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Geschäftsführende Direktorin: PD Dr. rer. nat. Barbara Fritz
des Fachbereichs Medizin der Philipps-Universität Marburg

Functional Characterization of the BCR/JAK2 Fusion Gene in a Murine Pro B Cell Line

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Felix Marbach aus Hanau

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1. Korreferent: Prof. Dr. T. Stiewe

2. Korreferent: PD Dr. H. Garn

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1 Introduction

1.1 Introduction part one – general characteristics of cancer and molecular mechanisms.

1.11 Cancer

In Western, industrialized countries, cancer is the second most common cause of death, with only cardiovascular diseases having a higher percentage of overall mortality. Cancer has severe effects on many aspects of a patient's life, often significantly reducing life expectancy and quality. The knowledge of suffering from a life-threatening illness also poses a great psychological challenge, sometimes leading to depression, anxiety, fear, and panic attacks. The course of the disease is often protracted, prompting social and, eventually, financial problems to arise as the patient's ability to participate in former activities may be impaired. Besides affecting physical and emotional health on the individual basis, the frequency of cancer, in line with the high cost of current therapies, also poses a challenge to a nation's healthcare system. Since there is a clear correlation between age and the incidence of cancer, the rising life expectancy is expected to lead to a worldwide increase in the incidence of cancer and cancer-related deaths. Additionally, the mortality from cardiovascular diseases has decreased significantly faster than the mortality from malignancies in the last decades. If the current trends continue, cancer will become the primary cause of death in industrialized countries within the next 15 to 20 years (RKI 2013).

Therefore, research regarding the causes, the evolution, the behaviour and the therapy of the different entities of cancer is of great importance to many current and future patients, as well as the society in general. Since Johannes Peter Müller showed that cancer tissue is composed of cells, similar to normal tissue in 1850, the scientific development has accelerated. New technologies have been generating new insights with increasing frequency over the past 160 years. Of this multitude of discoveries, it may have been Theodor Boveri's observation of chromosomal aberrations in cancer cells in 1914, and Peter Nowel's and David Hungerford's discovery of the Philadelphia chromosome in 1960, which created an awareness towards the role of chromosomal abnormalities in the malignant transformation of cells. It was in 1972, along with the introduction of new chromosome banding techniques, that Janet Rowley was able to demonstrate that the Philadelphia chromosome was the result of a translocation between chromosome 9 and 22. In 1983, it was discovered that this translocation led to the fusion of the 5' part of the *BCR* gene and the 3' part of the *ABL1* gene, marking the first description of an oncogenic fusion gene (Heisterkamp et al., 1983). Since then, an increasing number of such fusions have been described. The functional characterization of the

proteins originating from these fusion genes has greatly contributed to the understanding of the pathology of the malignant transformation on the cellular, as well as on the molecular level. During the last decade, these proteins have become preferred targets for the development of new treatments, with the first clinical trial of a BCR-Abl kinase-inhibitor, Imatinib, being conducted in 1998.

1.12 Alterations of the genome and cancer

A wide variety of findings support the assumption that cancer is caused by alterations of the genome. Firstly, the clonal nature of malignant cells strongly suggests that they are derived from one origin, a single cell, which underwent a malignant transformation. Secondly, a strong correlation between the onset of cancer and the presence of substances causing DNA damage has been observed.

Throughout the life of a cell, its DNA is subjected to a multitude of endogenous and exogenous hazards, such as reactive oxygen species or ultraviolet radiation. But it is also believed that many mutations occur as a consequence of normal DNA metabolism like DNA transcription and replication. While the extent of the damage may vary from a single base substitution to chromosomal aberrations, such as balanced or imbalanced translocations or even aneuploidy, the resulting phenotype will usually be determined by the genes which are involved.

Usually, the DNA damage recognition and repair mechanisms of a mammalian cell are extremely effective. *In vivo* DNA replication has an error rate of roughly 1 in 10^9 base pairs, a figure which is many times lower than the error rate a purely thermodynamic model would suggest (Wagner C, Müller O., 2010). While minor damages like single base mutations or single- and double-strand breaks can be repaired, more extensive damage, especially on a chromosomal scale, will most likely lead to apoptosis. Even if a mutation remains undetected, its chances of yielding a tumorigenic effect are relatively slim. Often, introns or noncoding regions will be affected and in other instances, the mutation of non-essential genes will leave the cell functional. However, if a mutation occurs within or in the vicinity of a proto oncogene or tumour suppressor gene, the consequences can be profound. This may result in a gain of function (GOF) or a loss of function (LOF) of the gene, triggering what could be the first step towards the creation of a cancer cell.

It is believed that, in most cases, several events within a single cell are required for the development of cancer. According to this model, an initial mutation may impair the ability to control DNA damage or to initiate apoptosis, leaving the cell more vulnerable to further mutations. The result is a lineage of cells undergoing what is called a somatic evolution involving the activation of oncogenes and the deactivation of tumour suppressor genes. During this process, the biological properties of the cell will

change, such as the disappearance of senescence and apoptosis or the acquisition of the capacity to induce angiogenesis. Also, the dependence on external growth factors will be reduced or even disappear completely.

1.13 The cell cycle

The common characteristic of all known types of cancer is the uncontrolled growth of pathological tissue, caused by an imbalance between mitosis and apoptosis. Simply put, the portion of proliferating cells in cancer tissue is greater than it is in healthy differentiated adult tissue. A large part of the various mutations within a cell's genome, which have been identified as promoting malignant transformation, are directly or indirectly linked to cell cycle control. Therefore, the understanding of the cell cycle is crucial to understanding the pathological changes within the cancer cell.

In preparation of mitosis, a cell will go through the interphase, which can be divided into 3 parts: The G_1 , S and G_2 phase. During the G_1 , or growth phase, the cell will synthesize mRNA and proteins needed for DNA replication, while the number of mitochondria will rise, indicating an increase in metabolic activity. The S phase or synthesis phase, is characterized by the replication of the cell's genomic DNA. During G_2 , the cell will continue to grow while a final checkpoint will ensure its readiness to enter the mitotic phase. Finally, the mitotic or M phase is the process of cell division: The nuclear DNA will be condensed to form the chromosomes and the nuclear membrane will disintegrate. Afterwards, the chromosomes will align at the metaphase plate, to be then separated, pulled apart by the shortening microtubules of the spindle apparatus. A new nuclear membrane will form around the two sets of chromosomes and the process of cytokinesis, the division of the cellular body, will commence until two daughter cells have been formed. After the successful completion of mitosis, the daughter cells will either repeat the cycle by entering the G_1 phase, or enter the G_0 phase, a quiescent stage, which is the state of most differentiated cells. Despite the fact that the cell cycle machinery is dismantled in G_0 , these cells still carry the potential to enter the cell cycle again, should the opportunity arise. However, some terminally differentiated cells remain in the quiescent G_T state for the remainder of their existence, which can range from 5 to 6 days in the case of neutrophil granulocytes, to an entire human lifespan in the case of neurons.

Several checkpoints, which depend on control proteins such as cyclins, cyclin-dependent kinases (CDKs) and adaptor proteins, regulate the proper and orderly progression through the cell cycle. Often triggered by DNA damage, their primary function is to either arrest the cell cycle to allow the damage to be repaired, or to initiate cellular apoptosis if the damage proves to be irreparable. Other

checkpoints include the G1 checkpoint, or restriction point, which will only permit the cell to enter the S phase if extracellular growth factors are present, and the metaphase checkpoint, which will inhibit the separation of the daughter chromosomes as long as their kinetochores are not properly attached to the spindle fibres. Despite such control mechanisms, the highly complex process of mitosis is vulnerable to error, which will sometimes result in mutations or the occurrence of chromosomal aberrations in the next generation of cells (Alberts et al., 2002; Lodish et al., 2012).

1.14 Tyrosine kinases

It is no coincidence that protein kinases constitute the largest proportion within the heterogeneous group of oncoproteins. Many external factors promoting cell proliferation will do so by making use of a signalling pathway which includes protein kinases attached to a transmembrane receptor. These kinases, once activated, will phosphorylate cytoplasmatic proteins such as transcription factors. In turn, the phosphorylated transcription factor will be able to travel inside the nucleus to alter the expression of certain genes, representing a simple signalling pathway. There are two main groups of kinases, the serine/threonine kinases and the tyrosine kinases. I will focus on the mechanisms associated with cytoplasmatic tyrosine kinases.

Non-receptor tyrosine kinases are enzymes which do not have an extracellular or transmembrane domain. Therefore, the kinases are located in the cytoplasm or, in some cases, inside the nucleus. The enzymes vary in function and purpose. Often activating the cellular pathways of extracellular signals, others will promote cell migration or adhesion (In the case of the SRC family) (Wagner C, Müller O., 2010). Notable tumour-associated tyrosine kinases are the ABL1 kinase and the families of CSK- SRC- and Janus kinases (JAKs).

In the case of the ABL kinase, an involvement in the processes of cell differentiation, proliferation, adhesion and stress response (NCBI Gene, 2014) has been described. Apart from its physiological functions, ABL is more commonly known for being a part of the BCR/ABL fusion gene, which is associated with malignancies of the bone marrow such as CML and ALL. While the ABL and BCR moieties of the fusion protein may vary in size, the fusion protein will always include the coiled-coil dimerisation domain of BCR and the kinase domain of ABL. This arrangement allows the dimerisation of the ABL kinase, leading to cross-phosphorylation and subsequent activation of the kinase activity (McWhirter et al., 1993). In the case of Janus kinases, a family of receptor-associated tyrosine kinases, a similar mechanism has been proposed by Griesinger et al. (Griesinger et al., 2005). Usually activated by the dimerisation of cytokine receptors, a BCR/JAK2 fusion may yield a similar effect through dimerisation of the fusion protein via the coiled-coil domain of BCR. In recent years, JAKs

have been best known for their involvement in the JAK/STAT pathway, which has been shown to be over-activated in different haematological diseases such as leukaemia, myelofibrosis and polycythaemia vera (Baxter et al., 2005; Kralovics et al., 2005).

1.141 Tyrosine kinases and the cell cycle

The JAK/STAT pathway has a number of genomic targets, such as MYC and CCND2. The protein encoded by the latter gene, cyclin D2, is known to bind and thereby activate the cyclin-dependent kinases 4 and 6. These CDKs are both active during the G₁ phase, facilitating the entry into the cell cycle (de Alboran et al., 2001). Additionally, the cyclin D-CDK4/6 complexes, together with a cyclin E-CDK2 complex will promote the G₁/S phase transition. The whole process is regulated by extracellular growth factors binding to transmembrane receptors, which then activate specific JAKs. Simply put, the growth factors are inducing the entry into the cell cycle, and therefore cell proliferation, by triggering the JAK/STAT pathway.

MYC forms homodimers and also heterodimers with MAX, which function as transcriptional activators or transcriptional repressors for many different genes. Regarding the cell cycle, MYC upregulates the expression of CCND2 and CDK4 promoting cell cycle progression. In addition to supporting other vital functions (the inactivation of MYC will cause embryonic lethality in mice), the wild type MYC gene has been shown to induce the proliferation of murine B cells *in vitro* (de Alboran et al., 2001).

1.142 JAK/STAT pathways

JAKs are known to be involved in the transduction of signals from type 1 cytokine receptors, including the receptors for IL-2, IL-3, IL-5, IL-6, IL-7 and GM-CSF, and type 2 cytokine receptors including the receptors for IFN α , IFN β , IFN γ and IL-10 and the receptors of growth hormone, prolactin, erythropoietin and thrombopoietin (Universal Protein Resource (UniProt)). The family of Janus tyrosine kinases consists of 4 proteins, JAK1, JAK2, JAK3 and TYK, each associated with certain cytokine receptors. After binding to these receptors, the Janus kinases activate downstream pathways via phosphorylation of cytosolic proteins, as soon as the receptor itself has been activated. In turn, STATs bind the cytosolic domain of a specific cytokine receptor, ensuring that only the receptor-specific STATs will be phosphorylated. The specificity is achieved by the SH2 (Src-homology 2) domain of the STAT protein, which binds to a certain phosphotyrosine of the cytosolic domain of the receptor. For example, STAT5 is specific to the EPO- and IL-2 receptors, while STAT1 will be

activated by the IFN α - and IFN γ receptors. The following table contains an overview of the different cytokines, receptor-associated kinases and the associated STAT proteins (Wagner C, Müller O., 2010).

Interleukin, Interferon	Kinase	STAT Protein
IFN α	TYK2, JAK1	STAT1, STAT2
IFN γ	JAK1, JAK2	STAT1
IL-6, IL-10, GM-CSF	JAK1, JAK2, TYK2	STAT3
IL-12	JAK2, TYK2	STAT4
IL-2	JAK1, JAK3	STAT5
EPO	JAK2	STAT5
IL-4	JAK1, JAK3	STAT6

Tab1: Cytokines and receptor-specific STAT proteins. Source: Wagner C, Müller O. Molekulare Onkologie - Entstehung, Progression, Klinische Aspekte, 3. Auflage. Georg Thieme Verlag 2010.

The genes regulated by each STAT differ, depending on the tissue-specific epigenetic modifications. STAT5, for example, will act as a transcription factor for genes encoding for milk proteins in the mammary gland, while upregulating the expression of the anti-apoptotic Bcl-xl gene in erythroid progenitor cells (Lodish et al., 2012).

1.143 Tyrosine kinase inhibitors

A tyrosine kinase inhibitor, Imatinib (marketed as Gleevec by Novartis), was the first cancer drug specifically designed for a distinct molecular target, the BCR/ABL fusion protein in patients with chronic myeloid leukaemia (CML). With clinical trials beginning in 1998, the therapeutic results were impressive, prompting the development of further kinase inhibitors and leading the way for several other targeted therapies in the field of oncology. The target of Imatinib is the ATP binding domain of the ABL kinase. By binding in the vicinity of the ATP-binding site, Imatinib locks the active site in a closed conformation, thereby inhibiting the ABL kinase activity semi-competitively (Wagner C, Müller O., 2010). However, resistances to Imatinib have been observed, especially in later stages of CML. Mutations of the ATP binding domain or amplification of the BCR/ABL fusion gene have both been described as the cause of resistance to Imatinib (Azam et al., 2003). Newer, so-called second generation inhibitors of ABL like dasatinib or nilotinib, which have a much higher affinity (as much as 300 times) towards the target structure, are currently used in cases of Imatinib resistant CML, with significant, but rather limited success (Kantarjian et al., 2006).

Apart from kinase inhibitors targeting the BCR/ABL protein, additional molecular targets for the treatment of various diseases have been identified. Other tyrosine kinase inhibitors include erlotinib and gefitinib, targeting the epidermal growth factor (EGF) receptor in the treatment of non-small cell lung cancer (NSCLC) and pancreatic cancer and lapatinib, targeting the erbB2 (HER2/neu) epidermal

growth factor receptor in breast cancer (Wagner C, Müller O., 2010). Table 2 contains a compilation of some tyrosine kinase inhibitors currently in use.

Inhibitor	Target	Indication
Dasatinib	ABL	CML, BCR/ABL positive ALL
Erlotinib	EGF receptor	NSCLC, pancreatic cancer
Gefitinib	EGF receptor	NSCLC
Imatinib	ABL, ARG, Kit, PDGFR α/β	CML, BCR/ABL positive ALL, GIST
Lapatinib	erbB2	HER2/neu-positive breast cancer
Nilotinib	ABL, ARG, Kit, PDGFR α/β	CML
Ruxolitinib	JAK1/2	Myelofibrosis, polycythaemia vera, plaque psoriasis
Tofacitinib	JAK3 and others	Rheumatoid arthritis

Table 2: A selection of tyrosine kinase inhibitors in clinical use. Source: Wagner C, Müller O. Molekulare Onkologie - Entstehung, Progression, Klinische Aspekte, 3. Auflage. Georg Thieme Verlag 2010.

Ruxolitinib, an inhibitor of the Janus kinases 1 and 2, gained FDA approval in 2011 for the treatment of myelofibrosis. During drug development, cell lines containing a mutant, activated JAK2 were found to be responsive to ruxolitinib. In cells treated with the substance, a reduction of phosphorylated JAK2 and STAT5 proteins was observed, indicating an inhibitory effect on the JAK/STAT pathway. During clinical trials involving patients with myelofibrosis, the group receiving ruxolitinib had a significant advantage in terms of survival and symptom control, as the drug was shown to reduce splenomegaly and anaemia (Harrison C, Vannucchi AM., 2012). Other inhibitors of JAK kinases have recently been developed or are still under development. Tofacitinib, originally designed as an inhibitor of JAK3, has demonstrated clinical efficiency in the treatment of rheumatoid arthritis. It was found to have an inhibitory effect on the cytokine-mediated phosphorylation of STAT1, STAT3 and STAT5, blocking JAK/STAT pathways downstream of the JAK-dependent cytokine receptor. In line with these findings, the production of inflammatory mediators was found to be significantly reduced (Ghoreschi et al., 2011).

1.2 Introduction part two – Chronic myeloid leukaemia and a case report from 2005.

1.21 Leukaemia

Leukaemia, which derives from the Greek words for “white blood”, is a malignant disease of the bone marrow, characterized by the autonomous proliferation of a single lymphatic or myeloid cell clone. The name leukaemia was introduced by Rudolf Virchow in 1847, in order to describe the unusually thick layer of white blood cells, which could be observed above the erythrocyte fraction after centrifugation of a patient’s blood sample. The clonal expansion of a malignant cell within the bone marrow leads to the displacement of normal haematopoietic tissue into extramedullary organs, especially the spleen and liver. In later stages, the accumulation of leukaemic cells within the blood will promote clotting as well as the displacement of normal bone marrow will lead to anaemia, immunodeficiency and excessive bleeding (Herold, 2013). Leukaemia can be divided into two large diagnostic groups, acute and chronic leukaemia. Each of these groups can be further subdivided into myelogenous and lymphocytic leukaemia, with a total of four subgroups emerging: Acute lymphoblastic and acute myelogenous leukaemia (ALL and AML) and chronic lymphoblastic and chronic myelogenous leukaemia (CLL and CML). Each group in turn contains several subtypes, differentiated by cytology, clinical characteristics or specific genetic aberrations, with distinct therapeutic regimen and different prognoses.

A graph portraying the relationship between age and incidence for all types of leukaemia will have an initial peak during childhood and a steady decline towards the age 25, followed by a prolonged plateau of relatively low risk and finally a steady increase of incidence above the age of 50. While ALL is the most common type of leukaemia in early life, the chronic leukaemia forms become more frequent in higher age. Although ALL is more common during childhood, AML becomes the most frequent type of acute leukaemia in adults.

