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Molecular signature and functional analysis of leukemic and normal CD34+ hematopoietic stem and progenitor cells

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Molecular signature and functional analysis of leukemic and normal CD34+ hematopoietic stem and progenitor cells

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Summary

Hematopoiesis physiologically depends on a precisely regulated equipoise of self-renewal, differentiation, and migration of more or less mature hematopoietic stem and progenitor cells. Understanding these key features provides the basis for different clinical and scientific applications of these cells.

The knowledge gained from stem cell biology can also give novel insights into cancer research. The best-studied malignant stem cell disorder is the chronic myelogenous leukemia (CML) which is characterized by the specific t(9;22) chromosomal translocation resulting in the expression of the BCR-ABL fusion oncogene and by a clonal expansion of hematopoietic progenitor cells. In this work we provide a molecular signature of highly enriched CD34+ hematopoietic stem and progenitor cells from bone marrow of untreated patients with CML in chronic phase in comparison with normal CD34+ cells using microarrays covering 8746 genes. Expression data reflected several BCR-ABL-induced effects in primary CML progenitors such as transcriptional activation of the classical MAPK pathway and the PI3 kinase/AKT pathway as well as down-regulation of the pro-apoptotic gene IRF8. Additionally, genes associated with early hematopoietic stem cells (HSC) and leukemogenesis such as HoxA9 and MEIS1 were transcriptionally activated. Moreover, several novel transcriptional changes in comparison with normal CD34+ cells, which were not described so far in CML and which might be of therapeutic relevance, were identified. These include an up-regulation of components of the TGFβ signaling pathway, fetal hemoglobin genes, sorcin, TIMP1, the neuroepithelial cell transforming gene 1 and down-regulation of selenoprotein P.

The differential expression of leptin receptor and genes involved in fatty acid synthesis, which were up-regulated comparing malignant CML CD34+ cells with normal CD34+ cells, suggested an important role of the lipid metabolism pathway in the pathophysiology of the disease. The functional analysis of candidate genes revealed that this up-regulation plays an active role in differentiation and proliferation of malignant hematopoietic progenitor cells. Incubation of CD34+ CML cells with

the fatty acid synthesis inhibitor Cerulenin resulted in a dose-dependent decreases in leukemic cell viability, as well as reduced clonogenic growth. Interestingly, comparing this effect with the one in normal CD34+ cells, the inhibition was not as strong as in leukemic cells. This opens novel therapeutic avenues in the treatment of the CML.

Beside the central role in malignant hematopoiesis, an ongoing set of investigations has led to claim that hematopoietic stem cells have the capacity to differentiate into a much wider range of tissues than previously thought possible, and recent interesting studies have shown a potential molecular interrelation of neuronal and hematopoietic signaling mechanisms. A previous report of our group could show the expression of neurobiological genes in human CD34+ hematopoietic stem and progenitor cells. Those include the GABA B receptor which had been primarily assigned to the nervous system. In this work, the functional role of this receptor on CD34+ cells is investigated: We could show that the GABA B receptor is functional active in hematopoietic stem and progenitor cells and influences the intracellular cAMP. Further functional assays could assess that the modulation of the GABA B receptor does not influence the proliferation, differentiation or migration of CD34+ progenitor cells. Our data showed a loss of expression of this receptor during differentiation of hematopoietic stem cells and suggests a functional role only in early hematopoiesis. This is different in neuronal stem cells, where the GABA B receptor has been described to play a central role in differentiation.

To summarize, in this work, we provide a molecular and functional study of malignant and normal CD34+ cells giving novel insights into the biology of hematopoietic stem and progenitor cells.

Part I.

Introduction

1. Hematopoietic stem and progenitor cells

1.1. Definition and general concepts about stem cells

A stem cell is a special kind of cell that has the unique capacity to reproduce itself (*self-renewal*) and to give rise to specialized cell types (*multipotency*) [1].

Multidifferentiative potential

Conventionally, two classes of stem cells have been defined, depending on their multidifferentiative capacity. On the one hand, the so-called *pluripotent stem cells* have the ability to give rise to types of cells that develop from the three germ layers (mesoderm, endoderm and ectoderm) from which all the cells of the body arise. The only known sources of human pluripotent stem cells are those isolated and cultured from early human embryos and from fetal tissue [2]. The other type, *adult stem cells* are undifferentiated (unspecialized) cells that occur in a differentiated (specialized) tissue, renew themselves, and become specialized to yield all of the specialized cell types of the tissue from which they originated. For example, adult hematopoietic stem cells, with their distinctive capabilities of self-renewal and differentiation, furnish a constant supply of blood cells of all hematopoietic lineages throughout life [3].

Self-renewal

To ensure self-renewal, stem cells undergo two types of cell division (Fig. 1.1, page 5). Symmetric division gives rise to two identical daughter cells both endowed with stem cell properties. Asymmetric division, on the other hand, produces only one stem cell and a *progenitor cell* with limited self-renewal potential. Progenitor cells are relatively immature cells that are precursors to a fully differentiated cell of the same tissue type.

They are capable of proliferating, but they have a limited capacity to differentiate into more than one cell type as stem cells do [1].

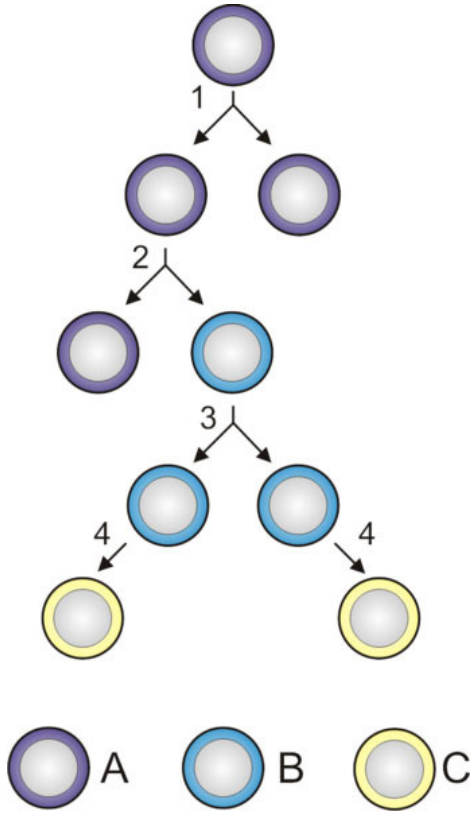


Figure 1.1: Stem cell division and differentiation. A - stem cell; B - progenitor cell; C - differentiated cell; When a stem cell divides, one of the two new cells is a stem cell capable of replicating itself again. In contrast, when a progenitor cell divides, it can form more progenitor cells or specialized cells, neither of which is capable of replicating itself. *Image released under the GNU Free Documentation License.*

1.2. Stem cells and hematopoiesis

A hematopoietic stem cell (HSC) is an adult stem cell isolated from the blood or bone marrow that can renew itself, can differentiate to a variety of specialized cells, can mobilize out of the bone marrow into circulating blood, and can undergo programmed cell death or apoptosis [4]. In adult humans, HSCs occur in the bone marrow, peripheral blood, liver and spleen [3]. The production of blood cells, or *hematopoiesis*, takes place in the bone marrow. A HSC can either replicate and remain a stem cell or differentiate into myeloid or lymphoid stem cells, which in turn can further proliferate and mature, ultimately giving rise to all the circulating blood cells [5] (Fig. 1.2, page 6).

Two kinds of adult stem cells have been defined. *Long-term stem cells*, which proliferate for lifetime, and *short-term progenitor or precursor cells*, which proliferate for a limited time, possible a few months [6].

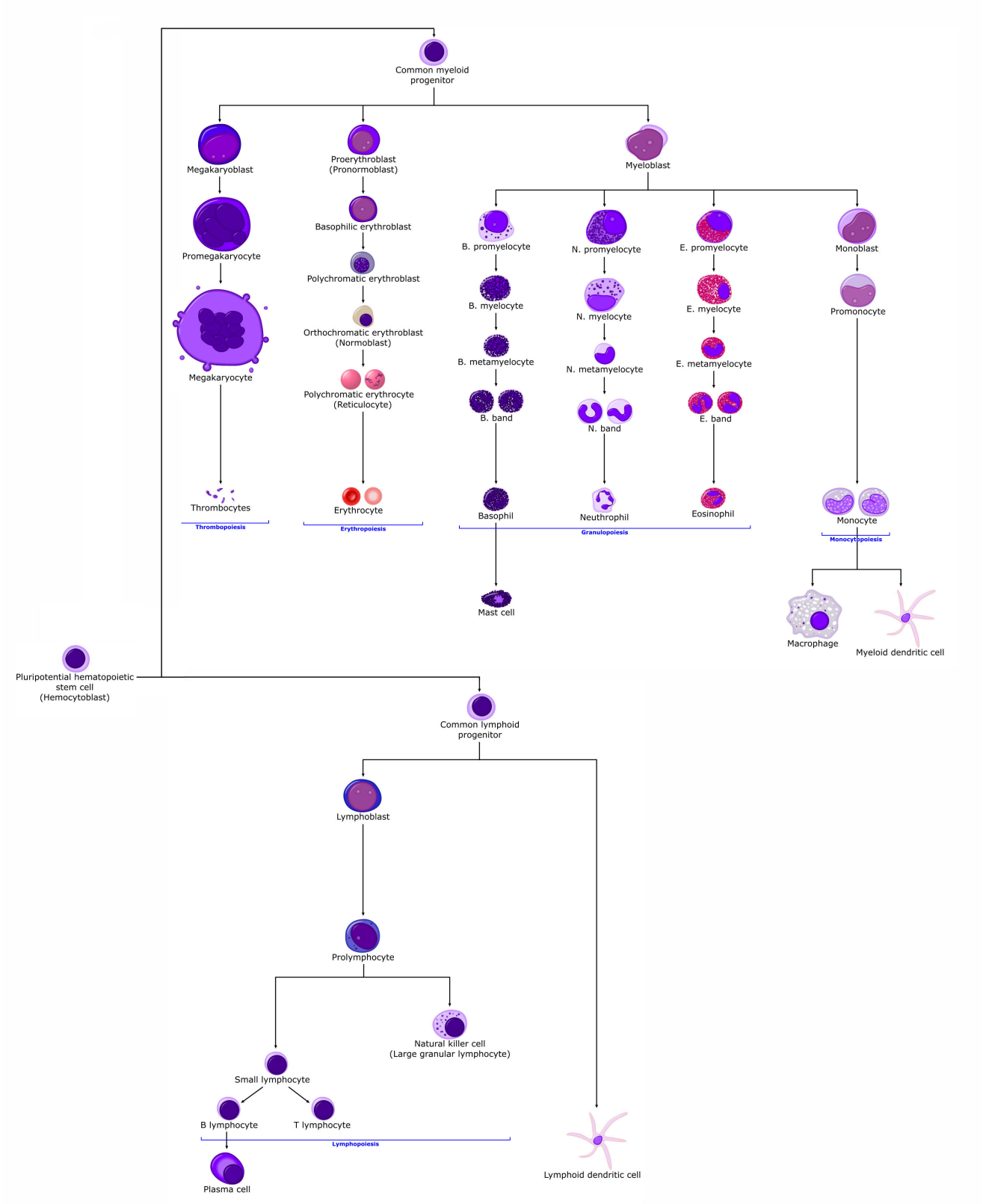


Figure 1.2: Hematopoiesis. A hematopoietic stem cell can replicate and differentiate into myeloid or lymphoid progenitor cells, which in turn can further proliferate and mature, ultimately giving rise to all the circulating blood cells. *Image released under the GFDL-self license.*

Long-term stem cells are capable of self- renewal. These cells have high levels of telomerase activity. Telomerase is an enzyme that helps to maintain the length of the ends of chromosomes, called telomeres, by adding on nucleotides. Active telomerase is a characteristic of undifferentiated, dividing cells and cancer cells. Differentiated, human somatic cells do not show telomerase activity [7]. Despite telomerase activity, the telomeres of HSCs shorten physiologically during ontogenesis as well as on replicative stress. Thus, HSCs age moleculary, resulting in diminished proliferation potential with the time [8].

Short-term hematopoietic stem cells differentiate into lymphoid and myeloid precursors. They are called common lymphoid progenitor cells (CLP) and common myeloid progenitor cells (CMP), the two classes of progenitors for the two major lineages of blood cells. Lymphoid precursors differentiate into T cells, B cells, and natural killer cells. The mechanisms and pathways that lead to their differentiation are still being investigated [9] [10]. Myeloid precursors differentiate into monocytes and macrophages, neutrophils, eosinophils, basophils, megakaryocytes, and erythrocytes [11].

In this way, hematopoietic stem cells are able to maintain a life-long supply of the entire spectrum of blood cells, dependent on the varying systemic demands of the individual.

1.3. Methods of characterization of hematopoietic stem cells

1.3.1. Immunological characterization: CD34 as a stem cell marker

The expression of surface molecules is one of the methods which have been used to characterize hematopoietic stem and progenitor cells. A widely used phenotypic marker of hematopoietic stem cells is the CD34 antigen [12], a highly glycosylated transmembrane protein of 115 kDa, which can be phosphorylated by a variety of kinases such as protein kinase C and tyrosine kinases [13]. On average, the CD34 antigen is present on 0.5-3% of cells in human bone marrow (CD34+ cells). This antigen is expressed not only on hematopoietic progenitor and stem cells but also on endothelial cells and some stromal cells, which might suggest a common origin from

a mesenchymal stem cell [14].

The population of hematopoietic CD34+ cells is heterogeneous regarding phenotype and function. Further subset analysis use monoclonal antibodies directed against differentiation- or lineage-specific antigens (collectively referred to as *Lin*) to recognize cell surface proteins present on differentiated hematopoietic lineages, such as the red blood cell and macrophage lineages. This types of analysis can divide the CD34+ cell compartment into primitive hematopoietic stem cells and more mature lineage-committed progenitor cells [15]. The subset of early CD34+ hematopoietic stem cells with high self- renewal and multilineage differentiation capacity is characterized by low, or absent, coexpression of HLA-DR or CD38 without expression of lineage- specific antigens, while the antigenic profile of more differentiated CD34+ hematopoietic progenitors is characterized by the coexpression of CD38 and lineage-specific antigens [16] [17] [18].

1.3.2. Molecular signature: Gene expression analysis

Recent progress in genomic sequencing and genome- wide expression analysis at the RNA and protein levels has greatly accelerated our progress in understanding stem cell physiology. This includes the sequencing of the human and mouse genomes and the development of techniques such as array-based analysis. Several research groups have combined cDNA cloning and sequencing with array-based analysis to begin to define the full transcriptional profile of hematopoietic stem cells from different species and developmental stages and compare these to other stem cells [19] [20] [21] [22] [23] [24]. Many of the data are available in online databases, such as the NIH/NIDDK Stem Cell Genome Anatomy Projects [1].

Numerous studies examined gene expression of human CD34+ cells, in most cases focusing on a limited number of genes that are hematologically interesting. Phillips et al. measured for the first time a gene expression profile of hematopoietic stem cells in mice [21]. However, hematopoiesis of humans considerably differs from hematopoiesis in mice. Our group measured gene expression profiles of primary human CD34+ stem cells derived from steady-state bone marrow (BM) or from peripheral blood (PB) after mobilization with G-CSF (Granulocyte colony-stimulating factor). Comparison of gene expression of BM-CD34+ cells and PB-CD34+ cells molecularly confirmed and explained the model that CD34+ cells residing in the bone mar-

[1] http://www.scgap.org

row are cycling more rapidly, whereas circulating CD34+ cells consist of a higher number of quiescent stem and progenitor cells [25].

In summary, this kind of studies and databases provides a gene expression profile of hematopoietic stem cells, identifying transcriptional changes that play a role in the regulation of self-renewal, differentiation, mobilization, and migration.

1.3.3. Functional assessment of hematopoietic stem cells

Candidate adult stem cells can be isolated, grown *in vitro* and investigated, by performing different functional experiments. Those functional assays permit the investigator to address basic features of the HSCs such a *self-renewal* with long-term culture initiating cell (LTC-IC) assays, *multilineage differentiation* with semisolid colony-forming cells cultures or *migration* with chemotaxis experiments.

1.4. Clinical and biological relevance of hematopoietic stem cell research

Hematopoiesis physiologically depends on a precisely regulated equipoise of self-renewal, differentiation, and migration of more or less mature hematopoietic stem and progenitor cells. The knowledge of these key features provides the basis for different clinical and scientific applications of these cells. HSCs have been used clinically since 1959 and are used increasingly routinely for transplantations. In addition, stem cells are a major area of investigation in cancer research. Even more, an ongoing set of investigations has led to claims that HSCs, as well as other stem cells, have the capacity to differentiate into a much wider range of tissues than previously thought possible.

1.4.1. Stem cells as a therapeutical tool

Currently the main therapeutical use of HSC is the transplantation in either hematopoietic malignancies, or after high-dose cytotoxic chemotherapy for non- hematopoietic malignancies (cancers in other organs). In both cases HSC transplantation permits hematological recovery after myeloablative therapy. Other indications include diseases that involve genetic or acquired bone marrow failure, such as aplastic anemia, thalassemia sickle cell anemia, and increasingly, autoimmune diseases [8].

There are two major types of stem cell transplantation maneuvers. *Autologous HSC transplantation* involves isolation and storage of HSC from the patient. This method allows cytotoxic high-dose therapies for patients with multiple myeloma, non-Hodgkin's lymphoma, breast cancer, lung cancer, or sarcoma [26]. In the *allogeneic HSC transplantation*, the patient's own cancerous hematopoietic cells are destroyed via radiation or chemotherapy, then replaced with a transplant of HSCs collected from a matched donor [26]. A matched donor is typically a sister or brother of the patient who has inherited similar human leukocyte antigens (HLAs) on the surface of their cells [27]. This kind of transplantation not only permits potentially curative treatment of malignant diseases involving hematopoietic stem and progenitor cells, but also bears an immunotherapeutic capacity to control or eradicate residual malignant cells of the recipient. Those therapeutic principles are used in the treatment of acute and chronic myelogeneous leukemia, myelodysplastic syndromes, and aplastic anemia [28] [29].

The most common sources of stem cells for transplantation are the bone marrow or the peripheral blood. In the case of a *bone marrow* transplant, the HSC are removed through a bone marrow puncture while *peripheral blood* stem cells are collected from the blood through a process known as apheresis. HSC from bone marrow have been used over the last three decades, whereas transplantation of blood-derived HSC was performed for the first time in 1986 [30] and has become more and more popular [31] [32] [33] [34].

1.4.2. Molecular biology of stem cells: a way of understanding cancer

Due to their life-long persistence and self-renewal capacity stem cells have a high probability to accumulate mutations that eventually result in malignant transformation. In addition, stem cells and malignant cells share several features such as the ability to self-renew, similar mechanisms involved in migration and mechanisms for prevention of cellular aging such as telomerase expression [35]. Both normal stem cells and tumorigenic cells give rise to phenotypically heterogeneous cells that exhibit various degrees of differentiation. Thus, tumorigenic cells can be thought of as cancer stem cells that undergo an aberrant and poorly regulated process of organogenesis analogous to what normal stem cells do. Knowledge gained from stem cell biology can also give novel insights into cancer research. Recent studies have pro-

posed that conventional therapies may shrink tumours by killing mainly cells with limited proliferative potential. If the putative cancer stem cells are less sensitive to these therapies, then they will remain viable after therapy and re-establish the tumour. By contrast, if therapies can be targeted against cancer stem cells, then they might more effectively kill the cancer stem cells, rendering the tumours unable to maintain themselves or grow [36].

The best-studied malignant stem cell disorder is chronic myelogenous leukemia (CML) which is characterized by a clonal expansion of hematopoietic progenitor cells [37]. Important clinical and molecular aspects of this disease are explained in detail in the second chapter of this work (*Chronic myelogenous leukemia: A stem cell disease*).

1.4.3. Adult stem cell plasticity

Recently, it has been reported that adult stem cells from one tissue appear to be capable of developing into cell types that are characteristic of other tissues. For instance, several pivotal experiments imply that hematopoietic stem cells might be, beyond their hematopoietic potential, able to differentiate into a variety of nonhematopoietic cell types, such as hepatocytes [38], cardiomyocytes [39] [40], endothelial cells [41], and cells of the nervous system [42]. So, a new concept and a new term emerged-*adult stem cell plasticity*. These observations could be the basis for the development of new treatments for patients with myocardial or cerebral infarction as well as degenerative disorders.

However, in interpreting these data it is necessary to be aware of experimental and methodical limitations. Novel studies have challenged the transdifferentiation model by suggesting cell fusion rather than plasticity of stem cells [43] [44] [45].

Still, a better understanding of the molecular framework underlying transdifferentiation and plasticity will be a prerequisite for a purposeful use of the entire therapeutic potential of hematopoietic stem cells. In the last years, experimental results have suggested a possible neuronal regulation of immature hematopoietic progenitors, insinuating a close molecular and ontogenetic propinquity of neuro- and hematopoietic cells [8]. Recent interesting studies showing a potential molecular interrelation of neuronal and hematopoietic signaling mechanisms are further described in the third chapter of this work (*Neuroreceptors in hematopoietic stem and progenitor cells*).

2. Chronic myelogenous leukemia: A stem cell disease

2.1. Definition and epidemiology

Chronic myelogenous leukemia (CML) is a malignant hematological disorder that results in an increase in myeloid cells as well as platelets in the peripheral blood and marked myeloid hyperplasia in the bone marrow [46]. The origin of CML is a malignant hematopoietic stem cell clone which replaces the normal stem cell population and gives rise to a myelocytic series which is the source of abnormal proliferation into both intra- and extramedullary spaces [47].

Following the classification of myeloid neoplasms of the World Health Organization, CML is included in the group of myeloproliferative diseases, besides Polycythemia vera, essential thrombocytosis and myelofibrosis [48].

CML occurs in all age groups, but most commonly in the middle-aged and elderly. Its annual incidence is 1 to 2 per 100,000 people, and slightly more men than women are affected. CML represents about 15-20% of all cases of adult leukemia and 2-3% of childhood leukemias [37].

