost, and mature blood cells have a limited life span. HSCs depend on both internal signals and external stimuli from the microenvironment and surrounding cells in the stem cell niche, when deciding the cell's fate; to undergo cell division to self-renew or to differentiate, to migrate, or to undergo

programmed cell-death through apoptosis, all in order to maintain homeostasis (Adams and Scadden 2006).

Leukemia and leukemic stem cells

Leukemia is the name for a variety of malignant disorders in the hematopoietic system. Traditionally, leukemia is divided into two groups, acute and chronic leukemia depending on the clinical course of the disease. Each of these groups is further divided into lymphoid and myeloid leukemia, depending on the blood lineage manifestation of the leukemia. The malignant cells show enhanced proliferation and/or abnormal differentiation that suppresses normal hematopoiesis. In 2008, approximately 450 persons in Sweden were newly diagnosed with chronic or acute myeloid leukemia (Socialstyrelsen 2010). Myeloproliferative neoplasms (MPNs) are conditions in which one or several myeloid cell lineages show an increased expansion, but in contrast to acute leukemia the differentiation is relatively normal. The result is an overload of myeloid cells at different maturation stages.

Leukemia and MPN arises as the clonal expansion of a single cell (Fialkow et al. 1987; Warner et al. 2004). The initiating event is thought to be the acquisition of genetic aberrations i.e. disruption/deregulation of genes or formation of fusion genes (Mitelman et al. 2004). Similar to normal hematopoiesis, the cells within a leukemic clone are heterogeneous (Warner et al. 2004), and it has been shown that only a minor subset of the leukemic cells in acute myeloid leukemia (AML) are capable of engrafting and inducing leukemia in immunodeficient mice (Bonnet and Dick 1997; Dick 2008). These SCID leukemia initiating cells (SL-ICs) typically have a $CD34^+CD38^-$ immunophenotype similar to normal HSCs, suggesting that the cellular origin of AML is a transformed HSC. SL-ICs are also commonly referred to as leukemia stem cells (LSCs). However, the transforming event resulting in leukemia could possibly take place in a committed progenitor cell that acquires the potential for self renewal. It should be noted that the immunophenotype of leukemic cells might be aberrant and not necessarily reflect the normal cell of origin. For example, LSC in acute lymphoid leukemia (ALL) might have a $CD34^+CD38^-CD19^+$ immunophenotype (Castor et al. 2005), although this cell population is not seen in normal hematopoiesis. Recently, a number of studies have challenged the $CD34^+CD38^-$ phenotype of AML and ALL LSCs as cells with more mature

immunophenotypes have been demonstrated to engraft immunodeficient mice (le Viseur et al. 2008; Taussig et al. 2008). In acute promyelocytic leukemia (APL), which is caused by the *PML/RARA* fusion gene, the $CD34^{+}CD38^{-}$ cells fail to engraft NOD/SCID mice, indicating that the LSC in this leukemia subtype has a different and more mature immuno-phenotype (Turhan et al. 1995). Accordingly, it has been shown in mice that the LSCs in APL are myeloid-committed (Guibal et al. 2009). Similar to HSCs, the LSCs have the ability for self renewal, as shown in serial transplantation studies (Bonnet and Dick 1997; O'Brien et al. 2010). During the course of an MPN, additional mutations can occur in the clone that gives rise to the initiating chronic phase, thereby resulting in a fully transformed LSC with a more mature immuno-phenotype that give rise to a leukemic clone found in the acute phase of the disorder. In the acute phase of chronic myeloid leukemia (CML), also called the blast crisis, the bulk of cells have a more mature phenotype similar to granulocyte/macrophage progenitors (GMP) and these cells were shown to have self-renewal capacity (Jamieson et al. 2004; Abrahamsson et al. 2009).
CML is the most well-studied MPN, and is in most cases recognized by the characteristic Philadelphia chromosome (Ph) (Nowell and Hungerford 1960) (Table 1). The Ph chromosome is caused by a reciprocal translocation, t(9;22)(q34;q11), involving chromosomes 9 and 22 (Rowley 1973), in which the tyrosine kinase *ABL1* and the *BCR* gene are fused to form a *BCR/ABL1* fusion gene (Heisterkamp et al. 1985). The classical MPNs also include polycythemia vera (PV), essential thrombocytosis (ET), and primary myelofibrosis (PMF) (Table 1). Almost all cases of PV, and about half of the ET and PMF cases, harbor an activating point mutation in the *JAK2* tyrosine kinase gene (Baxter et al. 2005; James et al. 2005; Kralovics et al. 2005; Levine et al. 2005). In the 2008 revision of the World Health Organization (WHO) classification of myeloid neoplasms, a new group was added; 'myeloid neoplasms associated with eosinophilia and abnormalities of *PDGFRA*, *PDGFRB*, or *FGFR1'*, in which 'myeloid neoplasms associated with rearrangements of the tyrosine kinase receptor *FGFR1* gene' constitutes a subgroup. Fusion genes involving *FGFR1* is the molecular cause of the 8p11-myeloproliferative syndrome (EMS) (Goradia et al. 2008; Jackson et al. 2010).

As exemplified above, MPNs often harbor a genetic aberration that involves a gene encoding a protein with tyrosine kinase activity. These proteins function as internal signal transducers. Normally, in response to different stimuli

Table 1. Abbreviated outline of the WHO classification scheme of myeloid neoplasms, 2008 revision (Tefferi et al. 2009).

Myeloid neoplasms and acute leukemia
1. Myeloproliferative neoplasms (MPN)
1.1 Chronic myelogenous leukemia, *BCR-ABL1*-positive (CML)
1.2 Polycythema vera (PV)
1.3 Essential thrombocytosis (ET)
1.4 Primary myelofibrosis (PMF)
1.5 Chronic neutrophilic leukemia
1.6 Chronic eosinophilic leukema, not otherwise specified
1.7 Mast cell disease
1.8 MPN, unclassifiable
2. Myeloid and lymphoid neoplasms associated with eosinophilia and abnormalities of *PDGFRA*, *PDGFRB*, and *FGFR1*
3. Myelodysplastic syndromes/myeloproliferative neoplasms
4. Myelodysplastic syndromes
5. Acute myeloid leukemia (AML)

they become activated through phosphorylation of tyrosine residues and subsequently mediate signals within the cell. An activating point mutation or the formation of a fusion gene, results in a constitutively active tyrosine kinase, with aberrant downstream signaling as a consequence (Cross and Reiter 2002; Goldman and Melo 2008; Levine and Gilliland 2008).

MPNs may eventually progress into, or at least predispose for, acute leukemia. The mechanism for disease progression is not fully understood, but additional genetic aberrations are commonly found in later stages of these disorders. One model for disease progression is the two-hit model, which postulates that the induction of AML requires two cooperating transforming events (Dash and Gilliland 2001). A class I mutation conferring proliferative and/or survival advantages could give rise to a chronic phase CML-like disorder, but it takes an additional class II mutation that severely impairs hematopoietic differentiation and subsequent apoptosis to induce AML. Typically, class I mutations results in constitutive activation of a tyrosine kinase protein or its downstream effectors, whereas class II mutations alters the function of a

transcription factor (Kelly and Gilliland 2002). Although attractive, this two-hit model is likely to be oversimplified, given the vast numbers of mutations currently being discovered in acute myeloid and lymphoid leukemia using genome-wide technologies (Mullighan et al. 2007; Ley et al. 2008).

CML and EMS

Genetic and clinical characteristics

CML is molecularly characterized by the presence of the *BCR/ABL1* fusion gene, that in most cases arises from the reciprocal chromosome translocation t(9;22) (Goldman and Melo 2008). EMS is characterized by rearrangements of chromosome band 8p11 harboring the *FGFR1* gene. In EMS, *FGFR1* has so far been reported to fuse with 10 different partner genes (Table 2). Among the roughly 65 EMS cases reported so far, t(8;13) is the most common translocation, found in about one third of the cases (supplemental Table). The t(8;22), t(8;9), and t(6:8) are equally common with about half of the 65 cases harboring one of these translocations. The rest of the fusions have been reported in only a few cases each.