1.22 Chronic myelogenous leukaemia

Chronic myelogenous leukaemia is the most common chronic myeloproliferative disorder, characterized by high numbers of mature neutrophil granulocytes and granulocytic precursors in the peripheral blood. It usually occurs in elderly patients, with a slight predilection of males (Alison, 2007). The disease can be divided into three phases – a chronic, relatively stable phase of variable duration, which often yields few symptoms, followed by a symptomatic acceleration phase in which an increasing number of myeloblasts and basophils can be observed as the disease undergoes a

cytogenetic evolution acquiring additional genomic alterations. The final, mostly lethal stage known as blast crisis will see high numbers of blasts flooding the peripheral blood and bone marrow, even accumulating in extramedullar locations such as the skin. The prognosis depends heavily on the genetic characteristics of the malignancy, with an overall survival of five to ten years. The t(9;22)(q34;q11) translocation is a frequent observation, forming the well-known Philadelphia chromosome. The translocation, which is present in roughly 85% of cases, usually leads to the formation of the BCR/ABL fusion gene, which encodes for a protein containing the coiled-coil dimerisation domain of BCR and the kinase domain of ABL (Heim S, Mitelman F., 2009). The high tyrosine kinase activity of the fusion protein is regarded to be both bad, since it is the driving force behind the development and acceleration of the disease, and beneficial, since its vulnerability to tyrosine kinase inhibitors can be exploited. A publication in 2006 by Druker et al. showed that 70 to 90% of the patients receiving Imatinib displayed a complete cytogenetic response after 60 months, with an overall survival rate of 89%, which illustrates the effectiveness of TKI treatment in CML (Druker et al., 2006).

Besides the iconic Philadelphia chromosome, additional chromosomal aberrations, like a loss of the Y chromosome, trisomy 8, i(17q), or a second Ph chromosome can be detected during progression into the accelerated phase or blast crisis. These additional changes can be found during the accelerated and blast crisis phase of 60-80% of the cases. Their presence at the time of diagnosis is a negative prognostic factor (Sokal et al., 1988). During the cytogenetic evolution, a great number of other rearrangements involving all chromosomes are known to occur, sometimes even related to the treatment the patient receives, but their prognostic and therapeutic implications have yet to be fully understood.

1.23 Atypical CML – a case report by Griesinger et al.

In 2005, a 63-year-old female patient presented to the outpatient clinic of Braunschweig Hospital with fatigue and left upper abdominal pain. Initial blood tests showed several abnormalities, including anaemia, leukocytosis and a left shift in the differential blood count. Table 3 contains the abnormal parameters found at the time of her first visit at Braunschweig Hospital.

<i>Parameter</i>	<i>Reference</i>	<i>Patient</i>
Metabolic panel		
LDH (U/l)	<250	478 ↑
Blood count		
Hb (g/dl)	12-16	10.4 ↓
Leukocytes (/μl)	3.800 – 10.500	42.000 ↑
Platelets (/μl)	140.000-345.000	250.000
Differential blood count		
Immature neutrophil granulocytes (%)	0-5	11 ↑
Segmented granulocytes (%)	30-80	58
Eosiniphils (%)	0-6	7 ↑
Basophils (%)	0-2	3 ↑
Lymphocytes (%)	15-50	14 ↓
Monocytes (%)	1-12	3
Additional tests		
LAP (leukocyte alkaline phosphatase) (Index points)	10-110	4 ↓

Table 3: Blood test results of the 63-year-old patient. Source: Griesinger F, Hennig H, Hillmer F, Podleschny M, Steffens R, Pies A, Wörmann B, Haase D, Bohlander SK. A BCR-JAK2 fusion gene as the result of a t(9;22)(p24;q11.2) translocation in a patient with a clinically typical chronic myeloid leukemia. *Genes Chromosomes Cancer*. 2005 Nov;44(3):329-33.

A bone marrow examination was performed, revealing hyperplastic bone marrow, a left-shifted myelopoiesis and eosinophilia. The blast count was less than 5% and megakaryopoiesis was increased. The megakaryocytes were described as discretely dysplastic. A trephine biopsy confirmed these findings and, additionally, bone marrow fibrosis was observed. Based on these results, the diagnosis of CML was made. The patient was initially treated with hydroxyurea and interferon alpha, and a complete hematologic response was achieved. A blast crisis was diagnosed 20 months after the first diagnosis and in response, the patient was treated with Imatinib for eight weeks. However, as it became apparent that the disease was not responding to Imatinib, the patient was administered high-dose Ara-C and mitoxantrone. Despite these efforts, the patient died in myeloid blast crisis, 24 months after the initial diagnosis of CML (Griesinger et al., 2005).

At the time of diagnosis, conventional cytogenetics were performed. The outcome was unexpected, as it did not show the CML typical t(9;22)(q34;q11.2), but instead a t(9;22)(p24;q11.2) in 21 of 21 metaphase cells. It was the sole chromosomal aberration at the time. Conventional cytogenetics was performed again at the time of the blast crisis, revealing cytogenetic evolution with a 7q deletion [del(7)(q22q36)] in 21, and an additional trisomy 19 in five metaphase cells. A fluorescence in situ

hybridization (FISH) using a BCR and an ABL gene probe was performed to analyze the unknown (9;22)(p24;q11.2) translocation. Three signals were observed with the BCR probe, but only two signals for the ABL probe. These results ruled out any variant of a hidden BCR/ABL translocation, indicating that BCR had a novel, yet undetermined fusion partner.

The JAK2 gene is located on 9p24, and therefore a fusion between BCR and JAK2 seemed to be quite likely. To confirm this hypothesis, a RT-PCR assay was performed, using a 5' sense BCR and a 3' antisense JAK2 primer. The primers were:

5' BCR sense primer: 5'-CAG AAC TCG CAA CAG TCC-3' (bp 1602 – 1622)

3' JAK2 antisense primer: 5'-TC ATA CCG GCA CAT CTC CAC AC-3' (bp 3100 – 3081)

Gel-purification and sequencing of the PCR product showed an in-frame fusion between exon 1 of BCR and exon 19 of JAK2. An amplification product of roughly 300 bp, spanning the BCR/JAK2 breakpoint region, could be seen in gel electrophoresis. Based on the sequence, a BCR/JAK2 fusion protein of 747 amino acids in length, incorporating the coiled-coil dimerisation (CC) domain of BCR and the kinase (JH1) domain of JAK2 was proposed. Fig. 1 shows a diagram of the BCR and JAK2 proteins and fusion protein as published by Griesinger et al.

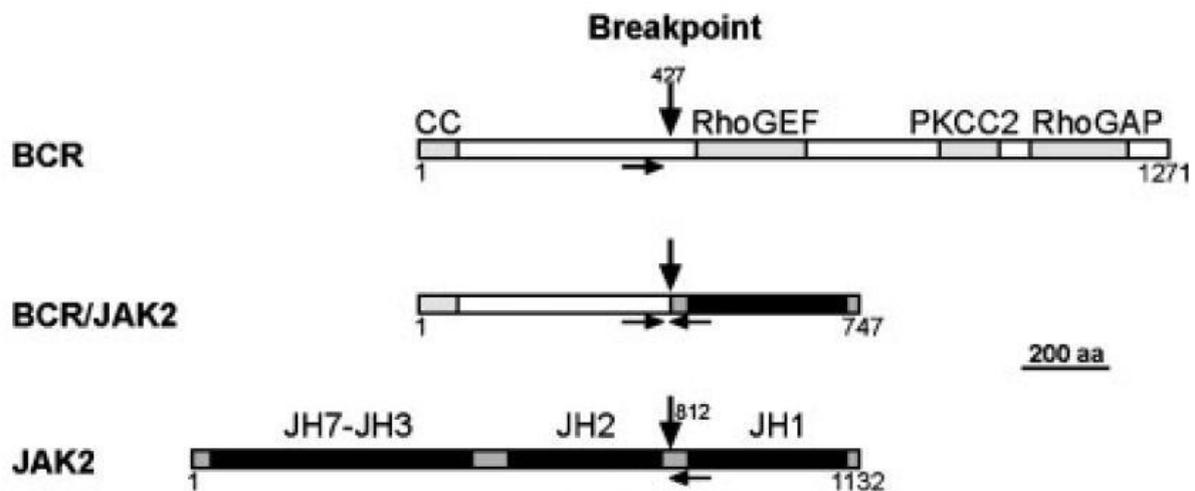


Figure 1: The BCR and JAK2 proteins and the proposed BCR/JAK2 fusion protein. Source: Griesinger F, Hennig H, Hillmer F, Podleschny M, Steffens R, Pies A, Wörmann B, Haase D, Bohlander SK. A BCR-JAK2 fusion gene as the result of a t(9;22)(p24;q11.2) translocation in a patient with a clinically typical chronic myeloid leukemia. *Genes Chromosomes Cancer*. 2005 Nov;44(3):329-33.

It is worth noting that the breakpoint of BCR, as described by Griesinger et al., is identical to the breakpoint in the BCR/ABL variant leading to the p190 BCR/ABL fusion protein which is common in ALL and infrequently observed in CML.

1.24 Case reports of BCR/JAK2 fusions

Case reports of different malignancies of the bone marrow, exhibiting a t(9;22) translocation, but without any sign of a BCR/ABL fusion protein, have occasionally been published in the recent years. A total of nine cases have been reported worldwide. This involved cases of myeloproliferative disease (MD), ALL, AML and CML (Griesinger et al., 2005; Cirmena et al., 2008; Lane et al., 2008; Tirado et al., 2010; Impera et al., 2011; Elnaggar et al., 2012; Cuesta-Domínguez et al., 2012, Bellesso et al., 2013, Schwaab et al., 2014). The publication by Cirmena et al. contains the case report of a 67-year old female patient with AML M1 with the a t(9;22)(p24;q11) as the only cytogenetic abnormality at the time of diagnosis. This time, the breakpoint of BCR was identical to the breakpoint of the BCR/ABL p210 variant. Sequencing revealed that the BCR gene was truncated in exon 14, followed by a 17 bp insertion of unknown origin and the 3' rest of JAK2 beginning with the first nucleotide of exon 11. Despite intensive treatment including high-dose chemotherapy and allogeneic peripheral blood stem cell transplantation, the patient died 23 months after diagnosis. Lane et al. reported a t(9;22)(p24;q11.2) translocation in a 44-year-old male patient with CML, leading to a BCR/JAK2 fusion. This time, the breakpoint was located between exon 1 of BCR and exon 17 of JAK2. Two years later, Tirado et al. published the case of a 14-year-old male patient with B-acute lymphoblastic leukaemia, also featuring a BCR/JAK2 fusion gene. In 2011, Impera et al. published a case report of a 49-year-old woman with an unclassifiable myeloproliferative neoplasm (MPN) with a three-way t(9;18;22)(p23;p11.3;q11.2) translocation. Two variants of the BCR/JAK2 fusion gene could be detected: A fusion between BCR exon 1 and JAK2 exon 17, and a longer variant fusing BCR exon 1 to JAK2 exon 15. The patient, presenting with leukocytosis and hypercellular bone marrow, was administered imatinib and dasatinib, which failed to induce a haematological response. The treatment was then switched to PEG-interferon therapy, achieving a complete haematological response over the course of 10 months. In 2012, a publication by Elnaggar et al. described the case of an 84-year-old male patient with CML-like myeloproliferative disease. Similar to the first case described by Griesinger et al., sequencing revealed a fusion between exon 1 of BCR and exon 19 of JAK2. During the same year, Cuesta-Domínguez et al. published a case description of a 58-year old male patient diagnosed with ALL over six years earlier, exhibiting a similar BCR/JAK2 fusion (BCR exon 1, JAK2 exon 19). After diagnosis, the patient received a "standard high-risk ALL protocol" followed by a autologous peripheral blood stem cell transplant and remained in complete remission (at the time of the publication in 2012). In 2013, Bellesso et al. reported a 54-year-old male patient with CML, featuring the t(9;22)(p24;q11.2) translocation. Again, treatment with imatinib and dasatinib proved to be unsuccessful, and the patient died of acute graft-versus-host disease after receiving allogeneic bone marrow transplantation. Finally, in 2014, Schwaab et al. published a case report featuring the use of the janus kinase-inhibitor Ruxolitinib on two patients with myeloproliferative

neoplasms involving JAK2. One of these patients exhibited the BCR/JAK2 (BCR exon 1, JAK2 exon 16) fusion and the TKI treatment proved to be initially successful leading to a short-term haematological remission (see 4.22). The following table contains a compilation of some key features of these case reports.

Age	Gender	Phenotype	BCR/JAK2 variant	Published by:
63	F	CML	BCR exon 1, JAK2 exon 19	Griesinger et al., 2005
67	F	AML M1	BCR exon 14, JAK2 exon 11	Cirmena et al., 2008
44	M	CML	BCR exon 1, JAK2 exon 17	Lane et al., 2008
14	M	B-ALL	No data provided	Tirado et al., 2010
49	F	MPN (unclassifiable)	BCR exon 1, JAK2 exon 15/17	Impera et al., 2011
84	M	CML-like MD	BCR exon 1, JAK2 exon 19	Elnaggar et al., 2012
58	M	ALL	BCR exon 1, JAK2 exon 19	Cuesta-Domínguez et al., 2012
54	M	CML	No data provided	Bellesso et al., 2013
-	-	MPN	BCR exon1, Jak2 exon 16	Schwaab et al., 2014

Table 4: A compilation of case reports featuring the BCR/JAK2 fusion gene in patients diagnosed with diseases of the bone marrow.

1.25 Characterisation of the BCR/JAK2 fusion gene

In the field of leukaemia research, *JAK2* is not an unknown gene. In fact, the ETV6/JAK2 fusion in B- and T-ALL, first described in 1997, has been the subject of several publications over the last 15 years. The product of the *ets* variant gene 6 (*ETV6*), has a helix-loop-helix (HLH) oligomerization domain prompting the mutant ETV6/JAK2 fusion protein to oligomerize, leading to autophosphorylation and consecutive activation of the JAK2 kinase domain (Peeters et al., 1997). Furthermore, activating mutations of *JAK2* are a frequent observation in several diseases. The JAK2V617F mutation is a nucleotide exchange within exon 14 of the *JAK2* gene, resulting in a consecutive overactivation of the JAK2 kinase domain. The JAK2V617F is highly prevalent in cases of polycythaemia vera (PV) (up to 95%), essential thrombocythemia (ET) (up to 50%) and primary myelofibrosis (PMF) (up to 50%), and has also been described in rare cases of lymphoblastic leukaemia (James et al., 2005; Baxter et al., 2005; Tefferi et al., 2007). There are other, more uncommon JAK2 fusion genes linked to a broad spectrum of myeloproliferative neoplasms, such as the PCM1-JAK2, SSBP2-JAK2, RPN1-JAK2, NF-E2-JAK2, RUNX1-JAK2 and PAX5-JAK2 fusions (Patnaik et al., 2010).

Because of several case reports the BCR/JAK2 fusion gene, a distinct type of tumorigenic JAK2 fusion gene, has been brought to attention. Given the broad spectrum of clinical phenotypes and the paucity of BCR/JAK2 case reports to date, it is a rewarding subject for clinical and experimental research. This fusion gene appears to be relatively rare, however, it might be more common than the number of descriptions would suggest. Although a rare finding, it may yield important prognostic and therapeutic implications. While some clinicians suggested a negative impact on the patient's prognosis (Tirado et al., 2010), the introduction of TKIs, especially the JAK1/2-inhibitor ruxolitinib, may lead to a new therapeutic option for these patients.

Working with material acquired from the first patient described by Griesinger et al., it is the intention of this doctoral thesis to study the transformation potential and functional characteristics of the BCR/JAK2 fusion protein. Assuming a connection between the elevated activity of a mutant JAK2 and unrestrained, accelerated cell proliferation, I conducted experiments to characterize and quantify the effects of the gene *in vitro*.

2 Materials and Methods

2.1.1 Reagents

Reagent	Source
1-kb-DNA Ladder	Invitrogen, Karlsruhe, Germany
80 -1000 bp Ladder	Invitrogen, Karlsruhe, Germany
Acetic acid	Merck, Darmstad, Germany
Acrylamid Rotiphorese® Gel 30 (37, 5:1)	Carl Roth, Karlsruhe, Germany
Agar select	Carl Roth, Karlsruhe, Germany
Agarose	Invitrogen, Karlsruhe, Germany
Amino acids	Sigma-Aldrich, St. Louis, USA
Ampicillin	Ratiopharm, Ulm, Germany
APS (Ammonium persulfate)	Sigma-Aldrich, St. Louis, USA
Aqua ad iniectabilia	Braun B Melsungen AG, Germany
β-Mercaptoethanol	Sigma-Aldrich, St. Louis, USA
Big Dye	Thermo Fisher Scientific Inc., Waltham, USA
Bovine serum albumine (BSA)	Sigma-Aldrich, St. Louis, USA
Calcium chloride (CaCl ₂)	Sigma-Aldrich, St. Louis, USA
d ₂ H ₂ O	Millipore, Eschborn, Germany
DAPI (4',6-diamidino-2-phenylindole)	Sigma-Aldrich, St. Louis, USA
DMSO (dimethyl sulfoxide)	Merck, Darmstadt, Germany
DPBS	Thermo Fisher Scientific Inc., Waltham, USA
dNTP mix	Invitrogen, Carlsbad, USA
EDTA (ethylenediaminetetraacetic acid)	Carl Roth, Karlsruhe, Germany
Ethanol	Merck, Darmstadt, Germany
Ethidium bromide	Carl Roth, Karlsruhe, Germany
FBS (Fetal bovine serum)	Invitrogen Cell Culture, Karlsruhe, Germany
Ficoll DNA loading buffer	Sigma-Aldrich, St. Louis, USA
Formamide	Carl Roth, Karlsruhe, Germany
Glycerol	Carl Roth, Karlsruhe, Germany
Glycine	Merck, Darmstadt, Germany
HEPES (N-(2-Hydroxyethyl) piperazine-N'-(2 ethanesulfonic acid))	Sigma-Aldrich, St. Louis, USA
Hydrochloridric acid 1M	Carl Roth, Karlsruhe, Germany
Hydrochloridric acid 2M	Carl Roth, Karlsruhe, Germany
Isopropanol	Carl Roth, Karlsruhe, Germany
Klenow Fragment	Fermentas, St. Leon-Rot, Germany
Magnesium Chloride (MgCl ₂)	Sigma-Aldrich, St. Louis, USA
Methanol	Sigma-Aldrich, St. Louis, USA
Milk powder	Carl Roth, Karlsruhe, Germany
Page Ruler Prestained Protein Ladder	Thermo Fisher Scientific Inc., Waltham, USA