2.2. Phases and clinical characteristics

The disease is characterized by a biphasic or triphasic clinical course in which a benign chronic phase is followed by transformation into an accelerated and blastic phase [49].

About 50% of patients in the chronic phase remain asymptomatic until the accelerated or blastic stages and the diagnosis is found on a routine laboratory test, presenting incidentally with an abnormally elevated white blood cell count. In this setting, CML must be distinguished from a leukemoid reaction, which can have a similar appearance on a blood smear. In the remaining patients, most present with fatigue,

anorexia and weight loss. Approx. 50% of patients have palpable splenomegaly. Other symptoms of CML may include: malaise, fever, increased susceptibility to infections, anemia, and thrombocytosis or thrombocytopenia [50].

The natural history of the disease is a progression from the chronic phase, that may last 3-5 years, to an accelerated myeloproliferative phase manifested by worsening anemia and poor response to therapy, that may last up to 12 months [51].

Finally, there is a short terminal blastic or acute transformation stage characterized by elevated numbers of blast cells and multiple complications such a sepsis and bleeding. This stage clinically behaves like an acute myelogenous leukemia, with rapid progression and short survival [52].

2.3. Molecular biology

CML was the first malignancy to be linked to a distinctive cytogenetic abnormality. Approx. 95% of patients with CML have a chromosomal translocation known as the Philadelphia chromosome (Ph), which is demonstrable in all hematopoietic precursors and which appears in all bone marrow-derived cells [37].

This is a new mutation arising in patients rather than an inherited abnormality and it is due to a reciprocal translocation between the long arms of chromosome 9 and chromosome 22. A segment of the c-abl gene from chromosome 9q34 is transposed onto the bcr gene on chromosome 22q11, creating a hybrid BCR-ABL oncogene located on the resulting, shorter chromosome 22 [53] (Fig. 2.1).

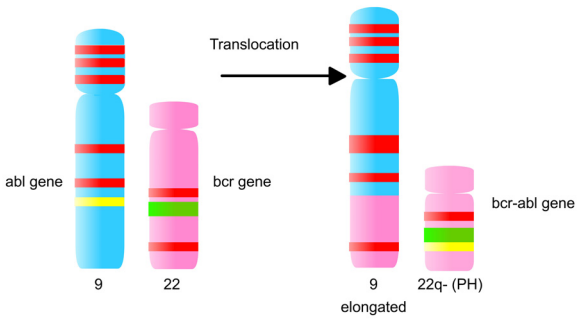


Figure 2.1: Pathophysiology of CML. A chromosomal defect called Philadelphia chromosome is responsible for the pathophysiology of the CML. Parts of two chromosomes, 9 and 22, swap places. Part of the BCR gene from chromosome 22 (region q11) is fused with part of the ABL gene on chromosome 9 (region q34). The result of the translocation is the p210 BCR-ABL protein which induces adhesive and cytoskeletal abnormalities resulting in deregulation of proliferation, apoptosis and release of progenitors from bone marrow.

The result of the translocation is the production of an abnormal protein, the BCR-ABL oncoprotein, directly related to the malignant proliferation of hematopoietic stem and progenitor cells. ABL stands for *Abelson*, the name of a murine leukemia virus which carries a similar protein, and BCR is the *breakpoint cluster region* of the gene on the chromosome 22. Following translocation, the tyrosine kinase activity of the ABL protein is constitutively activated in the BCR-ABL fusion protein, i.e. it does not require activation by other cellular messaging proteins.

Numerous mechanisms are involved in the malignant transformation orchestrated by the BCR-ABL oncoprotein. For one side, BCR-ABL activates a number of cell cycle-controlling proteins and enzymes, speeding up cell division. It constitutively activates mitogenic signaling pathways such as RAS/mitogen-activated protein kinase (MAPK) pathways, the JAK/STAT pathway, phosphoinositide-3 (PI3) kinase pathway and the MYC pathway [54]. In addition, BCR-ABL protects CML cells from apoptosis [55] and induces adhesive and cytoskeletal abnormalities which might facilitate the release of progenitors from the bone marrow[56] [57]. Moreover, it inhibits DNA repair, causing genomic instability and making the cell more susceptible to developing further genetic abnormalities, potentially causing the blast crisis in CML [54].

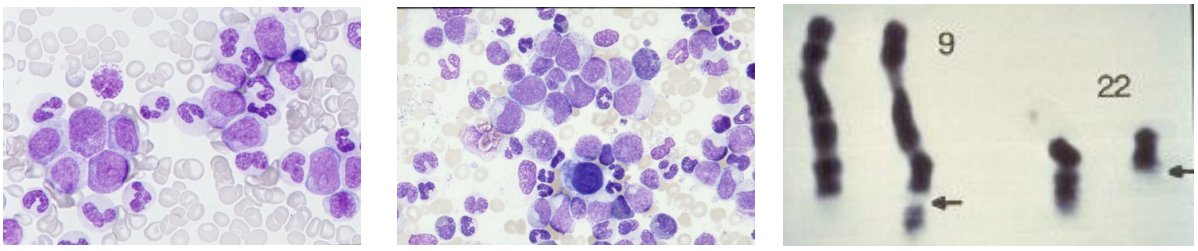
2.4. Diagnosis

CML is often suspected by means of the complete blood count, which shows increased granulocytes of all types, typically including immature myeloid cells (Fig. 2.2 (a), page 15). Since basophils and eosinophils are almost universally increased and the leukocyte alkaline phosphatase is low in CML, these features may help to differentiate CML from a leukemoid reaction. A bone marrow biopsy is often performed as part of the evaluation for CML, but bone marrow morphology alone is insufficient to diagnose CML (Fig. 2.2 (b), page 15).

Ultimately, CML is diagnosed by detecting the Philadelphia chromosome. This characteristic chromosomal abnormality can be detected by routine cytogenetics, by fluorescent in situ hybridization (FISH), or by reverse transcription-polymerase chain reaction (RT-PCR) for the BCR-ABL fusion transcript (Fig. 2.2 (c), page 15).

Controversy exists over so-called Ph-negative CML, or cases of suspected CML in which the Philadelphia chromosome cannot be detected. Many such patients in fact have complex chromosomal abnormalities which mask the (9;22) translocation,

or have evidence of the translocation by FISH or RT-PCR in spite of normal routine karyotyping [50]. The small subset of patients without detectable molecular evidence of bcr-abl fusion may be better classified as having an undifferentiated myelodysplastic/myeloproliferative disorder, as their clinical course tends to be different from patients with CML [48].



(a) Peripheral blood smear (b) Bone marrow aspirate (c) Philadelphia chromosome

Figure 2.2: Diagnosis of chronic phase CML. Peripheral blood smear demonstrates leucocytosis, reflected in a numerical increase in all stages of myeloid differentiation and pronounced basophilia. Bone marrow aspirates shows profound hypercellularity and increased myeloid:erythroid ratio (>25:1). Philadelphia chromosome is present in all myeloid cells.

2.5. Treatment

The treatment of CML depends on the age of the patients and on the clinical evolution of the disease. Conventional chemotherapy with hydroxyurea or busulfan can achieve hematologic control but cannot modify the natural disease course, which inevitably terminates in a rapidly fatal blastic phase. Since its introduction in the 1980s, allogeneic stem cell transplantation has provided the groundwork for a cure of CML. However, not all the patients are eligible for this treatment because of donor availability and age restrictions. Therapy with interferon-α alone or in combination with cytarabine suppresses the leukemic clone, produces cytogenetic remissions, and prolongs survival. It is an effective alternative first-line treatment for patients ineligible for transplantation [49].

With improved understanding of the nature of the BCR-ABL protein, new drugs have been developed and marketed in the last years. They are part of a new class of agents that act by inhibiting particular tyrosine kinase enzymes, instead of nonspecifically inhibiting rapidly dividing cells. Chronic phase of CML can be treated with imatinib mesylate (marketed in 2001 as *Gleevec* in the US or *Glivec* in Europe by

the pharmaceutical company Novartis). Imatinib is a new agent, which specifically targets the constitutively activated tyrosine kinase BCR-ABL. It works by binding to the ATP binding site of BCR-ABL and inhibiting the enzyme activity of the protein competitively [58]. Imatinib has passed through Phase III trials for CML, and has been shown to be more effective than the previous standard treatment of interferon-α, cytarabine and hydroxyurea. Another new drug, dasatinib (marketed as *Sprycel*), which has a similar mechanism of action to imatinib but inhibiting a broader spectrum of tyrosine kinases, was approved in 2006 for use in patients with CML who cannot tolerate the drug or who are no longer responding to therapy with imatinib [59]. Other drugs, as ceflatonin (homoharringtonine) and nilotinib (AMN 107) are currently in active clinical trials in patients with CML who have developed resistance to imatinib [60].

2.6. Current status of gene expression analysis of leukemic cells

Large-scale gene expression analyses of leukemic cells from patients with Ph+ CML have been used to identify a genomic profile associated with response to therapy with the tyrosine kinase inhibitor imatinib [61] and have broadened the knowledge about alterations of gene expression in BCR-ABL positive cells in comparison with normal cells [62] [63]. While Ohmine and coworkers examined CD133+ hematopoietic progenitor cells in the study of Nowicki and coworkers mononuclear CML cells were used. However, mononuclear cells are a heterogenous cell population consisting of a broad spectrum of partially and completely differentiated cell types. Therefore, the expression pattern might only reflect the different proportions of the leukocyte subtypes in normal and CML mononuclear cells [64]. The cell population for studying the pathophysiology of CML are certainly primary hematopoietic stem and progenitor cells from patients with CML.

In order to compare similar cell subsets, to analyse a more homogenous cell population and to examine cells which are closer to the cell of origin in CML, our group examined highly enriched CD34+ hematopoietic stem and progenitor cells by means of cDNA array technology using an array covering 1185 genes, identifing a distinct gene expression pattern of malignant CD34+ cells from patients with untreated newly diagnosed CML [65]. Several recent studies have reported an assessment of primary

CML cells by means of gene expression analyses to identify expression profiles associated with response to therapy [66] [67] [68] [69] or progression to advanced phases of disease [70] [71] but no study exists to date which provides a large-scale expression analysis of chronic phase CML CD34+ stem and progenitor cells in comparison with the normal counterparts.

3. Interrelation of neuronal and hematopoietic signaling in stem and progenitor cells

3.1. Relevant previous studies

Several studies imply that hematopoietic progenitors might be able to transdifferentiate into nonhematopoietic cells, which could open novel therapeutic avenues in the treatment of various degenerative diseases [38] [39] [40] [41] [42].

In line with this theory, studies in animal models showed the presence of sensory and autonomic nerves in the bone marrow as a morphologic correlate of a possible neural regulation of hematopoiesis [72] [73] [74]. In addition, after bone marrow transplantation cells derived from the marrow were shown to migrate into the brain and to express neuronal antigens in mice [42]. Even more, intracerebral transplantation of adult hematopoietic progenitors into neonatal mouse brain resulted in expression of oligodendroglia-specific markers [75], while intracranial transplantation of bone marrow resulted in better functional restoration in rats with traumatic brain injury [76]. However, the idea that neuromediators might directly influence hematopoietic progenitors is controversially discussed [77] [78] [79] [80].

A better molecular understanding of the signal perception pathways of hematopoietic stem and progenitor cells seems to be required to understand the conditions under which transdifferentiation of hematopoietic cells may occur.

3.2. Neuroreceptors in hematopoietic stem and progenitor cells

Recent studies have demonstrated a potential molecular interrelation of neuronal and hematopoietic signaling mechanisms, supporting the model of a possible neuronal regulation of immature hematopoietic progenitors. Several investigators have described partly overlapping genetic programs of hematopoietic and neuropoietic cells in mice [22] [81] and, interestingly, the detection of a potential human neurohematopoietic stem cell population has been reported [82]. Supporting this view, our group examined human hematopoietic cells by means of specialized cDNA arrays, quantitative real-time reversetranscription-polymerase chain reaction (RT-PCR), and fluorescent-activated cell sorter analysis, focusing on gene expression known to be involved in neurobiologic functions. We found that primary human CD34+ cells express numerous genes that are primarily assigned to the nervous system, among them G protein-coupled receptors of neuromediators such as the GABA B receptor, receptor tyrosine/serine kinases, receptor tyrosine phosphatases, ligand- and voltage-gated ion channels, as well as genes involved in vesicle fusion, motility, and adhesion [83] (Fig. 3.1).

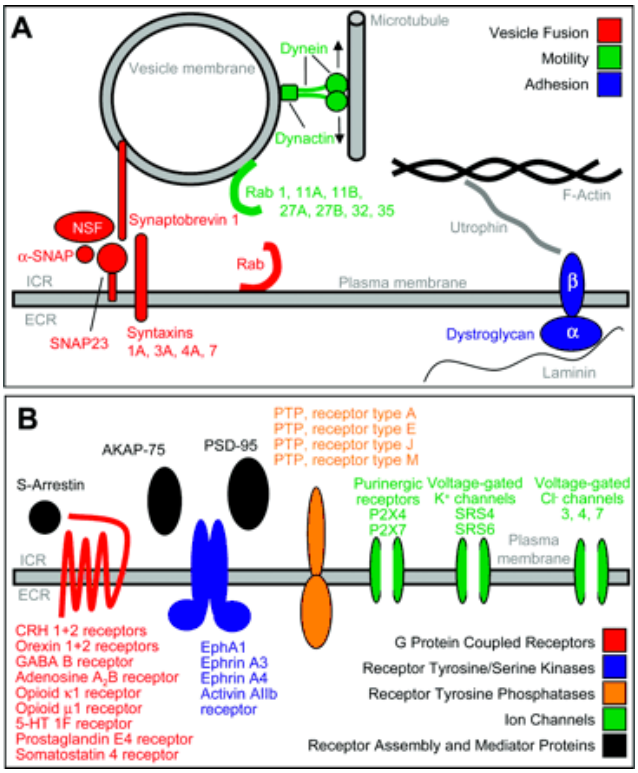


Figure 3.1: Schematic display of neurobiologic genes expressed in primary human CD34+ hematopoietic stem and progenitor cells (Steidl et al., 2004). Intracellular rooms (ICR) and extracellular rooms (ECR) are indicated. (A) Motility and cytoskeletal genes. (B) Receptors and ion channels.

Focusing on the expression of G protein-coupled receptors for neuromediators on malignant hematopoietic stem and progenitor cells, we found that the GABA B receptor was upregulated in CD34+ cells from patients with CML in chronic phase in comparison to normal CD34+ cells as shown by flow cytometry on single cells [65].

The functional role of the newly detected GABA B and other G protein-coupled receptors in human hematopoietic stem and progenitor cells needs to be investigated.

In the following, some basic concepts about G protein-coupled receptors and more concretely, about the GABA B receptor are described.

3.3. G protein-coupled receptors

Numerous hormones, neurotransmitters, chemokines, local mediators, and sensory stimuli exert their effects on cells by binding to heptahelical membrane receptors coupled to heterotrimeric G proteins. These highly specialized transducers can modulate the activity of multiple signaling pathways leading to diverse biological responses [84].

The so-called G protein-coupled receptors (GPCRs) or seven transmembrane receptors (7-TM) constitute a large and diverse family of proteins whose primary function is to transduce extracellular stimuli into intracellular signals. They are predicted to contain seven membrane-spanning helices, an extracellular N-terminus and an intracellular C-terminus. [85]. The structural determinants of receptor-G protein-effector specificity are not completely understood and, in addition to involving interaction domains of these primary acting proteins, also require the participation of scaffolding and regulatory proteins [84].

The first step in signal transduction is ligand binding. When a ligand interacts with a heptahelical receptor on the surface of the cell, the ligand either stabilizes or induces a conformation in the receptor that activates a heterotrimeric G protein (composed of α-, β-, and γ-subunits) on the inner membrane surface of the cell [86]. This change in the conformation of the receptor may involve disruption of a strong ionic interaction between the third and sixth transmembrane helices [87] [88], which facilitates the activation of the G-protein heterotrimer. In the inactive heterotrimeric state, GDP is bound to the Gα-subunit. Upon activation, GDP is released, GTP binds to Gα, and subsequently Gα-GTP dissociates from G$\beta\gamma$ and from the receptor (Fig. 3.2, page 21).

G-proteins are named by their α subunit: Gs, which activates adenylyl cyclase, Gi, which inhibits adenylyl cyclase and Gq which activates phospholipase C. Both Gα-

GTP and G$\beta\gamma$ subunits are free to activate downstream effectors. Depending on the type of G protein to which the receptor is coupled, a variety of downstream signalling pathways can be initiated [89]. A well characterized example of a G protein-triggered signalling cascade is the cAMP pathway. The enzyme adenylate cyclase is activated by Gα-GTP and synthesizes the second messenger cyclic adenosine monophosphate (cAMP) from ATP (Adenosine 5'-triphosphate). Second messengers then interact with other proteins downstream to cause a change in cell behavior [90]. Finally, signalling, desensitization and eventual resensitization are regulated by complex interactions of various intracellular domains of the GPCRs with numerous intracellular proteins [91] [92].

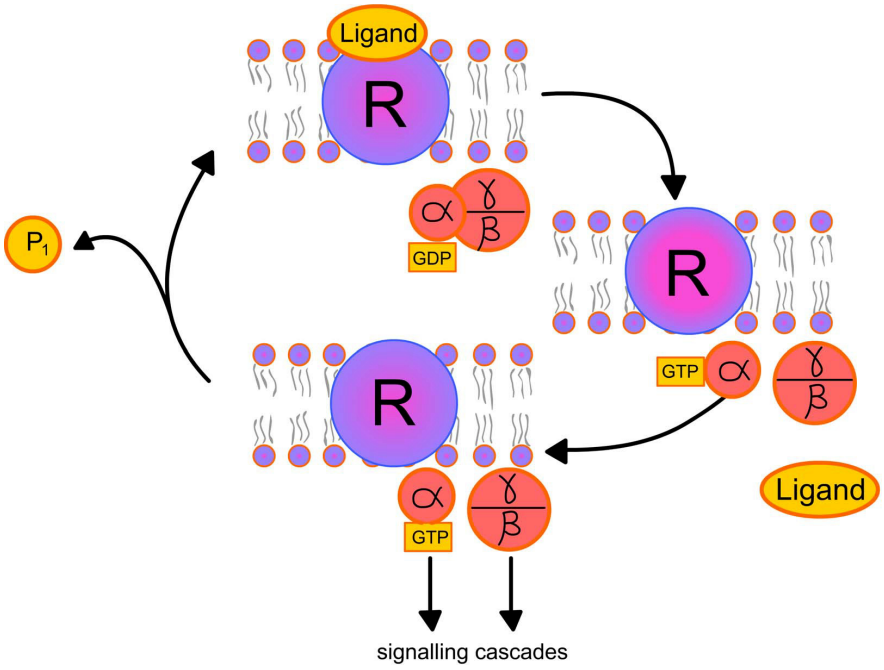


Figure 3.2: Activation cycle of G proteins by G protein-coupled receptors. When a ligand activates the G protein-coupled receptor, it induces a conformation change in the receptor that allows the heterotrimeric G protein to now bind to the receptor. The G protein then releases its bound GDP from the Gα subunit, and binds a new molecule of GTP. This exchange triggers the dissociation of the Gα subunit, the G$\beta\gamma$ dimer, and the receptor. Both, Gα-GTP and G$\beta\gamma$, can then activate different signalling cascades. The duration of the signal is determined by the intrinsic GTP hydrolysis rate of the G-subunit and the subsequent reassociation of G-GDP with G$\beta\gamma$.

3.4. The GABA B receptor

3.4.1. The GABA B receptor in the nervous system

Based on their structural and functional characteristics, neurotransmitter receptors can be classified into two broad categories: metabotropic and ionotropic receptors. In contrast to the latter, metabotropic receptors do not form an ion channel pore; rather, they are indirectly linked with ion-channels on the membrane of the cell through signal transduction mechanisms. This class of receptors includes the metabotropic glutamate receptors, muscarinic acetylcholine receptors, most serotonin receptors, as well as receptors for norepinephrine, epinephrine, histamine, dopamine, neuropeptides, endocannabinoids and GABA B receptors [93] [94] [95].

Within the nervous system the GABA B receptor is defined as a seven transmembrane receptor for gamma-aminobutyric acid (GABA) that is linked via G-protein to ion-channels as well as adenylate cyclase and hence is classified as a metabotropic receptor [96]. This receptor was first demonstrated on presynaptic terminals where it serves as an autoreceptor and also as a heteroreceptor to influence transmitter release by suppressing neuronal Ca(2+) conductance. Subsequent studies showed the presence of the receptor on postsynaptic neurones where activation produces an increase in membrane K(+) conductance and associated neuronal hyperpolarization [97].

Structure

G protein-coupled receptors can form homo- and/or heterodimers and possibly more complex oligomeric structures, and indeed heterodimerization has been shown to be essential for the function of receptors such as the metabotropic GABA B receptors.

The first indication of the structure of the GABA B receptor emerged in 1997 when Bettler and colleagues identified a large molecular weight (130 kDa), seven transmembrane spanning receptor protein subunit, termed GABA B1 [98]. A year after this initial discovery, it was realized that GABA B1 is not expressed on the surface of cells without the support of a second receptor protein, referred to as GABA B2, which appears to couple to GABA B1 at the level of the endoplasmic reticulum in order to facilitate surface expression [99]. GABA B2 also has a seven transmembrane spanning motif and links to GABA B1 at its intracellular carboxyl-terminals (C-termini). The combination of these two proteins forms a heterodimer that is essential for full receptor function. However, no GABA binding has been associated with GABA B2,

although this subunit is crucial for the coupling of GABA B receptor to G proteins [100] (Fig. 3.3).