EMS is evenly distributed among males and females, with patients' age ranging between 3 and 84 years, and with a median age of presentation of 44 years (Jackson et al. 2010). CML has a slight male predominance of 1.3-1.4, and a somewhat higher median age of presentation of 55 years (Cortes 2004; Rohrbacher and Hasford 2009). Clinically, both CML and EMS often present with leukocytosis and splenomegaly. Basophilia is common in CML, but is rarely found in EMS, which more often displays eosinophilia. In both CML and EMS, the disease begins with a relatively indolent chronic phase that subsequently progress into an aggressive acute phase, i.e. blast crisis, a condition that resembles acute leukemia with accumulation of immature blast cells in BM and blood. Whereas the chronic phase of CML commonly last for several years, in EMS the disease usually progresses into an acute phase within a few months, hence many EMS patients are not diagnosed until the disease has already entered the acute phase (Jackson et al. 2010). In about two-thirds of the CML cases, the blasts in the acute phase are of myeloid phenotype, whereas in the remaining third they are lymphoid (Randolph 2005). In the majority of EMS cases, the acute phase manifests itself as AML, but in rare cases it is defined as ALL, with a B-cell phenotype being more common than T-cell leukemia (supplemental Table). In

Table 2. Different *FGFR1* fusion partner gene described to date.

Genetic aberration	Fusion partner gene[a]	Reference
t(8;13)	*ZMYM2* (*ZNF198*)	(Popovici et al. 1998; Reiter et al. 1998; Smedley et al. 1998; Xiao et al. 1998)
t(6;8)	*FGFR1OP (FOP)*	(Popovici et al. 1999)
t(8;9)	*CEP110*	(Guasch et al. 2000)
t(8;22)	*BCR*	(Demiroglu et al. 2001; Fioretos et al. 2001)
t(8;19)	*HERVK*	(Guasch et al. 2003)
ins(12;8)	*FGFR1OP2*	(Grand et al. 2004)
t(7;8)	*TRIM24*	(Belloni et al. 2005)
t(8;17)	*MYO18A*	(Walz et al. 2005)
t(8;12)	*CPSF6*	(Hidalgo-Curtis et al. 2008)
t(2;8)	*LRRFIP1*	(Soler et al. 2009)

[a]Commonly used alternative gene symbols are presented within parenthesis.

some cases the lymphoid cells also express myeloid markers. Although the different fusion partner genes fuse to the same breakpoint in *FGFR1*, they induce at least partly dissimilar kinds of disease pattern in patients; in cases harboring *ZMYM2/FGFR1* the myeloid disorder is often accompanied by T-cell lymphoma, whereas this has not been found in cases with *BCR/FGFR1* or *FGFR1OP/FGFR1*. Monocytosis is more common in cases with *CEP110/FGFR1*, and to some extent cases with *ZMYM2/FGFR1*, than what has been described in cases harboring *BCR/FGFR1* or *FGFR1OP/FGFR1* (supplemental Table).

The mechanism for disease progression in CML and EMS is still unclear. In CML trisomy 8, isochromosome 17q, and duplication of the Ph chromosome are the most common secondary genetic aberrations found upon progression into blast crisis (Fioretos and Johansson 2009). There are however no mechanistic explanations for how these chromosomal changes induce blast crisis. One interesting theory for disease progression in CML is that the increased activity of *BCR/ABL1* found in blast crisis patient cells, would induce a maturation block in myeloid cells, possibly in combination with additional mutations that confer subsequent activation of β-catenin (Gaiger et al. 1995; Jamieson et al. 2004;

Abrahamsson et al. 2009). Also in EMS, additional chromosomal aberrations are commonly found in the acute phase of the disorder. Roughly one third of the reported EMS cases harbors an additional chromosome 21 (Article I)(Mitelman et al. 2009). Myeloid disorders with trisomy 21 have been reported to harbor mutations in *RUNX1* at high frequencies (Preudhomme et al. 2000; Taketani et al. 2003). A detailed investigation of an EMS case in a progressed phase, also harboring an additional t(9;22), revealed that *RUNX1* was translocated, with haploinsufficiency of *RUNX1* as a possible outcome (Article I). Since *RUNX1* is a transcription factor important for cellular differentiation, this could confer a class II mutation, which would be in line with the two-hit model discussed earlier.

The introduction of the tyrosine kinase inhibitor (TKI) imatinib mesylate (Gleevec) as first line treatment for chronic phase CML has greatly increased the survival rate among patients with this disorder (O'Brien et al. 2003; Druker et al. 2006; Roy et al. 2006). The imatinib molecule binds the ATP-binding pocket of BCR/ABL1, thereby preventing activation of the tyrosine kinase and abrogating downstream signaling (Deininger et al. 2005). Resistance to imatinib may develop through mutations in the *ABL1* kinase domain, and the effect of treatment in the advanced phase of CML is modest. This has led to the development of second generation TKIs, such as dasatinib and nilotinib, which have demonstrated improved response in recent clinical trials (Kantarjian et al. 2010; Saglio et al. 2010). None of the compounds provides a cure for CML as they do not target the quiescent leukemia stem cells (Graham et al. 2002; Copland et al. 2006; Jorgensen et al. 2007), thus life-long treatment is probably necessary to control the disease. In patients with EMS, there is no available targeted therapy. In fact, no drug has been successfully used in treatment of EMS patients for a longer period of time. A few TKIs, including PKC412 and TKI258, have been shown to inhibit the proliferation of cells expressing *FGFR1* fusion genes in vitro, or to prolong the survival of mice transplanted with *FGFR1* expressing cells (Chen et al. 2004; Chase et al. 2007), but the low incidence of EMS and the lack of a relevant in-vivo model have hampered further development. At present the only permanent cure for EMS is allogenic BM transplantation.

BCR/ABL1 and FGFR1 fusion genes and their biological effects

The BCR moiety of BCR/ABL1 contains a coiled-coil domain that facilitates oligomerization and subsequent activation of the ABL1 tyrosine kinase (McWhirter et al. 1993) (Figure 3). It has been suggested that *BCR/ABL1* induces

increased cellular proliferation and decreased sensitivity to apoptosis, provokes genetic instability, and interferes with DNA-repair (Melo and Barnes 2007; Quintas-Cardama and Cortes 2009). Most BCR/ABL1-induced effects are abrogated in kinase deficient mutants (Zhang and Ren 1998; Wertheim et al. 2002; Ramaraj et al. 2004), demonstrating that the kinase activity is crucial for mediating the leukemogenic signals. Depending on the breakpoint in *BCR*, there are three variants of the *BCR/ABL1* fusion gene. The two most common are P190 *BCR/ABL1*, which mainly are found in Ph-positive ALL, and P210 *BCR/ABL1* that is most common in CML, but also found in a fraction of Ph-positive ALL (Figure 3) (Melo 1996). The third is P230 *BCR/ABL1*, a rare variant mainly associated with chronic neutrophilic leukemia (CNL). It is currently uncertain whether the reason for differences in disease manifestation between P190 and P210 *BCR/ABL1* depend on intrinsic properties of the fusion proteins, or that their different clinical

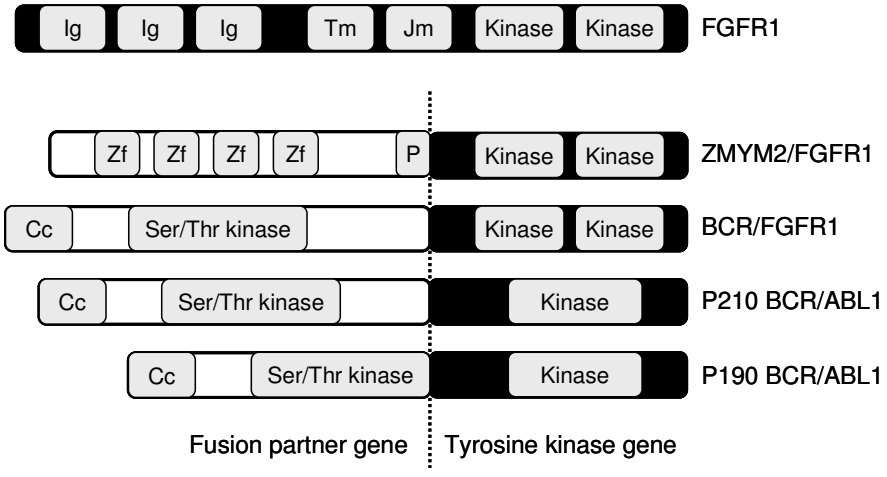

Figure 3. Schematic overview of normal FGFR1 and the ZMYM2/FGFR1, BCR/FGFR1, P210 BCR/ABL1 and P190 BCR/ABL1 fusion proteins. FGFR1 consists of an extracellular domain with an N-terminal signal peptide and three immunoglobulin-like domains (Ig), followed by a transmembrane domain (Tm), a juxtramembrane domain (Jm), an intracellular tyrosine kinase domain split by a non-catalytic interkinase domain, and a short C-terminal tail. The BCR and ZMYM2 moieties involved in the fusion genes contribute with N-terminal oligomerization domains in the shape of zink finger-like (Zf) and proline rich (P) domains or a coiled-coil domain (Cc), respectively. The C-terminal tyrosine kinase domains of FGFR1 and ABL1 are retained in the fusion proteins. The different proteins and the various domains are not drawn to scale.