Penicillin/streptomycin	Thermo Fisher Scientific Inc., Waltham, USA
Pepsin	Sigma-Aldrich, St. Louis, USA
Peroxidase	Sigma-Aldrich, St. Louis, USA
Polyethylenglycol (PEG) 4000	Merck, Darmstadt, Germany
Potassium chloride (KCl)	Carl Roth, Karlsruhe, Germany
Protamine sulphate	Sigma-Aldrich, St. Louis, USA
Proteinase K (recombinant), PCR grade	Fermentas/ Thermo Fisher Scientific Inc., Waltham, USA
Qiagen Master Mix for PCR	Qiagen, Venlo, Netherlands
Recombinant murine IL3 (rm IL-3)	Immunotools GmbH, Germany
Restriction enzymes	Fermentas/ Thermo Fisher Scientific Inc., Waltham, USA
Restriction enzymes	NewEngland Biolabs, Schwalbach, Germany
SDS (sodium dodecyl sulfate)	Carl Roth, Karlsruhe, Germany
Sequencing buffer for PCR	Thermo Fisher Scientific Inc., Waltham, USA
S.O.C. medium	Invitrogen, Carlsbad, USA
Sodium acetate, Anhydrous (NaAC)	Merck, Darmstadt, Germany
Sodium chloride (NaCl)	Merck, Darmstadt, Germany
Sodium hydroxide (NaOH)	Merck, Darmstadt, Germany
Sodium phosphate dibasic anhydrous (Na ₂ HPO ₄)	Sigma-Aldrich, St. Louis, USA
Tris (2-Amino-2-hydroxymethyl-propane-1,3-diol)	Carl Roth, Karlsruhe, Germany
Trypan blue	Invitrogen, Karlsruhe, Germany
Trypsin-EDTA	Thermo Fisher Scientific Inc., Waltham, USA
Tween® 20	Sigma-Aldrich, St. Louis, USA
X-alpha-gal (5-Bromo-4-Chloro-3-indolyl a-Dgalactopyranoside)	Sigma-Aldrich, St. Louis, USA
Yeast extract	Sigma-Aldrich, St. Louis, USA

2.1.2 Materials and kits

Material	Source
Blotting paper GB003 and GB002	Schleicher & Schuell, Dassel, Germany
Coverslips	Menzel-Gläser®, Braunschweig, Germany
Cryotube™	Vials Nalge Nunc International, Denmark
Endofree® Plasmid Maxi kit	Qiagen, Hilden, Germany
Eppendorf tubes	Eppendorf, Hamburg, Germany
FACS Polystyrene round-bottom tubes	Becton Dickinson, Meylan, France
Falcon™ Round-Bottom Polypropylene Tubes	Thermo Fisher Scientific Inc., Waltham, USA
Falcon™ 50ml conical tubes	Thermo Fisher Scientific Inc., Waltham, USA
Ficoll type 400	Sigma-Aldrich, St. Louis, USA
Filter unit 0.22 µm	Millipore, Belford, USA
Filter unit 0.45 µm	Millipore, Belford, USA
Glass beads	Sigma-Aldrich, St. Louis, USA
Glassware	Schott, Jena, Germany
Hemocytometer/ Neubauer counting chamber	Merck, Darmstadt, Germany
Microcentrifuge tube (1.5 ml)	Eppendorf, Hamburg, Germany
Microscope slides	Menzel-Gläser®, Braunschweig, Germany
Parafilm	SPI supplies, West Chester, USA
PCR Mycoplasma Test Kit	AppliChem Inc., St- Louis, USA
Pipettes (manual)	Eppendorf, Hamburg, Germany
Pipette tips	Eppendorf, Hamburg, Germany
Pipette tips	Starlab Group, Hamburg, Germany
Plasmid Quiaquick Miniprep-kit	Qiagen, Hilden, Germany
Plastic cuvettes for biophotometer	Carl Roth, Karlsruhe, Germany
Plastic ware for cell culture	Sarstedt, Nümbrecht, Germany
Plastic ware for cell culture	Corning Inc., New York, USA
Plastic ware for cell culture	Sigma-Aldrich, St. Louis, USA
PVDF-Membrane Hybond-P Amersham	Thermo Fisher Scientific Inc., Waltham, USA
QIAquick gel extraction kit	Qiagen, Hilden, Germany
Qproteome Mammalian Protein Prep Kit	Qiagen, Hilden, Germany
S.O.C. medium	Invitrogen, Carlsbad, USA
Super Signal West Pico Chemiluminescent Substrate Kit	Thermo Fisher Scientific Inc., Waltham, USA
Surgical blades	B. Braun Melsungen, Melsungen, Germany
Syringes	B. Braun Melsungen, Melsungen, Germany
Tween® 20	Sigma-Aldrich, St. Louis, USA
Whatman paper	Sigma-Aldrich, St. Louis, USA
Wipes	Kimberly Clark, Dallas, USA

2.1.3 Restriction enzymes and buffers

Enzyme or Buffer	Composition		Source
BamH1	BamH1, 10U/ μ l		Fermentas/ Thermo Fisher Scientific Inc., Waltham, USA
10xBuffer BamH1	Tris-HCl (pH8.0)	100mM	Fermentas/ Thermo Fisher Scientific Inc., Waltham, USA
	MgCl ₂	50mM	
	KCl	1M	
	TritonX-100	0.2%	
	BSA	1mg/ml	
EcoR1	EcoR1, 10U/ μ l		Fermentas/ Thermo Fisher Scientific Inc., Waltham, USA
10xBuffer EcoR1	Tris-HCl (pH7.5)	500mM	Fermentas/ Thermo Fisher Scientific Inc., Waltham, USA
	MgCl ₂	100mM	
	NaCl	1M	
	TritonX-100	0.2%	
	BSA	1mg/ml	
Esp31 (BsmB1)	Esp31, 10U/ μ l		Fermentas/ Thermo Fisher Scientific Inc., Waltham, USA
10xBuffer Tango	Tris-acetate (pH7.9)	330mM	Fermentas/ Thermo Fisher Scientific Inc., Waltham, USA
	Magnesium acetate	100mM	
	Potassium acetate	660mM	
	BSA	1mg/ml	
	DTT	10mM	

Enzyme or Buffer	Composition	Source
FastAP Thermosensitive Alkaline Phosphatase	Alkaline phosphatase, 1U/ μ l	Fermentas/ Thermo Fisher Scientific Inc., Waltham, USA
10xFastAP Buffer	Tris-HCl (pH8.0) 100mM MgCl ₂ 50mM KCl 1M TritonX-100 0.2% BSA 1mg/ml	Fermentas/ Thermo Fisher Scientific Inc., Waltham, USA
Fse1	Fse1, 2U/ μ l	New England Biolabs GmbH, Ipswich, USA
10xNEBuffer 4	Potassium acetate 500mM Tris-acetate 200mM Magnesium acetate 100mM DTT 10mM	New England Biolabs GmbH, Ipswich, USA
Klenow Fragment	Klenow Fragment, 10U/ μ l	Fermentas/ Thermo Fisher Scientific Inc., Waltham, USA
10xReaction Buffer	Tris-HCl (pH 8.0) 500mM MgCl ₂ 50mM DTT 10mM	Fermentas/ Thermo Fisher Scientific Inc., Waltham, USA
Not1	Not1, 10U/ μ l	Fermentas/ Thermo Fisher Scientific Inc., Waltham, USA
10xBuffer 0	Tris-HCl (pH 7.5) 500mM MgCl ₂ 100mM NaCl 1M BSA 1mg/ml	Fermentas/ Thermo Fisher Scientific Inc., Waltham, USA
Sac2	Sac2, 20U/ μ l	New England Biolabs GmbH, Ipswich, USA
T4 DNA Ligase	T4 DNA Ligase, 5U/ μ l	Fermentas/ Thermo Fisher Scientific Inc., Waltham, USA

10xT4 DNA Ligase Buffer	Tris-HCl	400mM	Fermentas/ Thermo Fisher Scientific Inc., Waltham, USA
	MgCl ₂	100mM	
	DTT	100mM	
	ATP	5mM	

2.1.4 Buffers and Solutions

2.1.4.1 Buffers and Solutions used for plasmid preparation

Buffer	Composition		Source
10x TE buffer	Tris-HCl pH 7.5	100 mM	Qiagen, Hilden, Germany
	EDTA	10 mM	
Alkaline lysis buffer for plasmid preparations (Buffer P2, Qiagen)	NaOH	200 mM	Qiagen, Hilden, Germany
	SDS	1%	
Neutralization buffer for plasmid preparations (Buffer P3, Qiagen)	Potassium acetate	3 M	Qiagen, Hilden, Germany
	pH 5.5		
Resuspension buffer for plasmid preparations (Buffer P1, Quiagen)	Tris-HCl, pH 8.0	50 mM	Qiagen, Hilden, Germany
	EDTA	10 mM	
	RNase A	100 µg/ml	

2.1.4.2 Buffers and solutions used in cell culture

Solution	Composition		Source
2x HBS Type A	HEPES	50 mM	
	KCl	12 mM	
	NaCl	280 mM	
	Na ₂ HPO ₄	1.5 mM	
	pH 7.05		
2x HBS Type B	HEPES	50 mM	
	NaCl	280 mM	
	NaP	1.5 mM	
	pH 7.2		
Bufen SE	EDTA	10 mM	Qiagen, Hilden, Germany
	Tris-HCl pH 8.0	100 mM	
	NaCl	1 M	

2.1.4.3 Buffers and solutions used for western blotting

Solution	Composition	
10x Electrophoresis buffer	Tris-HCl	250 mM
	Glycine	1.92 M
	SDS	1 %
Transfer Buffer	Tris-HCl	20 mM
	Glycine	15.4 mM
	SDS	0.8 %
	Methanol	20 %
1x TBS	Tris-HCl pH 8.0	10 mM
	NaCl	150 mM

Separating gel (8,55%)	HPLC grade H ₂ O	5.55 ml
	Bisacrylamide 38%	2.25 ml
	1.5 M Tris-HCl pH 8.8	1.875 ml
	SDS 10%	100 µl
	APS 10%	110 µl
	Temed	12 µl
Stacking gel	HPLC grade H ₂ O	3.685 ml
	Bisacrylamide 38%	630 µl
	1 M Tris-HCl pH 6.8	625 µl
	SDS 10%	50 µl
	APS 10%	65 µl
	Temed	12 µl
1x TBS-T	Tris-HCl pH 8.0	10 mM
	NaCl	150 mM
	Tween® 20	0.1 %

2.1.4.4 Antibodies for western blotting

Antibody	Source
JAK2 (D2E12)XP® Rabbit mAb	Cell Signaling Technology, Cambridge, UK
α Rabbit IgG Peroxidase	Sigma-Aldrich, St. Louis, USA

2.1.5 Bacteria culture medium

Medium	Composition	
LB medium (liquid)	Tryptone	1 %
	Yeast extract	0.5 %
	NaCl	1 %
LB medium for agar plates	Tryptone	1 %
	Yeast extract	0.5 %
	NaCl	1 %
	Agar select	1.5 %
	Ampicillin	50 µg/ml

The ingredients for culture medium or agar plates were dissolved in dH₂O and then autoclaved. In order to select transformants, 50µg/ml ampicillin was added to the LB medium for agar plates. It was then poured into Petri dishes, dried overnight and stored at +4°C. Similarly, 50µg/ml ampicillin was added to LB medium used for liquid cultures of transformed bacteria.

2.1.6 Eukaryote culture medium

Medium	Composition	Source
Gibco® DMEM (Dulbecco's Modified Eagle medium) cell culture medium		Thermo Fisher Scientific Inc., Waltham, USA
DPBS (Dulbecco's Phosphate-Buffered Saline)		Thermo Fisher Scientific Inc., Waltham, USA
Gibco® RPMI (Roswell Park Memorial Institute culture medium) cell culture medium		Thermo Fisher Scientific Inc., Waltham, USA
Culture Medium for BaF3 cells	Gibco® RPMI 80 % FBS 10 % WEHI-medium 10 % Penicillin/streptomycin 100 µg/ml	
Culture medium for Phoenix Ecotrophic cells	Gibco® DMEM 85 % FBS 15 % Penicillin/streptomycin 100 µg/ml	
Culture medium for WEHI cells	Gibco® RPMI 90 % FBS 10 % Penicillin/streptomycin 100 µg/ml	

2.1.7 Bacterial strains

Strain	Usage	Genotype	Source
DH5 α TM competent cells	Plasmid amplification	<i>F⁻ ϕ80lacZΔM15 Δ(lacZYA-argF) U169 deoR recA1 endA1 hsdR17 (r_k⁻, m_k⁺) phoA supE44 λ^- thi-1 gyrA96 relA1</i>	Invitrogen, Carlsbad, USA
XL10-gold ultracompetent cells	Plasmid amplification	<i>Tet^r Δ(mcrA)183 Δ(mcrCB-hsdSMR-mrr) 173 endA1 supE44 thi-1 recA1 gyrA96 relA1 lac Hte [F' proAB lacI^rZΔM15 Tn10 (Tet^r) Amy Cam^r].</i>	Agilent Technologies, Santa Clara, USA

2.1.8 Mammalian cell lines

Cell line	Detail
Ba/F3	Mouse pro B-cells, IL-3 dependent line derived from the C3H mouse strain.
Phoenix ecotropic	Packaging cell line producing gag-pol and envelop proteins for ecotropic viruses such as pMIG; derivative of 293T cell line.
WEHI	A myelomonocytic leukemia cell line established from inbred BALB/c mice, capable of producing IL-3.

2.1.9 Human sample

Human cDNA derived from the blood sample of a 63-year-old female patient with chronic myelogenous leukemia at the Klinikum Grosshadern medical facility, was used to amplify the BCR/JAK2 breakpoint fragment via PCR. The amplification product was cloned into the pCR2.1 TOPO[®] vector to serve as a starting point for further cloning steps.

2.1.10 Plasmids

Plasmid	Usage	Resistance	Source
pcDNA3	Cloning vector	Ampicillin	Invitrogen, Carlsbad, USA
pCR2.1 TOPO [®]	Cloning vector with 3'-thymidine (T) overhangs for direct ligation of PCR products.	Ampicillin	Invitrogen, Carlsbad, USA
pMIG	Retroviral vector containing GFP, used for gene expression in mammalian cells.	Ampicillin	Addgene, Cambridge, USA

2.1.11 Constructs

Construct	Usage	Source
pcDNA3 BCR/ABL	Expression construct for BCR/ABL, used during the cloning process of the BCR/JAK2 fusion gene.	Prof. Dr. Stefan K Bohlander
pcDNA3 ETV6/JAK2	Expression construct for ETV6/JAK2, used during the cloning process of the BCR/JAK2 fusion gene.	Prof. Dr. Stefan K Bohlander

2.1.12 Oligonucleotides

Primer	Sequence	Purpose
5' BCR sense primer	5'-CAGAACTCGCAACAGTCC-3'	Amplification of the BCR/JAK2 breakpoint fragment and verification of the cloning process
3' JAK2 antisense primer	5'-TCATACCGGCACATCTCCACAC-3'	Amplification of the BCR/JAK2 breakpoint fragment and verification of the cloning process
BCR reverse primer	3'-GCTCGAAGTTGGACCTGAG-5'	Verification of the cloning process
pcDNA3 reverse primer	3'-GCGATGCAATTCCTCATTT-5'	Verification of the cloning process
pMIG reverse primer	3'-GAGGAACTGCTTCCTTCACG-5'	Verification of the cloning process
pMIG reverse primer 2	3'- TGCCGGTCATAGCTCTTCTT-5'	Verification of the cloning process

2.1.13 Equipment

Equipment	Source
Analytical balance Mettler AE 240	Mettler-Toledo, Greifensee, Switzerland
Analytical balance Mettler PM 2000	Mettler-Toledo, Greifensee, Switzerland
Centrifuge Eppendorf 5417C	Eppendorf, Hamburg, Germany
Centrifuge Heraeus Megafuge 16R	Heraeus, Hanau, Germany
Centrifuge Labofuge 400	Thermo Fisher Scientific Inc., Waltham, USA
Centrifuge Sorvall® GLC-2B General Laboratory Centrifuge	DuPont Instruments, Wilmington, USA
Electrophoresis power supply Gibco® BRL	Life Technologies, Carlsbad, USA
Flow cytometer DakoCytomation MoFlo-Cellsorter	Blue Lion Biotech, Carnation, USA
Freezer (+4°C)	Liebherr-International AG, Bulle, Switzerland
Freezer (-20°C)	Liebherr-International AG, Bulle, Switzerland
Freezer (-80°C) Hera Freeze	Heraeus, Hanau, Germany
Illumination unit Vilber Luroma	Vilber Lourmat Deutschland GmbH, Eberhardzell Germany
Incubator (Used for cell culture)	Heraeus, Hanau, Germany
Incubator Cytoperm (Used for cell culture)	Heraeus, Hanau, Germany
Incubator (Used for bacterial culture)	Heraeus, Hanau, Germany
Incubator (Including a shaker, used for bacterial culture)	New Brunswick Scientific Inc., New Jersey, USA

Laboratory bench SCALA Secuflow	Waldner Laboreinrichtungen GmbH & Co. KG, Wangen, Germany
Laboratory bench Safe 2020	Thermo Fisher Scientific Inc., Waltham, USA
Laboratory bench Heraeus LaminAir® HA 2248GS	Heraeus, Hanau, Germany
Laboratory water bath GFL® 1083	GFL - Gesellschaft für Labortechnik mbH, Burgwedel, Germany
Laboratory water bath GFL® 1086	GFL - Gesellschaft für Labortechnik mbH, Burgwedel, Germany
Magnetic stirrer Ikamag REO	Heidolph, Schwabach, Germany
Microscope Axioplan	Carl Zeiss, Jena, Germany
Microscope Diavert	Leitz, Wetzlar, Germany
Microwave	Robert Bosch GmbH, Gerlingen, Germany
PCR machine GeneAmp PCR System 9600	PerkinElmer Inc., Waltham, USA
pH meter	Mettler-Toledo, Greifensee, Switzerland
Pipette (automatic) Accu-Jet® Pro	Brand GMBH + CO KG, Wertheim, Germany
Plate shaker	Heidolph, Schwabach, Germany
Printer Mitsubishi P93	Mitsubishi, Japan
Sequencer ABI PRISM™ 310 Genetic Analyzer	Applied Biosystems, Foster City, USA
Spectrophotometer Gene Quant II RNA/DNA Calculator	Pharmacia Biotech, Uppsala, Sweden
Thermal cycler Biometra Trio Thermoblock	Biometra GMBH, Goettingen, Germany
Transilluminator 302nm	Ultra-Violet Products Ltd, Cambridge, UK
Vortex Mixer	Janke & Kunkel (IKA), Staufen im Breisgau, Germany
Vortex Mixer Heidolph REAX 2000	Heidolph, Schwabach, Germany

2.2 Methods

2.2.1 Microbiology techniques

2.2.1.1 Bacterial cultures for plasmid amplification

In order to amplify plasmids, *E. coli* strain DH5 α were transformed with the plasmids to be amplified. Cultures of *E. coli* DH5 α [™] competent cells stored at -80°C were thawed on wet ice and gently stirred. A 50 μ l aliquot of the culture was transferred to a 1.5ml epi tube, 10ng of plasmid DNA were added to the tube and the contents were gently mixed. After incubation on wet ice for 30 minutes, a heat shock was applied by dipping the tube into a 37°C water bath for 20 seconds. Afterwards, the tube was cooled on wet ice for another 2 minutes before adding 950 μ l of liquid LB medium pre-warmed to 37°C. The tubes were then kept in a 37°C shaking incubator at 150 rpm for 1 hour. A total of 4 plates of LB agar containing 50 μ g/ml ampicillin were used to select transformant colonies. 100 μ l, as well as 250 μ l of the culture were streaked out on 2 plates each, in order to increase the probability of single, distinguishable cultures on the following day. A negative control of *E. coli* DH5 α [™], which was previously treated like the transformed culture with the exception of not receiving any plasmid DNA, was streaked out on two additional agar plates (100 μ l and 250 μ l) to confirm the effectiveness of the antibiotic-based selection process. A total of 6 plates were incubated at 37°C overnight or for at least 16 hours. The next day, single colonies were picked and transferred to 14ml Falcon[™] tubes containing 5ml of liquid LB medium with 50 μ g/ml ampicillin. Again, the tubes were kept in a 37°C incubator at 150rpm for 16 hours. The next day, the liquid cultures were harvested for plasmid preparation.