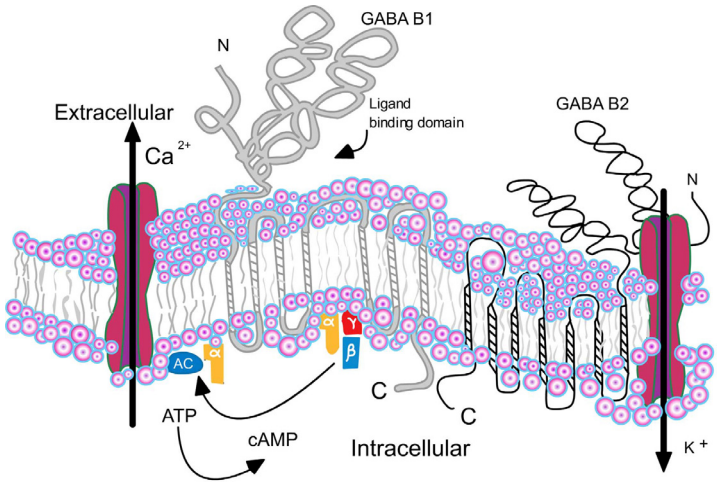


Figure 3.3: The GABA Receptor The GABA B receptor is an heterodimer composed of two, seven transmembrane-spanning units that are linked by their carboxyl-termini. The ligand binding domains are on the extracellular surface of the two subunits. Activation of the heterodimer modulates adenylate cyclase (AC) activity via G-proteins. This activation influence K+ and Ca2+ conductance.

The cloning of GABA B1 uncovered the existence of at least three spliced forms of human GABA B1 protein (B1a, B1b and B1c). However, whether different combinations of these isoforms produce different pharmacological characteristics is not known. A more detailed study of the in vivo function and anatomical distribution of the various isoforms needs to be carried out in order to resolve these issues with respect to receptor diversity [101].

3.4.2. The GABA B receptor in the hematopoietic system

Recently, using gene expression analysis performed on CD34+ hematopoietic stem and progenitor cells, our group could show that the GABA B receptor gene is expressed on hematopoietic stem and progenitor cells and even more, fluorescence-activated cell sorter analysis of highly enriched CD34+ cells could confirm that this receptor is also expressed at the protein level on the surface of these cells [83]. In a further study, we found that the GABA B receptor was up-regulated on CML CD34+ cells compared to normal hematopoietic stem and progenitor cells [65]. The higher expression of neurobiologic receptors in immature, developmentally early hematopoietic cells or in malignant cells and their undetectable expression in fully differentiated

blood cells might furthermore suggest a developmental affinity of human hematopoietic and neural cells [83].

More detailed studies need to be carried out to further dissect the functional significance of the GABA B receptor in hematopoietic regulation, stem cell signaling, differentiation, transdifferentiation and cancer development.

4. Aims of this work

4.1. Assessment of the gene expression signature of CML stem and progenitor cells

Investigating the molecular biology of stem cells can give novel insights into cancer research. CML is one of the best-studied malignant stem cell disorder, which is characterized by a clonal expansion of hematopoietic progenitor cells. The cell population for studying the pathophysiology of CML are certainly primary hematopoietic stem and progenitor cells from patients with this disease. Thus, the first objetive of this work was to provide a large-scale expression analysis of highly enriched CD34+ stem and progenitor cells from patients with CML in chronic phase in comparison with the normal counterparts. A further aim was to compare the expression profile of leukemic CD34+ cells from bone marrow with those of circulating cells. For this propose, CD34+ stem and progenitor cells should be isolated and studied using microarrays analysis, quantitative real-time RT-PCR, and flow cytometry.

4.2. Functional examination of candidate genes important for the pathophysiology of CML

Following this analysis, candidate genes important for the pathophysiology of CML should be identified and further investigated by performing different functional *in vitro* experiments. Those functional assays should permit to address basic features of the hematopoietic stem and progenitor cells such as *proliferation* with suspension cultures and [3H] uptake assays, and *differentiation* using colony-forming cells cultures.

4.3. Assessment of the functional role of GABA B receptor on hematopoietic stem and progenitor cells

Within the nervous system, GABA plays a central role as a neurotransmitter mediating inhibitory postsynaptic potentials either by increasing the permeability of potassium channels or by blocking voltage-dependent calcium channels. The GABA B receptor is G protein-coupled with mixed effects on adenylate cyclase activity. Since GABA B receptor mRNA as well as surface protein was also expressed in human hematopoietic stem and progenitor cells, this receptor could be viewed as a mediator between neuronal and hematopoietic cells. Hence, a further objective of this work was to study the functional role that the GABA B receptor plays in the regulation of *proliferation*, *differentiation* and *migration* of primary CD34+ hematopoietic stem and progenitor cells and the study of which *intracellular pathways* are activated following receptor modulation.

Part II.

Materials and Methods

5. Cells

Human CD34+ hematopoietic stem and progenitor cells

After informed consent MNCs were obtained from bone marrow (BM) or peripheral blood (PB) of CML patients or healthy volunteers. The CD34+ hematopoietic stem and progenitor cells used in this work were highly enriched from mononuclear cells (MNCs). For gene expression analyses, MNCs were obtained by density centrifugation from BM aspirate of eight healthy volunteers and of nine patients with untreated newly diagnosed Ph+ CML in chronic phase. Patients characteristics are indicated in table 5.1. For performing functional assays, MNCs were obtained from peripheral blood of healthy volunteers who donated hematopoietic stem cells for allogeneic transplantation and were submitted to leukapheresis or from peripheral blood of patients with untreated newly diagnosed Ph+ CML in chronic phase.

Table 5.1: Patient's characteristics: Age, sex (female: F, male: M), peripheral blood counts (leukocytes (Leuko), hemoglobin (Hb), platelets (Plt), BCR-ABL transcript levels (BCR-ABL/G6PDH ratio) and purity of enriched CD34+ cells is given for each patient included in the gene expression study. BCR-ABL/G6PDH ratio was assessed as previously described [102]. All patients had a newly diagnosed Ph positive CML in chronic phase without previous treatment.

No.	age	sex	Leuko (/nl)	Hb (g/dl)	Plt (/nl)	BCR-ABL/G6PDH ratio (%)	purity (%)
1	26	F	25.7	13.9	915	9.3	98.9
2	42	F	180	12.1	476	5.4	99.9
3	40	F	129	9.4	261	9.5	98.9
4	52	M	30.5	12.8	359	4.4	n.a.
5	69	F	9.0	14.3	283	8.2	n.a.
6	64	M	38.3	14.1	212	7.1	99.5
7	28	F	164	9.5	842	11.2	99.7
8	55	M	206	10.1	331	25.8	99.1
9	54	M	52.2	9.3	388	5.7	99.9

K562 cell line

A human cell line called K562 was chosen for performing suspension culture experiments. These cells were established from the pleural effusion of a 53-year-old woman with CML in blast crisis in 1970 and they are Ph+ with a BCR-ABL b3-a2 fusion gene. Our group obtained this cell line from the *Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH* (DSMZ).

6. Materials

In the following the different materials used in this work are exposed in tabularly form.

6.1. Instruments and software

Instruments and Software	**Provider**
ABI PRISM 7900HT Sequence Detection System Instrument with *ABI PRISM* Software	Applied Biosystems, Applera Deutschland GmbH, Darmstadt, D
Axiovert 25 CFL Microscope with Sony Power HAD 3CCD Camera	Carl Zeiss AG, Oberkochen, D
Axioskop 2 Microscope	Carl Zeiss AG, Oberkochen, D
Systec 2540EL Autoclave Machine	Tuttnauer, Wesel, D
Biofuge fresco Centrifuge with *Heraeus* Rotor #3325	Thermo Electron Corporation, Langenselbold, D
Canon Power Shot G5 camera	Canon Deutschland GmbH, Krefeld, D
Cell counter machine Coulter MD II Series Analyzer	Beckman Coulter GmbH, Krefeld, D
Cell sorter *FACSCalibur* with *CellQuest* Software	Becton Dickinson GmbH, Heidelberg, D
Electric Motor pipette *Accu-Jet*	Brand, Wertheim, D
HERAcell CO2 - Incubator	Thermo Electron Corporation, Langenselbold, D
HERAsafe HS 12 Flow	Thermo Electron Corporation, Langenselbold, D
Megafuge 1.0R Centrifuge with Heraeus Rotor #2704	Thermo Electron Corporation, Langenselbold, D
PCR Mastercycler gradient	Eppendorf GmbH, Wesseling-Berzdorf, D
Thermo mixer *Comfort*	Eppendorf GmbH, Wesseling-Berzdorf, D
Wallac 1420 Victor2 Microplates-Reader	EG&G, Wallac, Turku, FIN

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Instruments and Software	Provider
Vortex mixer *Neolab*	Neolab, Heidelberg, D
Aglient Bioanalyzer 2100	Agilent Technologie, Waldbronn, D

6.2. Consumable materials

Consumable Materials	Provider
96-Well Plates with round bottom	Corning,New York, USA
24 Transwell-Plates (5 µm Pores)	Corning Life Sciences, Schiphol-Rijk, NL
96-Well Nylonmembran	PerkinElmer, Shelton, CT, USA
12-Well Plates	Corning, New York, USA
Cell culture bottles	BD Falcon, Franklin Lakes, USA
5 ml FACS- Tubes	SARSTEDT AG, Nümbrecht, D
1.5 ml Reaction Tubes	Eppendorf , Hamburg, D
2ml Reaction Tubes	Eppendorf , Hamburg, D
14ml Tubes	Greiner Cellstar,Frickenhausen, D
50ml Tubes	BD Falcon, Franklin Lakes, USA
Human Genome Focus Array	Affymetrix, Santa Clara, USA
LS Separation Columns	Miltenyi Biotec GmbH, Bergisch Gladbach, D
MACS MultiStand	Miltenyi Biotec GmbH, Bergisch Gladbach, D
MidiMACS Separation Unit	Miltenyi Biotec GmbH, Bergisch Gladbach, D
MicroAmp Fast Optical 96 Well-Platte	Applied Biosystems, Foster City, USA
MicroAmp Optical Adhesive Cover	Applied Biosystems, Foster City, USA
Neubauer improved hemocytometer	Paul Marienfeld GmbH, Lauda-Königshofen, D
QIAShredder spin Column	Qiagen GmbH, Hilden, D
RNAeasy spin Column	Qiagen GmbH, Hilden, D
Gene Chip Human genome focus array	Affymetrix, High Wycombe, UK

6.3. Kits

Kits	Provider
BioArray HighYield RNA Transcript Labeling System	ENZO Life Sciences, Farmingdale, USA
cAMP Enzym Immunoassay Kit	Sigma, Taufkirchen, D
High Capacity cDNA Archive Kit	Applied Biosystems, Foster City, USA
Human CD34 MicroBead Kit	Miltenyi Biotec GmbH, Bergisch Gladbach, D
MessageAmp aRNA Kit	Ambion, Austin, USA
RNeasy Mini Kit	Qiagen GmbH, Hilden, D

6.4. Buffers, mediums and supplements for mediums

Buffers, Mediums and supplements for Mediums	Provider
Dulbeccos PBS (phosphate-buffered saline puffer)	PAA Laboratories GmbH, Cölbe, D
FCS (fetal calf serum)	PAA Laboratories GmbH, Cölbe, D
HBSS-Buffer	PAA Laboratories GmbH, Cölbe, D
Hepes-Buffer (1 M)	PAA Laboratories GmbH, Cölbe, D
L-Glutamin	PAA Laboratories GmbH, Cölbe, D
Methocult SFBIT H4439 (Methylcellulose)	CellSystems, St. Katharinen, D
Penicillin / Streptomycin (100 x)	PAA Laboratories GmbH, Cölbe, D
RLT-Puffer	Qiagen. Hilden, D
RPMI-1640 Medium	Sigma-Aldrich GmbH, Seelze, D
RDD Buffer	Qiagen. Hilden, D
RPE Buffer	Qiagen. Hilden, D
RW Buffer	Qiagen. Hilden, D

6.5. Chemicals

Chemicals	Provider
AlbuRx 5 Human albumin-Solution (5%)	ZLB Behring GmbH, Marburg, D
ß-Mercaptoethanol	Sigma-Aldrich GmbH, Seelze, D
β-Scintillation Liquid	Wallac, Turku, FIN
BSA-Solution (30%)	PAA Laboratories GmbH, Cölbe, D
CaliBRITE- Microbeads	Becton Dickinson GmbH, Heidelberg, D
DMSO-Solution	Sigma-Aldrich GmbH, Seelze, D
EDTA-Solution (0.5 M)	Sigma-Aldrich GmbH, Seelze, D
Ethanol	Merck, Darmstadt, D
Ethidiumbromid-Solution (10 mg/ml)	Sigma-Aldrich GmbH, Seelze, D
FACS-Flow	Becton Dickinson GmbH, Heidelberg, D
Fibronectin- Solution (1 mg/ml)	Sigma-Aldrich GmbH, Seelze, D
HCl 0.1M	Merck, Darmstadt, D
LymphoPrepTM	PROGEN Biotechnik GmbH, Heidelberg, D
Fluo4-AM-Solution (1 mM)	Invitrogen GmbH, Karlsruhe, D
Glucose	AppliChem GmbH, Darmstadt, D
Human Interleukin-3 (IL3)	Promocell, Heidelberg, D
Human Interleukin-6 (IL6)	Promocell, Heidelberg, D
Human Stem Cell Factor (SCF)	Promocell, Heidelberg, D
Human SDF-1ß/CXCL12	R&D Systems GmbH, Wiesbaden, D
Loading Dye (6 x)	Promega GmbH, Mannheim, D
NaOH	Merck, Darmstadt, D
TaqMan Universal PCR Master Mix	Applied Biosystems, Foster City, USA
Tris-Base	Merck KGaA, Darmstadt, D
Thymidin-labeled Tritium (3[H]-Thymidin) 1mCi / ml	Amersham Biosciences, Braunschweig, D
Trypanblau-Solution (0.5%)	BIOCHROM AG, Berlin, D

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Chemicals	Provider
RNase-Free DNase Set	Qiagen GmbH, Hilden, D
100 bp DNA Ladder (0.1 µg/µl)	GeneCraft GmbH, Lüdinghausen, D

6.6. Genome Assays for the quantitative real-time PCR

Genome assays (Assays-on-demand) were provided by Applied Biosystems (Foster City, USA).

Assay-on-demand	reference number
GATA1	Hs00231112_m1
LEPR	Hs00174497_m1
TAL1	Hs00268434_m1
TIMP1	Hs00171558_m1
ACSL5	Hs00212106_m1
CRHBP	Hs00181810_m1
SEPP1	Hs00193657_m1
ELA2	Hs00236952_m1
GAPDH	Hs99999905_m1

6.7. Antibodies

Antibodies (Clone)	Provider
CD34 PE coupled (8G12)	Becton Dickinson GmbH, Heidelberg. D
CD38 FITC coupled (HB7)	Becton Dickinson GmbH, Heidelberg, D
Mouse IgG1 Isotyp-Control, FITC coupled (X40)	Becton Dickinson GmbH, Heidelberg, D
Mouse IgG2a Isotyp-Control, PE coupled (G155-178)	Becton Dickinson GmbH, Heidelberg, D

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Antibodies (Clone)	Provider
FITC-conjugated monoclonal anti-CD36 (NL07)	Becton Dickinson GmbH, Heidelberg, D
Goat IgG Isotyp control	Santa Cruz Biotechnology, Heidelberg, D
Ob-r C-20 and N-20 goat polyclonal IgG antibodies	Santa Cruz Biotechnology, Heidelberg, D
Donkey IgG FITC coupled	Santa Cruz Biotechnology, Heidelberg, D
Donkey anti goat IgG FITC coupled	Santa Cruz Biotechnology, Heidelberg, D

6.8. Receptors agonists and antagonists

Receptors Agonists and Antagonists	Provider
GABA B Agonist Baclofen	Sigma-Aldrich GmbH, USA
GABA B Antagonist Hydroxisaclofen	Sigma-Aldrich GmbH, USA
fatty acid synthesis inhibitor Cerulenin	Sigma-Aldrich, USA
human Leptin	Sigma-Aldrich GmbH, USA
human Leptin antagonist triple mutant	Biovendor Laboratory Medicine, Heidelberg, D

The protein human Leptin was reconstituted by adding 0.5 ml of 15mM HCL, 0.3 ml of 7.5 mM NaOH to bring the pH up to approximately 5.2 and the protein human leptin antagonist with 0.4% NaHCO3 adjusted to pH 8-9. Cerulenin was dissolved in ethanol and after the same volume of PBS was added for diluting. Baclofen and Hydoxisaclofen were diluted in PBS.

7. Methods

7.1. Selection of CD34+ Cells

7.1.1. Density centrifugation: Isolation of mononuclear cells

The mononuclear cell fraction from human peripheral blood (lymphocytes and monocytes) has a density below 1.077 g/ml. The granulocytes (polymorphonuclear leukocytes) comprises principally neutrophils with relatively small numbers of basophils and eosinophils. They have densities pre-dominantly above 1.080 g/ml and the density of erythrocytes significantly overlaps that of neutrophils. With this background, it is possible to use a simple discontinuous gradient to resolve the mononuclear cells (MNCs). Layering leukocyte rich plasma (LRP) over a barrier whose density is 1.077 g/ml, allow the granulocytes to pellet while the MNCs are retained at the interface.

Description of the process After informed consent, LRP was obtained by performing a *bone marrow* puncture in the pelvis of CML patients or healthy volunteers, or it was collected from the *peripheral blood* through a method known as leukapheresis. In this process, healthy donors who donated hematopoietic stem cells for allogeneic transplantation received 12 μg human recombinant G-CSF (Granulocyte colony-stimulating factor) per kilogram body weight for 4 to 5 days. Afterward cells were harvested using the CobeSpectra Apheresis System (Gambro BCT, Planegg-Martinsried, Germany). Donors mobilized at least 4 x 10^6 CD34+ cells per kilogram body weight, as determined by flow cytometry. We also obtained LRP from human peripheral blood from patients with untreated newly diagnosed Ph+ CML in chronic phase.

The LRP was diluted with PBS (Phosphate-buffered saline buffer, PAA laboratories GmbH, Coelbe, Germany) until a final volume of 30 mL, underlayed with 15 mL Ficoll- LymphoPrepTM (Progen biotechnik GmbH, Heidelberg, Germany) and centrifuged at 400g without break for 30 minutes. The cells of the interface were removed

and resuspended in 30 mL of PBS, centrifuged at 400g for 10 minutes, followed by a second washing procedure. The cell pellet was suspended in 20 mL PBS and to determine the end concentration of MNCs, the Coulter MD II Series Analyzer (Beckman Coulter GmbH, Krefeld, Germany) was used.

7.1.2. Immunomagnetical selection of CD34+ cells

From the pool of MNCs, CD34+ cells were positively selected using the MiniMACS immunomagnetic separation system (Miltenyi Biotec, Bergisch Gladbach, Germany).

To be separated the cells were first magnetically labeled with MACS MicroBeads. These beads are superparamagnetic particles that are coupled to the specific monoclonal mouse anti-human CD34 antibody. After magnetically labeling, the cells are passed trough a column (LS Separation Columns) which is placed in a strong magnet (midiMACS Separation Unit). The magnetizable column matrix serves to create a high-gradient magnetic field. The magnetically labeled CD34+ cells are retained on the column and separated from the unlabeled cells, which passed trough. After removal of the column from the magnetic field, the retained fraction of CD34+ can be eluted.

According to the manufacturer's instructions, 10^6 mononuclear cells were suspended in 300 μL sorting buffer. This sorting buffer was prepared by mixing 1 x PBS supplemented with 2 mmol/L EDTA (Sigma-Aldrich GmbH, Selze, Germany) and 0.5% bovine serum albumine (PAA laboratories GmbH, Coelbe, Germany). Afterwards, 100 μL human Ig FcR blocking antibody, and 100 μL CD34 Microbeads (both from Miltenyi Biotec, Bergisch Gladbach, Germany) were added and incubated for 30 minutes at 4°C. Cells were washed with 30 mL sorting buffer and centrifuged at 400g for 10 minutes.

The pellet was suspended in 500 μL sorting buffer and passed through a separation column exposed to the magnetic field of the MACS device. The column was washed four times with 3 ml sorting buffer and removed from the separator, and the retained cells were eluted with 5 ml sorting buffer using a plunger. Eluted cells were subjected to a second round of separation.

7.2. Immunofluorescence staining and flow cytometry

Flow cytometry is a technique for counting, examining and sorting microscopic particles suspended in a stream of fluid. It allows simultaneous multiparametric analysis of the physical and/or chemical characteristics of single cells flowing through an optical and/or electronic detection apparatus. A beam of laser light of a single frequency (colour) is directed onto a hydro-dynamically focused stream of fluid. A number of detectors are aimed at the point where the stream passes through the light beam; one in line with the light beam (Forward Scatter or FSC) and several perpendicular to it (Side Scatter or SSC) and one or more fluorescent detectors. The physical (morphological) profile of a cell can be observed by combining forward light scatter (FS) and orthogonal or side light scatter (SSC). In forward light scatter the laser beam is interrupted by the cell and the light that passes around the cell is measured. This measurement is an indication of the cell's unique refractive index which depends on a cell's size, organelles, water and molecular contents. Cellular side scatter is the light that is reflected 90°to the laser beam (all fluorescence is emitted and therefore collected at this angle) and is an indication of cytoplasmic density or cell surface granularity. Cells express proteins (antigens) on their surface which are unique to that cell. Antibodies (immunoglobulins) are proteins that bind to cell surface antigen epitopes very specifically. By tagging each antibody with a colored fluorochrome it is easy to distinguish the cell type and quantity of antigens expressed by each cell.

In this work Fluorescence-activated cell-sorting (FACS) was performed using a FACSCalibur cell sorter (Becton Dickinson GmbH, Heidelberg, Germany) in order to analyze antigene expression in CD34+ cells and K562 cells.

Immunomagnetically selected CD34+ cells were stained with a phycoerythrin (PE)-conjugated monoclonal anti-CD34 antibody (clone 8G12; Becton Dickinson, Heidelberg, Germany) in 50μL PBS at 4°C for 30 minutes. An isotype-identical monoclonal antibody (immunoglobulin G1 [IgG1]-PE; Becton Dickinson) served as control. For detection of the CD36 receptor, FITC-conjugated monoclonal anti-CD36 (clone NL07; Becton Dickinson) was applied and an isotype-identical monoclonal antibody (IgG1-FITC) was used as a control.