disease characteristics depend on the type of hematopoietic cell in which transformation occurs. In favor of the theory of intrinsic properties is that P190 has been shown to harbor a higher tyrosine kinase activity than P210 (Lugo et al. 1990), and in primary mouse BM cells P190 induces lymphoid expansion, whereas P210 under the same conditions induces myeloid expansion (Quackenbush et al. 2000). Also, in transgenic mice, P190 induces a distinct disease with shorter latency than P210 (Voncken et al. 1995). In contrast, P190 and P210 induces a similar phenotype in mouse B-lymphoid precursor cells and in murine BM transplant assays, however, P190 confers a higher proliferative advantage and elicits a more aggressive disease than P210 (McLaughlin et al. 1989; Kelliher et al. 1991). In another murine BM transduction/transplant model, P190 induces lymphoid disease and P210 myeloid disease, but when 5-fluorouracil (5-FU) treated donors are used both variants induce a CML-like disorder (Li et al. 1999). Since 5-FU treatment favors the survival of more quiescent cells, this could imply that the differences in disease manifestation are a result of different precursor cells being transformed. An indirect support for the model of different cellular origins is that in BM from *BCR/ABL1* positive ALL cases, P190 is only found in B progenitor cells whereas P210 is also found in the HSC compartment (Castor et al. 2005). Interestingly, in human primitive hematopoietic cells derived from cord blood (CB), expression of P210 and P190 induce similar proliferation and differentiation characteristics as well as high similarities in their global gene expression profiles. This suggests that intrinsic differences in the fusion proteins are limited (Article II).

Whereas the biological effects of *BCR/ABL1* have been extensively studied, only few studies have more thoroughly investigated fusion genes involving *FGFR1*. The normal *FGFR1* gene is located on the short arm of chromosome 8, and comprises 19 exons. Numerous splice variants have been found, involving secreted receptor isoforms lacking the kinase domain, with the different isoforms being differentially expressed in various cells and tissues (Johnson et al. 1990; Johnson and Williams 1993). Upon ligand binding, a conformational change is induced in FGFR1, leading to dimerization and subsequent activation of FGFR1 by autophosphorylation of the intracellular domain (McKeehan et al. 1998). To induce dimerization, the FGF/FGFR1 complex must bind to heparin or heparin sulfate proteoglycans, which regulate the ligand binding and the activation of the receptor (Rapraeger et al. 1991; Yayon et al. 1991; Schlessinger et al. 2000). In *FGFR1* fusion genes, the partner genes always constitute the 5’ part, fused to exon

9 of *FGFR1*. Thus, the N-terminal immunoglobulin-like domains facilitating normal FGFR1 ligand binding, with subsequent dimerization and activation, are lost. In ZMYM2/FGFR1, the ZMYM2 moiety contains a proline rich domain and several zinc finger-like domains, predicted to facilitate protein-protein interactions (Figure 3). ZMYM2/FGFR1 has been shown to dimerize or oligomerize in several cell lines (Ollendorff et al. 1999; Smedley et al. 1999; Xiao et al. 2000). Although it has not been experimentally proven that the other FGFR1 fusion proteins dimerize or oligomerize, they are predicted by their amino acid sequence to function in a similar manner. Since the tyrosine kinase domain of FGFR1 is retained in the fusion proteins, the functional outcome of the dimerization or oligomerization is a constitutively activated FGFR1 tyrosine kinase. This has been experimentally shown for ZMYM2/FGFR1 and FGFR1OP/FGFR1, both of which have constitutively activated kinase activity when expressed in cell lines (Smedley et al. 1999; Guasch et al. 2001). Similar to BCR/ABL1, in kinase defect mutants the transforming ability of FGFR1 fusion proteins is lost, in vitro and in vivo (Guasch et al. 2001; Roumiantsev et al. 2004). Thus, the most important contribution of the *FGFR1* partner gene seems to be its ability to induce dimerization or oligomerization of the fusion protein, thereby enabling the critical constitutive activation of the FGFR1 tyrosine kinase. This results in increased cellular proliferation and decreased sensitivity to apoptosis (Jackson et al. 2010).

Additional roles for FGFR1 fusion partner genes?

There are indications that the fusion partner protein contributes to more than just dimerization. Similar to BCR/ABL1, BCR/FGFR1 contains a binding site for GRB2 through phosphorylation of Tyr177 on the BCR moiety, and the downstream signaling events possibly direct the disease in transplanted mice into a more CML-like variant (Roumiantsev et al. 2004). The fusion partner protein could also influence the cellular localization of the FGFR1 fusion protein. Whereas the normal human FGFR1 protein localize to the plasma membrane, ZMYM2/FGFR1 instead localizes to the cytoplasm (Ollendorff et al. 1999; Smedley et al. 1999). In contrast, CEP110/FGFR1 and FGFR1OP/FGFR1 localizes to the centrosomes in mouse cells (Delaval et al. 2005). Phosphorylated FGFR1OP/FGFR1, but not CEP110/FGFR1, recruits and activates PI3K and PLCγ at the centrosome, thereby possibly affecting centrosome duplication (Lelievre et al. 2008). Gene expression profiling of CB cells has revealed that cells expressing *BCR/FGFR1* or *ZMYM2/FGFR1* are not more similar than cells

expressing *BCR/ABL1*, indicating a role for the fusion partner in downstream gene regulation (Article III). A more empirical indication that the fusion partner protein contributes to signaling is the fact that although they share the same breakpoint in *FGFR1*, the different fusion genes induces at least partly dissimilar kinds of disease pattern in patients (Jackson et al. 2010). However, given the relatively few published cases of EMS with various fusion partners, this remains to be validated in future investigations.

Signaling induced by BCR/ABL1 and FGFR1 fusion genes

The molecular actions of the *BCR/ABL1* fusion gene have been extensively studied, and although all the *BCR/ABL1*-induced effects are not fully understood, it has been demonstrated that *BCR/ABL1* perturbs many intracellular signaling pathways, including RAS/ERK, PI3K/AKT, and JAK/STAT (Melo and Deininger 2004; Quintas-Cardama and Cortes 2009). Fewer studies have focused on signaling induced by FGFR1 fusion proteins. In normal FGFR1-mediated signaling, autophosphorylation of Tyr766, in combination with Tyr653 and Tyr654, result in activation of the PKC pathway via PLCγ, resulting in stimulation of RAF/ERK signaling pathway (Mohammadi et al. 1991; 1996) (Figure 4). Depending on ligand binding and cellular context, signaling through the MAPK pathway results in cell proliferation and/or differentiation. The ability to bind and activate PLCγ via Tyr766 is retained in ZMYM2/FGFR1 and FGFR1OP/FGFR1 expressing murine cells (Guasch et al. 2001; Roumiantsev et al. 2004). In a murine BM transplant assay, FGFR1OP/FGFR1 rapidly induces a fatal myeloproliferative disorder without progression to blast phase, whereas a FGFR1OP/FGFR1 variant with mutated PLCγ binding site induces a milder myeloproliferative disorder, where some mice develop thymic lymphoma (Guasch et al. 2004). This indicates that PLCγ signaling is important in FGFR1 mediated leukemogenesis.