When working with bacteria transformed with the pCR2.1 TOPO[®] plasmid, 80 μ l of a 2% x-alpha-gal solution were streaked out on each agar plate prior to the addition of bacteria. The plasmid itself contains the lacZ gene coding for the enzyme β -galactosidase, which spans the polylinker region. Any insert within this region will lead to the disconnection of lacZ and therefore a lack of β -galactosidase activity. The enzyme cleaves the colorless X-gal into galactose and a product which turns blue after dimerisation and oxidation. This process was utilized to distinguish colonies containing empty plasmids, which would still be capable of the production of β -galactosidase and will therefore be blue, and colourless colonies containing plasmids with an insert.

2.2.1.2 Bacterial cultures for the amplification of clones from a ligation reaction

When working with the product of a ligation reaction, a more efficient bacterial cell line was sometimes required to obtain a reasonable number of transformant colonies. In these cases, the XL10-gold ultracompetent cells were put to use. After thawing on wet ice, an aliquot of 25 μ l was transferred to an 1.5ml epi tube, 1 μ l of β -mercaptoethanol was added and the mixture was gently stirred. The tube was then kept on wet ice for 10 minutes. Afterwards, 5 μ l of the ligation reaction mixture were added, the contents of the tube were gently stirred and the mixture was incubated on ice for another 30 minutes before a heat shock was applied by dipping the tube into a 42°C water bath for 30 seconds. The tube was cooled down on ice for 2 minutes and 975 μ l of pre-warmed SOC medium were subsequently added. After incubating the epi tubes at 37°C and 150rpm for 40 minutes, the content was streaked out on LB agar plates containing 50 μ g/ml ampicillin as described in 2.11.

2.2.1.3 Preparation of glycerol stocks

In order to prepare the long-term archiving of plasmid-bearing bacteria, 800 μ l of a 16-hour liquid culture (As described in 2.11) were mixed with 200 μ l of glycerol. The mixture was then frozen and stored in a -80°C freezer.

2.2.2 Molecular biology techniques

2.2.2.1 Preparation of plasmids from bacterial cultures

5ml liquid cultures were harvested by centrifugation at 3000rpm for 10 minutes. The plasmid DNA was isolated with Quiagen's Quiaquick Miniprep-kit, utilizing the buffers and solutions included in the kit and closely following the instructions provided by the manual. During the final step, the DNA obtained from a 5ml liquid culture was usually diluted in 50 μ l TE buffer and stored at -20°C for further use.

2.2.2.2 Isolation of the pMIG plasmid prior to the transfection of mammalian cell lines

Since the transfection of mammalian cell lines required highly purified, endotoxin-free DNA, Qiagen's Endofree® Plasmid Maxi kit was used to obtain plasmid DNA from 20ml liquid overnight cultures. The purified plasmids were diluted in HPLC grade H₂O and stored at -20°C.

2.2.2.3 Isolation of genomic DNA from mammalian cell lines

To isolate genomic DNA, the contents of a 15ml culture were centrifuged, the supernatant was discarded and the pellet was frozen at -20°C for 10 minutes. After thawing, the cells were resuspended with PBS and transferred to a 1.5ml Eppendorf tube. After 2 additional washing steps of centrifugation and resuspension with PBS, the pellet was resuspended with 150µl SE (Saline-EDTA) Buffer, 30µl 10% SDS and 2.5µl 10mg/ml Pronase. The mixture was then incubated in a 55°C water bath for 3 hours, before 150µl of SE buffer were added and the mixture was incubated for another 10 minutes at 55°C. Then, 100 µl of 5M NaCl solution were added and the solution was vortexed gently and centrifuged. The supernatant, now containing the DNA, was transferred to another Eppendorf tube and precipitated by the addition of 750µl of ice-cold 98% ethanol. After centrifugation, another washing step containing 70% ethanol was applied and the pellet was then air-dried for several minutes. Finally, the DNA was resuspended in TE buffer on a plate shaker at 4°C overnight and stored at -20°C for further use.

2.2.2.4 Agarose gel electrophoresis

During the cloning process, new plasmid samples acquired from bacterial cultures were usually subjected to a series of experiments to verify the presence of an insert or to determine the orientation of the insert. The plasmid DNA was cleaved by different restriction enzymes and the fragments were visualized by agarose gel electrophoresis. Depending on the size of these fragments, different concentrations of agarose were used. Agarose concentrations of 0.8%, 1% or 2% allowed to visualize and distinguish fragments ranging from 50 bp to several kb. Depending on the requirements of the experiment, 0.8g, 1g or 2g of agarose were diluted in 100ml TE buffer and heated until reaching the boiling point at least twice. The solution was cooled down in a 55°C water bath for several minutes, before 5µl of 10g/l ethidium bromide solution were added, aiming at a final concentration of 0.5µg/ml ethidium bromide. The gel was poured into the gel tray and left to solidify at room temperature for at least 1.5 hours. The gel was then put into the electrophoresis chamber, filled with TAE buffer. The DNA samples were prepared with Ficoll DNA loading buffer and carefully

pipetted into the gel pockets. A DNA size marker was added in the form of a 1-kb, or 80-1000 bp DNA ladder and the fragments were separated by the application of an electrical current. Usually, an electric potential of 80V was applied for 10 minutes and subsequently increased to 110V for the following 30 to 90 minutes. After separation, DNA fragments were visualized using a 302nm transilluminator.

2.2.2.5 Isolation of DNA fragments for cloning

Given its mutagenous effects, DNA fragments intended for cloning purposes were required to be isolated without the application of ethidium bromide. To achieve this, an agarose gel was prepared according to 2.224 but without the addition ethidium bromide. The digested DNA was pipetted into 3 gel pockets: The largest quantity, usually the bulk of the reaction, was placed in the middle, while smaller quantities were placed to the left and right to indicate the position of the desired DNA fragment later on. A normal gel electrophoresis was performed and the gel was then sliced into 3 parts: Two parts, each containing one of the outer gel pockets and the narrow part in the middle. The two outer parts were stained in TAE buffer containing 2µg/ml EtBr for 5 minutes. The gel was subsequently put back together on the transilluminator, and the visible EtBr-stained bands could be used as indicators for cutting out the desired DNA band of the EtBr-free middle part. The DNA was then isolated using the QIAquick gel extraction kit, the weight of the cut-out gel fragment was determined and the quantities of buffers and solutions were adjusted accordingly. The DNA was resuspended in TE buffer and stored at -20°C.

2.2.2.6 Cloning reactions

Cloning reactions were planned with regard to several features of the DNA fragments involved. Depending on the size of the fragments, different quantities of plasmid and insert were required to gain a maximum number of correctly ligated plasmids. Again, these numbers changed depending on the nature of the fragment's endings (i.e. blunt or sticky ends). Sambrooks and Russel's *Molecular Cloning: A Laboratory Manual* (Green MR, Sambrook S, 2001) was of great help, providing the tables needed to determine the optimal ratio of plasmid and insert and information regarding the cloning process in general.

Prior to the cloning reaction, the ends of the plasmid fragment were usually dephosphorylated using alkaline phosphatase to prevent intramolecular ligation and to minimize the number of empty plasmids. T4 DNA ligase was used for the ligation reaction, which was kept on ice overnight. The ligase was inactivated the next day and the sample was stored at -20°C .

2.2.2.7 Enzymatic digestion

During the cloning process, a variety of experiments involving different restriction enzymes were conducted. The enzymes and the corresponding buffers, as listed under 2.1.3, were applied according to the manual provided by the producer. After incubation, the enzymes were inactivated and the samples were stored at -20°C for further use.

2.2.2.8 Elimination of cutting sites

In order to remove a BamH1 cutting site of the pCR2.1 plasmid, the plasmid was digested by BamH1, the enzyme was inactivated and a new reaction with Klenow fragment and dTNPs was carried out to fill up the overhangs. With the ends blunted,, the plasmid was religated using T4 DNA ligase, thus eliminating the BamH1 site. Accordingly, after amplification by bacterial culture, the plasmid could no longer be linearized by BamH1.

2.2.2.9 Polymerase chain reaction (PCR)

Polymerase chain reaction was used to amplify the BCR/JAK2 breakpoint fragment from the cDNA patient sample prior to cloning it into the pCR2.1 plasmid. A master mix containing Taq polymerase and dTNPs supplied by Quiagen was used for the reaction. The 5' BCR sense primer and the 3' JAK2 antisense primer (see 2.1.12) were applied and the PCR machine was programmed with the protocol shown in table 5.

Steps	Temperature (°C)	Time (min)	Frequency
Initial denaturation	95	5	Once
Denaturation	95	1	40 cycles
Annealing	58	1	
Elongation	72	1	
Final elongation	72	3	Once
Cooldown	4	∞	Once

Table 5: PCR protocol used for the amplification of the BCR/JAK2 breakpoint fragment.

The amplification product of 358 bp was then visualized by agarose gel electrophoresis as shown in figure 8.

2.2.2.10 Sequencing

During the cloning process, it was necessary to assure the integrity and completeness of the fusion gene, especially since mutations are known to occur during plasmid amplification in bacterial hosts. While sequencing of larger DNA segments was usually performed by the SEQLAB Sequence Laboratories GmbH in Göttingen, the breakpoint fragment was sequenced in our laboratory after cloning into the pCR2.1 plasmid. The modified Sanger sequencing method was applied, utilizing fluorescent dyes and a sequencing machine capable of capillary electrophoresis. Two sequencing reactions, containing the plasmid DNA, sequencing buffer, big dye and either the 5' BCR sense primer or the 3' JAK2 antisense primer were prepared and the thermal cycler was programmed with the protocol shown in table 6.

Steps	Temperature (°C)	Time (s)	Frequency
Initial denaturation	95	180	Once
Denaturation	95	20	28 cycles
Primer annealing	58	20	
Elongation	60	180	
Final elongation	60	120	Once
Cooldown	4	∞	Once

Table 6: Thermal cycling protocol used for linear amplification of single strands prior to sequencing.

In order to cover the region of interest, a relatively small number of primer oligonucleotides were required, given the long range and high quality of the sequencing performed by the SEQLAB Sequence Laboratories Göttingen GmbH.

2.2.3 Cell culture techniques

2.2.3.1 Cultivation of mammalian cells

All cell lines, which were utilized during the cell culture stage, were kept in an incubator at 37°C and only removed for maintenance or to conduct experiments. The components of the culture medium of each cell line can be found in 2.1.6. In both RPMI and DMEM medium, a depletion of nutrients was indicated by a colour change from red to yellow. When the colour change occurred, four fifths of the flask's volume were usually discarded or used to acquire cells for storage (see 2.2.3.3), new medium was added to the rest and the flask was returned to the incubator. The cells were frequently counted using a Neubauer counting chamber. Depending on the estimated density of cells, the medium was diluted with trypan blue at two different ratios (For example 1:5 and 1:10), and 10µl of each dilution were transferred to one of the two slots of the Neubauer plate. In order to determine the number of cells per ml, the average value of both counts was used in the calculation.

Cultivation of the adherent Phoenix Ecotrophic packaging cell line required harvesting of the cells for storage, counting, and medium changes. The cell culture consisted of a thin, continuous layer of cells adhering to the bottom of the flask. The original medium was discarded and the cells were washed with pre-warmed (37°C) PBS. After the PBS was discarded, a trypsin-EDTA solution was added and the flask was returned to the 37°C incubator for 4 minutes. After the cells had detached from the

surface, they were suspended in pre-warmed culture medium and residual cell clusters were dissolved by pipetting the medium up and down repeatedly. The cell solution could now be split between flasks in order to expand the culture.

Maintenance of the semi-adherent WEHI cell line, which was cultivated for the production of IL-3 conditioned medium, required similar measures. While the majority of cells were growing in suspension, a number of cells were attached to the flask's surface. After incubation with DPBS (without Ca²⁺ or Mg²⁺) for 10 minutes at 37°C, these were usually detached using a scraper and residual chunks were dissolved mechanically by pipetting.

The IL-3 dependent BaF3 cell line, which was the main object of study, proved to be robust and rather simple to maintain. Since the cells grew unattached to a surface, they were mixed simply by pipetting the medium up and down prior to counting, freezing, medium change or expansion of the cultures.

2.2.3.2 Production of IL-3 conditioned culture medium

The WEHI cell line, capable of the production of IL-3, was cultivated for the single purpose of harvesting IL-3 conditioned medium required to maintain the BaF3 cell line. WEHI cultures were harvested by centrifugation and the supernatant was filtered through a 0.22µm filter and stored at -20°C.

2.2.3.3 Freezing and thawing of cells

In order to create long-time stocks of a certain cell line, the contents of a culture flask were mixed thoroughly and the number of cells per ml was determined as described in 2.2.3.1. Based on the cell number, a volume containing approximately 10⁷ cells was transferred to a 50ml Falcon tube and centrifuged. The supernatant was discarded and the pellet was resuspended with 1ml of freezing medium consisting of 90% FBS and 10% DMSO (dimethyl sulfoxide). The solution was pipetted into a 1.5ml Cryotube™, which was placed in a freezing container. The container was put into a -80°C freezer overnight and the next day, the Cryotube™ was transferred to a regular storage box and kept at -80°C. This method was often modified: Medium containing a multiple of 10⁷ cells was centrifuged, a greater volume of freezing medium was added and the solution was split up between several freezing tubes, each containing roughly 1x10⁷ cells.

Since slow thawing could potentially damage the cells, the process was accelerated by dipping the tubes, recovered from storage at -80°C , in a 37°C water bath. After thawing, the cells were transferred to a 50ml falcon tube, filled with 25ml of the corresponding culture medium and the contents were mixed and centrifuged. The supernatant was discarded and the pellet was resuspended in culture medium, now ready to be transferred to appropriate culture flasks. The washing step served to reduce the exposure to DMSO, which would be harmful to the cells at room temperature.

2.2.3.4 Transfection of adherent cells

The Phoenix Ecotrophic cells, derived from the 293T human embryonic kidney cell line, were used as a packaging cell line for the retroviral pMIG plasmid. In short terms, Phoenix-ECO cells express the Pol gene, coding for reverse transcriptase, the Gag gene, coding for retroviral group antigens, and genes coding for viral packaging proteins. The cells are therefore capable of producing fully-functional retroviral particles as soon as they are transfected with a retroviral vector.

For transfection with the pMIG vector, the CaPO_4 precipitation method was chosen. The day before the transfection, 10cm cell culture plates were filled with 10ml DMEM medium supplemented with 10% FBS and 1% penicillin/streptomycin mixture and 6.5×10^6 cells were seeded on each plate. These were incubated at 37°C overnight, and the next day, approximately 4 hours before the transfection, they were given a whole medium change. The transfection was performed as follows: A $500\mu\text{l}$ mixture was prepared in a 1.5ml tube consisting of 10-15 μg Plasmid DNA, $50\mu\text{l}$ of a 2M CaCl_2 and a variable volume of HPLC grade water. The mixture was added dropwise to another tube containing $500\mu\text{l}$ of 2x HBS Type A (See 2.1.4.2), the contents were mixed by gently shaking the tube and incubated at room temperature for 4 minutes. Drop by drop, the mixture was then evenly distributed on each of the culture plates, which were by now covered by a monolayer of Phoenix-ECO cells. After swirling, the plates were put back into the incubator and kept at 37°C overnight. On the following morning, the culture medium was changed to remove calcium crystals which could be toxic to the cells. Starting 12 hours after the medium change, virally-conditioned medium (VCM) was ready to be harvested.

2.2.3.5 Production of virally-conditioned medium

12 hours after the last medium change, the supernatant of the transfected Phoenix-ECO cultures was harvested with a 10ml syringe and passed through a 0.45µm filter unit into a Falcon tube. 10ml of pre-warmed, fresh medium was added to the cells and the plates were returned to the incubator, while the collected VCM was stored at -20°C. The procedure was repeated twice, each time after a minimum interval of 12 hours. After the third VCM collection, the culture plates were discarded.

2.2.3.6 Transduction of BaF3 cells

To examine the transforming potential of the BCR/JAK2 fusion, two stable cell lines had to be established: A BaF3 cell line transduced with pMIG carrying the BCR/JAK2 fusion gene, and a BaF3 line carrying the empty pMIG vector, serving as a negative control for further experiments.

Prior to the transduction, BaF3 cell cultures were counted and harvested. An aliquot of WEHI-conditioned culture medium, virally-conditioned medium, protamine sulphate and BaF3 cells was gently mixed in a falcon tube and distributed between 4 wells of a 6-well plate. In total, 1.5ml of culture medium, 1.5ml of VCM, 0.6µl of a 5g/l protamine sulphate solution and 0.75×10^6 cells were added to each well. In the absence of an appropriate centrifuge with temperature-control, the 6-well plate was centrifuged at 1200rpm for 90 minutes at room temperature, possibly reducing the efficiency of the transduction protocol (Originally, the protocol required centrifugation with 2500rpm for 90 minutes at 30°C). Afterwards, the cells were put back into the 37°C incubator for 2.5 hours and 2ml of fresh medium were subsequently added to each well. The next day, the cell cultures were transferred to culture flasks and expanded, until a sufficient quantity of cells was available for sorting.