For detection of Leptin receptors, indirect immunofluorescence was used. Highly enriched CD34+ cells were incubated for 30 minutes at 4°C with primary goat polyclonal IgG antibodies raised against Leptin N, Leptin C termini of the receptor (Santa Cruz Biotechnology, Heidelberg, Germany). Normal goat IgG served as a control for

the primary staining. After washing with PBS, secondary staining was performed for 15 minutes at 4°C using a FITC-conjugated donkey anti-goat IgG antibody (Santa Cruz Biotechnology). FITC-conjugated normal donkey IgG was used as a control for the secondary staining. For 2-color immunofluorescence staining of CD34+ cells, we used fluorescein isothiocyanate (FITC)-conjugated monoclonal anti-CD38 (clone HB7; Becton Dickinson) antibodies.

After antibody staining, cells were washed and suspended in PBS buffer. The cells were analyzed using the FACSCalibur cell sorter. Resultant data were analyzed using the CellQuest software (Becton Dickinson) after gating on viable cells.

7.3. Gene Expression Analysis

Gene expression is the process by which a gene's DNA sequence is converted into the structures and functions of a cell. It is a multi-step process that begins with transcription of DNA into messenger RNA. Indirectly, the expression of particular genes may be assessed with DNA microarray technology, which can provide a rough measure of the cellular concentration of different messenger RNAs, or with reverse transcription through real-time polymerase chain reaction, a technique used to amplify and quantify a specific part of a given mRNA molecule.

7.3.1. DNA microrarray technology

In oligonucleotide microarrays, the probes are designed to match parts of the sequence of known or predicted mRNAs. There are commercially available designs that cover complete genomes from companies such as Affymetrix. The GeneChips from Affymetrix consist of small DNA fragments (referred to here as probes), chemically synthesized at specific locations on a coated quartz surface. The precise location where each probe is synthesized is called a feature, and millions of features can be contained on one array. By extracting and labeling nucleic acids from experimental samples, and then hybridizing those prepared samples to the array, the amount of label can be monitored at each feature, enabling gene level expression analysis.

Measuring gene expression using microarrays can be used to identify disease genes by comparing gene expression in diseased and normal cells. In this work, immunomagnetically enriched CD34+ hematopoietic stem and progenitor cells from BM of nine patients with untreated BCR-ABL-positive CML in chronic phase and from eight

healthy volunteers were examined and their gene expression profile was compared using Affymetrix HG Focus arrays covering 8746 genes. Total RNA was first isolated from CD34+ cells and used to generate biotin labeled cRNA. For this purpose, total RNA was reversely transcribed using a T7-Oligo(dT) Promoter Primer in the first-strand cDNA synthesis reaction. Following second-strand cDNA synthesis, the double-stranded cDNA was purified and served as a template in the subsequent in vitro transcription (IVT) reaction. The IVT reaction was carried out in the presence of T7 RNA Polymerase and a biotinylated nucleotide analog for complementary RNA (cRNA) amplification and biotin labeling. The biotinylated cRNA targets were then cleaned up, fragmented, and hybridized to GeneChip expression arrays. Measuring the quality of the RNA (total RNA and cRNA) was essential to the overall success of the analysis. In the following, these steps are described in detail.

RNA isolation

After stimulation, CD34+ cells were pelleted and then stored at -70°C until required for RNA purification. For the isolation of the RNA two steps are needed. First, complete disruption of cell walls, plasma membranes and organelles, is required to release all the RNA contained in the sample. Afterwards, homogenization is necessary to reduce the viscosity of the lysates produced by disruption. Homogenization shears high-molecular-weight genomic DNA and other high-molecular-weight cellular components to create a homogeneous lysate. In addition, DNase digestion may be required for certain RNA applications that are sensitive to very small amounts of DNA (e.g., TaqMan RT-PCR analysis). After it, the DNase is removed in subsequent wash steps.

Description of the process

Isolation of total RNA from 1 x 10^6 CD34+ cells was performed with the RNeasy Mini Kit (Qiagen AG, Hilden, Germany) according to the manufacturer's instructions. Cells were pelleted by centrifuging for 5 min at 300 x g and disrupted with 600 μL of Lysis Buffer RLT. This Buffer was made by adding 10 μL of β-Mercaptoethanol (Sigma-Aldrich GmbH, Seelze, Germany) to 1 ml of RLT Buffer (Qiagen AG). The lysate was pipeted into a QIAshredder spin column placed in a 2 ml collection tube, and centrifuged for 2 min at full speed. 600 μL of 70% ethanol were added to the lysate, which was transferred to an RNeasy spin column placed in a 2 ml collection

tube and centrifuged for 15 s at 14000 x g. The lysate was homogenized as it passes through the spin column. The RNeasy spin column was washed with 350 μL Buffer RW1 by centrifuging for 15 s at 14000 x g. For eliminating genomic DNA contamination, 10 μL of DNase I Solution and 70 μL of RDD Buffer (Qiagen AG) were mixed and added to the spin column membrane. After 15 minutes of incubation it was washed again with Buffer RW1 and two times more with 500 μL Buffer RPE (both from Qiagen AG). The RNeasy spin column was placed in a new 2 ml collection tube and centrifuged at full speed for 1 minute to dry the spin column membrane, ensuring that no ethanol was carried over during RNA elution. The spin column was placed in a new 1.5 ml collection tube and 30 μL RNase-free water were added directly to the spin column membrane and centrifuged for 1 min at 14000 x g to elute the RNA.

RNA amplification

The procedure consists of reverse transcription with an oligo(dT) primer bearing a T7 promoter and in vitro transcription of the resulting DNA with T7 RNA Polymerase to generate hundreds to thousands of antisense RNA copies of each mRNA in a sample.

The aRNA amplification procedure is a 5 step process:

1. First strand cDNA synthesis is primed with the T7 Oligo(dT) Primer to synthesize cDNA with a T7 promoter sequence from the poly(A) tails of messages by reverse transcription.

2. Second strand cDNA synthesis converts cDNA with the T7 promoter primer into double-stranded DNA (dsDNA) template for transcription.

3. cDNA purification removes RNA, primers, enzymes, and salts from the dsDNA that inhibit in vitro transcription.

4. In vitro transcription generates multiple copies of aRNA from the double-stranded cDNA templates; it is the amplification step.

5. aRNA purification removes unincorporated NTPs, salts, enzymes, and inorganic phosphate to improve the stability of the aRNA and facilitate subsequent enzymatic manipulations.

For the cDNA synthesis and purification the MessageAmpTM aRNA Kit from Ambion (Austin, USA) was used.

Description of the process

1. First strand cDNA synthesis.

Total RNA (350 ng- 650 ng isolated RNA) was mixed with 1 μL of T7 Oligo(dT) Primer. Nuclease free Water was added to a final volume of 12 μL and the mixture was incubated for 10 minutes at 70°C, centrifuged for 5 minutes and placed into ice. In a second tube, the Reverse Transcription Master Mix was assembled and incubated for 2 minutes at 42°C.

Amount	Component
2μL	10X First Strand Buffer
1μL	Ribonuclease Inhibitor
4μL	dNTP Mix

Working quickly, 7 μL of Reverse Transcription Master Mix and 1 μL of Reverse Transcriptase were transferred to each RNA sample and incubated for 2 hours at 42°C.

2. Second-Strand cDNA Synthesis.

After the 2 incubation, the tubes were centrifuged for 5 seconds to collect the reaction at the bottom. The tubes were placed on ice and immediately incubated with the second strand cDNA synthesis reagents for 2 hours at 16°C.

Amount	Component
20μL	cDNA sample
63μL	Nuclease-free Water
10μL	10X Second Strand Buffer
4μL	dNTP Mix
2μL	DNA Polymerase
1μL	RNase H

3. cDNA Purification.

Before starting the cDNA purification the Filter Cartridges from the kit were equilibrated for each sample by adding 50 μL cDNA Binding Buffer and incubated at room temperature for 5 minutes. After it, 250 μL of cDNA Binding Buffer were added to each cDNA sample. Afterwards, the samples were placed onto the equilibrated Filter

Cartridges. After centrifuging for 1 minute at 10,000 x g, the flow-through was discarded and the Filter Cartridges were washed with 500 μL of cDNA Wash Buffer and once more centrifuged for drying. Filters were transferred to fresh tubes. For eluting the RNA, nuclease-free water (10 μL) that was preheated to 50°C was added, incubated for 2 minutes at room temperature and then centrifuged for 1 minute at 10,000 x g. The elution process was repeated a second time. The cDNA was kept on ice or at -20°C until it was added to an in vitro transcription reaction.

4. In Vitro Transcription to Synthesize aRNA.

The In Vitro Transcription Reaction (IVT) was made with the Enzo Bioarray High-Yield RNA Transcript Labeling Kit according to the manufacturer's instructions. With this process large amounts of hybridizable biotin-labeled RNA targets can be produced in vitro from bacteriophage T7 RNA polymerase promoters. This kit utilizes two different labeled nucleotides, Biotin-CTP and Biotin-UTP, to label the RNA. The following components were assembled at room temperature:

Amount	Component
16 μL	Template DNA
12 μL	Distilled or deionized water
4 μL	10X HY Reaction Buffer
4 μL	10X Biotin-Labeled Ribonucleotides
4 μL	10X DTT
4 μL	10X RNase Inhibitor Mix
2 μL	20X T7 RNA Polymerase
40 μL	Total Volume

The reaction was incubated for 6-14 hours at 37°C.

5. aRNA Purification.

The purification removes enzymes, salts and unincorporated nucleotides from the aRNA. It was done using the same Filter Cartridges that were used for the cDNA purification, but with different Binding and Wash Buffers (aRNA Binding and wash Buffer). Each aRNA sample, product of IVT, was mixed with 60 μl of aRNA Elution Solution, 350 μL of aRNA Binding Buffer and 250 μL of ACS grade 100% ethanol. The mixture was applied to equilibrated Filter Cartridges and centrifuged for 1 minute at 10,000 x g. The flow-through was discarded and the Filter Cartridges were washed

with 500 μL of cDNA Wash Buffer. The process followed as explained above and at the end of the purification, the aRNA was eluted from the filter with 50 μL aRNA Elution Solution.

Fragmentation of RNA

Fragmentation of cRNA target before hybridization onto GeneChip probe arrays is critical in obtaining optimal assay sensitivity. In this step, the full-length cRNA is broken down to 35 to 200 base fragments by metal-induced hydrolysis. For the fragmentation reaction, the cRNA samples were adjusted at a final concentration of 0.5 μg /μL in a volume of 24 μL with RNase-free Water, and 6 μL of 5x Fragmentation Buffer (Affymetrix GeneChip Sample Cleanup Modul, Affymetrix, Santa Clara, USA) were added. After 35 minutes incubation at 94°C, the samples were centrifuged and put on ice.

Affymetrix GeneChip Expression Analysis

For the Gene Expression analysis, the Affymetrix HG-Focus GeneChips were used. Hybridization, washing, staining and scanning steps of the biotinylated cRNA were performed at the *Institut fuer Onkologische Chemie* of the *Heinrich Heine University* (HHU) in Duesseldorf, Germany. Quantification, normalization, and statistical analysis of the data were processed at the *Institut fuer Bioinformatik* of the HHU.

Following the manufacturers instructions, a hybridization cocktail was prepared, including 10μg (at least 5μg) Biotin-labeled RNA fragments as the target, probe array controls, BSA, and herring sperm DNA. It was then hybridized to the probe array during a 16-hour incubation at 45°C in a Hybridization Oven. The hybridized probe array was washed and stained with streptavidin phycoerythrin conjugate on the fluidics station and scanned by the Agilent GeneArray Scanner 2500A. The amount of light emitted at 570 nm was proportional to the bound target at each location on the probe array.

For quality control, normalization and data analysis, we utilized the affy package of functions of statistical scripting language 'R' integrated into the Bioconductor project[1]. Using histograms of perfect match intensities, 5' to 3' RNA degradation side-by-side plots, or scatter plots, we estimated the quality of probes and hybridizations.

[1] http://www.bioconductor.org/

To normalize raw data, we used a method of *variance stabilizing transformations*. For identification of differentially expressed genes, we used the *Significance Analysis of Microarrays*[2] algorithm v2.23, which contains a sliding scale for *false discovery rate* (FDR) of significantly up- and downregulated genes. All data were permuted 1000 times by using the two classes, unpaired data mode of the algorithm. As cut-off for significance an estimated FDR of 0.1% (comparison CML vs. normal) or 5% (comparison CML BM vs. CML PB) was chosen by the tuning parameter delta of the software. The significance level of each gene was given by the q-value (lowest FDR at which the gene is called significant). Moreover, a cut-off for fold-change of differential expression of 1.2 was used.

Quantification and qualification of RNA

Spectrophotometric quantification of RNA. The concentration of RNA was determined by measuring the absorbance at 260nm (A260) in a spectrophotometer. An absorbance of 1 unit at 260 nm corresponds to 44 μg of RNA per ml (A260=1 $\rightarrow$ 44 μg /ml). The ratio of the readings at 260 nm and 280 nm (A260/A280) provides an estimate of the purity of RNA with respect to contaminants that absorb in the UV spectrum, such as proteins. Pure RNA has an A260/A280 ratio of 1.9-2. RNase-free water was used to calibrate the spectrophotometer. The RNA samples were measured in RNase-free cuvettes.

Integrity of RNA. The integrity and size distribution of extracted RNA and cRNA was checked at the *Biomedizinische Forschungszentrum* (BMFZ) from the HHU by using an Agilent 2100 bioanalyzer (Agilent Technologies, Boeblingen, Germany) according to the manufacturers instructions. The RNA was transferred onto miniature glass chips that contain a network of interconnected channels and reservoirs. The channels are filled with a gel matrix and the wells with buffer or sample, allowing electrophoresis to be carried out on a miniaturized scale. This enables interpretation of an electropherogram where the ribosomal RNAs appears as sharp bands or peaks. The apparent ratio of 28S rRNA to 18S RNA should be approximately 2:1. If the ribosomal bands or peaks of a specific sample are not sharp, but appear as a smear towards smaller sized RNAs, it is likely that the sample suffered major degradation either before or during RNA purification. For this work, the RNA 6000 Nano Chip (for 5 ng of total RNA) or the RNA 6000 Pico Chip (for 200 pg of total RNA) were used and the Agilent 2100 Bioanalyzer Software automatically calculated the over-

[2] http://www-stat.stanford.edu/_tibs/SAM/

all RNA concentration and showed the percentage of ribsomal impurities in mRNA samples.

7.3.2. Quantitative real-time PCR

Quantitative real-time PCR was performed in order to confirm the data of the Affymetrix HG Focus arrays comparing gene expression analysis between CD34+ hematopoietic stem and progenitor cells from CML patients and healthy volunteers. Real-time PCR is a sensitive and accurate method of relative gene expression measurement, based up on the quantification of DNA fragments that are exponentially amplified.

General principle of PCR

Polymerase chain reaction (PCR) is technique that allows a small amount of DNA to be amplified exponentially. The DNA fragment to be amplified is determined by selecting primers. Primers are short, artificial DNA strands that are complementary to the beginning or the end of the DNA fragment to be amplified. The PCR process usually consists of a series of twenty to forty cycles. Each cycle consists of three steps:

Denaturing: The double-stranded DNA has to be heated to 94-96°C in order to separate the strands.

Annealing: The temperature is lowered so the primers can attach themselves to the single DNA strands.

Elongation: A DNA polymerase copies the DNA strands.

A basic PCR run can be broken up into three phases:

Exponential: Exact doubling of product is accumulating at every cycle (assuming 100% reaction efficiency). The reaction is very specific and precise.

Linear (High Variability): The reaction components are being consumed, the reaction is slowing, and products are starting to degrade.

Plateau (End-Point: Gel detection for traditional methods): The reaction has stopped, no more products are being made and if left long enough, the PCR products will begin to degrade.

Real-time PCR

As a PCR progresses, some of the reagents are being consumed as a result of amplification. This depletion will occur at different rates for each replicate. The reactions start to slow down and the PCR product is no longer being doubled at each

cycle. For that reason, it is more precise to take measurements during the exponential phase, where the replicate samples are amplifying exponentially because there is a quantitative relationship between amount of starting target sample and amount of PCR product at any given cycle number. Real-Time PCR detects the accumulation of amplicon (PCR product generated from a DNA or cDNA template during the reaction) at the exponential phase of the PCR reaction. The DNA is quantified after each round of amplification; this is the *real-time* aspect of it. There are two common methods of quantification: one, the use of fluorescent dyes that intercalate with double-strand DNA, or another, modified DNA oligonucleotide probes that fluoresce when hybridized with a complementary DNA. The real-time PCR technique used for this work is built on 5′ nuclease chemistry. A probe is designed to anneal to a specific sequence of template between the forward and reverse primers. The probe sits in the path of the enzyme Polymerase as it starts to copy DNA or cDNA. When the enzyme reaches the annealed probe, the 5′ exo-nuclease activity of the enzyme cleaves the probe. Cleavage of the probe separates the reporter dye and the quencher dye, which results in increased fluorescence of the reporter. Accumulation of PCR products can be detected in real-time directly by monitoring the increase in fluorescence of the reporter dye.

Description of the process

Real-time PCR involves first isolating mRNA from a particular cell sample before producing a DNA copy of complementary DNA of each mRNA molecule using the enzyme reverse transcriptase. The gene of interest is then further amplified from the cDNA mixture together with a *housekeeping* gene. Housekeeping genes are those whose expression levels remain roughly constant in cells. In this work, real-time RT-PCR was performed for corroboration of RNA expression data by using the chemicals and instruments from Applied Biosystems, Applera Deutschland GmbH (Darmstadt, Germany) and GAPDH was chosen as housekeeping gene. First, in the reverse transcription (RT) step, cDNA was reversely transcribed from total RNA samples using random primers from the High- Capacity cDNA Archive Kit. Second, in the PCR step, PCR products were synthesized from cDNA samples using the PCR master mix and the Assays-on-Demand Gene Expression Products, a collection of predesigned primer and probe sets to perform quantitative gene expression studies. Finally, a signal was captured by the ABI PRISM 7900HT Sequence Detection System Instrument

and displayed by the ABI PRISM software. In the following the different steps of the process are explained in detail.

1. RNA isolation.

Total RNA was first isolated from CD34+ hematopoietic stem and progenitor cells from BM of five patients with untreated BCR-ABL-positive CML in chronic phase and from five healthy volunteers as described before.

2. cDNA synthesis.

The High-Capacity cDNA Archive Kit of Applied Biosystems was used to synthesize single stranded cDNA from total RNA samples by reverse transcription. Following components were mixed and incubated, first for 10 minutes at 25°C and after, for 2 h at 37°C:

3. Real-Time RT-PCR.

The real-time PCR Reaction components from Applied Biosystems included:

Amount	Component
12.5 μL	2 x qPCR Mastermix Plus
1.5 μL	Primer Probe Mix
1.65 μL	cDNA from the reverse transcription
9.6 μL	RNase-free water

The following Primer Probe Mixes or Assay-on-Demand Gene Expression Products were used: GATA1 (Hs00231112_m1), LEPR (Hs00174497_m1), TAL1 (Hs00268434_m1), TIMP1 (Hs00171558_m1), ACSL5 (Hs00212106_m1), CRHBP (Hs00181810_m1), SEPP1 (Hs00193657_m1), ELA2 (Hs00236952_m1), and GAPDH (Hs99999905_m1). Each assay consists of two unlabeled PCR primers for amplifying the sequence of interest and a TaqMan probe for detecting the sequence of interest. The oligonucleotide called TaqMan Probe was added to the PCR reagent master mix in a MicroAmp Fast Optical 96 Well-Platte, covered with a MicroAmp Optical Adhesive and centrifuged. The different runs of real-time RT-PCR were performed by the ABI PRISM 7900HT Sequence Detection System Instrument from Applied Biosystems:

Step	Time	Temparature	Function
1.	2 min	50 °C	AmpErase UNG activation
2.	10 min	95 °C	AmpliTaq DNA-Polymerase activation
3.	15 sec	95 °C	cDNA desnaturalization
4.	1 min	60 °C	Primer-Annealing and -Extension

The steps 3 and 4 were forty times repeated (forty cycles).

4. Quantification of the real-time PCR.

When the fluorescent signal Reporter increased to a detectable level, it was captured and displayed as an Amplification Dot Plot which contained valuable information for the quantitative measurement of DNA. The Threshold line is the level of detection or the point at which a reaction reaches a fluorescent intensity above background. The threshold line was set in the exponential phase of the amplification for the most accurate reading. The cycle at which the sample reached this level was called the Cycle Threshold (CT). This relative quantification of gene expression allowed to quantify differences in the expression level of a specific target gene between different samples. CT values were calculated by the ABI PRISM software and relative gene expression levels were expressed as the difference of CT values of target gene and the hauskeeping gene GAPDH (ΔCT). The data output was expressed as a fold-change of expression levels. Given a PCR efficacy of 2 a ΔCT value of 1 reflects a fold-change of 2.

7.4. Cell culture experiments

7.4.1. Cell suspension culture

After cells are placed in culture, it is possible to study the effect that different chemicals have on the proliferation, just by determination of the cell number with Trypan blue and a Hemocytometer. Trypan blue is a vital stain that colours dead tissues or cells blue. Live cells or tissues with intact cell membranes will not be coloured. Hence, dead cells are shown as a distinctive blue colour under a microscope. The hemocytometer is a device that consists of a thick glass microscope slide with a rectangular indentation that creates a chamber of certain dimensions. This chamber is etched with a grid of perpendicular lines. The device is crafted so that the area bounded by the lines is known, and the depth of the chamber is also known. Therefore it is

possible to count the number of cells in a specific volume, and thereby calculate the concentration of cells in the fluid overall.

Determination of the cell number with Trypan blue and a hemocytometer

For determining the concentration of the CD34+ cells suspension product after selection, a sample of 20 μL was taken and mixed with 20 μL 0.5% Trypanblau (Biochrom AG, Berlin, Germany). 10 μL of this mix were placed on the chamber of a hemocytometer (Neubauer improved, Paul Marienfeld GmbH, Lauda-Koenigshofen, Germany). It was covered with a cover glass, and capillary action completely fills the chamber with the mix. Looking at the chamber through a microscope, the number of cells in the chamber was used to calculate the concentration of the cells in the initial cell suspension product after the selection, following this formel: Cells per ml in suspension = Counted cells/ Number of counted chambers x Dilution factor x 10^4.