The juxtamembrane domain of normal FGFR1 contains binding sites for the FRS2 family of docking proteins, which are lipid anchored adaptor proteins linking FGFR1 to downstream signaling pathways (Ong et al. 2000). Tyrosine phosphorylated FRS2α activates the RAS/ERK and PI3K signaling pathways, resulting in signals for cellular proliferation/differentiation and survival (Kouhara et al. 1997; Ong et al. 2001; Zhang et al. 2008). Ligand dependent stimulation of the RAS/ERK signaling pathway could also be induced independently of FRS2, and is at least partly dependent on Tyr766 (Klint et al. 1995). In FGFR1 fusion proteins, the juxtamembrane domain is disrupted, thus binding and signaling via

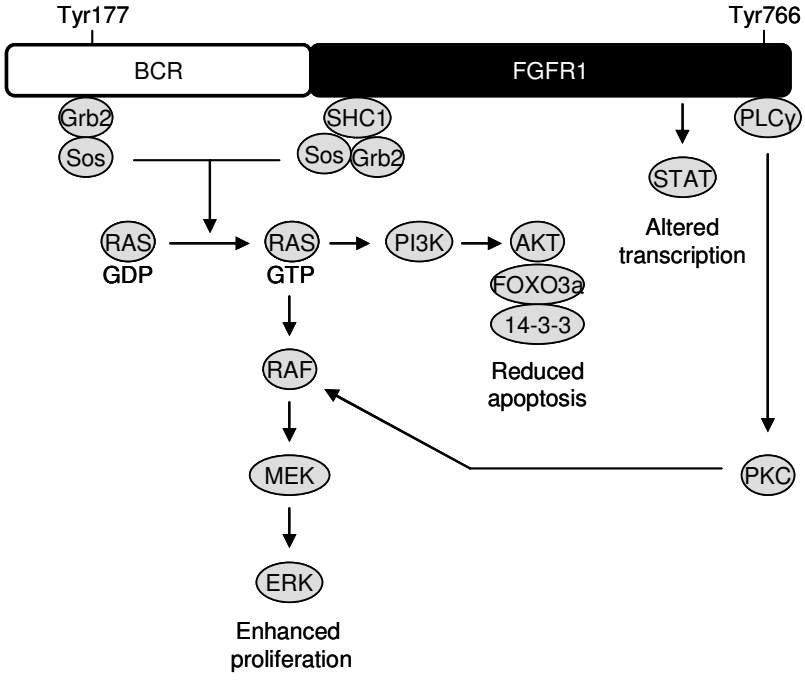

Figure 4. Schematic overview of signaling pathways activated by FGFR1 fusion proteins. The FGFR1 moiety has binding sites for SHC1 and PLCγ, resulting in downstream activation of RAS/ERK, RAS/PI3K, and PKC/RAF. Furthermore, signaling through STAT seems to be important, but the exact mechanisms for STAT activation or which genes that are targeted by STAT have not been further elucidated. In BCR/FGFR1, the BCR moiety has a binding site for GRB2, also resulting in activation of RAS/ERK. The fusion protein figure is not drawn to scale.

FRS2 is lost. However, expression of FGFR1OP/FGFR1 has been shown to promote signal transduction through ERK, although FGFR1OP/FGFR1 act as a less potent activator of ERK than FGFR1 (Guasch et al. 2001). BCR/FGFR1 and ZMYM2/FGFR1 expressing murine cells are sensitive to farnesyltransferase inhibitors, indicating that RAS is important, but inhibiting MEK had no effect on transformed cells, suggesting that the classical MAPK pathway (RAF-MEK-ERK) is not activated; instead proliferation is blocked by inhibitors against p38 and PI3K (Demiroglu et al. 2001).

FGFR1OP/FGFR1 has been shown to activate PI3K and AKT, thereby possibly protecting the cells from apoptosis (Guasch et al. 2001).

ZMYM2/FGFR1 was reported to activate both AKT and ERK, resulting in phosphorylation and 14-3-3 dependent sequestration of the AKT-target FOXO3A, potentially leading to reduced apoptosis by preventing FOXO3A induced transcription of pro-apoptotic genes such as FAS ligand (Porter et al. 2006; Dong et al. 2007). In murine cells, ZMYM2/FGFR1 induces expression of the serine proteinase inhibitor SERPINB2, and the level of SERPINB2 protein correlates with resistance to TNFα mediated apoptosis (Kasyapa et al. 2006).

Several studies have reported phosphorylation of STAT proteins in response to the expression of *FGFR1* fusion genes. FGFR1OP/FGFR1 phosphorylates STATs 1 and 3, but not STAT5 in murine cells (Guasch et al. 2001). Several studies reported that ZMYM2/FGFR1 induces phosphorylation of STATs 1, 3, and 5 (Smedley et al. 1999; Baumann et al. 2003; Heath and Cross 2004). The activation of STAT5 by FGFR1 fusion proteins suggests that STAT5, in contrast to what has been reported for normal FGFR1, could be one of the key effectors in FGFR1 fusion protein signaling. Indeed, silencing of STAT5 with shRNA results in decreased cellular proliferation (Article III). However, the downstream effects of STAT5 activation in FGFR1 fusion gene expressing cells have not been investigated, though it seems likely that the role of STAT5 in EMS leukemogenesis depends on transcriptional activation of STAT5 target genes.

Experimental models of EMS and CML in human cells

A majority of the studies on EMS and CML has been performed in cell lines, often of murine origin, or murine BM transplant assays. Although these studies have provided valuable insights into possible disease mechanisms, the cellular context is distant from the human immature blood cell in which the initiating genetic event in MPN is believed to take place (Kennedy and Barabe 2008; Mulloy et al. 2008). One approach to more faithfully study human MPN cells in vivo is to transplant primary patient cells into immunodeficient mice. This has been done in a number of studies using chronic phase CML cells. In the early studies crude mononuclear cell fractions were used for transplantation into SCID mice. The average engraftment of human cells was around 1%, with a majority of the engrafted cells being Ph^-, suggesting that growth of normal stem cells was favored (Sirard et al. 1996). Transplantation of mononuclear cells into NOD/SCID instead of SCID mice resulted in an approximate 10-fold increase in engraftment of human cells, with the proportion of Ph^+ cells being increased to 40-70% (Lewis et al. 1998;

Wang et al. 1998). Compared to the use of crude mononuclear cell fractions, isolation of $CD34^+$ cells prior to transplantation result in higher engraftment of human cells in relation to the number of transplanted cells, indicating an enrichment of leukemic stem cells in the $CD34^+$ cell fraction (Lewis et al. 1998; Verstegen et al. 1999). Typically, the leukemic cells in the murine BM express a shift towards a myeloid phenotype in contrast to normal HSC that predominantly give rise to lymphoid cells in NOD/SCID mice (Sirard et al. 1996; Lewis et al. 1998; Verstegen et al. 1999). Surprisingly, in one study the mice displayed high levels of human Ph^+ mast cells (Dazzi et al. 2000). It has been shown that P210 BCR/ABL1 forms a complex with the mast cell receptor c-kit in cell lines (Hallek et al. 1996), and that the ligand of c-kit, stem cell factor (SCF), induces preferential proliferation of the Ph^+ fraction of primary $CD34^+$ cells (Moore et al. 1998). These results could indicate a role for *BCR/ABL1* dependent c-kit activation in promoting mast cell proliferation of the human Ph^+ cells in murine BM. A minor proportion of mice transplanted with primary cells from chronic phase CML patients display MPN-like changes in BM and spleen, including hypercellularity and increased levels of megakaryocytes and eosinophils, as revealed by histopathologic analysis, but no transformation into an acute phase is displayed (Lewis et al. 1998). Altogether, transplantation of primary CML cells into immunodeficient mice yield highly variable results, as only cells from selected patients can repopulate the BM of immunodeficient mice, and in most cases the engraftment of human cells is below 10% and often less than 1%. In EMS, the rareness of this disorder in combination with its rapid progression into acute leukemia, further complicate these types of studies.

An alternative approach to model CML and EMS would be to use primitive hematopoietic cells derived from human CB, transduce them with the fusion gene of interest, and study the fusion gene expressing cells in vitro. Such studies have predominantly been performed for over-expressed genes or fusion genes found in acute leukemia (Kennedy and Barabe 2008; Mulloy et al. 2008). A few studies have focused on MPN, and mainly on *BCR/ABL1*. P210 *BCR/ABL1* induces increased cellular proliferation and erythropoietin (EPO)-independent erythroid differentiation in cells cultured in vitro (Chalandon et al. 2002; Modi et al. 2007). Expression of P190 or P210 *BCR/ABL1* induces STAT5-dependent expansion of erythroid cells, and similar gene expression signatures (Article II). Interestingly, similar to *BCR/ABL1*, expression of *BCR/FGFR1* and *ZMYM2/FGFR1* in human primitive CB cells results in increased proliferation and erythroid differentiation

(Article III). The erythroid differentiation is somewhat surprising, since this is not a characteristic feature in CML and most EMS cases. One explanation for the erythroid differentiation may be an inherent propensity for CB cells to erythroid expansion. However, following expression of *BCR/ABL1*, *BCR/FGFR1*, or *ZMYM2/FGFR1* in $CD34^+$ cells derived from adult BM, a similar erythroid phenotype is seen, indicating that the erythroid phenotype is not dependent on the CB origin of the $CD34^+$ cells (Article III). Moreover, chronic phase CML patients display an increase of megakaryocyte/erythrocyte progenitor (MEP) cells, and primary cells from CML patients have been shown to form EPO-independent burst-forming units erythroid (BFU-E) colonies (Eaves and Eaves 1979; Jamieson et al. 2004).