2.2.3.7 Testing for mycoplasma contamination

Before the cells could be sorted, the cultures had to be tested for mycoplasma infection in order to prevent contamination of the flow cytometer. To ensure a high sensitivity, a PCR kit (AppliCem's PCR Mycoplasma Test Kit) was put to use. Cell culture supernatant from the transduced BaF3 cell lines was prepared according to the protocol and with these samples, as well as a positive control sample, a PCR amplification was carried out. The amplification products were analyzed by conventional agarose gel electrophoresis, which did not reveal any kind of mycoplasma-specific amplification product in the cell culture samples. Therefore, mycoplasma contamination was ruled out and the transduced cell lines could be purified by sorting.

2.2.3.8 Establishing a stable transduced cell line

To acquire a stable cell line for further experiments, successfully transduced cells had to be sorted out and purified. The sorting process was based on the expression of GFP, located on the retroviral pMIG vector. GFP (green fluorescent protein) exhibits green fluorescence when exposed to blue or ultraviolet light, indicating the successful integration of the vector into the genome of the cell. While the outcome of the retroviral transduction described in 2.2.3.6 could not be determined right away, the flow cytometry was providing valuable data regarding the efficiency of the process.

In preparation of the sorting process, the cells were harvested by centrifugation and 10^7 - 10^8 cells of each culture were resuspended in 1ml of PBS. Additionally, the cells were stained with DAPI, allowing the exclusion of dead cells. The samples were then sorted in the Flow Cytometry Core Facility of the Philipps University Marburg's clinic for hematology and oncology based on the expression of GFP and DAPI uptake, using the DakoCytomation MoFlo-Cellsorter. In total, three rounds of sorting were necessary to acquire sufficient numbers of GFP positive cells. It is worth notice, that the first round of sorting revealed a rather small ratio of GFP expression in the transduced cultures: Only 0.14% of the BaF3 cells transduced with pMIG-BCR/JAK2 were GFP positive, along with a GFP expression of 0.75% in the culture transduced with the empty pMIG vector. The second round yielded significantly higher numbers, with a GFP expression ratio of 93.1% within the pMIG-BCR/JAK2, and 82.77% within the empty vector cell line, demonstrating the efficiency of the process.

2.2.3.9 Proliferation assays

To examine the transforming potential of the BCR/JAK2 gene, proliferation assays containing virally transduced BaF3 cell lines were carried out. Since the growth of the BaF3 cell depends on the presence of IL-3, autonomous growth in the absence of IL-3 can be attributed to the presence of a transforming oncogene. Furthermore, being associated with the IL-3 receptor, the wild type JAK2 kinase plays a vital role in the transduction of the pro-proliferative signal. Therefore, we speculated that a mutant, autonomously active JAK2 would continuously trigger the downstream pathway and promote proliferation, even in the absence of the otherwise obligatory growth factor.

In order to compare the behaviour of native BaF3 cells, cells carrying the empty vector-DNA and cells expressing the fusion gene (Subsequently referred to as “Native, pMIG and BCR/JAK2”, respectively) in the presence and the absence of IL-3, the following experiment was set up: The three cultures were harvested and washed by three cycles of centrifugation and resuspension with DPBS to remove any IL-3 originating from WEHI medium. Simultaneously, two aliquots of RPMI medium containing 10% FBS and 1% penicillin/streptomycin solution were prepared. To one of these aliquots, 10ng/ml of recombinant murine IL-3 (rm IL-3) was added. Native, pMIG and BCR/JAK2 cells were counted and subsequently suspended in culture medium with- and without IL-3. In total, six 6ml aliquots, each containing 180.000 cells were prepared and distributed between four 6-well plates. Finally, each cell line was represented by six wells: Three wells, each containing 80.000 cells suspended in 2ml medium with IL-3, and three wells containing 80.000 cells in 2ml medium without IL-3. The basic pattern of the experiment is shown in Figure 2:

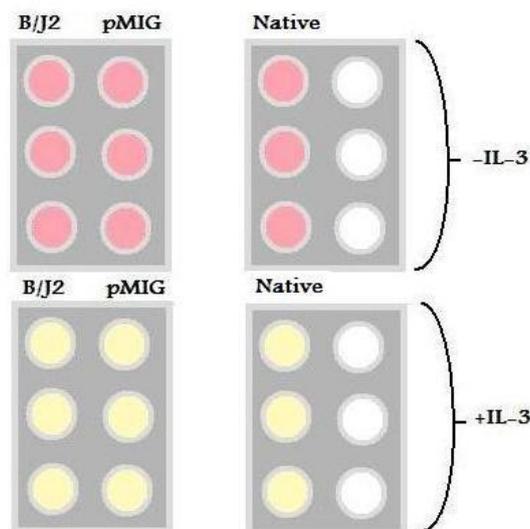


Figure 2: Set-up of the proliferation assay. The figure shows the distribution of 2ml cultures of the three BaF3 lines “Native, pMIG and BCR/JAK2 (B/J2)” between four 6-well plates.

Except for the time required to obtain samples for counting, the 6-well plates were kept in an incubator at 37°C. 24 hours after the assay had been set up, a cell count of each well was obtained using a Neubauer counting chamber. Again, the samples were diluted and stained with trypan blue at two different ratios, and the average of both counts was used to calculate the number of cells per ml. Dead or dying cells, recognizable by their irregular shape and high uptake of trypan blue, were excluded from the count. For the first proliferation assay, cultures were counted six times, with the last count being performed 144 hours after the experiment was started.

The second proliferation assay, while performed in a similar fashion, was carried out using BaF3 BCR/JAK2 cells from the first assay, which were still vital and expanding despite the prolonged absence of IL-3. The aim of the second assay was to compare the cytokine-mediated proliferation activity of native cells, to the autonomous IL-3-independent proliferation of the transduced BaF3 line. The set-up of the second assay was identical, distributing Native, pMIG and BCR/JAK2 cell cultures between four 6-well plates. This time, the last cell count was obtained at 96 hours.

2.2.4 Protein biochemistry

2.2.4.1 Protein extraction from mammalian cells

BaF3 cells transduced with the pMIG retroviral vector carrying the BCR/JAK2 fusion gene were harvested by centrifugation and washed twice by resuspension with PBS and subsequent centrifugation. The proteins were extracted using Qiagen's Qproteome Mammalian Protein Prep Kit utilizing the buffers and solutions included in the kit and following the instructions provided by the manual. The protein samples were stored at -80°C for further use.

2.2.4.2 Western blot analysis

To determine the presence of the BCR/JAK2 fusion protein, the protein extracts of stably transduced BaF3 cells as well as native BaF3 cells were examined. Standard Western blot analyses were performed using the SDS polyacrylamide gel electrophoresis method, closely following a well-established protocol. An electric potential of 110V was applied until the stained bands of the protein ladder, which served as a scale on the separating gel, had travelled a satisfactory distance. The proteins were then transferred to a polyvinylidene fluoride (PVDF) membrane by electroblotting, applying an electric potential of 48V for 180 or 240 minutes. The next day, the membrane was incubated with the primary and secondary antibody. The JAK2 (D2E12)XP[®] Rabbit mAb, intended to bind the JAK2-rest of the fusion protein was the primary, the peroxidase-linked α Rabbit IgG was the secondary antibody, intended to bind and later stain the first one. The membrane was treated with peroxidase and enhancer solution from the Super Signal West Pico Chemiluminescent Substrate Kit, photographic developer solution was added and the membrane and photographic film paper were placed together in an appropriate box for 15, 30 and 45 minutes, successively.

3 Results

3.1 Cloning the BCR/JAK2 expression construct

3.11 Development of a cloning strategy

The cloning strategy was based on the availability of DNA fragments from three different sources: The BCR/JAK2 breakpoint fragment, derived from the patient sample, and two pcDNA3 plasmids, one containing BCR/ABL, the other one ETV6/JAK2. The BCR/JAK2 sequence published by Griesinger et al. served as a template for the planning, as well as the validation of the cloning process.

Starting from the 358bp breakpoint fragment (BP), the 5' part of BCR and the 3' part of JAK2 had to be attached to its 5' and 3' ends in order to recreate the fusion gene (Figure 3). After identifying cutting sites for restriction enzymes within the BP fragment and the respective plasmids, it became clear that the *Bam*H1 cutting site in the pcr2.1 polylinker region had to be eliminated prior to the other cloning steps. Similarly, a *Bsm*B1 site in pcDNA3 BCR/ABL had to be cut out using *Sac*2 and *Nse*1, before the 3' part of the BCR/JAK2 fusion gene could be cloned into the plasmid. During the first step of the cloning process, the 358 bp BP fragment, which was amplified from patient cDNA by PCR, was cloned into the linearized pcr2.1 vector. Then, the breakpoint fragment had to be cut out using *Eco*R1, and inserted into the modified pcr2.1 plasmid without the *Bam*H1.

During the second step, *Bam*H1 and *Not*1 were used to cut out the 3' portion of JAK2 from the pcDNA3 ETV6/JAK2 plasmid, and this 944 bp fragment was inserted into pcr2.1, thus completing the 3' part of the fusion gene.

For the third step, a fragment of 1030 bp, spanning a short part of *BCR* close to the breakpoint, as well as the 3' end of *JAK2*, was cut out of pcr2.1 using *Bsm*B1 and *Not*1. It was subsequently cloned into the modified pcDNA3 BCR/ABL plasmid (after the removal of a second *Bsm*B1 site located 5' of BCR).

For the final cloning step, the full length fusion gene was released from pcr2.1-BCR/JAK2 using *Eco*R1 and cloned into the pMIG retroviral vector prior to the transfection of mammalian cells. Figure 3 shows an overview of the cloning strategy.

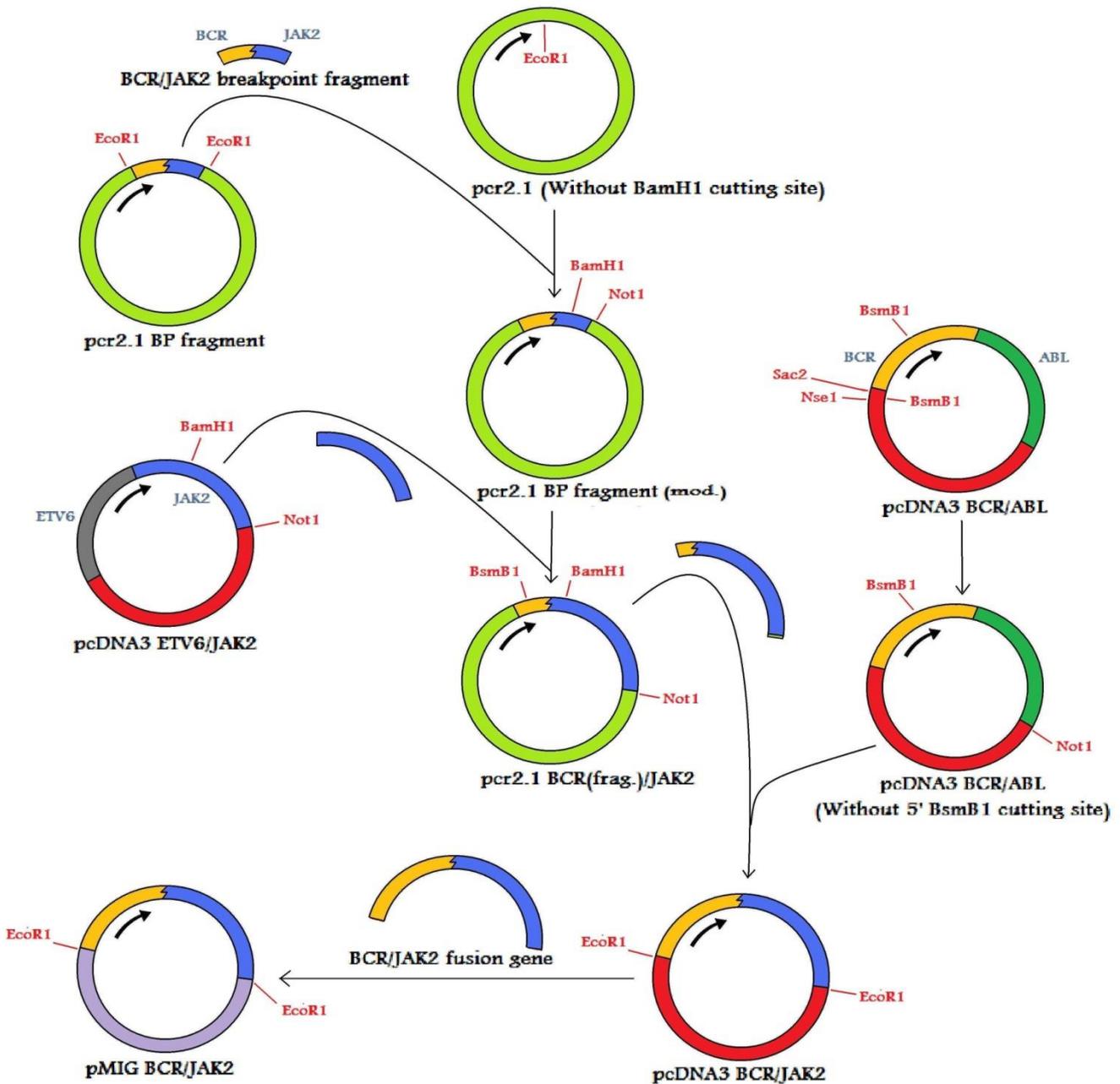


Figure 3: Cloning strategy for the BCR/JAK2 fusion gene. BCR is pictured in yellow- JAK2 is pictured in blue colour. Starting with the 358bp BP fragment amplified by PCR, this figure illustrates the steps used to clone the complete BCR/JAK2 fusion gene. The final construct, pMIG BCR/JAK2, was used for the transfection of the murine BaF3 cell line, in order to perform a functional characterisation of the BCR/JAK2 gene.

3.12 Cloning process

After the *Bam*H1 site of pcr2.1 had been removed (see 2.2.2.8), the BP fragment was successfully cloned into a linearized pcr2.1 vector (see 2.2.2.6). Figure 4 shows the vector and the 358bp insert visualized after digestion with *Eco*R1. After that, the plasmid was again digested using *Eco*R1, the 358bp insert was isolated by agarose gel electrophoresis (see 2.2.2.5) and inserted into the pcr2.1 vector from which the *Bam*H1 site had been removed. Since two *Eco*R1 sites meant that the breakpoint fragment could be inserted in two different orientations, the plasmids containing the inserts in the correct orientation had to be identified. This was done by control digestions with different restriction enzymes and the visualisation of the resulting DNA fragments by agarose gel electrophoresis. Figure 5 depicts the result of two control digestions performed with plasmids obtained from several *E. coli* DH5 α colonies after transformation with the ligation reaction.

For the next step, the pcDNA3 ETV6/JAK2 plasmid was digested using *Bam*H1 and *Not*1, and a 944bp fragment corresponding to the 3' portion of the *JAK2* coding region was isolated. The pcr2.1 vector containing the BP fragment was also cut with *Bam*H1 and *Not*1, and a big fragment containing the vector and the BP fragment up to the *Bam*H1 site was gel purified. The 3' *JAK2* fragment was then cloned into the pcr2.1 vector with the BP fragment, completing the *JAK2* part of the fusion gene. Figure 6 shows a control digestion of plasmids derived from 8 *E. coli* DH5 α colonies after transformation, using *Eco*R1 and *Not*1 to determine the correct length of the insert. After the successful ligation had been validated by a series of control digestions, the bulk of the insert, spanning from within the BCR segment of the breakpoint region to the 3' end of *JAK2*, was cut out using *Bsm*B1 and *Not*1, and ligated with the modified pcDNA3 BCR/ABL plasmid (one *Bsm*B1 site removed) after digestion with *Bsm*B1 and *Not*1. This plasmid had been digested with *Sac*2 and *Nse*1, blunted and religated, eliminating one of the *Bsm*B1 sites. By digestion with *Bsm*B1 and *Not*1, a large section containing the ABL part of the fusion gene, had been removed – allowing the insertion of the BP fragment and the 3' end of *JAK2*. Figure 7 shows the new insert of 2294 bp, consisting of the BCR/*JAK2* fusion gene after digestion with *Eco*R1. The insert was then sequenced using the “BCR forward”, “*JAK2* reverse” and “pcDNA3 reverse” primers to confirm the integrity of the expression construct (see 2.2.2.10).

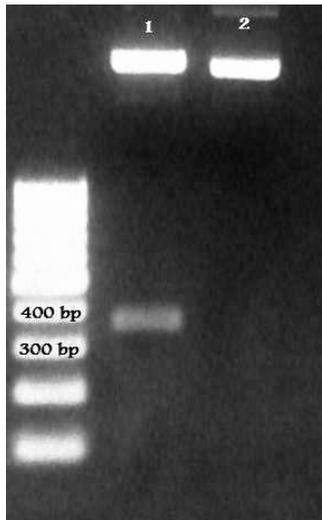


Figure 4: The pcr2.1 plasmid and the 358bp insert containing the BCR/JAK2 breakpoint fragment, visualized by agarose gel electrophoresis after digestion with *EcoR1* (Lane 1). The uncut plasmid (lane 2) is supercoiled and migrates a little faster than the linearized vector.