CD34+ cells culture

After the determination of the concentration of the immunomagnetically selected CD34+ cells, 1 x 10^6 cells per milliliter were cultured in RPMI 1640 medium (Sigma-Aldrich GmbH, Seelze, Germany) containing 10% heat-inactivated fetal calf serum (FCS, PAA Laboratories, Coelbe, Germany), 100 U/mL penicillin and 100 μg /mL streptomycin (both from Sigma-Aldrich GmbH) and 2 mM L-glutamine (PAA Laboratories). The medium was supplemented with a mix of cytokines obtained from PromoCell (Heidelberg, Germany): 20 ng/mL human Interleukin-3 (IL3), 20 ng/mL human Interleukin-6 (IL6) and 50 ng/mL human stem cell factor (SCF) were added.

K562 cells culture

1 x 10^6 cells per milliliter were cultured in RPMI 1640 medium (Sigma-Aldrich GmbH) containing 20% heat-inactivated fetal calf serum (PAA Laboratories), 100 U/mL penicillin and 100 μg /mL streptomycin (both from Sigma-Aldrich GmbH) and 2 mM L-glutamine (PAA Laboratories).

Cell stimulation

CD34+ and K562 cells were cultured in 12 wells Plates Corning, NY, USA) at the concentration of 2 x 10^5 cells/ml in 1ml per well. Daily, different chemicals were added to

culture : 100, 50 or 10 ng/ml of human Leptin or human Leptin antagonist, 10 μg /ml of cerulenin, and GABA B agonist, antagonist or both of them at the concentrations of 60 μM, 5 μM and 1 μM. Every sample was performed in duplicate and the number of cells calculated with Trypan blue and a Hemocytometer. After knowing the concentration of cells for every sample, the results were related to the non-stimulated control.

7.4.2. Semisolid clonogenic assay

In vivo, hematopoietic progenitor cells proliferate and differentiate resulting in the generation of mature blood cells. The semisolid clonogenic test is an in vitro assay developed to quantify multi-potential progenitors and lineage-restricted progenitors of the erythrocyte, granulocyte, monocyte-macrophage, and megakaryocyte myeloid cell lineages. When cultured in a semi-solid matrix, individual progenitors called colony-forming units (CFUs) proliferate to form discrete cell clusters or colonies. In our work, CFU assays were performed by placing a cell suspension into a semi-solid medium, such as methylcellulose, supplemented with nutrients and cytokines, followed by incubation at 37°C for 14 to 16 days. The CFUs were then classified and enumerated based on the morphological recognition. Colony evaluation and enumeration were done in situ by light microscopy.

Cell stimulation

First, immunomagnetically selected CD34+ stem and progenitor cells of healthy donors or untreated patients with CML in chronic phase were incubated with 100, 50 or 10 ng/ml of human Leptin or human Leptin antagonist, 10 μg /ml of Cerulenin, and GABA B agonist, antagonist or both of them at the concentrations of 60 μM, 5 μM and 1 μM. Every sample was made in duplicate with 2 x 10^3 cells/ml in 300μL .

Culture in methylcellulose

The stimulated cells were seeded in semisolid methylcellulose growth medium containing stem cell factor (SCF), granulocyte macrophage colony-stimulating factor (GM-CSF), granulocyte colony-stimulating factor (G-CSF); interleukin-3 (IL3), interleukin-6 (IL6) and erythropoietin. This is a ready-to-use medium called MethoCult H4436 SFBIT H4436 (CellSystems, St. Katharinen, Germany). In order to yield duplicate cultures of 1 mL each, a final volume of 3 ml was prepared: In 5ml tubes, 2.7ml of

Methocult was added to the 300μL of stimulated cell suspension. After capping the tubes, all components were thoroughly mixed with the vortex and 1ml was dispensed for each duplicated sample into petri dishes of 35 mm of diameter. For avoiding a possible drying of the methylcellulose, an additional petri dish with 3 ml water was prepared. The three small dishes were placed in a bigger one (94 mm of diameter) and incubated for 14-16 days in humidified incubator at 37°C and 5% CO2.

Counting of the colonys

Count and evaluation of colony types were made by using the Axiovert 25 CFL microscope (Carl Zeiss AG, Oberkochen, Germany) and the Canon Power Shot G5 Camera (Canon Germany GmbH, Krefeld, Germany). From every duplicate the median number of colony forming units granulocyte/macrophage (CFU-GM) and burst-forming unit erythrocyte (BFU-E) was calculated and related to the non-stimulated control.

7.4.3. Proliferation assay

Tritium (symbol T or [3H]) is a radioactive isotope of hydrogen that can be used as an isotopic tracer, e.g. tritiated thymidine is used in cell proliferation assays. The nucleoside thymidine is incorporated into the DNA of cells as they are replicated during cell division. The extent of cell proliferation may then be determined by liquid scintillation counting, a standard method for measuring radiation from beta-emitting nuclides. Beta particles emitted from the sample transfer energy to the solvent molecules, which in turn transfer their energy to the fluors; the excited fluor molecules dissipate the energy by emitting light. In this way, each beta emission results in a pulse of light.

Cell stimulation

Immunomagnetically selected CD34+ stem and progenitor cells of healthy donors or untreated patients with CML in chronic phase were incubated in a round-bottomed 96-well plate (Corning, NY, US) in RPMI 1640 medium supplemented with FCS, penicillin, streptomycin, L-glutamine and the mix of cytokines at the previously described concentrations. Every well contained 2 x10^4 cells in 100 μL Medium. The different samples were incubated with 100, 50 or 10 ng/ml of human Leptin or human Leptin antagonist, 10 μg /ml of Cerulenin, and GABA B agonist, antagonist or both of them at the concentrations of 60 μM, 5 μM and 1 μM. Every sample was made in

duplicate. In addition, 1 μCi/ml of 3[H]-Thymidin (Amersham Biosciences , Braunschweig, Germany) was added to each well.

Measure of the proliferation

After 18-20h of culture, cells were harvested using a pressure pump and sated on a 96-Well Nylonmembran (PerkinElmer, Shelton, CT, USA). Non incorporated nucleotides were washed up with distil water. After 1 day allowing the membrane to dry up, 25μL of β-scintillation liquid (Wallac, Turku, Finnland) was added to every sample. 3[H]-Thymidin uptake was measured on a β-scintillation counter (Perkin Elmer, Shelton, CT, US). The measured counts per minute (cmp) were proportional to both, the intensity of the radiation and the amount of tritiated thymidine incorporated to DNA. Stimulatory capacity was expressed by the Proliferation Index (PI = cpm of stimulated CD34+ cells/cpm of unstimulated CD34+ cells.

7.4.4. cAMP assay

Adenosine 3', 5'-cyclic monophosphate (cyclic AMP; cAMP) is one of the most important *second messengers* involved as a modulator of physiological processes. Since changes in cAMP levels can result in the activation of proteins known to play key roles in signal transduction cascades, quantitative measurement of cAMP can serve to identify signaling events.

Intracellular cAMP levels of CD34+ cells were measured by a competitive enzyme immunoassay (EIA; Sigma, Taufkirchen, Germany). The assay is based on the competition between cAMP present in the sample and a fixed quantity of a cAMP-peroxidase conjugate that binds to rabbit anti-cAMP-specific antibodies. Samples or standards, alkaline phosphatase conjugate, conjugate and antibody are simultaneously incubated at room temperature in a secondary antibody coated microwell plate. The excess reagents are then washed away and substrate is added. After an incubation time the enzyme reaction is stopped and the yellow color generated read on a microplate reader at 405 nm. The intensity of the bound yellow color is inversely proportional to the concentration of cAMP in either standards or samples. The measured optical density is used to calculate the concentration of cAMP.

Description of the process

Immunomagnetically selected CD34+ cells were cultured at 1 x 10^6 cells per milliliter in RPMI 1640 medium supplemented with FCS, penicillin, streptomycin, L-glutamine and the mix of cytokines at the previously described concentrations in 5% CO2 at 37°C for 15 hours. Subsequently, the cells were incubated with either GABA B agonist, antagonist or both of them at the concentrations of 60 μM, 5 μM and 1 μM, or PBS alone (control) for 30 minutes at 37°C. After centrifugation, the supernatant was completely removed and the cell pellets were treated with 100 mM HCl for 20 minutes to lyse the cells and decrease endogenous phosphodiesterase activity. After centrifugation at 600g at room temperature, cell lysates were acetylated by adding 5% of acetic anhydrate in order to prevent cAMP degradation and thereby improving the sensitivity of the assay. There were 5 samples containing defined amounts of cAMP (0.078 pmol/mL, 0.312 pmol/mL, 1.25 pmol/mL, 5 pmol/mL, or 20 pmol/mL) that served as standards and were treated in the same manner with HCl followed by acetylation as described. Following acetylation, standards as well as samples were incubated in separate wells of a microtiter plate coated with goat anti-rabbit IgG (EIA, Sigma). Alkaline phosphatase (50 μL) covalently linked to cAMP as well as 50 μL of a polyclonal rabbit anti-cAMP antibody (EIA, Sigma) were added. The sealed microtiter plate was incubated at room temperature on a shaker at 300 rpm for 2 hours to allow competitive binding of the anti-cAMP antibody, and afterwards wells were treated with 200 μL wash buffer (EIA, Sigma) 3 times. Then, 200 μL of a solution of p-nitrophenyl phosphate (EIA, Sigma), serving as a substrate for alkaline phosphatase, was added to every well and incubated at room temperature without shaking. After one hour, the enzyme reaction was stopped and the optical density at 405 nm (inversely proportional to the concentration of cAMP in either standards or samples) was determined using the Wallac 1420 Victor multilabel plate reader (EG&G; Wallac, Turku, Finland). The concentration of cAMP in the samples was calculated using the standards as a reference and expressed as percentage of the non stimulated controls.

7.4.5. Measurement of intracellular calcium mobilization

Cytoplasmic free calcium (Ca2+) is a key intracellular messenger in many cell types. Fluo-4 AM is a fluorescent Ca2+ indicator that is used for in-cell measurement of agonist-stimulated and antagonist- inhibited calcium signaling. This indicator is avail-

able as a acetoxymethyl (AM) ester that can permeate cell membranes. Once inside the cell, the lipophilic blocking groups are cleaved by nonspecific esterases, resulting in a charged form that leaks out of cells far more slowly than its parent compound. If not bound to Ca2+, Fluo-4 has an Absorption maximum at 416nm that increases upon to 494nm if bound to Ca2+. The emission maximum in both cases is 516nm. The large fluorescence increase upon binding Ca2+ of Fluo-4 has made it the indicator of choice for characterizing G protein-coupled receptor function, permitting analysis of Ca2+ in flow cytometers with a 488 nm argon laser excitation source.

Incubation of the CD34+ cells with Fluo-4

For every sample, 3 x10^5 immunomagnetically selected CD34+ cells were incubated in Hanks balanced salt solution (HBSS-Buffer, PAA Laboratories GmbH, Coelbe, Germany) containing 10 μM Fluo-4 (Fluo-4-AM-Solution, Invitrogen GmbH, Karlsruhe, Germany) for 30 minutes at 37°C. After a 1:5 dilution in HBSS-Buffer, 1% fetal calf serum (PAA Laboratories) and a further incubation for 40 minutes at 37°C, the cells were washed 3 times, suspended in HEPES-buffered saline (137 mM NaCl, 5 mM KCl, 1mM Na2HPO4, 5 mM glucose, 1 mM CaCl2, 0.5 mM MgCl2, 1 g/L bovine serum albumin and 10 mM HEPES, pH 7.4, PAA Laboratories), and incubated for 10 minutes at 37°C.

Intracellular free Ca2+ measure

The fluorescence (FITC channel) was continuously analyzed during 80 seconds using a FACSCalibur flow cytometer (Becton Dickinson) in 8-second acquisition intervals. After determining the baseline level of every sample during 16 seconds, the samples were stimulated with 100 ng/ml of SDF-1 (recombinant human SDF-1, R&D Systems GmbH, Wiesbaden, Germany) or/plus GABA B agonist, antagonist or both of them at the concentrations of 60 μM, 5 μM and 1 μM. The fluorescence was analyzed until the 80 seconds were over. Measured time dependent changes of Fluo-4 fluorescence intensity by continuous flow cytometry were expressed as relative mean fluorescence compared to the baseline level.

Variant form of the experiments

Before the measure with the flow cytometer, and during the incubation of Fluo-4, the cells were co-incubated with GABA B agonist, antagonist or both of them in similar

molar concentrations as described previously. The fluorescence from every sample was analyzed by continuous flow cytometry after determining the baseline level and adding 100 ng/ml of SDF-1.

7.4.6. Migration Experiments

Migratory capacity of cells may be tested using a transwell microporous membrane. This is a tool consisting in two chambers isolated by a membrane with pores of a determined diameter size. After coverage of the membrane with fibronectin, cells are placed into the upper chamber. Following an incubation time, motile cells actively transmigrate through the pores of the membrane to the lower chamber. In this way, positive responder cells can be harvested and counted for accurate determination of migration.

Stimulation of the CD34+ cells prior to migration assay

In selected experiments, human SDF-1β/CXCL12 (R&D Systems GmbH, Wiesbaden, Germany) was added to the lower chamber of the transmigration system at a final concentration of 250 ng/mL. Furthermore, the effect of the GABA B receptor on the cellular migration in response to SDF-1 was assessed. The GABA B agonist Baclofen and the GABA B antagonist Hydroxisaclofen were added to the upper chamber of the transmigration system at a final concentration of 60 μM, 5 μM and 1 μM. In additional experiments, CD34+ cells were preincubated with the GABA B agonist and antagonist during 30 minutes at 4°C prior to the migration assay.

Migration assay

Migration assays were performed in 6.5-mm diameter 5-μm pore transwells (Corning Life Sciences, Schiphol-Rijk, NL). Transwell filters were coated with 30 μg /cm2 Fibronectin (Fibronectin Solution 1mg/ml, Sigma-Aldrich GmbH, Seelze, Germany) in 1ml PBS (PAA laboratories). Coating was done overnight at 4°C. The coating solution was aspirated and replaced by a 1% BSA solution (PAA laboratories) diluted in PBS at 37°C for 30 minutes to block nonspecific binding sites. After 2 washes with HEPES 25 mM (PAA laboratories), 1.5 x 10^5 cells were plated in 150 μL RPMI-1640 Medium (Sigma-Aldrich GmbH) in the upper chamber of the transwell. The bottom compartment was filled with 500 μL medium. After incubation at 37°C during 8 hours, migrating cells were harvested from the bottom compartment. Enumeration

of migrated cells was accomplished by addition of a fixed number (20.000) of FITC-CaliBRITE beads (BD Biosciences) per sample and subsequent FACS analysis. The gate 1 for data acquisition was established for low forward scatter (FSC) and high FL-1, which is specific for FITC beads. The gate 2 was also generated from the gated events of intermediate FSC and low side scatter (SSC) for CD34+ cells. To obtain absolute values of migratory cells, flow cytometric counts for each sample were obtained during a period of time determined by the count of a fixed number (5000) of beads. The number of transmigrating CD34+ hematopoietic progenitor cells was calculated from the ratio of cell per bead multiplied by the number of beads added (counted cells x 20000/5000). Data were expressed as a relative index (migrated cells/total number of cells plated).

7.5. Statistical Analysis

For calculation of statistically significant differences between two groups in quantitative real time RT-PCR, flow cytometry and cell culture experiments, the t-test for unpaired or paired samples was used. A p value <0.05 was considered to be statistically significant.

Part III.

Results

8. Gene expression signature of CD34+cells from patients with CML in chronic phase

Immunomagnetically enriched CD34+ hematopoietic stem and progenitor cells from bone marrow (BM) of nine patients with untreated BCR-ABL-positive CML in chronic phase and from eight healthy volunteers were examined using Affymetrix HG Focus arrays covering 8746 genes. Significantly differentially expressed genes were identified using the *Significance Analysis of Microarrays* algorithm, which contains a sliding scale for false discovery rate of significantly up- and down-regulated genes (Fig. 8.2, page 61). The complete results of the array experiments are available in the *Gene expression Omnibus* database [1], according to the *Minimum Information About a Microarray Experiment*, a proposal of the European Bioinformatics Institute, that describes the minimum of information required to ensure that microarray data can be easily interpreted and that results derived from its analysis can be independently verified.

8.1. Comparison of gene expression profiles between normal and CML bone marrow CD34+ cells

Comparing malignant bone marrow (BM) CD34+ cells from patients with CML with normal BM CD34+ cells 1539 genes were significantly differentially expressed (fold change: >1.2 or <0.83; q value: <0.1%) indicating a distinct molecular phenotype of chronic phase CML CD34+ cells. A total of 1420 genes had a higher and 119 genes a lower expression in CML (Fig. 8.2.A, page 61).

A hierarchical cluster analysis was performed using an average linkage clustering algorithm. The analysis showed the homogeneity of the samples and could clearly

[1] www.ncbi.nlm.nih.gov/geo/; accession no.: GSE5550

distinguish between normal and CML CD34+ cells. (Fig. 8.1)

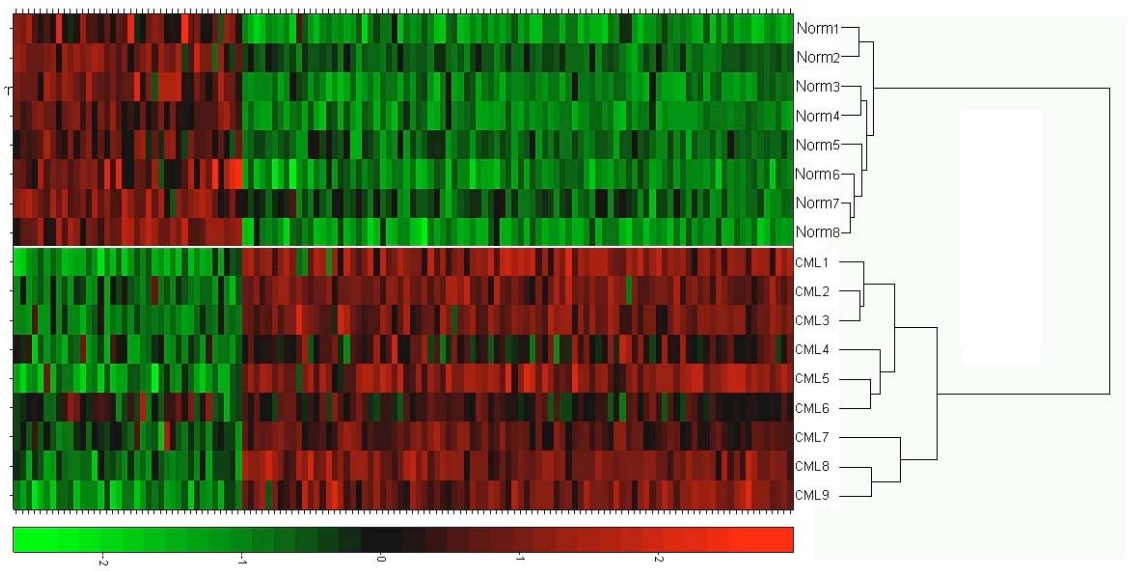


Figure 8.1: Hierarchical cluster analysis distinguishes bone marrow- and peripheral blood-derived CD34+ cells. All 17 CD34+ cells samples derived from bone marrow either of healthy donors or CML patients were subjected to hierarchical cluster analysis on the basis of 288 differentially expressed genes which were at least 1.5-fold differentially expressed. Median-centered and normalized data are displayed using a color code, shown at the bottom of the figure. Red fields indicate higher values than the median, green fields indicate lower values than the median, and black fields indicate values that are equal to the median. The dendrogram visualizes the similarity of the different subgroups. Branch nodes connect closely related samples. The branch length indicates the degree of relationship, so that the shorter a branch is, the more similar are the connected samples. Two main clusters are clearly distinguishable, one containing all 8 normal control CD34+ cell samples and one containing all 9 CML CD34+ cell samples.

8.2. Comparison of gene expression profiles between bone marrow and peripheral blood CML CD34+ cells

In a recent study, significant differences were shown between the molecular phenotypes of BM-derived and circulating normal CD34+ cells (Steidl et al., 2002). Therefore, this fact was investigated in this work for leukemic CD34+ cells. Comparing the expression profiles of sedentary leukemic CD34+ cells from BM with those of circulating CD34+ cells from peripheral blood of untreated patients with CML no differ-

entially expressed genes with sufficient significance (q value: <5%) were found (Fig. 8.2.B).

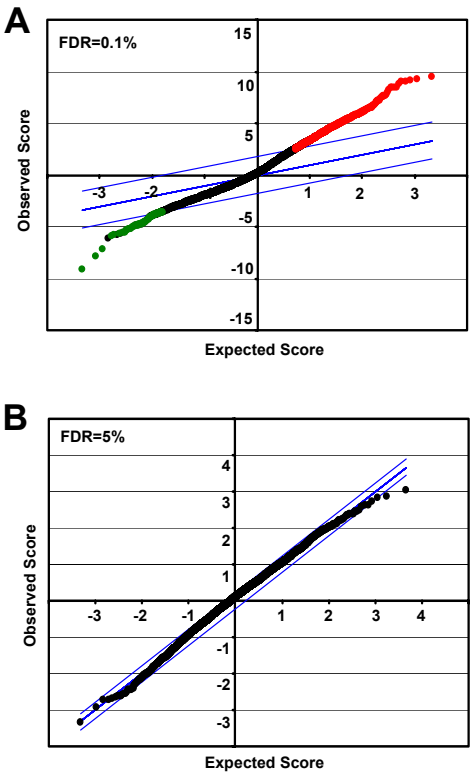


Figure 8.2: Identification of significantly differentially expressed genes. Significance Analysis of Microarrays (SAM) algorithm comparing normal BM with CML BM CD34+ cells (A) and CML BM with CML PB CD34+ cells (B). Significantly up- and down-regulated genes are indicated in red and green, respectively. Blue lines indicate cut-off levels for significant differential expression for estimated false discovery rates (FDR) of 0.1% (A) and 5% (B).