Transplantation of the transduced CB cells into immunodeficient mice allows in vivo investigations of fusion gene expressing cells. A number of studies using such xeno-transplantation protocols have been reported for fusion genes or over-expressed genes associated with AML or ALL. However, most mice do not develop a significant hematologic disorder (Buske et al. 2001; Mulloy et al. 2003; Shen et al. 2004; Vercauteren and Sutherland 2004; Bäsecke et al. 2005; Reynaud et al. 2005; Wunderlich et al. 2006; Hong et al. 2008). Two important exceptions are the *MLL/AF9(MLLT3)* and *MLL/ENL(MLLT1)* fusion genes, both of which are shown to fully transform primitive human cells when transplanted into immunodeficient mice, and to induce disease phenotypes closely resembling the corresponding human leukemia (Barabe et al. 2007; Mulloy et al. 2008; Wei et al. 2008).

A few retroviral xeno-transplantation studies have focused on fusion genes associated with human MPN. One such fusion gene is *TEL(ETV6)/JAK2,* which has been found in cases with atypical CML, but also in T-ALL and B-ALL (Lacronique et al. 1997; Peeters et al. 1997). In contrast to human disease, *TEL/JAK2*-expressing CB cells induced a PMF-like phenotype in immunodeficient mice (Kennedy et al. 2006), thereby more closely resembling characteristics of disorders associated with the JAK2 mutation V617F. JAK2V617F, which is commonly found in PMF, PV, and ET has not been studied this way. However, when primary cells from PV patients expressing JAK2V617F were transplanted to immunodeficient mice, increased erythroid differentiation was observed (Geron et al. 2008). *BCR/ABL1*-transduced human CB cells transplanted into immunodeficient mice results in an increased ratio of human myeloid or erythroid over B-lymphoid cells (Chalandon et al. 2005). However, in general, the level of

human cell engraftment was very low. At 5-6 months after transplantation only two mice displayed slightly increased white blood cell count and one mouse suffered from splenomegaly, indicative of MPN-like disease, but no animal was shown to develop blast crisis. It is questionable if the described model represents a reproducible model of CML. Interestingly in the closely related MPN EMS, upon transplantation of human CB cells expressing *BCR/FGFR1* or *ZMYM2/FGFR1* into immunodeficient mice, an expansion of the human myeloid cell compartment, together with an increase in erythroid cells, was seen. In a majority of *BCR/FGFR1* mice, human granulocytopoiesis displayed a left-shifted maturation pattern with marked expansion of myeloid cells at all stages of maturation, including eosinophilia, which is in agreement with findings described in the chronic phase of human EMS. In addition, splenomegaly was commonly noted, and several *BCR/FGFR1* and *ZMYM2/FGFR1* mice displayed blast accumulation indicative of disease progression (Article III).

In conclusion, the establishment of humanized mouse models that faithfully recapitulates human MPN has been difficult. Improvement of such models should be encouraged by the findings provided in this thesis, and, hopefully, lead to the development of relevant disease models of human MPN.

THE PRESENT INVESTIGATION

Aims of the study

The general aim of this thesis project was to study *BCR/ABL1* and *FGFR1* fusion genes in a relevant cellular context, that of primary human hematopoietic cells, in order to increase our understanding of disease mechanisms underlying CML and EMS. More specifically, the aims were:

- To identify genetic changes responsible for disease progression in human EMS. (Article I)

- To study and compare the effects of P190 and P210 *BCR/ABL1* expression in primary human hematopoietic cells, in order to better understand the basis for the different disease phenotypes associated with the two fusion gene variants. (Article II)

- To investigate the consequences of *ZMYM2/FGFR1* and *BCR/FGFR1* expression in primary human hematopoietic cells in vitro and to establish and characterize a model of human EMS in immunodeficient mice. (Article III).

Methods

This section contains a brief overview of the methods used in this thesis project. For a more detailed description of the material and methods used, the reader is referred to the individual articles (Articles I-III).

Molecular methods

In Article I, several molecular methods were used. In short, fluorescence in situ hybridization (FISH) was used to characterize a translocation involving chromosomes 9 and 21. Rapid amplification of cDNA ends (RACE) showed that *RUNX1* exon 4 was fused to repetitive chromosome 9 sequences, a finding that was confirmed by polymerase chain reaction (PCR) analysis. Sequencing and bioinformatics, including RepeatMasker and Open Reading Frame Finder analyses, were performed to further characterize the fusion. Southern blot analysis was used to identify the genomic breakpoint in *RUNX1*. Northern blot analysis was performed to analyze the expression of the fusion RNA in different tissues and cell lines. Standard PCR and western blot analyses were performed as outlined in Articles II and III.

Global gene expression analysis

Gene expression analysis using microarrays was used to compare the expression profiles induced upon retroviral transduction of umbilical CB $CD34^+$ cells using P210 and P190 BCR/ABL1 (Article II), and BCR/FGFR1 and ZMYM2/FGFR1 in relation to P210 BCR/ABL1 (Article III). Two different platforms were used. In Article II, oligonucleotide microarray slides containing 27,000 oligos representing approximately 16,000 genes were used on RNA isolated from cells collected at day 2, 3, and 4 after transduction. In Article III, Affymetrix U133 plus 2.0 microarrays, containing 54,000 probe sets corresponding to approximately 38,500 genes, were used on three biological replicates of RNA isolated from cells collected at two days after transduction. In both cases, unsupervised analysis was performed using hierarchal clustering analysis based on Pearson correlation or Euclidean distance and average linkage. Data analysis and visualization was performed using TIGR Multi Experiment Viewer (Saeed et al. 2003). Differentially expressed genes were extracted using the significance of microarrays (SAM) method with a false discovery rate of <1% (Tusher et al.

2001). The gene lists generated from the SAM analyses were subjected to gene ontology and pathway analysis using the EASE software (Hosack et al. 2003).

Viral vectors and retroviral transduction

For the introduction of fusion genes into human cells, the murine stem cell virus (MSCV) vector was used (Articles II and III). This retroviral bicistronic vector co-express IRES-GFP and a cDNA insert of choice. Six different inserts were used, including P210 *BCR/ABL1*, P190 *BCR/ABL1*, *BCR/FGFR1*, *ZMYM2/FGFR1*, and the kinase deficient variants *BCR/FGFR1* Y653/654F and *ZMYM2/FGFR1* Y653/654F. As a control the empty vector MSCV-IRES-GFP (MIG) was used. The viral vectors were produced by transient transfection of Phoenix-ampho or 293T cells and subsequent harvesting of the VSV-G or RD114 viral envelope pseudotyped vectors. Prior to transduction, $CD34^+$ cells isolated from human CB were thawed and prestimulated for 48 hours in medium supplemented with serum and the cytokines SCF, TPO, and FLT3 ligand, in order to induce cell cycling that is required for the integration of the desired gene into the hosts genome. The viral vectors were preloaded onto plates using retronectin, whereafter cells were added. Two days after transduction, GFP^+ cells were sorted using a FACSAria cell sorter (Becton Dickinson). For the in vivo studies, 2-4 x 10^5 unsorted transduced cells per mouse were transplanted one day after transduction (Article III). For the silencing of STAT5, lentiviral short-hairpin RNA (shRNA) scramble and anti-STAT5 expressing vectors (Scherr et al. 2006), co-expressing red fluorescent protein (RFP), were produced according to standard methods with VSV-G pseudotyping.

In vitro assays

Two different systems were used for studying the proliferation and differentiation characteristics of cells in vitro: suspension culturing and colony forming assays (CFA). For cell culturing in suspension media, the medium was supplemented with SCF, TPO, FLT3 ligand, IL3, and IL6 to promote myeloid differentiation. Analysis of the cells' proliferative capacity was assessed by manual counting. To study cellular differentiation characteristics, cells were stained with fluorophore-coupled antibodies against CD34, CD13, CD235a, and CD71, and analyzed by flow cytometry (FACS). To validate and further characterize the differentiation of the cells, cytospin slides were prepared from suspension culture cells, stained with

May-Grünwald-Giemsa. To study the lineage potential of more immature cells in CFA, cells were plated at low density in methylcellulose, and the number of primary and secondary erythroid and myeloid colonies was counted.