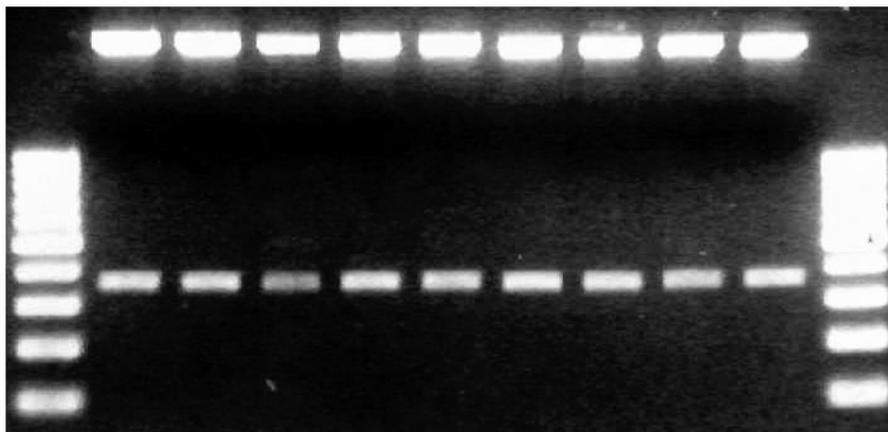
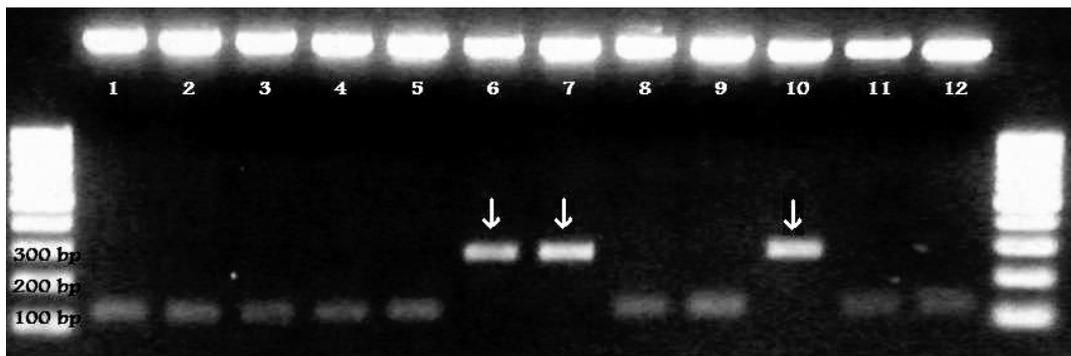


Figure 5: Agarose gel electrophoresis: Control digestions performed with plasmids obtained from several *E. coli* DH5 α colonies after transformation with the *BamH1*-depleted pcr2.1 plasmid ligated with the breakpoint fragment. The upper image shows plasmids extracted from 12 different *E. coli* DH5 α colonies cut with *BamH1* and *Not1*. The correct orientation of the insert, indicated by the presence of a 128 bp fragment, can be seen in most plasmids. However, the wrong orientation, indicated by a 284 bp fragment (white arrow), can be observed in three of the samples. The lower image shows the presence of the BCR/JAK2 breakpoint fragment in all of the plasmids with the correctly oriented insert.

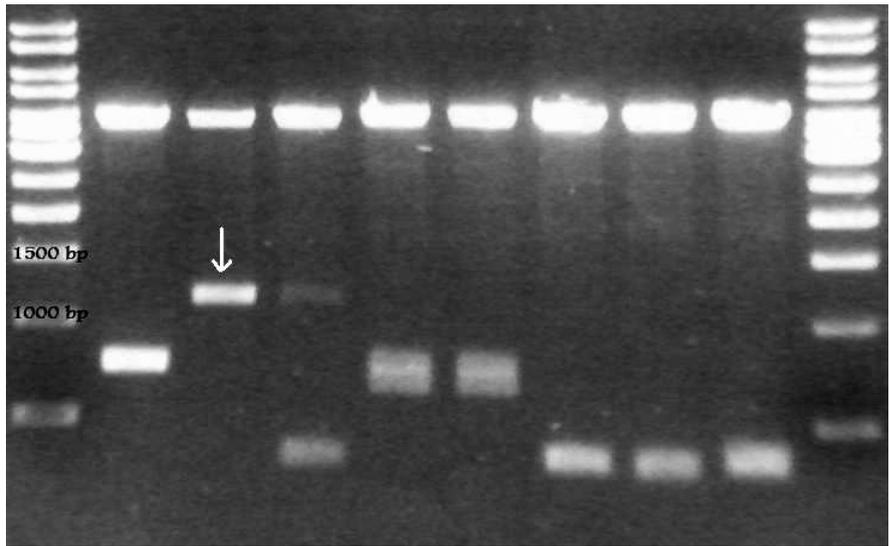


Figure 6: Agarose gel electrophoresis of plasmids obtained from 8 different *E. coli* DH5 α colonies after digestion with *Eco*R1 and *Not*I. A correct insert length of 1211bp can only be observed in the second sample (indicated by the white arrow). Further confirmation of the integrity of the insert was performed, but is not shown here.

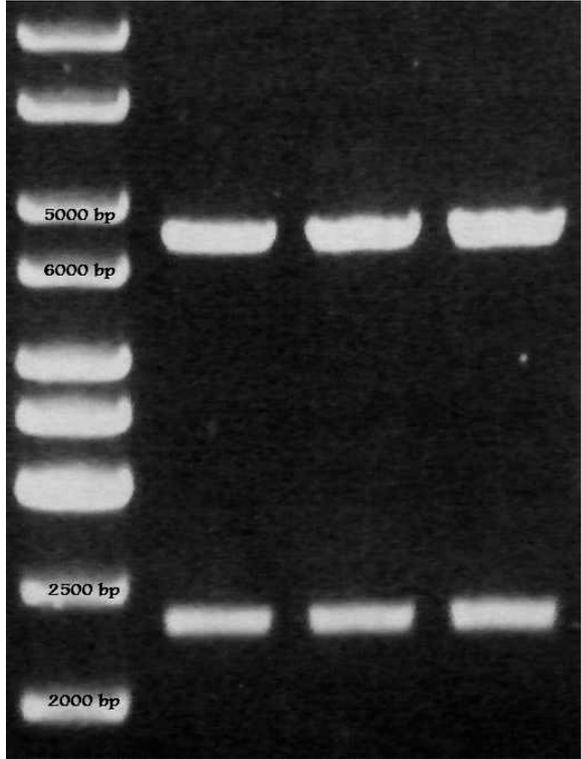


Figure 7: Agarose gel electrophoresis: Control digestion of pcDNA3 BCR/ABL with *Eco*R1. The 2294bp insert containing the complete BCR/JAK2 fusion gene can be seen in three plasmid samples extracted from *E. coli* XL10-gold colonies after transformation.

For the last cloning step, the complete BCR/JAK2 insert was cut out using *EcoR1*, and ligated with the pMIG vector, which was linearized by *EcoR1* digestion as well. Again, the possibility of two different orientations required a control experiment designed to distinguish both variants. Figure 8 depicts an agarose gel electrophoresis of a control digestion with *Xho1* performed with plasmids obtained from 7 different *E. coli* DH5 α colonies after transformation. Prior to the transduction of mammalian cells, the plasmid was again sequenced using the “BCR forward”, “JAK2 reverse”, “pMIG forward” and “pMIG reverse” primers, confirming the integrity of the fusion gene within the retroviral vector (see 2.2.2.10). The complete sequence can be found in the Appendix section.

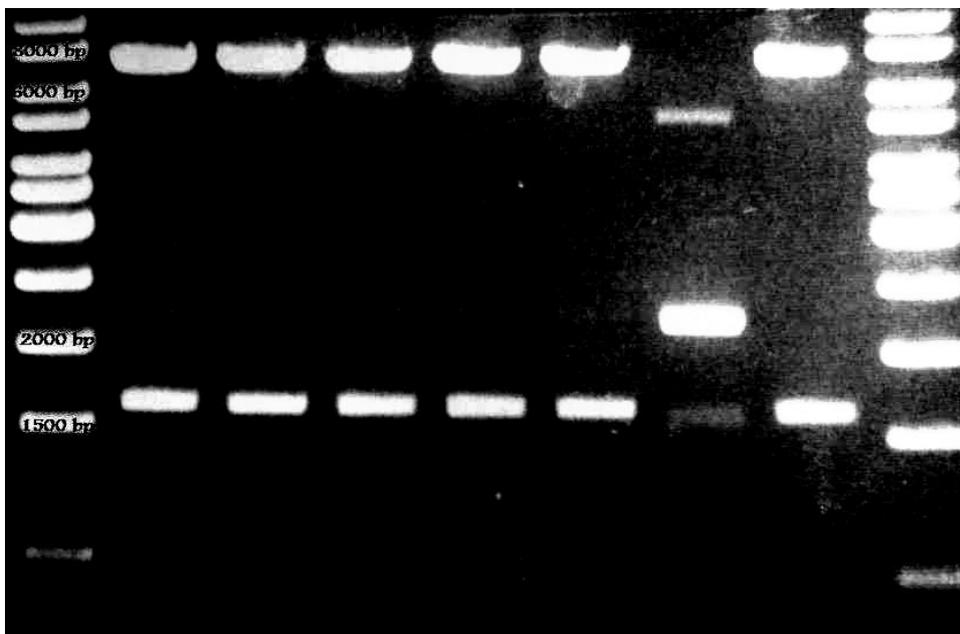


Figure 8: Agarose gel electrophoresis: Control digestion of 7 plasmid samples extracted from 7 different *E. coli* DH5 α colonies using *Xho1*. A correctly oriented insert is indicated by the presence of a 1652bp fragment, which is visible in 6 out of 7 samples. This result was rather unexpected, since the probability of both orientations should be at 50% each.

3.2 BCR/JAK2 induces IL-3 independent growth of BaF3 cells *in vitro*

In order to produce stably transduced cell lines of BaF3 cells, retroviral particles containing the BCR/JAK2 fusion gene in the pMIG vector, as well as retroviral particles containing the empty pMIG vector, were generated by transfection of the Phoenix-ECO cell line using the CaPO₄ precipitation method (see 2.2.3.4 and 2.2.3.5). The virally conditioned medium (VCM) was harvested and used for the transduction of BaF3 cells (see 2.2.3.6). Initially, flow cytometry revealed a very low transduction efficiency: At the time of the first FACS sorting, as little as 0.14% of cells transduced with pMIG-BCR/JAK2, and only 0.75% of cells transduced with the empty pMIG vector were GFP positive. This rate was then increased by repeated sorting (See 2.2.3.8). After a total of three rounds of FACS sorting, the proportion of GFP positive cells was > 98% in both transduced cell lines (BaF3 pMIG, BaF3 pMIG-BCR/JAK2).

After stably transduced cell lines had been established, the first proliferation assay was carried out as described in 2.2.3.9. The assay revealed an overwhelming advantage for BCR/JAK2 transduced and expressing cells over the native and empty vector transduced cell lines in the absence of IL-3. In the presence of 10ng/ml IL-3, the three cell lines did not exhibit any major differences in proliferation. However, in the wells containing IL-3-free medium, the picture was different: After 24 hours, the proliferation of all three cell lines was stagnating. 24 hours later, the number of dead cells, identifiable by intense staining and irregular shape, had increased significantly in all three cell lines. From 48 hours onwards, a difference between the BaF3 line transduced with the BCR/JAK2 retroviral construct and the native and empty vector transduced lines could be observed. While a fraction of the BaF3 pMIG-BCR/JAK2 cells was dying, another fraction was expanding, and the overall number of cells began to increase. When the assay was stopped at 144 hours, The BCR/JAK2 transduced cell line was rapidly expanding, while living cells were only sporadically observed within the native and empty vector transduced lines. Figure 9 depicts the growth of the three BaF3 cell lines (Native, pMIG and BCR/JAK2 transduced) in the presence of IL-3. Figure 10 depicts the growth of the three BaF3 cell lines (Native, pMIG and BCR/JAK2 transduced) in the absence of IL-3.

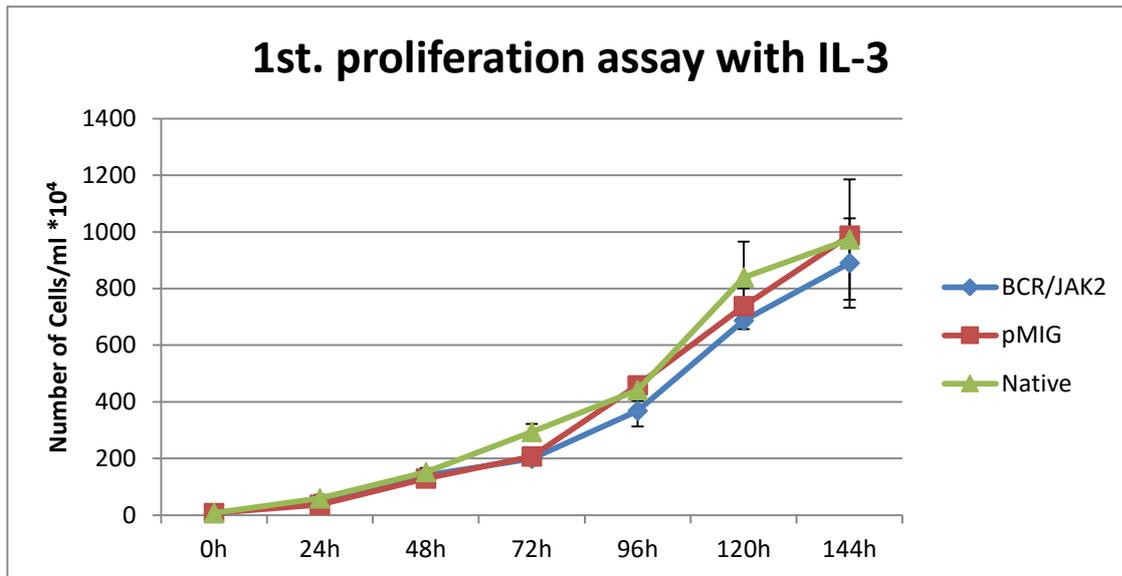


Figure 9: 1st proliferation assay: Growth of the three cell lines “Native, pMIG and BCR/JAK2 (B/J2)” over time in the presence of 10ng/ml IL-3. The graphs represent the mean value of the total number of cells from three independent cell culture wells, which were counted using a Neubauer counting chamber. Standard deviation is indicated by black bars.

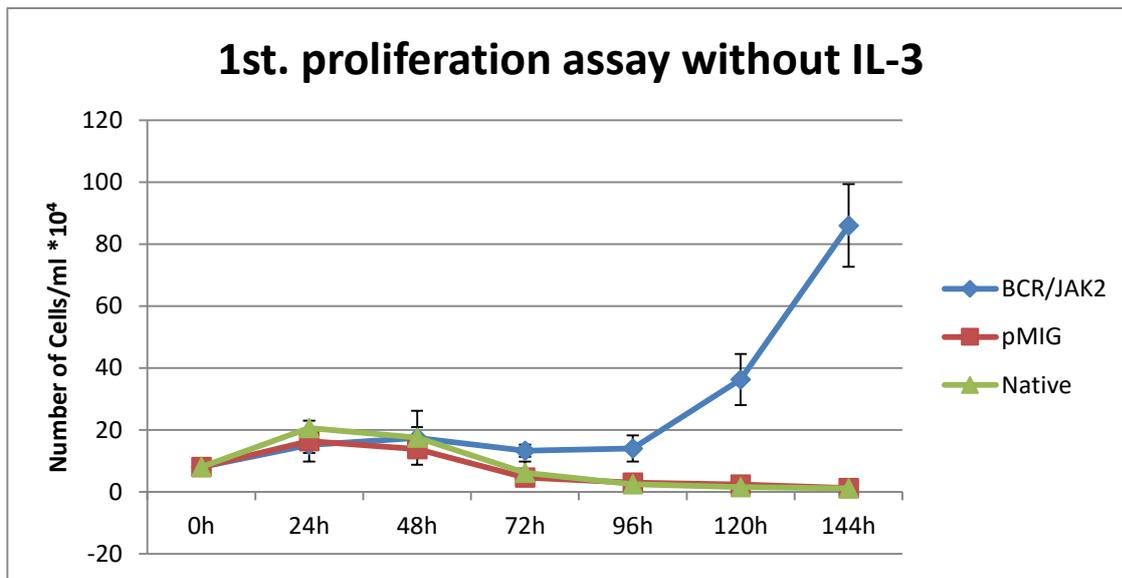


Figure 10: 1st proliferation assay: Growth of the three cell lines “Native, pMIG and BCR/JAK2” over time in the absence of IL-3. The graphs represent the mean value of the total number of cells from three independent cell culture wells, which were counted using a Neubauer counting chamber. Standard deviation is indicated by black bars.

The second proliferation assay, which was primarily intended to compare the IL-3-dependent and independent growth of the stably transduced BaF3 pMIG-BCR/JAK2 cell line, yielded similar results. This time however, the pre-selected BCR/JAK2 cells expanded from the beginning. Figure 11 and 12 depict the results of the second proliferation assay.

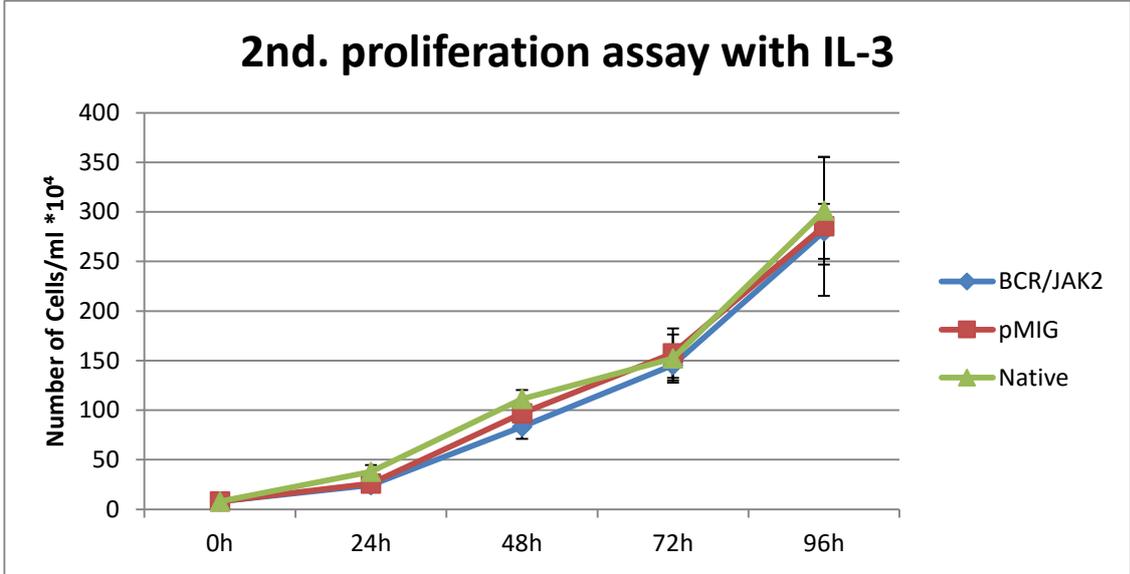


Figure 11: 2nd proliferation assay: Growth of the three cell lines “Native, pMIG and (pre-selected) BCR/JAK2 (B/J2)” over time in the presence of 10ng/ml IL-3. The graphs represent the mean value of the total number of cells from three independent cell culture wells, which were counted using a Neubauer counting chamber. Standard deviation is indicated by black bars.