8.3. Analysis of differentially expressed genes

To summarize, comparing malignant BM CD34+ cells from patients with CML with normal BM CD34+ cells several genes were significantly differentially expressed, indicating a distinct molecular phenotype of chronic phase CML CD34+ cells. In the following, a selection of differentially expressed genes is described, by allocating genes into functional groups (Table 8.1, page 66).

Those groups include genes involved in BCR-ABL signaling, proliferation and apoptosis, genes encoding adhesion proteins and cytoskeletal proteins, genes encoding

growth factors and their receptors, genes associated with early hematopoietic stem cells and their self-renewal, genes involved in fatty acid and lipid metabolism and genes encoding several surface receptors.

8.3.1. BCR-ABL signaling

First, an up-regulation of genes encoding substrates of BCR-ABL was found. The BCR-ABL adapter proteins Grb2 and Crkl, as well as the signaling molecules STAT3, STAT4, STAT5, VAV3 and the Ras GTPase activating protein G3BP2 were higher expressed.

Next, BCR-ABL-activated mitogenic signaling pathways such as Ras/ mitogen-activated protein kinase (MAPK), PI3 kinase and MYC pathways were studied. Of the classical Ras/MAPK pathway Ras, the Ras-related proteins Rac-1 and Rac-2, the downstream serine-threonine kinase MEK1 as well as ERK were significantly up-regulated whereas the ERK inhibitor dual specific phosphatase 1 (DUSP1) was down-regulated in primary BCR-ABL-positive cells. Of the alternative JNK MAPK pathway the germinal center kinase related protein (GCKR; MAP4K5) and Rac-1 as well as Rac-2 were higher expressed. The transcriptional target of MAPK pathways MYC was significantly up-regulated as well as the heterogenous nuclear ribonucleoprotein K (HNRPK). Out of the genes involved in the PI3 (phosphoinositide 3) kinase pathway, besides components of the PI3 kinase itself, its substrate AKT and the downstream molecules NF-κB and BclXL were significantly up-regulated in CML CD34+ cells.

In summary, several substrates of BCR-ABL as well as proteins of BCR-ABL signaling pathways were significantly up-regulated in primary CML CD34+ cells.

8.3.2. Proliferation and apoptosis

Several genes encoding for cell cycle promoting proteins were up-regulated in BCR-ABL-positive CD34+ cells such as the cell division cycle (CDC) proteins 16, 23, 27 and 37, the cyclins H, G1, the cyclin-dependent kinases (CDK) 2, 7 and 8, the CDK2-associated protein, the minichromosome maintenance protein 3, the origin recognition complex subunits 2, 4, 5, the DNA polymerase epsilon 2 (POLE2) and 3 (POLE3) as well as the transcription factor Dp-1. With respect to apoptosis-related proteins the pro-apoptotic key proteins caspases 3, 6 and 10 and the FAS receptor were significantly up-regulated in BCR-ABL-positive CD34+ cells. On the other hand, the anti-apoptotic protein BclXL was also up-regulated in CML. The interferon regula-

tory factor 8 (IRF8, interferon consensus sequence binding protein) was significantly down-regulated.

Thus, CML CD34+ cells showed a higher expression level of proliferation-associated genes and down-regulation of the pro-apoptotic interferon regulatory factor 8.

8.3.3. Focal adhesion and cytoskeleton proteins

Several differentially expressed genes involved in progenitor cell adhesion and migration were founded. Those included genes involved in focal adhesion, adhesion molecules, and cytoskeleton proteins.

The BCR-ABL adapter protein Crkl which facilitates complex formation with focal adhesion proteins and is involved in regulation of cellular motility was up-regulated in CML CD34+ cells. Among the Crkl-associated adhesion proteins vinculin, talin, parvin and filamin were also significantly up-regulated in CML.

Looking at changes in integrin expression, only the integrins αE and α2b were up-regulated. SRC kinase LYN was higher expressed in CML CD34+ cells. LYN positively regulates CD34+ cell movement and lowers adhesion of CD34+ cells to stromal cells by inhibiting the ICAM-1-binding activity of α2 integrins.

Interestingly, several proteins involved in regulation of the actin cytoskeleton were deregulated in CML CD34+ cells in comparison with normal cells. The BCR-ABL associated cytoskeletal protein, actin gamma 1, a component of f-actin, that regulates cell adhesion, had a significantly lower expression level, whereas the regulators of the actin cytoskeleton, Rho GTPases Rac1 and Rac2, were significantly up-regulated in CML CD34+ cells.

Additional adhesion molecules, ICAM-4, cadherin 1 (E-cadherin) and the cadherin-associated protein catenin α1 were up-regulated and L-selectin and cadherin 2 (N-cadherin), a molecule involved in the interaction of the stem cell and its niche, were down-regulated in CML CD34+ cells.

Finally, the chemokine receptors CXCR4 and CCR2, which are involved in stem cell and monocyte migration, respectively, were significantly down-regulated in CML CD34+ cells.

In summary, CML CD34+ cells had an increased expression of focal adhesion proteins and a reduced expression of L-selectin and N-cadherin.

8.3.4. Growth factors and their receptors

Looking at genes of the TGFβ signaling pathway, an up-regulation of macrophage colony-stimulating factor, IL1β, IL7 and TGFβ1 as well as of the GM-CSF receptor was observed. In turn, IL7 receptor and FLT3 were significantly down-regulated in leukemic progenitor cells. Looking in detail on the TGFβ signaling pathway, TGFβ1 itself as well as SMAD2, SMAD4 and the transcription factor Dp-1 (TFDP1) were significantly up-regulated in CML CD34+ cells. The neuroepithelial cell transforming gene 1 (NET1) which is induced upon TGFβ stimulation was also up-regulated in CML CD34+ cells.

To summarize, an up-regulation of genes of the TGFβ signaling pathway was observed.

8.3.5. Hematopoiesis and differentiation

Several genes associated with early hematopoietic stem cells and their self-renewal were significantly up-regulated in CML CD34+ cells. These genes include GATA2, homeo box genes HoxA9 and HoxA10, MEIS1, zinc finger protein Ikaros, Tal-1, lim-finger protein Lmo-2, member of the Polycomb group Bmi-1 and aldehyd dehygrogenase ALDH1A1.

Looking at genes involved in differentiation we found that GATA1 was significantly up-regulated and myeloperoxidase (MPO), neutrophil elastase 2 (ELA2) and C/EBPδ, genes expressed during later stages of myeloid differentiation, were down-regulated in CML CD34+ cells. Two of the central genes promoting lymphoid differentiation, GATA3 and IL-7 receptor α, as well as the B cell differentiation-associated genes terminal desoxynucleotidyltransferase, pre-B lymphocyte gene 1 (VPREB1), B-cell linker (BLNK) and BCL-6 were downregulated in CML CD34+ cells. On the other hand, erythropoiesis-associated genes such as erythropoietin receptor, Kruppel-like factor 1 and transferrin receptor were higher expressed in CML CD34+ cells.

An interesting finding was an up-regulation of fetal hemoglobin (Hb) components such as Hbγ1, Hbγ2 and Hbδ as well as Hbβ whereas the genes for adult hemoglobin chains Hbα1 and Hbα2 were not altered indicating an induction of fetal hemoglobin synthesis in CML progenitor cells.

In general, CML CD34+ cells showed an expression pattern characteristic for immature stem cells as well as megakaryocyte-erythrocyte progenitor cells.

8.3.6. Fatty acid, lipid and protein metabolism

Several genes involved in fatty acid and lipid metabolism were found to be up-regulated in CML CD34+ cells. For example, the isoenzymes of the long-chain fatty-acid-coenzyme A ligases ACSL (acyl-CoA synthetase long-chain family member) 1,4 and 5, were higher expressed in leukemic CD34+ cells in comparison to normal cells. Furthermore, fatty acid synthase (FASN) which catalyzes the synthesis of long-chain saturated fatty acids, and hydroxyacyl-coenzyme A dehydrogenase alpha (HADHA), which promotes oxidation of fatty acids, were also up-regulated in CML.

In addition, several genes involved in the ubiquitin-proteasome system such as the ubiquitin-conjugating enzymes E2M, E2G1, E2G2, E2G3, E2E1, E2N, E2D2, E2D3, the SUMO-1 activating enzyme (UBA2), the F-box protein 7, UBADC1, and the proteasome subunits alpha 1, 2, 3, 4, 7 and subunits beta 1, 2, 5 were significantly up-regulated.

In summary, an up-regulation of genes involved in fatty acid metabolism and in the ubiquitin-proteasome system was observed in CML cells.

8.3.7. Surface receptors and other genes

Several surface receptors, which have not been mentioned in the context of the previously addressed functional groups were also deregulated in CML CD34+ cells. As examples, the receptor for leptin and the surface molecule CD36 (Thrombospondin receptor) were up-regulated in CML CD34+ cells. Other interesting genes, which were up-regulated in CML CD34+ cells, were sorcin and the gene DLC1 (deleted in liver cancer 1), which activates Rho GTPases and modulates the organization of the cytoskeleton. Moreover, the tissue inhibitor of metalloproteinase 1 (TIMP1) was also up-regulated in CML CD34+ cells while the selenoprotein P (SEPP1), which is involved in response to oxidative stress, was down-regulated in CML CD34+ cells.

Table 8.1: Selection of genes with significantly differential gene expression in CD34+ cells from patients with CML in comparison with those from healthy volunteers

Gene Symbol	Gene Title	Fold Change (CML/normal)
BCR-ABL signaling		
AKT1	v-akt murine thymoma viral oncogene homolog 1	1.36
BCL2L1	BCL2-like 1 (BCL-XL)	1.35
CRKL	v-crk sarcoma virus CT10 oncogene homolog (avian)-like	1.42
G3BP2	Ras-GTPase activating protein SH3 domain-binding protein 2	1.39
GRB2	growth factor receptor-bound protein 2	1.20
HNRPK	heterogeneous nuclear ribonucleoprotein K	1.20
KRAS	v-Ki-ras2 Kirsten rat sarcoma viral oncogene homolog	1.44
LYN	V-yes-1 Yamaguchi sarcoma viral related oncogene homolog	1.56
MAP2K1	mitogen-activated protein kinase kinase 1 (MEK1)	1.21
MAP4K5	mitogen-activated protein kinase kinase kinase kinase 5 (GCKR)	1.31
MAPK1	mitogen-activated protein kinase 1 (ERK2)	1.42
MYC	v-myc myelocytomatosis viral oncogene homolog	1.85
NFKB1	NF kappa B 1 (p105)	1.21
NRAS	neuroblastoma RAS viral (v-ras) oncogene homolog	1.26
PIK3C3	phosphoinositide-3-kinase, class 3	1.28
PIK3CA	phosphoinositide-3-kinase, catalytic, alpha polypeptide	1.20
PIK3CB	phosphoinositide-3-kinase, catalytic, beta polypeptide	1.35
RAC1	ras-related C3 botulinum toxin substrate 1	1.27
RAC2	ras-related C3 botulinum toxin substrate 2	1.41
STAT3	signal transducer and activator of transcription 3	1.26
STAT4	signal transducer and activator of transcription 4	1.32
STAT5A	signal transducer and activator of transcription 5A	1.42
VAV3	vav 3 oncogene	1.25
DUSP1	dual specificity phosphatase 1	0.51
Proliferation and apoptosis		
CASP3,6,10	caspases 3,6,10	1.31 – 2.30
CCNG1,H	cyclins G1, H	1.36 – 1.72
CDC	cell division cycle proteins 16,23,27,37	1.30 – 1.32
CDK2,7,8	cyclin-dependent kinases 2,7,8	1.34 – 1.59
CDK2AP1	CDK2-associated protein 1	1.45
FAS	FAS	1.26
MCM3	minichromosome maintenance deficient 3	1.38
ORC2,4,5L	origin recognition complex, subunit 2-like (4-like, 5-like)	1.26 – 1.50
POLE2	DNA polymerase, epsilon 2 (p59 subunit)	1.47
POLE3	DNA polymerase, epsilon 3 (p17 subunit)	1.26
TFDP1	transcription factor Dp-1	1.37
IRF8	interferon binding protein 8	0.23
Cytoskeleton, adhesion and stem cell niche		
CADH1	E-cadherin	1.94
CTNNA1	catenin, alpha 1	1.33
FLNA	filamin A, alpha	1.37
ICAM4	intercellular adhesion molecule 4	1.88
ITGAE,2B	integrin, alpha E/alpha 2b	1.31 – 2.74
PARVB	parvin, beta	1.71
TEK	tyrosine-protein kinase receptor Tie-2	1,34

Continued on next page

Table 8.1 – continued from previous page

Gene Symbol	Gene Title	Fold Change(CML/normal)
TLN1	talin	1.38
VCL	vinculin	1.46
ACTG1	actin, gamma 1	0.71
CADH2	N-cadherin	0.72
FLNB	filamin B, beta	0.69
SELL	L-selectin	0.41
Growth factors and their receptors		
CSF1	macrophage colony stimulating factor	1.41
CSF2RB	granulocyte-macrophage colony-stimulating factor receptor	2.35
IL1B	interleukin 1, beta	1.63
IL7	interleukin 7	1.35
NET1	neuroepithelial cell transforming gene 1	2.66
SMAD2,4	mothers against DPP homolog 2,4	1.35 – 1.55
TGFB1	transforming growth factor, beta 1	1.94
FGFR1	fibroblast growth factor receptor 1	0.75
FLT3	fms-related tyrosine kinase 3	0.44
IL7R	interleukin 7 receptor	0.69
Self-renewal and early stem cells		
ALDH1A1	aldehyde dehydrogenase 1 family, member A1	3.28
GATA2	GATA binding protein 2	1.85
HOXA10	homeo box A10	1.36
HOXA9	homeo box A9	1.48
LMO2	LIM domain only 2 (rhombotin-like 1)	1.38
MEIS1	myeloid ecotropic viral integration site 1 homolog	1.44
PBX3	pre-B-cell leukemia transcription factor 3	1.79
PCGF4	polycomb group ring finger 4	1.38
TAL1	T-cell acute lymphocytic leukemia 1	1.95
ZNFN1A1	zinc finger protein, subfamily 1A, 1 (Ikaros)	1.28
Differentiation		
EPOR	erythropoietin receptor	1.22
GATA1	GATA binding protein 1	1.99
HBB	hemoglobin, beta	2.87
HBD	hemoglobin, delta	4.06
HBG1/G2	hemoglobin, gamma A / gamma G	3.94
KLF1	Kruppel-like factor 1 (erythroid)	2.32
TFR2	transferrin receptor 2	2.54
BCL6	B-cell CLL/lymphoma 6	0.59
BLNK	B-cell linker	0.29
CEBPD	CCAAT/enhancer binding protein (C/EBP), delta	0.54
DNTT	terminal deoxynucleotidyltransferase	0.24
ELA2	elastase 2, neutrophil	0.15
GATA3	GATA binding protein 3	0.81
MPO	myeloperoxidase	0.34
VPREB1	pre-B lymphocyte gene 1	0.33
Fatty acid and lipid metabolism		
ACSL1,4,5	acyl-CoA synthetase long-chain family members 1,4,5	1.29 – 1.50
FASN	fatty acid synthase	1.31

Continued on next page

Table 8.1 – continued from previous page

Gene Symbol	Gene Title	Fold Change(CML/normal)
HADHA	hydroxyacyl-Coenzyme A dehydrogenase, alpha subunit	1.32
Receptors		
CD36	thrombospondin receptor (CD36)	2.25
F2R	coagulation factor II (thrombin) receptor (PAR1)	1.45
FCGR2A	Fc fragment of IgG receptor IIa (CD32)	1.40
GPR56	G protein-coupled receptor 56	1.61
LEPR	leptin receptor	4.12
P2RX5	purinergic receptor P2X5	1.48
CCR2	chemokine (C-C motif) receptor 2	0.68
CXCR4	chemokine (C-X-C motif) receptor 4	0.65
INSR	insulin receptor	0.76
Miscellaneous		
DLC1	deleted in liver cancer 1	2.27
SRI	sorcin	1.40
TIMP1	tissue inhibitor of metalloproteinase 1	1.82
SEPP1	selenoprotein P, plasma, 1	0.44

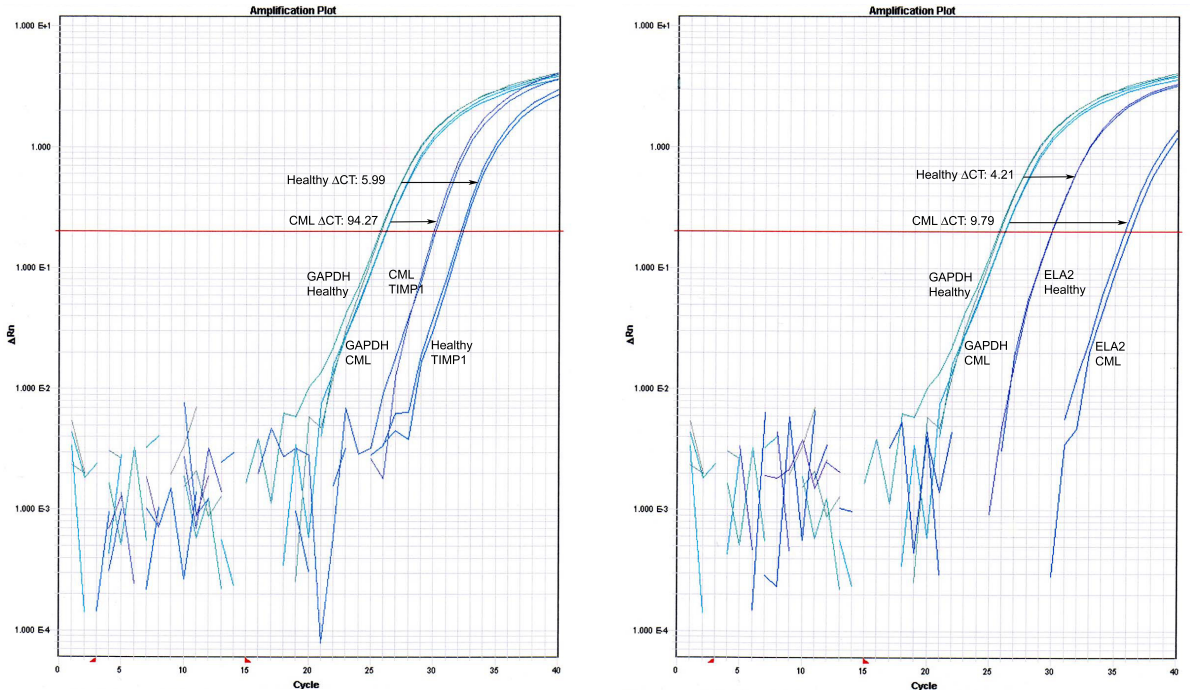
9. Corroboration of selected genes by RT-PCR and flow cytometry

To corroborate the data obtained by cDNA array technology, the expression of selected genes was surveyed by quantitative real-time reverse transcription-polymerase chain reaction (RT-PCR) and fluorescence-activated cell sorter analysis.

9.1. Corroboration by RT-PCR

For corroboration of the microarray data, the mRNA expression level of 8 genes was assessed by quantitative real-time reverse transcription-polymerase chain reaction (RT-PCR). The reverse transcribed mRNA of highly enriched CD34+ cells from five samples from CML and five samples from healthy volunteers was studied using the ABI PRISM 7900HT Sequence Detection System from Applied Biosystems. Following genes were studied: GATA binding protein 1 (GATA1), Leptin receptor (LEPR), T-cell acute lymphocytic leukemia 1(TAL1), Tissue inhibitor of metalloproteinase 1 (TIMP1), Acyl-CoA synthetase long-chain family member 5 (ACSL5), corticotropin releasing hormone binding protein (CRHBP), Selenoprotein P (SEPP1) and Elastase 2 (ELA2). The glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as reference gene (Fig. 9.1, page 70).

Quantitative real-time RT-PCR confirmed that TAL1 and GATA1 ($p<0.01$), as well as LEPR, TIMP1 and ACSL5 ($p<0.05$) were significantly higher expressed in CML-CD34+ cells than in normal-CD34+ cells. On the other hand, the genes CRHBP and SEPP1 ($p<0.01$) and ELA2 ($p<0.05$) were significantly lower expressed in CML-CD34+ cells. All these findings were in line with the microarray data (Fig. 9.2, page 71).



(a) Up-regulation of TIMP1 (b) Down-regulation of ELA2

Figure 9.1: Corroboration of differential mRNA expression by quantitative real-time RT-PCR. Real-time RT-PCR curves of two differentially expressed genes with the respective GAPDH control are displayed. A representative up-regulated gene is shown on the left, and a representative down-regulated gene is shown on the right side. For each target one normal- and one CML- derived samples were measured in duplicate in the same RT-PCR run and displayed in the same amplification plot to directly compare the results. The difference of the CT values of target and GAPDH control (Δ CT) reflect the relative amount of target mRNA in each sample. The smaller the delta CT, the higher the relative abundance of the target's mRNA.