Humanized mouse models

For the transplantation of human cells into mice, three different mouse strains were used: NOD/SCID (Prochazka et al. 1992), NOD/SCID $\beta 2m^{-/-}$ (Christianson et al. 1997), and NOD/SCID IL2-receptor deficient (NSG) (Shultz et al. 2005). All three mouse strains are heavily immuno-compromised with impaired T and B lymphocyte development, to allow the engraftment of human cells which would otherwise be rejected by the host's immune system. NOD/SCID $\beta 2m^{-/-}$ is a derivate of the conventional NOD/SCID mouse, but has an additional immune defect, a deleted $\beta 2$-microglobulin gene, resulting in reduction of functional NK cells. NSG mice combine the features of the NOD/SCID background and IL2 receptor gamma chain deficiency. As a result, the NSG mice lack mature T cells and B cells, functional NK cells, and are deficient in cytokine signaling, leading to better engraftment of human hematopoietic cells.

The day before transplantation, mice were subjected to a non-lethal dose of radiation (300-350 Rad) in order to reduce the number of hematopoietic cells in the BM. From this day on the mice were given antibiotics and powder food. Intravenous or intrafemoral injections were used, and each mouse was transplanted with 2-4 x 10^5 unsorted cells retrovirally transduced with *BCR/FGFR1*, *ZMYM2/FGFR1* or the MIG control. To analyze the engraftment of human cells, mice were sedated and BM was aspirated from the right femur. The BM cells were subject to FACS analysis as described above, using antibodies against CD45, CD34, CD19, CD33, CD15, and CD235a. Human cells were defined as cells expressing GFP and/or CD45. When found to be pre-moribund, or at end of study, mice were sacrificed. To investigate the morphological changes induced by the fusion genes, the left femur, spleen and liver were fixed in formalin. The samples were decalcified (femur), sectioned, and stained with hematoxilin/eosin (HE) to allow morphology analysis, silverstaining was used to analyze reticulin, and immunostaining to allow detection of human cells at various differentiation stages. Antibodies against CD3, CD4, CD15, CD20, CD34, CD45, CD68, CD79a, CD117, CD235a, and myeloperoxidase (MPO) were used for immunostainings, and the antibodies were analyzed for cross-reactivity using normal mouse cells. BM-sections were also examined using FISH with centromer-specific probes in

order to validate the human or murine origin of cells in the murine BM. All investigations on mice were approved by the regional ethics committee.

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 also referred to the original articles provided in the final section.

Article I

Fusion gene-mediated truncation of *RUNX1* as a potential mechanism underlying disease progression in the 8p11-myeloproliferative syndrome

One characteristic feature of EMS is the rapid progression of disease into an aggressive acute phase. The mechanisms underlying this disease progression in EMS remains unknown.

In this study, a case of EMS harboring a *BCR/FGFR1* fusion gene as well as a secondary t(9;21)(q34;q22) at the time of AML transformation, was investigated. FISH and RT-PCR analyses revealed that the t(9;21) leads to a fusion gene consisting of the 5' part of *RUNX1* (exons 1–4) fused to repetitive sequences including a stop codon of a gene with an unknown function on chromosome 9. The t(9;21) hence results in a truncation of *RUNX1*. No point mutation was found in the other *RUNX1* allele. The most likely functional outcome of this rearrangement is the haploinsufficiency of RUNX1. The autosomal dominant familial platelet disorder with predisposition to AML (FPD/AML) has been linked to haploinsufficiency of *RUNX1* (Song et al. 1999; Michaud et al. 2002), and point mutations in *RUNX1* are known to play an important role in leukemogenesis (Osato 2004). Furthermore, studies in knock-out mice have suggested that the function of Runx1 is dose dependent (Sun and Downing 2004). Haploinsufficiency of RUNX1 may thus be one mechanism by which EMS transforms to AML. The presence of such an alteration in a case that also harbored a *BCR/FGFR1* fusion gene is in agreement with the proposed two-step model of AML, in which two mutations cooperate in the leukemogenic process (Dash and Gilliland 2001). A survey of close to 50 published EMS cases revealed that roughly one third had trisomy 21 as a secondary change. In the present study, three additional cases of EMS were analyzed for possible *RUNX1* mutations, but none were identified. However, none of the additional cases harbored an extra chromosome 21. Thus, if

point mutations in *RUNX1* are frequent in EMS with trisomy 21 remains to be investigated.

Article II

Expression of P190 and P210 *BCR/ABL1* in normal human CD34$^+$ cells induces similar gene expression profiles and results in a STAT5-dependent expansion of the erythroid lineage

The P190 *BCR/ABL1* fusion gene is found in ALL, whereas P210 *BCR/ABL1* is mainly associated with CML. It is presently unclear whether these two variants of the *BCR/ABL1* fusion gene are functionally different following expression in primitive human hematopoietic cells.

In this study, the effects of retroviral P190 and P210 *BCR/ABL1* expression on cell proliferation, differentiation, and global gene expression in human CB derived CD34$^+$ cells, was investigated and systematically compared. Expression of either P190 or P210 *BCR/ABL1* resulted in expansion of erythroid cells and stimulated EPO-independent formation of erythroid colonies. Similar findings following retroviral expression of P210 *BCR/ABL1* have been reported previously (Chalandon et al. 2002). This was now also, for the first time, demonstrated for P190 *BCR/ABL1*. Both P210 and P190 BCR/ABL1 induced phosphorylation of STAT5. Following silencing of STAT5 by expression of anti-STAT5 short-hairpin RNA, it was found that both P190 and P210 BCR/ABL1-induced erythroid cell expansion were STAT5-dependent. Under in vitro conditions favoring B-cell differentiation, neither P190 nor P210 *BCR/ABL1*-expressing cells formed detectable levels of CD19-positive cells. Gene expression profiling revealed that P190 and P210 *BCR/ABL1* induced almost identical gene expression profiles.

In conclusion, the results obtained in Article II suggest that the early cellular and transcriptional effects of P190 and P210 *BCR/ABL1* expression are very similar when expressed in the same human progenitor cell population, and that STAT5 is an important regulator of BCR/ABL1-induced erythroid cell expansion. The findings do not support the idea of a functional difference between P190 and P210 *BCR/ABL1*, but instead indirectly support the idea that differences in clinical disease characteristics induced by the two variants depend on transformation of different hematopoietic precursor cells.

Article III

Modeling the human 8p11-myeloproliferative syndrome in immunodeficient mice

EMS is molecularly characterized by fusion of various partner genes to the *FGFR1* gene, resulting in constitutive activation of the tyrosine kinases of FGFR1. To date, no previous study has addressed the functional consequences of ectopic *FGFR1* expression in the potentially most relevant cellular context, that of normal primary human hematopoietic cells.

Upon retroviral transduction of $CD34^+$ cell derived from CB, cells expressing *ZMYM2/FGFR1, BCR/FGFR1* or *BCR/ABL1* displayed increased cellular proliferation and differentiation toward the erythroid lineage in an EPO-independent manner. Global gene expression profiling revealed that *BCR/FGFR1*, *ZMYM2/FGFR1*, and *BCR/ABL1* all upregulated several genes involved in JAK/STAT signaling. To study if STAT5 activation was equally important for the three different fusion oncogenes, in terms of their effects on cellular proliferation, we co-transduced cells with anti-STAT5 shRNA and the different fusion genes. A significant decrease in cell proliferation was observed for all three fusion oncogenes, suggesting that STAT5 activation is an important mechanism whereby FGFR1 fusion oncogenes elicit their transforming effects.

$CD34^+$ CB cells expressing *BCR/FGFR1* or *ZMYM2/FGFR1* had the ability to repopulate the BM of immunodeficient mice. In agreement with flow cytometric data of aspirated BM cells, analysis of stained BM sections revealed expansion of the human myeloid cell compartment in *BCR/FGFR1* and *ZMYM2/FGFR1* mice, and an increase in erythroid cells. In 14 of 15 investigated *BCR/FGFR1* mice, and in five of six *ZMYM2/FGFR1* mice, clusters of human histiocytes and mast cells were found, indicating the expansion of multiple myeloid lineages. In ten of 15 *BCR/FGFR1* mice, human granulocytopoiesis displayed a left-shifted maturation pattern with marked expansion of myeloid cells at all stages of maturation, including eosinophilia, which is in agreement with findings described in the chronic phase of human EMS. Three of the *BCR/FGFR1* mice displayed an accumulation of human blasts in the BM. In two of the three mice, the blasts were positive for CD235a, suggesting the emergence of an acute erythroleukemia. In the third mouse, the blast refrained staining with all antibodies used, suggesting an immature and primitive origin of these blasts. Eleven of the 15 *BCR/FGFR1* mice and one of the six *ZMYM2/FGFR1* mice suffered from BM

fibrosis. Similar to patients with MPN, splenomegaly, consistent with an increased extramedullary hematopoiesis, was detected in several *BCR/FGFR1* mice. In most cases the same abnormalities seen in the BM were also observed in the spleen. Of the 21 *BCR/FGFR1* mice, seven died during the study due to disease. Histopathological and immunohistochemical analysis was performed on five of these mice, all displaying the characteristic features of disease, i.e. left-shifted granulocytopoiesis with eosinophilia, BM fibrosis, and splenomegaly. Three of the dead mice showed accumulation of blasts in the BM. All examined MIG mice displayed a relatively normal BM hematopoiesis with dispersed lymphoid and single granulocytic and erythroid human cells.