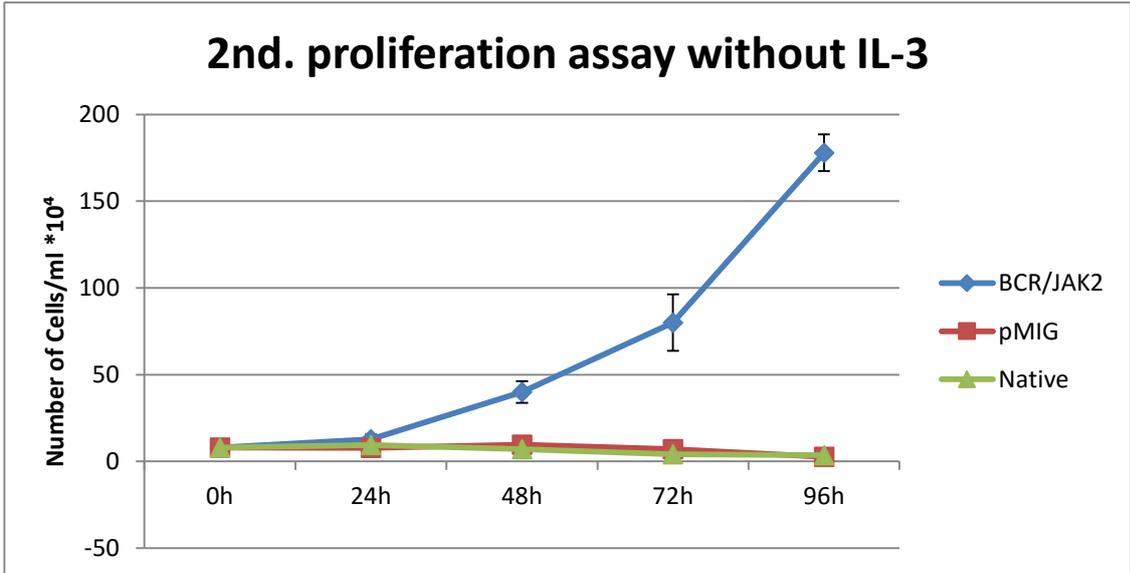


Figure 12: 2nd proliferation assay: Growth of the three cell lines “Native, pMIG and (pre-selected) BCR/JAK2” over time in the absence of IL-3. The graphs represent the mean value of the total number of cells from three independent cell culture wells, which were counted using a Neubauer counting chamber. Standard deviation is indicated by black bars.

Generally, the proliferation assays strongly support the assumption, that the presence of the BCR/JAK2 fusion gene promotes growth-factor independent cell proliferation. In the absence of IL-3, BaF3 cells containing the gene had a strong growth advantage over the native, as well as the empty vector cell line.

3.21 IL-3 independent proliferation is weaker than IL-3 dependent growth *in vitro*

When directly comparing the BaF3 pMIG-BCR/JAK2 cell line in terms of IL-3 mediated and IL-3 independent proliferation, the growth-factor independent growth was shown to be significantly weaker. At 96 hours, the average number of cells per ml was 1.7792×10^6 in the wells without IL-3, and 2.8042×10^6 in the wells containing the growth factor. A graph illustrating the growth of the BCR/JAK2 transduced BaF3 cell line under different conditions is shown in figure 13.

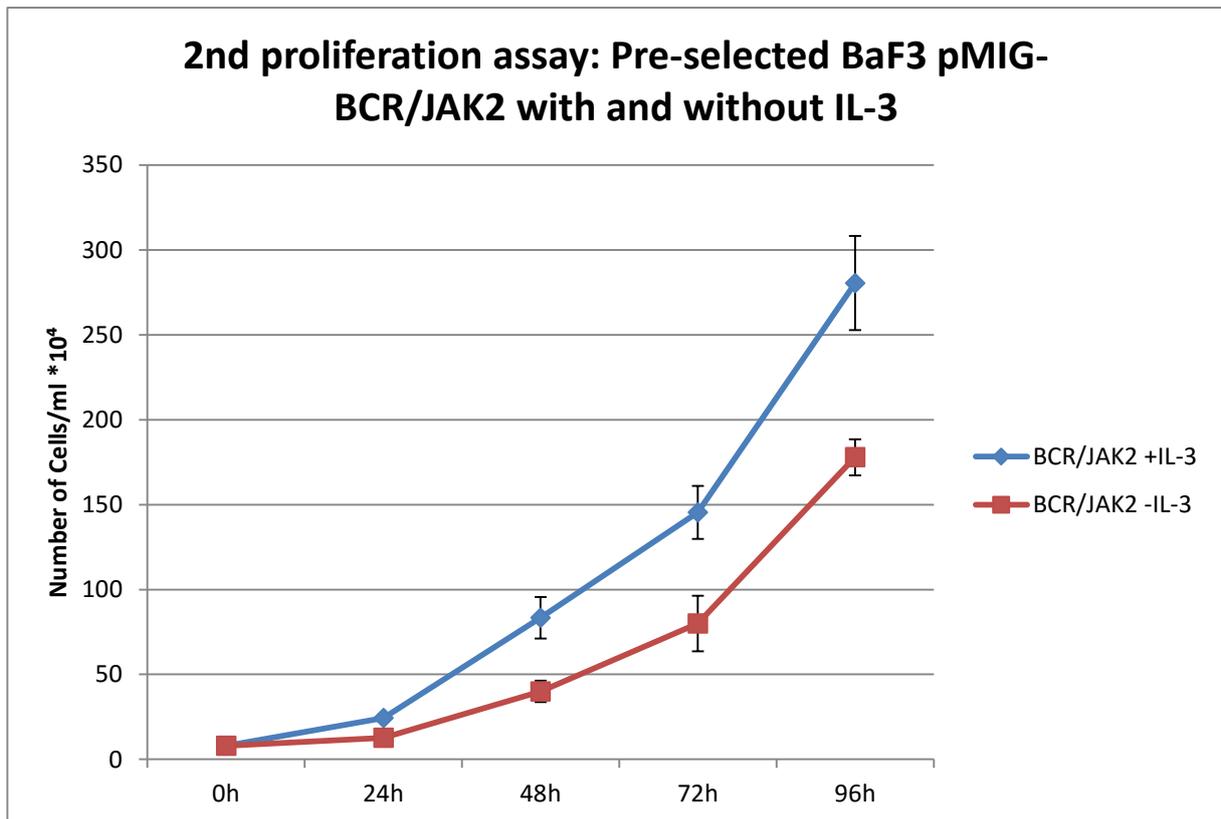


Figure 13: 2nd proliferation assay: Comparison of pre-selected BaF3 pMIG-BCR/JAK2 cells with- and without 10ng/ml IL-3. The graphs represent the mean value of the total number of cells from three independent cell culture wells, which were counted using a Neubauer counting chamber. Standard deviation is indicated by black bars.

However, these results should be interpreted with caution, since the growth of BaF3 cells in medium containing IL-3 depends on the concentration of the cytokine and is therefore variable within certain

limits. On the other hand, cell proliferation in IL-3 free medium is most likely linked to- and limited by- the expression of the BCR/JAK2 fusion gene and the activity of the corresponding protein. Additionally, the BaF3 cells, an immortalized murine B-cell precursor cell line, are not comparable to normal lymphocytes in terms of proliferation, resistance to apoptosis etc.. Although a comparison of receptor-mediated and autonomous growth can be made *in vitro*, the results should not lead to any conclusions regarding the *in vivo* proliferation of hematopoietic cells expressing a BCR/JAK2 fusion gene *in vivo*.

3.22 Validation of the fusion gene in retrovirally transduced BaF3 cells by PCR

A PCR using the 5' BCR sense primer and 3' JAK2 antisense primer (see 2.1.12) was carried out in order to validate the presence of the fusion gene in the stably transduced BaF3 pMIG-BCR/JAK2 cell line. The characteristic 358 bp amplification product was then visualized by agarose gel electrophoresis, confirming the presence of BCR/JAK2 fusion transcripts in the cells. Figure 14 shows the BCR/JAK2 breakpoint fragment after PCR amplification visualized by agarose gel electrophoresis.

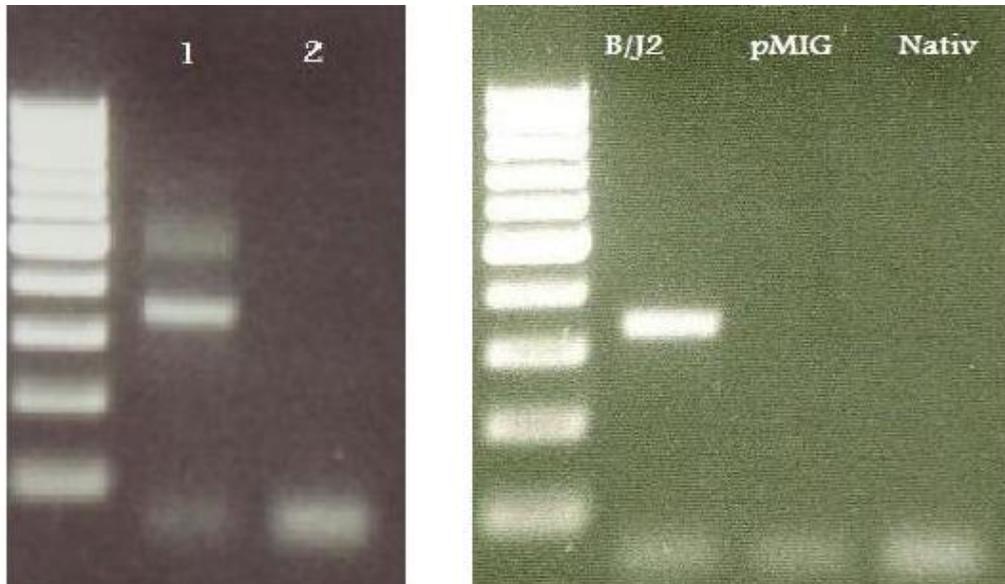


Figure 14:

Left picture: PCR of the cDNA patient sample, visualized by agarose gel electrophoresis. The 358 bp BCR/JAK2 breakpoint fragment (1) can be seen next to the 80-1000 bp ladder, and the PCR control (2).

Right picture: PCR of genomic DNA extracted from BaF3 cells of the second proliferation assay. The breakpoint fragment can be seen in the lane containing a PCR sample from the BaF3 pMIG-BCR/JAK2 cells (B/J2).

3.23 Western blot analysis

Despite several attempts, it was not possible to confirm the presence of the BCR/JAK2 fusion protein by Western blot analysis (see 2.2.4.2). Although the first antibody, a monoclonal JAK2 antibody, targets residues surrounding Pro841 of the JAK2 protein (see Figure 1), a section of JAK2 which should be present in the BCR/JAK2 fusion, neither the normal JAK2 protein, nor the mutated BCR/JAK2 fusion protein could be detected. Most likely, successful detection of these two proteins would have been a matter of time and refinement of the Western blotting technique. However, towards the end of the project, time was running short for various reasons and work on the Western blot analysis was discontinued. It is worth to mention that another group conducting similar experiments involving a retroviral expression model of this particular BCR/JAK2 variant in the BaF3 cell line was able to show Western blots of both the normal JAK2 protein of ~130 kDa, as well as the BCR/JAK2 fusion protein of ~90 kDa (Cuesta-Domínguez et al., 2012).

4 Discussion

4.1 The BCR/JAK2 fusion gene

4.11 Functional characterisation

According to the current model, the BCR/JAK2 fusion gene combines two key features of the *BCR* and the *JAK2* genes: The first one is the N-terminal coiled-coil dimerisation domain of BCR, an $\alpha 2$ helix superstructure capable of forming an antiparallel coiled-coil with the corresponding domain of another BCR protein, which is the basis of the formation of dimers and tetramers (Zhao et al., 2002). The second one, the JH1 domain of JAK2, has two lobes - an N-terminal lobe, containing a twisted β -sheet and an α -helix superstructure, and a C-terminal lobe containing several α -helices - in between of which the catalytic activity of the tyrosine kinase is thought to be located (Lindauer et al., 2001). Since it is believed that receptor-bound JAKs usually activate each other by cross-phosphorylation after dimerisation of cytokine receptors (Wagner C, Müller O, 2010), it is very likely that this mechanism also operates in the BCR/JAK2 fusion protein after di- or tetramerisation mediated by the coiled-coil dimerisation domain of the BCR portion.

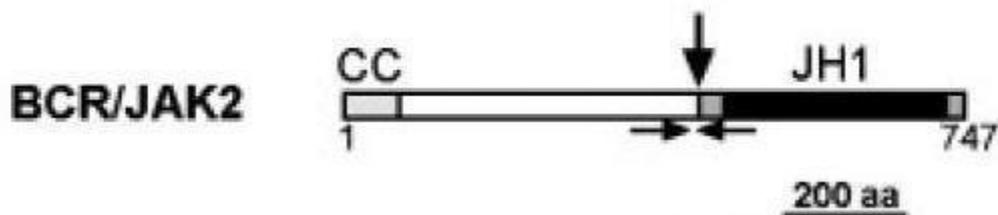


Figure 15: Diagram of the BCR/JAK2 (BCR exon 1, JAK2 exon 19) fusion protein according to Griesinger et al., indicating the positions of the N-terminal coiled-coil dimerisation domain of BCR (CC), and the JH1 tyrosine kinase domain of JAK2 (JH1) (Griesinger et al., 2005).

Presumably, the downstream pathways activated by the fusion protein are very similar to those activated by wild type JAK2, phosphorylating STAT1, 3, 4 and 5 proteins which, once activated, act as transcription factors for a number of genes (see table 1). Of these transcription factors, only STAT5 is activated solely by JAK2, and it is therefore a likely target of the BCR/JAK2 kinase. While activated STAT5 monomers can shuttle between nucleus and cytoplasm, STAT5 dimers are actively transported to and retained inside the nucleus (Basham et al., 2008). Interestingly, a mutant, constitutively activated STAT5 protein alone was shown to induce growth factor-independent proliferation in Ba/F3

cells *in vitro* (Nosaka et al., 1999). The transcriptional targets of STAT5 include Pim-1 (Proto-oncogene serine/threonine-protein kinase) and Bcl-xl (B-cell lymphoma-extra large), which may both have tumorigenic potential. The Pim-1 serine/threonine kinase, which is overexpressed in hematopoietic malignancies and prostate cancer, is able to phosphorylate several proteins involved in cell cycle progression and apoptosis (Bachmann et al., 2005). Bcl-xl, which is a member of the Bcl-2 family, prevents the activation of downstream effector caspases by blocking the release of cytochrome C from mitochondria, thereby providing some resistance to apoptosis (Shimizu et al., 2000, Tsujimoto et al., 2000). Considering these findings, our working theory for the cell culture experiments can be summarized as follows: The BCR coiled-coil domain mediated dimerization of the BCR/JAK2 fusion protein results in constitutive activation of the JAK2/STAT pathway, leading to enhanced cell proliferation and survival – in this case, the IL-3 independent survival of BaF3 cells.

It is worth to note that our results correspond well with the data published by Cuesta-Domínguez et al. (Cuesta-Domínguez et al., 2012). Here, BaF3 cells were transduced with a similar BCR/JAK2 variant derived from a 58-year-old male patient diagnosed with ALL. The proliferation assays performed by this group revealed a significant advantage for the transduced cell line over the native BaF3 cell line in the absence of IL-3. Elevated levels of phosphorylated STAT5 were reported in native BaF3 cells in the presence of IL-3, as well as in BCR/JAK2 transduced BaF3 cells in the absence of IL-3, supporting the assumption that STAT5 is an important substrate of the BCR/JAK2 tyrosine kinase. Accordingly, qPCR of the Bcl-xl gene expression revealed an increased expression of Bcl-xl by a factor of two to three. However, the results of the proliferation assays presented in this doctoral thesis and by Cuesta-Domínguez et al. differ slightly in one aspect – while BaF3 cells carrying the fusion gene seem to exhibit a very similar proliferation rate with- and without 10ng/μl IL-3 in the data shown by Cuesta-Domínguez et al., our assays suggest a significant advantage of cytokine-dependent BaF3 proliferation over the cytokine-independent proliferation mediated by the BCR/JAK2 fusion protein (see 3.11).

Going back further, in 1999, Liu et al. published data from proliferation assays showing proliferation and increased survivability in BaF3 cells, transduced with a chimeric JAK2 construct, in the absence of murine IL-3 (Liu et al., 1999). A chimeric, constitutively active (β_c /JAK2) molecule combining the β_c subunit of the GM-CSF receptor with the JAK2 tyrosine kinase, was utilized to induce cytokine-independent BaF3 proliferation very similar to the data presented by Cuesta-Domínguez et al., as well as in this doctoral thesis. This β_c subunit, which is also present in the receptors for IL-3 and IL-5, is capable of dimerisation, much like the coiled-coil dimerisation domain of BCR. Additionally, Liu et al. reported that the level of proliferation of transduced BaF3 cells without IL-3 was less than the level of proliferation observed in the presence of up to 10ng/ml IL-3.

Of course, the induction of factor-independent proliferation of BaF3 cells cannot be interpreted as direct proof of the tumorigenic properties of the BCR/JAK2 fusion gene. Firstly, the immortalized BaF3 murine pro B cell line is already a tumour cell line, which is often used as a model for- but does not completely match- the biology of normal murine lymphocytes. Secondly, *in vitro* models like the one used in this doctoral thesis are, per se, flawed by the absence of a cellular environment similar to a living body, in which a disease involving this gene would develop. This environment provides a multitude of cytokines and chemokines as well as the host's immune system trying to eliminate developing tumours. An experimental animal model for lymphatic tumours, which takes these factors into account, would – for example - have to include the transduction of murine bone marrow cells with a retroviral vector carrying BCR/JAK2, which would then be transplanted back into recipient mice. This way, it would be possible to observe the development of a malignant haematological disease in mice, assuming that the isolated presence of a BCR/JAK2 fusion gene would be sufficient to drive tumorigenesis. Despite solving many problems, such a model would still rely on a retroviral expression construct like pMIG, which features a constitutively active viral promoter instead of the physiological promoter of BCR, which would normally regulate the expression of a BCR/JAK2 fusion gene originating from a t(9;22)(p24;q11.2). It is possible that besides the BCR/JAK2 fusion, additional mutations need to be acquired prior to the onset of a full-blown malignant disease. However, this crucial question cannot be answered based on the data presented in this doctoral thesis.

4.2 Clinical aspects

4.21 Possible implications for diagnostics

It is highly interesting that the BCR/JAK2 fusion has been reported in conjunction with a broad spectrum of haematological diseases involving the myeloid, as well as the lymphoid lineage. These include CML, AML, ALL and an unclassifiable myeloproliferative neoplasm/MPN (see 1.24 and Table 4). The occurrence of BCR/JAK2 in both CML like malignancies and lymphoid acute leukaemia is strikingly similar to the BCR/ABL translocation which can also be found both in CML and ALL and sometimes AML (Kurzrock et al., 1987). As mentioned before, activating mutations of *JAK2* can induce other malignancies, such as the ETV6/*JAK2* fusion in ALL or the *JAK2*V617F mutation in Polycythaemia Vera (see 1.25). Although only a limited number of case reports describing the BCR/*JAK2* fusion gene in haematopoietic diseases have been published to date (A total of nine cases have been published as of 03/2015), the actual incidence might be higher than the rarity of publications suggests. It is unlikely that a t(9;22)(p24;q11.2), which results in the BCR/*JAK2* fusion,

can be mistaken for the well known t(9;22)(q34;q11.2) translocation in a CML like disease. However, in cases with a lymphoid phenotype a t(9;22)(p24;q11.2) could be hiding in a complex karyotype and be overlooked in some instances. FISH analysis with BCR and ABL1 probes, which is frequently performed in such cases, would show three signals for BCR, but only two signals for ABL1, indicating intact ABL alleles and therefore suggesting another fusion involving BCR.