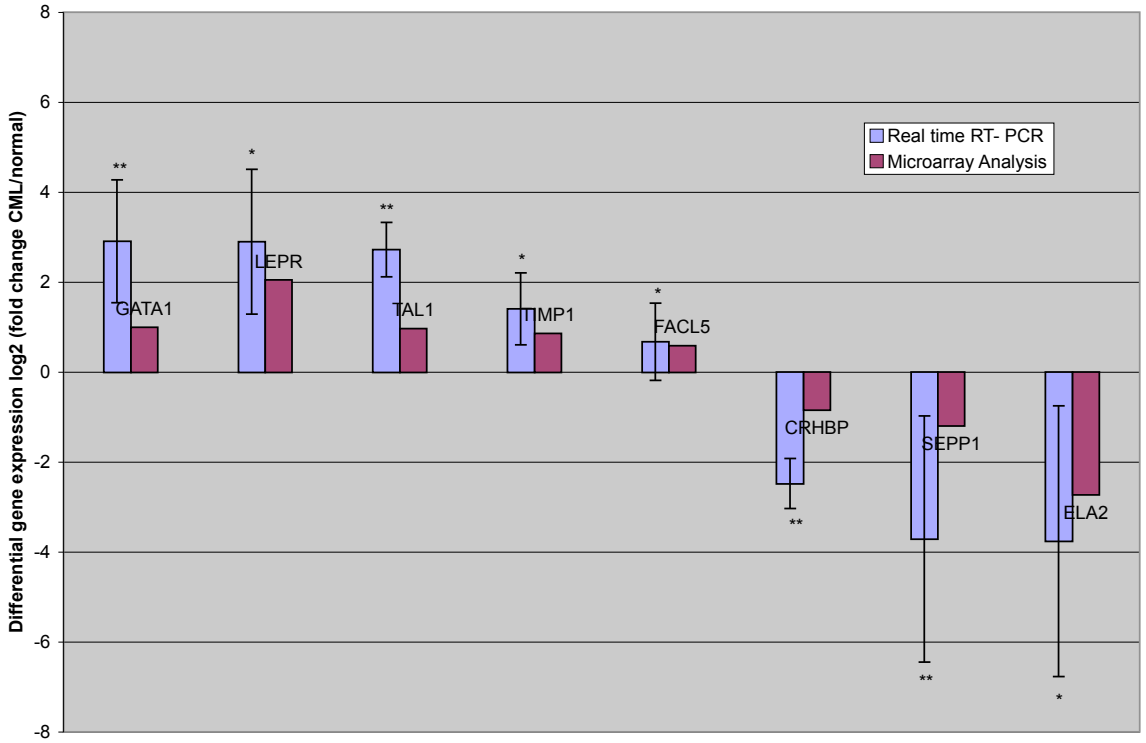


Figure 9.2: Comparison of differential gene expression measured by microarray technology and quantitative real-time RT-PCR. Fold changes determined using RT-PCR are shown in blue columns, fold changes assessed by the SAM software from microarray analysis are indicated in red columns. Upward-pointing bars indicate a higher expression in CML CD34 + cells than in normal CD34+ cells. Downward-pointing bars indicate a higher expression in normal CD34+ cells than in CML CD34 + cells. Y axis is scaled according to log base 2. Expression of all genes assessed by RT-PCR was significantly different (*P<0.05 and **P<0.01). Results from RT-PCR were based on five samples from CML and five samples from healthy volunteers. Each analysis was performed in duplicate.

9.2. Corroboration by flow cytometry

To corroborate the data obtained by cDNA array technology, the differential gene expression of selected genes was also surveyed by flow cytometry.

First, FACS analysis of highly enriched CD34+ cells was performed to confirm that the leptin receptor and the Thrombospondin receptor (CD36 antigen) were also expressed at the protein level. Goat polyclonal antibodies were raised against two peptides mapping at N and C termini of the surface receptor for leptin, and a FITC- conjugated monoclonal antibody was directed against the CD36 antigen surface molecule (Fig. 9.3).

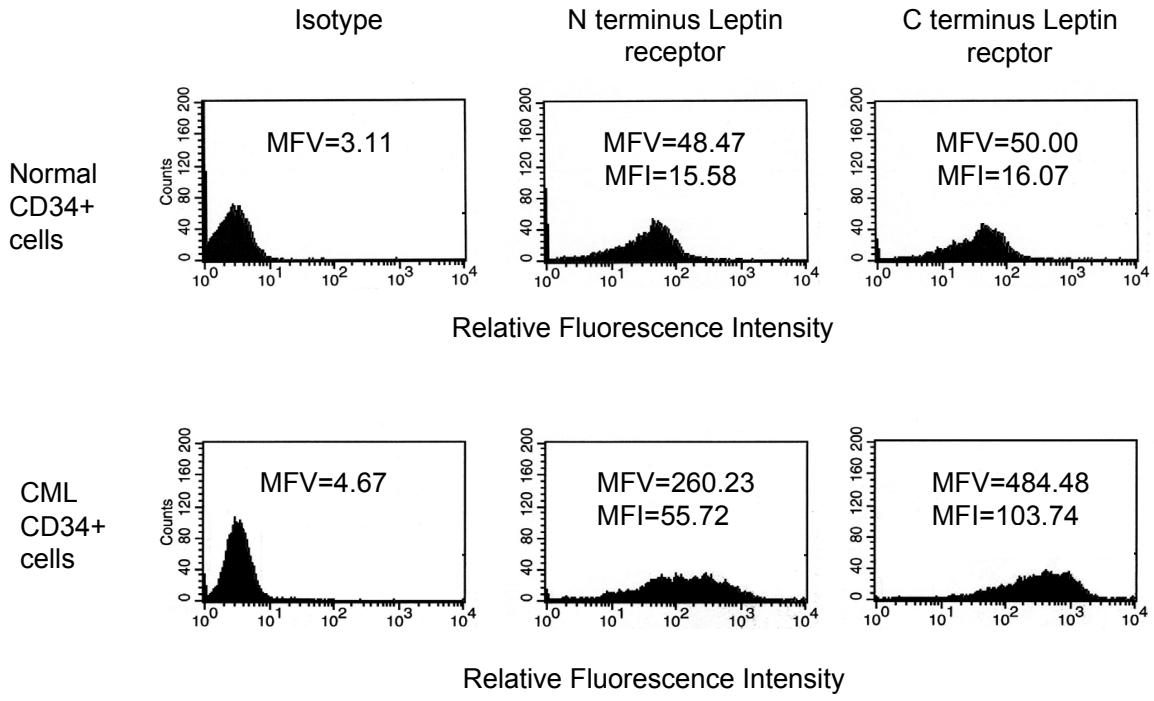


Figure 9.3: Representative experiments of fluorescence-activated cell sorter analysis of the leptin receptor on normal and malignant CD34+ cells. Goat polyclonal antibodies were directed the N and C termini of the surface receptor for leptin on highly enriched CD34+ cells from a healthy donor and a CML patient. Mean fluorescence value (MFV), which reflects the median fluorescence intesity per cell, is indicated for each histogram. Mean fluorescence Index (MFI), representing the surface receptor expression, was set according to the isotype control (MFI= MFV of receptor specific antibodies/ MFV of Isotype).

Afterwards, in order to examine a possible differential expression of these receptors between normal and leukemic CD34+ cells, FACS analysis of highly enriched CD34+ cells from four samples from CML and three samples from healthy volunteers were

subjected to FACS analysis. For the N and the C terminus of the leptin receptor the expression was 3.3-fold and 3.7-fold higher on CML CD34+ cells than in normal progenitor cells respectively, whereas no significant difference could be detected for the CD36 receptor (Fig. 9.4). The findings for the leptin receptor were in line with the microarray data.

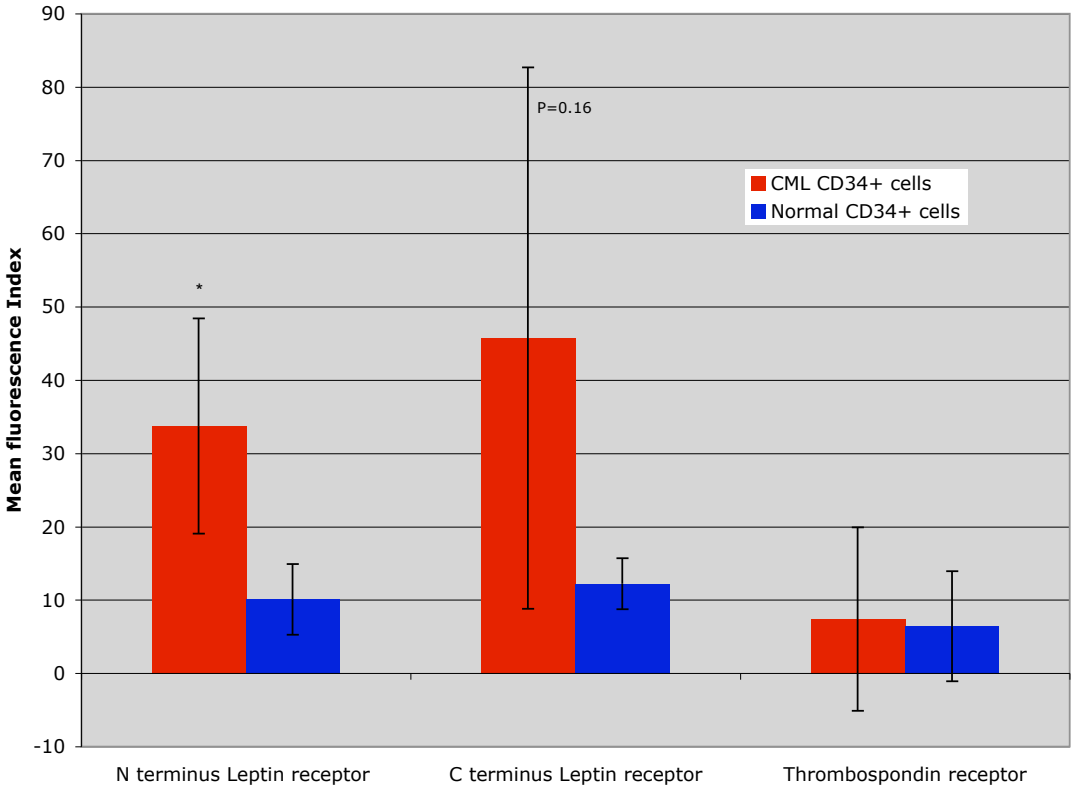


Figure 9.4: Summary of differential expression of leptin and Thrombospondin receptors on normal and malignant CD34+ cells by flow cytometry. FACS analysis of highly enriched CD34+ cells confirmed at the protein level a higher expression of N and C terminus of the leptin receptor in leukemic CD34+ cells compared to normal CD34+cells. Results were based on the median Mean Fluorescence Index (MFI) of 3 (normal CD34+ cell) and 4 (CML CD34+ cells) independent samples. The lines in each bar represent the standard deviation. Statistically significant differential expression of the N terminus of leptin in CML cells referring to normal cells is indicated (*P < .05). For the C terminus of leptin receptor, this difference could not be shown with sufficient statistical significance. P-value is indicated.

10. Assessment of selected genes in the leukemic cell line K562

At this point, a differential expression of 8 selected genes was demonstrated (GATA1, LEPR, TAL1, TIMP1, ACSL5, CRHBP, SEPP1 and ELA2) in CML CD34+ cells in comparison with normal CD34+ cells by microarray technology and quantitative real-time RT-PCR. In order to perform different assays to check for functional impact, it was necessary to investigate wether these genes may be present, not only in primary CML CD34+ cells, but also in a leukemic cell line. In order to answer this question, the leukemic cell line K562 was chosen. The total RNA of K562 cells was isolated, and reversely transcribed. Using the ABI PRISM 7900HT Sequence Detection System and aasays-on-demand from Applied Biosystems, the mRNA expression levels of all the selected genes except ELA2 could be assessed by quantitative real-time RT-PCR (Fig. 10.1).

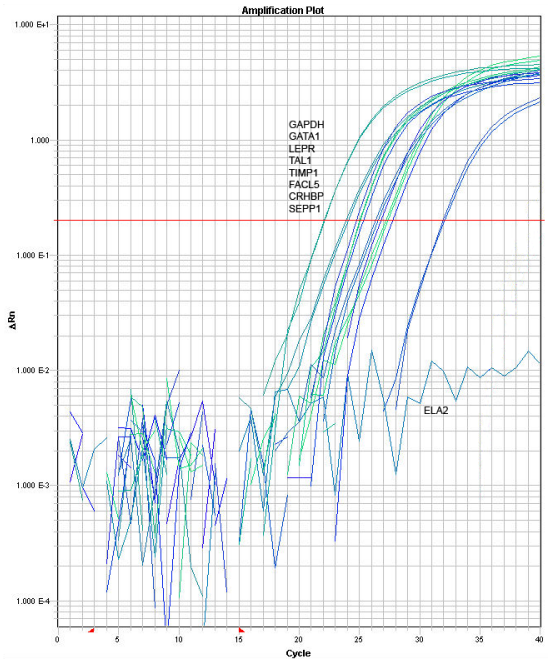


Figure 10.1: Assesment of mRNA expression of selected genes in the leukemic cellular line K562 by quantitative real-time RT-PCR. The mRNA expression level of GATA1, LEPR, TAL1, TIMP1, ACSL5, CRHBP, SEPP1 and GAPDH was assessed in leukemic K562 cells by quantitative real-time RT-PCR. The expression of the gene ELA2 remained undetermined. A representative amplification plot is displayed. Each target was measured in duplicate.

Even more, fluorescence-activated cell sorting analysis of K562 cells could confirm that the C terminus of leptin receptor was expressed at the protein level as well (Fig. 10.2).

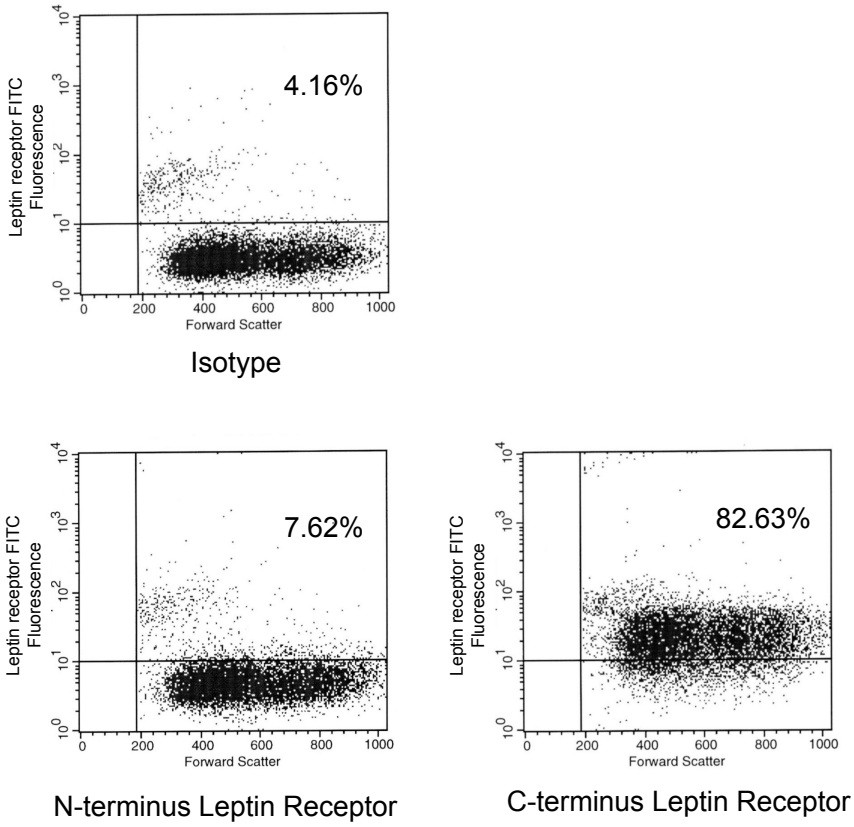


Figure 10.2: Fluorescence- activated cell sorting analysis of the N and C terminus of the leptin receptor on K562 cells. (Upper Dot plot) K562 cells stained with the isotype-identical control antibody. (Lower Dot plots) K562 cells stained with Goat polyclonal antibodies directed against peptides mapping at the N and C terminus of the leptin receptor. Percentages of positive cells above the threshold intensity are indicated.

11. Functional examination of candidate genes

Comparing malignant CD34+ cells from patients with CML with normal CD34+ cells, several genes were significantly differentially expressed, indicating a distinct molecular phenotype of chronic phase CML CD34+ cells. The next step in this work was trying to assess the functional meaning of this deregulation in leukemic cells.

Some candidates genes were chosen to perform different assays to check for functional impact. In particular, it was investigated the transcriptional activation of the lipid metabolism pathway including the leptin receptor (LEPR) and some genes involved in fatty acid and lipid metabolism, as the isoenzymes of the long-chain fatty-acid-coenzyme A ligases 1,4 and 5 (ACSL) and the fatty acid synthase (FASN).

There were specific decision criterions for the election of these candidates genes. For one side, the mRNA expression levels of both LEPR and ACSL5 genes had been assessed in normal and CML CD34+ cells and in the cellular line K562 by RT-PCR as described before, allowing the possibility of performing different cellular culture experiments with those cells. On the other hand, all these genes share the characteristic of being involved in energy expenditure and metabolical processes and they appeared to be of high interest due to their their relation with tumor formation. The isozyme encoded by ACSL5 has markedly increased levels in malignant gliomas, so that this gene has been described to mediate fatty acid-induced glioma cell growth [103] while the leptin receptor has been related to breast cancer [104] and myeloid leukemia [105][106]. Even more, high levels of FASN expression have been observed in several cancers, including breast, prostate, colon and lung carcinoma compared with their respective normal tissue [107].

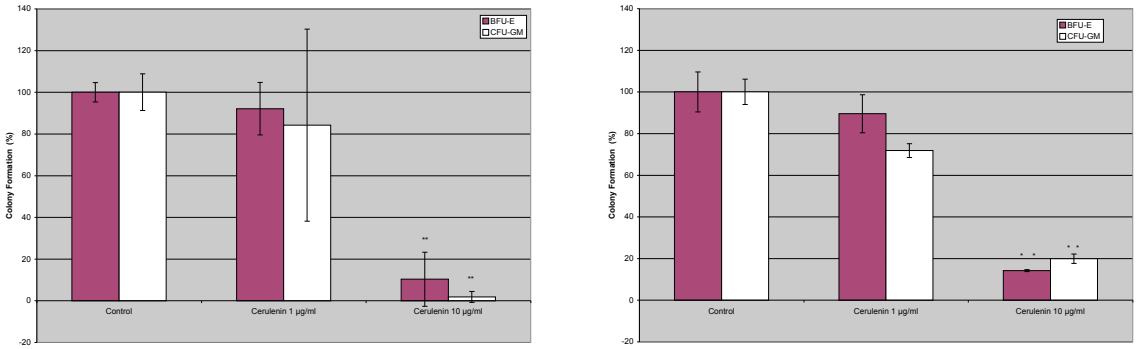
To investigate the functional meaning of the up regulation of these interesting genes in the chronic myeloid leukemia, they were studied in detail by performing different functional assays.

11.1. Clonogenic Growth

To asses if the upregulation of genes involved in fatty acid metabolism and the leptin receptor had a functional impact in the clonogenic growth of leukemic CD34+ cells, cells from PB of patients with CML were subjected to semisolid methylcellulose clonogenic assays in the presence or absence of the fatty acid synthesis inhibitor cerulenin and an specific agonist or antagonist of the leptin receptor. BFU-E and CFU-GM colonies were counted after two weeks.

11.1.1. Genes involved in fatty acid and lipid metabolism

Very interestingly, incubation of cells with 10μg /ml and 1μg /ml of cerulenin resulted in a significant ($p<0.01$) dose-dependent inhibition of clonogenic growth of CFU-GM by 84.21% and 1.82% as well as of BFU-E by 92.07% and 10.37 % (Fig. 11.1.a).



(a) CML CD34+ cells

(b) normal CD34+ cells

Figure 11.1: Differential clonogenic growth of CML and normal progenitor cells following receptor modulation with the fatty acid synthesis inhibitor cerulenin. Highly enriched CD34+ cells from PB of three patients with CML (a) and three healthy volunteers (b) were examined by semisolid methylcellulose clonogenic assays in the presence or absence of cerulenin. BFU-E (red) and CFU-GM (white bars) colonies were counted after two weeks. Each experiment was performed in duplicate. Administration of 1μg /ml and 10μg /ml of cerulenin resulted in a concentration-dependent inhibition of the clonogenic growth referring to the untreated control. Mean values and standard deviations are shown. Statistically significant ($P < .01$) inhibition is indicated by asterisks.

In order to compare this inhibitor effect of cerulenin on the clonogenic growth of leukemic CD34+ cells with the effect on normal cells, the same experiment was performed with immunomagnetically enriched CD34+ cells from healthy donors. By incubating the cells with 10μg /ml of cerulenin, a reduction of CFU-GM formation was observed, but interestingly, this inhibition was not as strong as in leukemic cells,

measuring a clonogenic growth of 19.89%. This dissimilarity reflected a differential expression of the genes of the fatty acid metabolism pathways in CML and normal CD34+ cells. The inhibition of CFU-GM formation after the incubation with 1μg /ml cerulenin was similar to the leukemic cells, showing a reduction of 71.82%. The inhibition of BFU-E formation showed similar results as in the leukemic cells: following incubation with 1μg /ml and 10μg /ml of cerulenin, a reduction of colony formation of 89.50% and 14.16% was measured respectively (Fig. 11.1.b, page 77).

Thus, incubation of CML CD34+ cells with the fatty acid synthesis inhibitor cerulenin resulted in a dose-dependent decrease in clonogenic growth. Even more, the inhibition of CFU-GM formation was stronger in leukemic than in normal samples.

11.1.2. Leptin receptor

Incubation of CML CD34+ cells with 100 ng/ml, 50 ng/ml and 10 ng/ml of agonist for LEPR alone or in the presence of a equimolar concentration of antagonist did not influence clonogenic growth of the cells, indicating that the modulation of the leptin receptor does not affect the growth and differentiation of CML hematopoietic progenitor cells (Fig. 11.2).