In a second set of experiments, the more severely immuno-compromised NSG mouse strain was used, resulting in higher engraftment of human cells. The increase in human myeloid and erythroid cells observed in *BCR/FGFR1* and *ZMYM2/FGFR1* NSG mice was typically more pronounced than that seen in NOD/SCID mice. Both fusion oncogenes induced an MPD-like disorder with left-shifted granulocytopoiesis, sometimes accompanied by eosinophilia and/or dysplastic megakaryocytes. *BCR/FGFR1* and *ZMYM2/FGFR1* NSG mice generally suffered from mild to severe BM fibrosis and displayed foci of human histiocytes and mast cells. Whereas the *BCR/FGFR1* mice all died of disease at day 23-32 after transplantation, most likely because of BM insufficiency caused by human myeloid cell expansion and/or fibrosis, the *ZMYM2/FGFR1* mice survived until day 38 or longer. Two *BCR/FGFR1* mice and one *ZMYM2/FGFR1* mouse showed blast expansion in their BM and/or spleen.

This study suggests that FGFR1 fusion oncogenes, by themselves, are capable of initiating an EMS-like disorder, and provides the first humanized model of a myeloproliferative disorder with blast accumulation indicative of transformation into acute leukemia in mice. The established in-vivo EMS model should constitute a valuable tool for obtaining further insights into *FGFR1* fusion gene mediated leukemogenesis and for the development and evaluation of new treatment strategies in EMS.

Conclusions

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

Article I

- *RUNX1* haploinsufficiency may be a mechanism underlying the disease progression of EMS.

- Trisomy 21 is a common secondary change at disease progression of EMS.

- The results support the proposed two-step model of AML in that an aberrant transcription factor gene (*RUNX1*) cooperates with a tyrosine kinase encoding fusion oncogene (*BCR/FGFR1*) in the leukemogenic process of EMS.

Article II

- Retroviral expression of P190 *BCR/ABL1* and P210 *BCR/ABL1* in primary human hematopoietic cells induce similar increased proliferative capacity and differentiation towards the erythroid lineage. The erythroid expansion for both P190 and P210 *BCR/ABL1*-expressing cells is partially STAT5-dependent as shown using shRNA-induced silencing.

- Retroviral expression of P190 and P210 *BCR/ABL1* in primitive human cells induces similar gene expression profiles.

- The findings indirectly support the notion that the difference in disease manifestation between P190 and P210 *BCR/ABL1* depends on separate cellular origins rather than intrinsic differences of the fusion proteins.

Article III

- Retroviral expression of *ZMYM2/FGFR1*, *BCR/FGFR1*, or *BCR/ABL1* in normal human hematopoietic cells results in increased cellular proliferation and erythroid differentiation in vitro, and similar global gene expression patterns.

- Primary human hematopoietic cells expressing *BCR/FGFR1* and *ZMYM2/FGFR1* have the ability to engraft immunodeficient mice.

- *BCR/FGFR1* and *ZMYM2/FGFR1* induce similar disorders in transplanted mice, and recapitulate human EMS in several ways, including the presence of eosinophilia, splenomegaly, and accumulation of blasts. The findings suggest that *FGFR1* fusion oncogenes, by themselves, are capable of initiating an EMS-like disorder.

- The established in-vivo EMS model is the first humanized model of a myeloproliferative disorder with blast accumulation indicative of transformation into acute leukemia in mice. As such it should constitute a valuable tool for obtaining further insight into *FGFR1* fusion gene mediated leukemogenesis and for the development and evaluation of new treatment strategies in EMS.

CONCLUDING REMARKS AND FUTURE PERSPECTIVES

Leukemias are characterized by the presence of chromosomal translocations generating fusion oncogenes (Mitelman et al. 2007; Pui et al. 2008). These fusion genes have been identified by careful investigations of primary cells obtained from patients. To obtain further insights in the functional consequences of the fusion proteins, different experimental systems are needed. During the last decades the investigations have mostly been performed using established non-hematopoietic cell lines and cells of murine origin. Lately, there has been an increasing awareness that the cellular context in which a fusion gene exerts its function is important (Kennedy and Barabe 2008; Mulloy et al. 2008). Thus, one of the main focuses of this thesis has been to use primary normal human cells to molecularly and functionally study fusion oncogenes.

EMS is a rare MPN that similar to CML is caused by a constitutively active tyrosine kinase and begins with a chronic phase. In EMS, however, the disease progression into aggressive leukemia is more rapid and there is a lack of effective treatment. In this thesis, investigations regarding disease progression in EMS were performed, and in support of the two-hit model proposed by Dash and Gilliland (2001), a mutation of a transcription factor, in this case a disruption of RUNX1, was suggested as a possible mechanism underlying disease progression. In addition, one third of the published EMS cases were found to harbor an extra chromosome 21 as a secondary genetic aberration. If trisomy 21 in EMS is associated with *RUNX1* mutations remains to be investigated, but it is interesting to note that myeloid malignancies with trisomy 21 have been shown to harbor only one *RUNX1* wild-type allele (Preudhomme et al. 2000). However, much work remains to elucidate the mechanisms underlying disease progression in both EMS and CML.

The rareness of EMS complicates studies of primary leukemic cells, and in particular in-vivo investigations that require large numbers of cells. This makes it critical to establish a suitable animal model for in vivo-studies of human EMS cells. Human primitive hematopoietic cells, retrovirally transduced with *BCR/FGFR1* and *ZMYM2/FGFR1*, were shown to have the ability to engraft immunodeficient mice. The two fusion oncogenes induce similar disorders in transplanted mice, with several features of human EMS, including the presence of

eosinophilia, splenomegaly, and accumulation of blasts. Notably, the established in-vivo EMS model provides the first humanized model of a myeloproliferative disorder transforming into acute leukemia in mice. In contrast to previous EMS-models, this model provides the possibility to study human EMS cells in vivo during the chronic phase as well as during progression into the acute phase, and finally the fully transformed leukemic cells in the most appropriate cellular context; that of normal human hematopoietic cells. As such it should constitute a valuable tool for obtaining further insights into *FGFR1* fusion gene mediated leukemogenesis. This model should also allow future investigations of mechanisms underlying disease progression, by for example analyzing the leukemic cells for secondary genetic, and possibly, epigenetic aberrations.

Our attempts to engraft secondary recipients with blasts from mice transplanted with *BCR/FGFR1*-expressing cells were unsuccessful. One reason for this could be that the cells were not fully transformed, perhaps requiring secondary genetic aberrations to become LSCs. The short latency could be an argument against this theory, and the problems to transplant the blast could possibly be solved by the use of intrafemoral injections instead of intravenous. The short latency, especially in the NSG-model, demonstrates the strong potency of *FGFR1* fusion genes to induce malignancy, but also complicates further studies of the disorder. Dilution experiments, with transplantation of fewer cell numbers than used in this study could possibly extend the time period from transplantation to fatal disease, thereby prolonging the window of time open for detailed studies of disease progression.

From the patients' point of view, the most alarming question is of course the lack of successful treatment strategies in EMS. Several compounds have shown promising effects on primary patients' cells or cell lines expressing *FGFR1* fusion genes in vitro, but further investigations have not been performed, with the exception of one patient who was successfully treated with PKC412 for a short time while awaiting BM transplantation (Chen et al. 2004). TKI258 (Dovitinib) is a TKI developed by Novartis, and is active against FGFR, VEGFR, PDGFR, FLT3, and KIT, and is currently in phase II-trials for multiple myeloma and solid tumors (NIH 2010). In vitro, TKI258 was shown to inhibit cellular proliferation of KG1-cells that harbor *FGFR1OP/FGFR1*, and primary cells from EMS patients (Chase et al. 2007). Our own preliminary data indicates that TKI258 reduces the expansion of primary human hematopoietic cells expressing *BCR/FGFR1* in vitro, and that TKI258-treatment of mice transplanted with *BCR/FGFR1*-expressing

cells delay the progression of disease (Ågerstam et al, unpublished data). Hence, the established EMS in-vivo model is an important tool for early evaluation of new treatment regimens of EMS. Future studies using the established model should further clarify the suitability of TKI258 as treatment for EMS.