Since the t(9;22)(p24;q11.2) does not lead to the formation of a BCR/ABL1 fusion protein, the disease will not respond to Imatinib or similar TKIs targeting ABL, leading to the administration of ineffective medication if diagnostics are limited to conventional cytogenetics or the wrong conclusions are drawn. In the case reports mentioned in 1.24, the atypical translocation was usually found by PCR, using different pairs of BCR forward and JAK2 reverse primers, and sequencing. During the experimental work for this publication, PCR amplification of a 358 bp BCR/JAK2 breakpoint fragment, as initially described by Griesinger et al., was used as a fast and reliable method to confirm the presence of the BCR/JAK2 fusion gene (see 2.2.2.9).

For clinicians and diagnosticians, awareness of the possibility of rare genomic alterations may be the key to detecting cases of uncommon translocations in haematopoietic diseases. In some cases, the perceived presence of the Philadelphia chromosome led to the administration of ABL kinase inhibitors over an extended period of time in BCR/ABL negative diseases (Griesinger et al., 2005; Impera et al., 2011; Bellesso et al., 2013). Since the BCR/JAK2 fusion is easily detectable by PCR when actively searched for, the appropriate testing should be performed to confirm its presence in the rather uncommon case of a Ph+, but ABL1 kinase inhibitor-resistant disease.

4.22 Possible clinical applications in therapy

Since JAK2-specific tyrosine kinase inhibitors have been approved by the US Food and Drug Administration for the treatment of intermediate and high-risk myelofibrosis, primary myelofibrosis, post-polycythaemia vera myelofibrosis and post-essential thrombocythaemia myelofibrosis, the use of these JAK1/JAK2 inhibitor in the case of a BCR/JAK2 positive disease is currently limited to clinical trials or off-label use (Mesa et al., 2012). Recently, a publication by Schwaab et al. described clinical data of a patient diagnosed with a BCR/JAK2 positive myeloproliferative neoplasm (MPN) treated with Ruxolitinib (Schwaab et al., 2014). TKI treatment was initially effective, achieving a complete haematologic response after 6 months and a complete cytogenetic response after 12 months, although the fusion gene was still detectable by PCR analysis. The patient suffered a relapse 18 months after the start of Ruxolitinib treatment and ultimately underwent allogeneic stem cell

transplantation. Nevertheless, Schwaab et al. could demonstrate clinical effectiveness of a JAK1/JAK2 inhibitor in a patient suffering from a haematologic disease with a BCR/JAK2 fusion gene.

Besides Ruxolitinib, several selective inhibitors of JAK2, like NS-018, BMS-911543, LY2784544, SB1518 and SAR302503/TG101348 are currently undergoing pre-clinical testing or clinical trials (Younes et al., 2012; Purandare et al., 2012; Ma et al., 2013; Zhang et al., 2014; Nakaya et al., 2014). Such developments will likely lead to the introduction of new, more selective JAK2 inhibitors, and the clinical use of these substances in the broad spectrum of JAK2 driven haematological malignancies. These trials will hopefully provide the data needed to determine which JAK2 inhibitors are appropriate for patients suffering from diseases driven by a BCR/JAK2 fusion gene.

5 Conclusion

Research in the field of cancer biology has rapidly accelerated over the past twenty years, revealing new details about the genetics and molecular pathways associated with a multitude of malignancies. Few things might underline the importance of basic research more impressively than the development of new drugs for the treatment of cancer, such as the advent of targeted therapies as exemplified by the development of the ABL tyrosine kinase inhibitor Imatinib for the treatment of BCR/ABL-positive malignancies, especially CML. This development led to a significant improvement of the prognosis of patients diagnosed with BCR/ABL positive leukaemia (Drucker et al., 1996, Drucker et al., 2006). The discovery and characterisation of chromosomal translocations and the resulting tumorigenic mutations, as the description of the BCR/JAK2 fusion gene, contributes to the development of more specific, individualized therapies.

The approach of this doctoral thesis was to establish a stable *in vitro* model and to demonstrate the pro-proliferative effects of the BCR/JAK2 fusion. This aim was successfully achieved as the fusion gene triggered strong, growth factor independent proliferation in BaF3 cells after retroviral transduction, which differed significantly from the cell line's growth factor-dependent proliferation. Although additional experiments, like the further characterisation of downstream pathways, or the development of a BCR/JAK2 bone marrow transplantation model were not possible, mostly due to logistical limitations and time restriction, the work will hopefully contribute to the ongoing effort to better understand this particular fusion gene, i.e. to provide accurate data regarding the proliferation of transduced BaF3 cells, to validate results published by other groups and to draw attention towards the occurrence of the BCR/JAK2 fusion in haematological diseases.

As a growing number of case reports with the BCR/JAK2 fusion gene have been published in recent years, and the first clinical attempt to administer an inhibitor of JAK2 to a patient diagnosed with BCR/JAK2 positive MPN has been made, more reports on this subject are likely to follow. The data collected by clinicians will help to improve treatment for these patients, and the continued development of targeted therapies, such as novel tyrosine kinase inhibitors, will likely contribute to this trend. Although basic as well as clinical research is often perceived to be rather slow and difficult, continuous advances in the field of malignant diseases have significantly improved the overall prognosis of cancer patients over the course of only a few decades.

6 Zusammenfassung

Auf dem Gebiet der Tumorbilogie wurden im Verlauf der letzten zwei Jahrzehnte bedeutende Fortschritte gemacht, die zum Verständnis der molekularen Signalwege und genetischen Grundlagen einer Vielzahl von Tumorerkrankungen beitragen. Dabei gibt es kaum bessere Beispiele für die Wichtigkeit medizinischer Grundlagenforschung als die Entwicklung von Pharmaka zur Krebstherapie, welche gegen molekulare Zielstrukturen der Tumorzelle gerichtet sind. So hat die Entwicklung von Imatinib, einem Inhibitor der ABL-Tyrosinkinase zur Behandlung BCR/ABL-positiver Leukämien wie der CML, zu einer deutlichen Verbesserung der Prognose der betroffenen Patienten geführt (Drucker et al., 1996, Drucker et al., 2006). Dementsprechend trägt die Identifikation und Charakterisierung chromosomaler Translokationen und der daraus resultierender Mutationen mit onkogenem Potential, wie die Beschreibung des BCR/JAK2 Fusionsgens, entscheidend zur Entwicklung neuer, spezifischerer Therapiestrategien für diese Patienten bei.

Ziel der experimentellen Arbeit zur Dissertation war die Klonierung des BCR/JAK2 Fusionsgens und die Etablierung eines stabilen *in vitro*-Modells zur Demonstration eines pro-proliferativen Effekts. Dieses Ziel wurde erreicht - wobei letztendlich sowohl die Zeit, als auch die nötigen Ressourcen für weitere Experimente wie der Untersuchung der assoziierten Signalwege, oder der Etablierung eines Tiermodells für BCR/JAK2-getriebene Leukämien, fehlten. Dennoch konnte gezeigt werden, dass das mittels eines retroviralen Vectors in BaF3 Zellen eingebrachte BCR/JAK2 Fusionsgen zu einer Wachstumsfaktor-unabhängigen Proliferation dieser Zellen führt, welche sich vom Wachstumsfaktor-abhängigen Proliferationsverhalten signifikant unterscheidet. Durch die Veröffentlichung von Referenzdaten zum Proliferationsverhalten mit BCR/JAK2 transduzierter lymphatischer Zellen sowie der Validation der Ergebnisse anderer Gruppen, soll die hier präsentierte Arbeit zum Verständnis dieses speziellen Fusionsgens beitragen und zudem auf eine seltene, aber hochinteressante chromosomale Translokation mit therapierelevanten Konsequenzen hinweisen.

Da in letzter Zeit eine kleine, aber wachsende Zahl an Berichten BCR/JAK2 positiver hämatologischer Erkrankungen, sowie der erste klinische Therapieversuch mit einem JAK2-spezifischen Tyrosinkinaseinhibitor veröffentlicht wurde, ist es wahrscheinlich, dass in den nächsten Jahren weitere Veröffentlichungen zu diesem Thema folgen werden. Die darin enthaltenen klinischen und experimentellen Daten werden, in Verbindung mit der Entwicklung neuerer Tyrosinkinaseinhibitoren, dazu beitragen die Behandlung dieser Patienten zu verbessern. Obwohl Fortschritte im Bereich der Tumor- und molekularbiologischen Grundlagenforschung langsam und umständlich erscheinen können, haben stetige Fortschritte in diesen Bereichen die Prognose von Krebspatienten innerhalb weniger Jahrzehnte dramatisch verbessert.

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Appendix

Appendix I: Sequence of the BCR/JAK2 fusion in pcDNA3

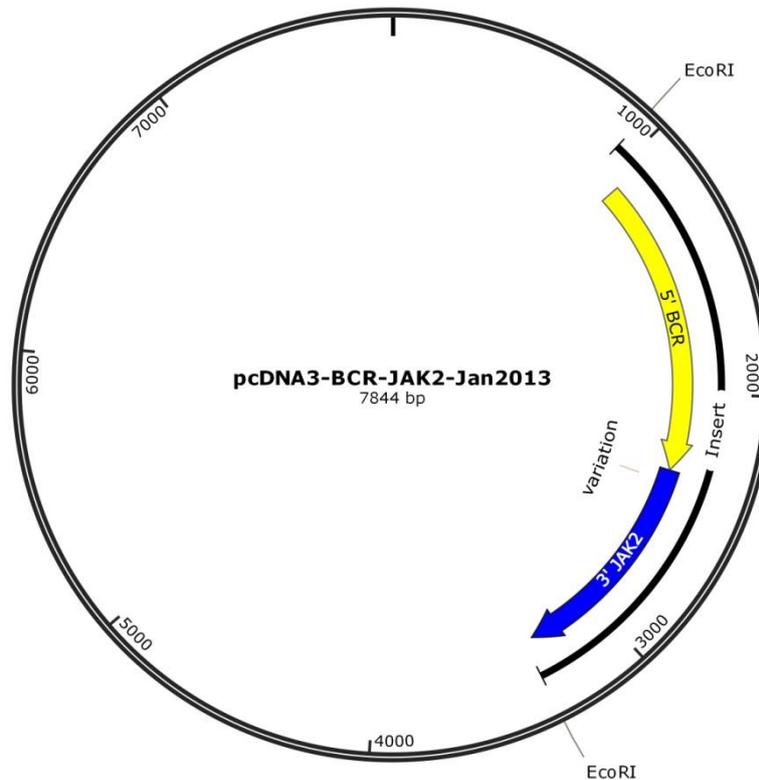


Figure 16: Vector map of the BCR/JAK2 fusion gene within the pcDNA3 plasmid. The 5' BCR and the 3' JAK2 sections of the 2241 BP fusion gene are highlighted in yellow and blue, respectively. A single nucleotide polymorphism is highlighted in red colour.

GACGGATCGGGAGATCTCCGATCCCCTATGGTCGACTCTCAGTACAATCTGCTCTGATGCCGCATAGTTAAGCCAGTATCTGCTCCCTGCTTGTGTGTTGG
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 GCTTCGCGATGTACGGGCCAGATATACGCGTTGACATTGATTATTGACTAGTTATTAATAGTAATCAATTACGGGGTCATTAGTTTCATAGCCCATATATGGA
 GTTCCGCGTTACATAACTTACGGTAAATGGCCCGCCTGGCTGACCGCCCAACGACCCCCGCCATTGACGTCAATAATGACGTATGTTCCCATAGTAACGCC
 AATAGGGACTTTCCATTGACGTCAATGGGTGGACTATTTACGGTAAACTGCCCACTTGGCAGTACATCAAGTGTATCATATGCCAAGTACGCCCCCTATTGA
 CGTCAATGACGGTAAATGGCCCGCCTGGCATTATGCCAGTACATGACCTTATGGGACTTTCCTACTTGGCAGTACATCTACGTATTAGTCATCGCTATTACC
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 CGGGCCCCGCGCGCAGCCGGCGCGCAGGTAAGGCCGGCCGCGCC**ATGGTGGACCCGGTGGGCTTCGCGGAGGCCTGGAAGG**
CGCAGTTCGCGACTCAGAGCCCCGCGCATGGAGCTGCGCTCAGTGGGCGACATCGAGCAGGAGCTGGAGC
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CGTGTTCATTTGATAGAATTTTGAAGAATAATGGAAGATTACCAAGACCAGATGGATGCCAGATGAGATC
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Appendix II: List of GMOs

Organism	Plasmid	Produced	Discarded
<i>E. coli</i> DH5α	pcDNA3 BCR-ABL	24.04.2012	31.07.2012
<i>E. coli</i> DH5α	pcDNA3 ETV6-JAK2	24.04.2012	31.07.2012
<i>E. coli</i> DH5α	pcr2.1 Vector	11.06.2012	14.09.2012
<i>E. coli</i> DH5α	pcr2.1 (minus BamH1 cutting site)	02.08.2012	14.09.2012
<i>E. coli</i> DH5α	pcr2.1 BCR-JAK2 breakpoint fragment	07.09.2012	17.12.2012
<i>E. coli</i> DH5α	pcDNA3 BCR-ABL (minus 5' BsmB1)	06.11.2012	08.01.2013
XL 10-Gold ultracompetent cells	pcr2.1 BCR-JAK2 BCR(fragment)-JAK2(complete)	06.11.2012	08.01.2013
XL 10-Gold ultracompetent cells	pcDNA3(mod.) BCR-JAK2	21.12.2012	01.02.2013
<i>E. coli</i> DH5α	pMIG Vector	23.01.2013	04.06.2013
<i>E. coli</i> DH5α	pMIG BCR-JAK2	31.01.2013	04.06.2013
Ba/F3 murine lymphocyte cell line	pMIG Vector	13.06.2013	-
Ba/F3 murine lymphocyte cell line	pMIG BCR-JAK2	13.06.2013	-

Table 5: List of genetically modified organisms (GMOs). All prokaryotic organisms which were created during the project have been discarded. The eukaryotic cell lines modified by retroviral transduction are stored at -80°C in the laboratory of the Institute for Human Genetics of the Philipps-University Marburg in Marburg.

Appendix III: Abbreviations

aa	Amino acids
ab	Antibody
ABL1	Abelson murine leukemia viral oncogene homolog 1
ALL	Acute lymphoblastic leukemia
AML	Acute myeloblastic leukemia
Ara-C	Cytosine arabinoside
ARG	Abl-related gene kinase
ATP	Adenosine triphosphate
B-ALL	B-cell acute lymphoblastic leukemia
Bcl-xl	B-cell lymphoma-extra large protein
BCR	Breakpoint cluster region protein
bp	Base pairs
CC (protein domain)	Coiled-coil (dimerisation) domain
CCND2	Cyclin D2
CDK	Cyclin-dependent kinase
cDNA	Complementary DNA
CLL	Chronic lymphocytic leukemia
CML	Chronic myelogenous leukemia
DNA	Deoxyribonucleic acid
EGF	Epidermal growth factor
EPO	Erythropoietin
erbB2	Avian erythroblastosis oncogene B (see HER2)
ET	Essential thrombocythemia
ETV6	<i>Ets</i> variant gene 6
FISH	Fluorescent In situ hybridization

GFP	Green fluorescent protein
GM-CSF	Granulocyte-macrophage colony-stimulating factor
Hb	Haemoglobin
HER2	Human epidermal growth factor receptor 2 (see erbB2)
i.e	Id est (Lat.: "That is")
IFN $\alpha/\beta/\gamma$	Interferon $\alpha/\beta/\gamma$
IL	Interleukin
JAK	Janus kinase
JH1 (protein domain)	Janus homology 1 domain
kb	Kilobases
kD	Kilodalton
Kit	Mast/stem cell growth factor receptor (SCFR)/ tyrosine-protein kinase Kit
LAP	Leukocyte alkaline phosphatase
LDH	Lactate dehydrogenase
MD	Myeloproliferative disease
MDS	Myelodysplastic syndrome
MPN	Myeloproliferative neoplasm
NF-E2	Erythroid cell-specific nuclear factor 2
Pax-5	Paired box protein 5
PCM1	Pericentriolar material 1 protein
PCR	Polymerase chain reaction
PDGFR	Platelet-derived growth factor receptor
Ph+/-	Philadelphia chromosome positive/negative
Pim-1	Proto-oncogene serine/threonine-protein kinase 1
PMF	Primary myelofibrosis
PV	Polycythemia vera
RKI	Robert Koch Institute
RT-PCR	Reverse transcription polymerase chain reaction
RPN1	Dolichyl-diphosphooligosaccharide-protein glycosyltransferase subunit 1
RUNX1	Runt-related transcription factor 1
SSBP2	Single-stranded DNA-binding protein 2
STAT	Signal Transducer and Activator of Transcription
T-ALL	T-cell acute lymphoblastic leukemia
TKI	Tyrosine kinase inhibitor
VCM	Viral conditioned medium

Meine akademischen Lehrer waren Damen/Herren in Marburg:

Adamkiewicz, Bartsch, Basler, Baum, Becker, Berger, Bette, Bien, Bolm, Brehm, Cetin, Czubayko, Daut, Dodel, Benes, Donner-Banzhoff, Efe, Ellenrieder, Engenhardt-Cabillic, Fendrich, Feuser, Fuchs-Winkelmann, Graz, Gress, Grundmann, Grzeschik, Hertl, Heverhagen, Hilt, Hofer, Höffken, Hofmann, Höglinger, Hoyer, Hundt, Jerrentrup, Kann, Kill, Kircher, Klose, Knipper, Koch, König, Konrad, Koolman, Krones, Kruse, Lill, Lingenfelder, Löffler, Lohoff, Lüers, Maier, Maisch, Moll, Möller, Mueller, Müller, Mutters, Neubauer, Neumüller, Nimsky, Oertel, Oliver, Pagenstecher, Pressel, Richter, Riera-Knorrenschild, Riße, Roelcke, Rohlf, Röhm, Ruchholtz, Schäfer, Schmidt, Schneider, Schofer, Schu, Schulze, Schütz, Schwarting, Seifart, Seitz, Sekundo, Steininger, Tackenberg, Thum, Toussaint, Vogelmeier, Vogt, Wagner, Weihe, Werner, Westermann, Wiegand, Wulf.

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