Since the discovery of the *BCR/ABL1* fusion gene, immense work has been done to elucidate the biological actions of *BCR/ABL1* fusion genes. Still many questions remain to be answered. One is the differences in disease manifestation induced by P190 and P210 *BCR/ABL1*. In primitive human hematopoietic cells, P190 and P210 *BCR/ABL1* induced similar biological effects, suggesting that differences in clinical characteristics depend on the type of hematopoietic cell in which transformation occurs, rather than on intrinsic differences between the fusion proteins. The result contradicts several studies that have been performed in murine cells, making further investigations in an appropriate cellular context desirable. To establish a xeno-transplantation model for CML has proven difficult. Such a model would be important for increased understanding of disease development and progression in CML. It would also provide a valuable tool in exploring the effects of new TKIs or antibody-based regimens in CML. One interesting candidate is IL1RAP, which was recently found to be expressed higher on primary immature *BCR/ABL1*-positive cells than on corresponding normal cells (Järås et al. 2010). A stable humanized CML animal model, similar to the EMS-model described in this thesis, would aid in the early evaluation of new treatment strategies in CML, such as targeting of IL1RAP expressing cells. The use of new immunodeficient mouse strains such as the NSG strain or NOD/SCID mice expressing human cytokines, may improve the establishment of a xeno-transplantation model.

The new possibilities of whole genome sequencing of cells from large cohorts of MPN patients will undoubtedly provide us with much new information about additional genetic changes in these disorders. However, functional studies of the candidate aberrations in vitro and in vivo in appropriate cellular contexts, such as those performed in this thesis, will be necessary to ascertain their relevance for disease phenotype and helpful to provide critical insights to the biology of the various MPNs. The studies performed in this thesis should, hopefully, contribute to an increased understanding of the mechanisms underlying human MPN, and to the future development of novel therapies for these malignancies.

SVENSK SAMMANFATTNING

I blodet cirkulerar olika typer av celler, var och en med sin uppgift. De röda blodkropparna (erytrocyter) transporterar syre till kroppens alla vävnader, blodplättarna (trombocyter) deltar vid koagulation för att stilla blodflödet efter en sårskada, och de vita blodkropparna (granulocyter och lymfoida celler) är viktiga i kroppens försvar mot bakterier och virus. Alla blodets celltyper utom de lymfoida cellerna kallas med ett gemensamt namn för myeloida celler. Alla celler i blodet härstammar från blodstamceller i benmärgen, som stegvis mognar ut till de olika celltyperna. Samtidigt med utmognaden sker också tillväxt av cellerna genom delning. Då cellen förbereder sig inför delning ordnas cellens DNA i kromosomer. Om två kromosomer bryts av och ändarna byter plats med varandra kallas det för en translokation. Om kromosombrotten sker i gener kan fusionsgener bildas. Till exempel kan början av gen *A* slås ihop med slutet av gen *B*, och bilda fusionsgenen *A/B*. Om translokationen sker i en blodstamcell, kan det ge upphov till en myeloproliferativ sjukdom eller leukemi. Till följd av den störda cellsignaleringen sker då en onormal produktion av en eller flera myeloida celltyper. Vid akut leukemi är dessutom utmognaden av celler störd, så att en ansamling av omogna celler sker i benmärg och blod. Detta är också vad som händer när den kroniska fasen i en myeloproliferativ sjukdom så småningom övergår i akut fas. Den mest välstuderade myeloproliferativa sjukdomen är kronisk myeloisk leukemi (KML). Den orsakas av *BCR/ABL1* fusionsgenen. En mer ovanlig myeloproliferativ sjukdom är 8p11-myeloproliferativt syndrom (EMS), som orsakas av fusionsgener där *FGFR1*-genen ingår. Båda dessa sjukdomar inleds med en kronisk fas, och kan idag endast botas genom benmärgstransplantation, vilket ofta är en svår och riskfylld behandling. I KML kan den kroniska fasen vara i flera år, och det finns numera bra läkemedel som i många fall förhindrar att sjukdomen övergår i akut fas. I EMS sker sjukdomsprogressionen ofta inom några månader och det finns ingen bra behandling.

I den första studien undersökte vi ett EMS-fall där det tidigare konstaterats att de leukemiska cellerna bar en *BCR/FGFR1* fusionsgen. Dessutom fanns en translokation mellan kromosomerna 9 och 21, som inte studerats närmare. Eftersom patienten var i akut fas av sin sjukdom då provet togs, var vår hypotes att denna extra genetiska förändring kunde kopplas till sjukdomsprogression. Vid

undersökningen framkom det att *RUNX1*, en gen som kodar för en del av en viktig transkriptionsfaktor som styr uttrycket av andra gener, genom translokationen bildat en fusionsgen med en hittills okänd gen på kromosom 9. Eftersom *RUNX1*-genen bröts på ett viktigt ställe kan man förutspå att de leukemiska cellerna får ett totalt sett för lågt uttryck av *RUNX1*. Det har tidigare påvisats att en för låg mängd *RUNX1* kan kopplas till en viss sjukdom som ökar risken för akut leukemi. Dessutom finns data som tyder på att sjukdomsprogression i myeloproliferativa sjukdomar kräver två genetiska förändringar, en i en tyrosinkinas som FGFR1 och en i en transkriptionsfaktor som RUNX1. Av våra resultat kan man därför dra slutsatsen att en avbruten *RUNX1* gen kan bidra till sjukdomsprogression i EMS.

I den andra studien använde vi stamceller från navelsträngsblod för att studera två typer av *BCR/ABL1* fusionsgenen, P210 och P190. Medan P210 *BCR/ABL1* främst orsakar KML, ger istället P190 *BCR/ABL1* upphov till akut lymfatisk leukemi (ALL) där sjukdomen istället påverkar de lymfoida cellerna. Om detta beror på att P210 *BCR/ABL1* och P190 *BCR/ABL1* i sig själva fungerar olika, eller skillnaden beror på att de uppkommer i olika typer av celler är inte klarlagt. Med hjälp av ett virus fördes P210 *BCR/ABL1* eller P190 *BCR/ABL1* in i navelsträngsceller, som odlades i plattor i laboratoriet. Oavsett vilken typ av *BCR/ABL1* som cellerna uttryckte, hade de en högre tillväxttakt än kontrollcellerna, och de utmognade till erytroida celler. Vid en genexpressionanalys, där man studerar nivåerna av uttryckta gener i cellerna, var de båda typerna mycket lika. Resultaten från denna studie pekar alltså på att skillnaden i sjukdom inducerad av P210 *BCR/ABL1* och P190 *BCR/ABL1* inte beror på att de båda varianterna i sig själva fungerar olika utan stödjer antagandet att skillnaden beror på att de uppkommer i olika typer av celler.

Även i den tredje studien använde vi oss av stamceller från navelsträngsblod, denna gång för att studera två olika typer av *FGFR1*-fusiongener, *ZMYM2/FGFR1* och *BCR/FGFR1*, som båda orsakar EMS. I likhet med *BCR/ABL1* gav dessa båda fusionsgener upphov till en högre tillväxttakt och erytroid utmognad i navelsträngscellerna när de odlades i en platta i laboratoriet. När cellerna tranplanterades till möss resulterade det i tillväxt av de humana cellerna i mössens benmärg. Genom att suga ut (aspirera) benmärg från mössen, kunde cellernas tillväxt och utmognad studeras. Även i mössen orsakade fusionsgenerna en ökning av erytroida celler, men mest påtagligt var ökningen av myeloida celler i förhållande till de lymfoida. Både *BCR/FGFR1* och *ZMYM2/FGFR1* orsakade en myeloproliferativ sjukdom hos mössen. I några fall

övergick sjukdomen till en akut fas med ansamling av omogna celler (blaster) i mössens benmärg. Mössen fick även förstorade mjältar samt en ökning av antalet eosinofila celler, som är en speciell myeloid celltyp, någon som är vanligt hos EMS-patienter. Därmed har den etablerade djurmodellen många likheter med human EMS, och kan utgöra ett viktigt verktyg vid fortsatta studier av sjukdomen, samt vid utvärderingen av nya läkemedel.

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