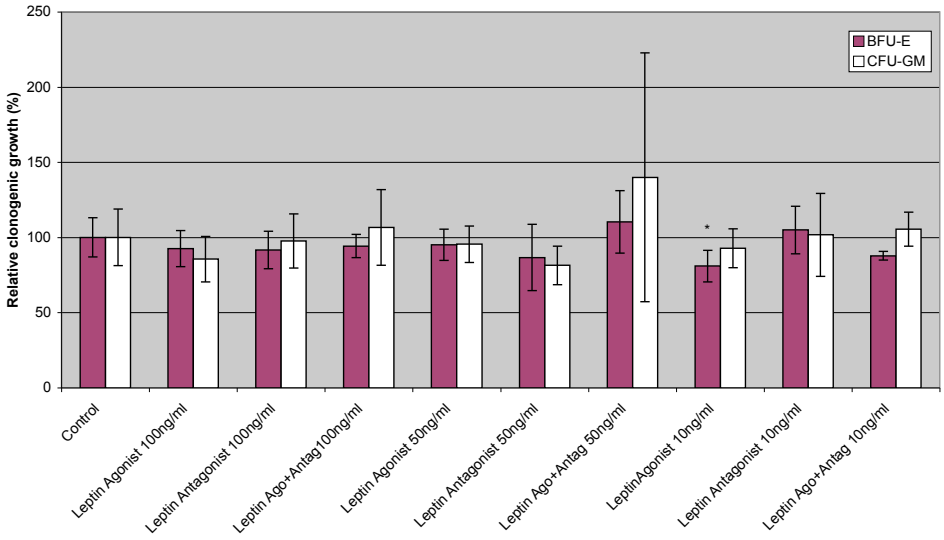


Figure 11.2: Clonogenic growth of CML progenitor cells following modulation of leptin receptor. The clonogenic growth of highly enriched CD34+ cells from PB of three patients with CML was examined by semisolid methylcellulose clonogenic assays incubating the cells with 100 ng/ml, 50 ng/ml and 10 ng/ml of leptin receptor agonist and/or antagonist. BFU-E (red) and CFU-GM (white bars) colonies were counted after two weeks. Every experiment was performed in duplicate. Mean values and standard deviations are shown. Statistical significance (*P < .05) is indicated.

11.2. Proliferation

The fatty acid synthesis inhibitor cerulenin has been shown to inhibit the proliferation of leukemic cell lines HL60 [108] and K562 [109] and leptin has been described to increase spontaneous AML blast proliferation [110]. To check whether a modulation of the fatty acid synthesis metabolism had a functional impact in the proliferation of leukemic cells, CD34+ cells from CML patients were stimulated with cerulenin. To asses the functional role of LEPR on cellular proliferation the cells incubated with agonists or antagonist of the leptin receptor. The proliferation was measured using the 3[H]-Thymidin (Tritium-Thymidin) uptake assay and additionally suspension cultures were performed along four days.

11.2.1. Genes involved in fatty acid and lipid metabolism

The inhibition of the proliferation of cells stimulated with 10 μg /ml of cerulenin could be shown after 3 hours incubation, where the cells showed a strong inhibition of the proliferation by 93.64 %. The incubation of the cells with only 1 μg /ml of cerulenin did not inhibit the proliferation of the cells which remained similar to the not stimulated control after this time (Fig. 11.3, page 80).

To investigate this results in detail, the effect of cerulenin on the proliferation of CML cells was studied along 4 days of suspension cultures. Even more, in order to compare the effect of cerulenin on CML cells with the effect on normal cells, CD34+ cells from healthy donors were used for these experiments as well. Moreover, the CML cell line K562 was also submited to this investigation. All the samples were stimulated daily with cerulenin and growth was measured determining the cell number with Trypan blue and a Hemocytometer. Incubation of the leukemic cells (CML and K562 cells) with the fatty acid synthesis inhibitor cerulenin (10 μg /ml or 1 μg /ml) resulted in a significant dose- and time- dependent inhibition of cell growth. After 4 days stimulation with 10 μg /ml, both cell growth from leukemic and normal cells was completely inhibited, founding no more vital cells at all in the suspension culture. Analyzing in detail day after day, it could be observed that this citotoxic effect following incubation with 10 μg /ml of cerulenin was not so strong in normal cells compared to the effect in leukemic cells.(Fig. 11.4, page 81).

To summarize, in this work it could be shown that the fatty acid synthesis metabolism plays an active functional role in the differentiation and in the proliferation of malignant hematopoietic progenitor cells.

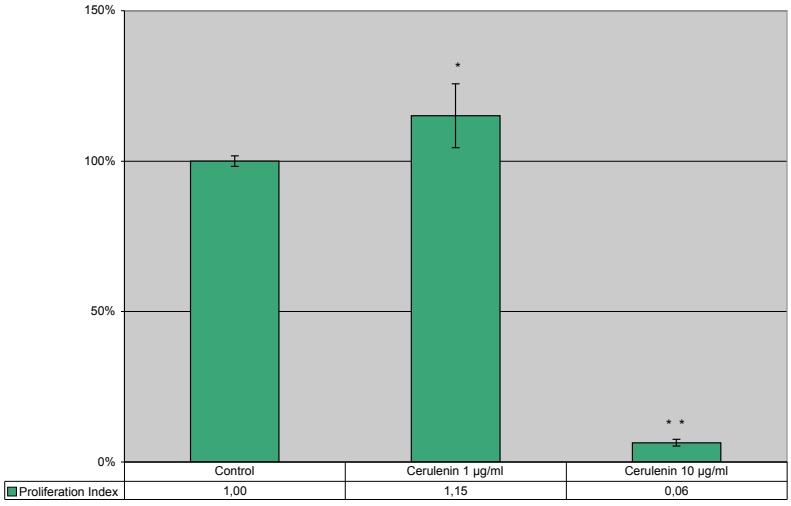
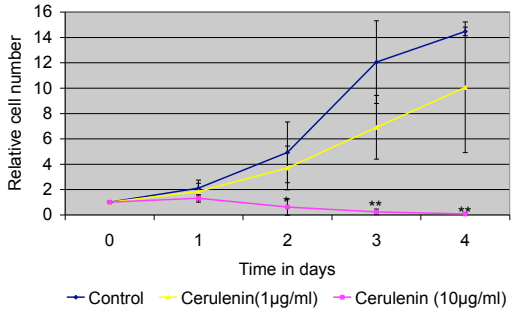
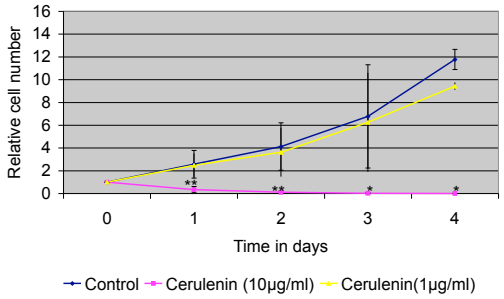


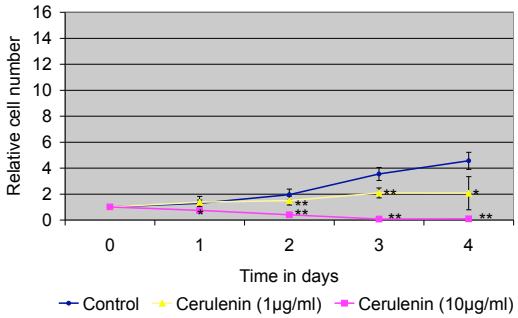
Figure 11.3: Effect of the fatty acid synthesis inhibitor cerulenin on Tritium Thymidine Incorporation. Highly enriched CD34+ cells from PB of three patients with CML were cultured with 1 μg /ml or 10 μg /ml of cerulenin. In addition, 1μCi of Tritium-Thymidin was added to each well. After 18-20h of incubation, cultures were harvested onto filter paper, immersed in scintillation fluid and quantitated in counts per minute. Each experiment was sampled in duplicate. The measured counts per minute (cpm) were proportional to both, the intensity of the radiation and the amount of Tritium-Thymidine incorporated to DNA. Proliferative capacity was expressed by the Proliferation Index (PI = cpm of stimulated CD34+ cells/cpm of unstimulated CD34+ cells). Mean values and standard deviations are shown. Statistically significant (**P < .01 and *P < .05) values are indicated.



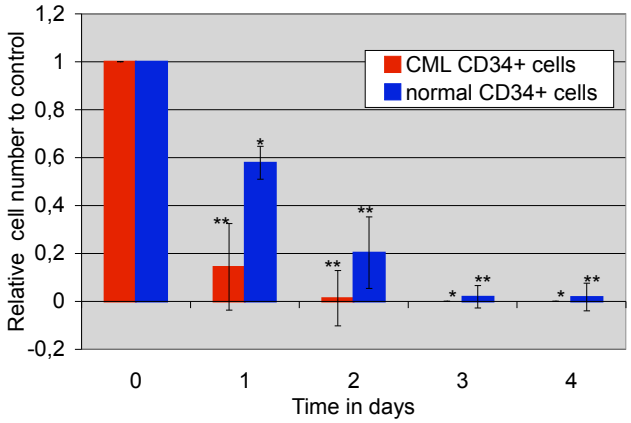
(a) K562 cells



(b) CML CD34+ cells



(c) normal CD34+ cells

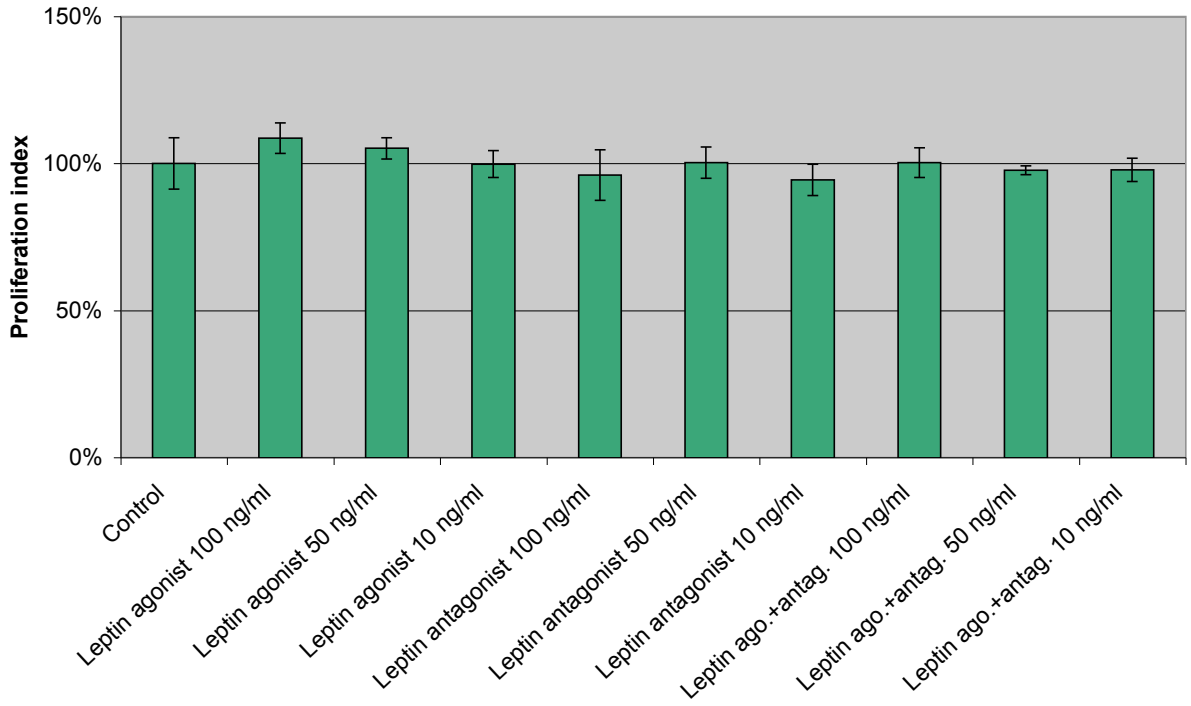


(d) Differential cytotoxic effect of cerulenin in CML and normal CD34+

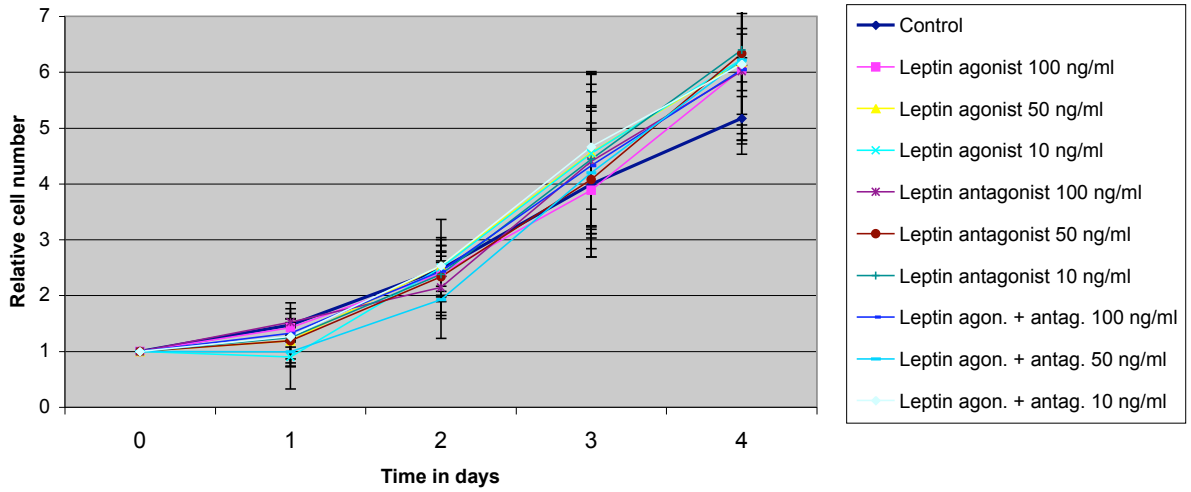
Figure 11.4: Effect of cerulenin on the cellular growth of leukemic and normal CD34+ cells. Three samples of K562 cells (a), CD34+ cells from PB of three patients with CML (b) and CD34+ cells from PB of three healthy donors (c), were stimulated with 10 μg /ml and 1 μg /ml of cerulenin. The cellular growth was examined over 4 days by determining the cellular number of the samples with Trypan blue and a Hemocytometer. Relative cell number (RCN) was determined daily for each experiment (RCN= concentration of stimulated cells daily/ concentration of unstimulated cells at day 0) and is displayed in the 3 curves. In (d), the results of the suspension cultures for CML and normal cells are compared. RCN values for each day and sample were referred to the control (not stimulated cells) and expressed as relative values. A significantly stronger inhibition of the proliferation rate of CML CD34+ cells compared to normal CD34+ cells could be detected by the stimulation with 10 μg /ml of cerulenin. Every experiment was performed in duplicate. Mean values and standard deviations are shown. Statistically significant (**P < .001 and *P < .05) values are indicated.

11.2.2. Leptin receptor

Incubation of CD34+ cells from CML patients with the agonist or the antagonist of the leptin receptor did not influence the proliferation of malignant cells as assessed by the 3H-Thymidin (Tritium-Thymidin) uptake assay (Fig. 11.5.a, page 83). This negative result was confirmed by determining the cell number with Trypan blue and a Hemocytometer along 4 days culture with leptin agonist and antagonist in different concentrations (Fig. 11.5.b, page 83). This results were in line with the clonogenic methylcellulose assays described above, indicating that the leptin receptor modulation does not influence the proliferation or differentiation of CML CD34+ progenitor cells.



(a) Tritium Thymidine incorporation measure



(b) Measure of cell growth with Trypan blue and a Hemocytometer

Figure 11.5: Leptin receptor modulation did not affect the proliferation of CML CD34+ cells . Highly enriched CD34+ cells from PB of three patients with CML were cultured with 100 ng/ml, 50 ng/ml and 10 ng/ml of leptin receptor agonist and/or antagonist. (A) Tritium Thymidine incorporation measure. Proliferative capacity was measured by adding 1μCi of Tritium-Thymidin to each well and measuring the amount of Tritium thymidine incorporated to DNA after 18-20h of incubation. Mean values and standard deviations are shown. (B) Measure of cell growth with Trypan blue and a Hemocytometer. Proliferative capacity was determined daily and expressed by the Relative cell number (RCN= concentration of stimulated cells daily/ concentration of unstimulated cells at day 0). Each experiment was performed in duplicate. Mean values and standard deviations are shown.

12. Functional examination of the GABA B Receptor

Recently, it was reported that the GABA B receptor mRNA as well as surface protein were expressed in human CD34+ hematopoietic stem and progenitor cells [83]. Even more, comparing leukemic CD34+ cells with normal CD34+ cells the GABA B receptor was found to be differentially expressed [65]. The GABA B receptor mRNA was upregulated in CD34+ cells from patients with CML in chronic phase in comparison to normal CD34+ cells as shown by flow cytometry on single cells.

Before, the GABA B receptor was thought to be almost exclusively expressed in the nervous system and the physiologic functions of this receptor in human CD34+ hematopoietic stem and progenitor cells were still not studied. For assessment of the functional role of the GABA B receptor in this work, immunomagnetically selected CD34+ cells from peripheral blood of G-CSF-mobilized healthy donors were subjected to different functional assays in the presence or absence of specific agonists or antagonist of the receptor.

12.1. cAMP concentration

The GABA B receptor is an heterodimer composed of two seven transmembrane-spanning units. Within the nervous system, binding of the neurotransmitter GABA-B to the ligand binding domain on the extracellular surface of the heterodimer, induces a conformational change in the receptor that leads to a modulation of the adenylate cyclase (AC) activity via G proteins [96]. It is well known that the stimulation of the AC catalyzes the conversion of ATP to cyclic 3′,5′-adenosine monophosphate (cAMP) which activates cAMP-dependent kinases eliciting a wide array of metabolic and functional processes [111] [112].

To investigate if the modulation of the GABA B receptor in human CD34+ stem and progenitor cells had an effect on the intracellular concentration of the second

messenger cAMP, immunomagnetically enriched CD34+ cells from peripheral blood of healthy donors were incubated with different concentrations of GABA B agonist and antagonist and the functional effects were examined, using a competitive enzyme immunoassay. Following incubation with 5 μM of the GABA B agonist Baclofen, a significant increase (345.52%) of the intracellular concentration of the second messenger cAMP could be detected. Incubation with 1 μM of GABA B agonist resulted in a significant decrease of cAMP by 59.49%. Even more, those effects could be prevented by incubating the cells with the GABA B agonist in combination with the same molar concentration of the antagonist (Fig. 12.1).

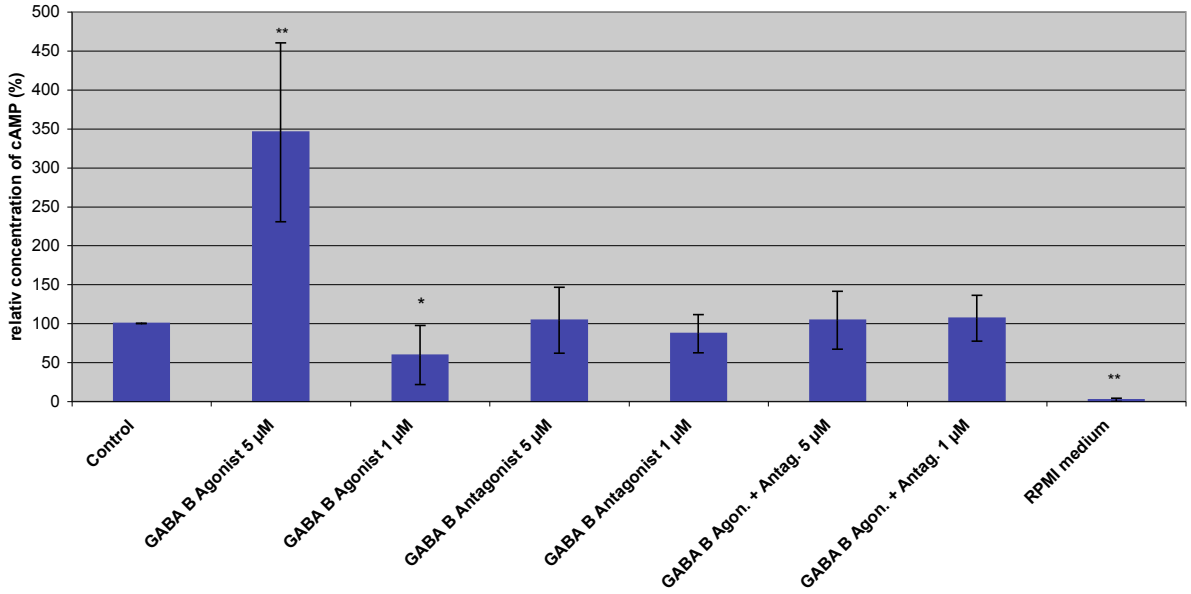


Figure 12.1: Measure of intracellular cAMP in human CD34+ cells. Highly enriched human CD34+ cells from healthy donors were stimulated with GABA B agonist and/or antagonist in the same molar concentrations (5 μM and 1 μM) for 30 min. Afterward, intracellular cAMP concentration was measured by using a competitive enzyme immunoassay. Results were compared with the intracellular cAMP concentration measured on not stimulated cells (control). Values for a negative control, without cells (RPMI Medium) are indicated. The lines in each bar represent the standard deviation of 4 independent experiments which were sampled in duplicate. Statistically significant changes are indicated (**P < .01 and *P < .05).

To summarize, modulation of the GABA B receptor specifically modified the intracellular concentration of cAMP in CD34+ cells, indicating this G protein-coupled receptor is functionally active in CD34+ cells.

12.2. Calcium concentration

Within the nervous system, activation of the GABA B receptor modulates adenylate cyclase activity via G proteins, influencing potassium and calcium conductances. The GABA B receptor was first demonstrated on presynaptic terminals, where it serves as an autoreceptor and also as a heteroreceptor, to influence transmitter release by suppressing neuronal calcium conductance [97].

In this work, it was investigated if the GABA B receptor plays a physiological role on the intracellular calcium release of human CD34+ stem and progenitor cells. PB-CD34+ cells were loaded with the calcium sensitive fluorescent dye Fluo-4 and time dependent changes of the fluorescence intensity were measured after receptor modulation by flow cytometry (Fig. 12.2). The samples were stimulated with SDF-1, which has been described to induce calcium release on CD34+ cells [113]. A clearly induced calcium flux was observed, while no significant effect could be assessed by the stimulation of the cells with GABA B agonist and/or antagonist (Fig.12.3.b, page 87).

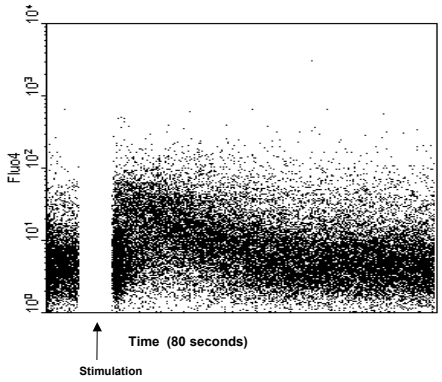
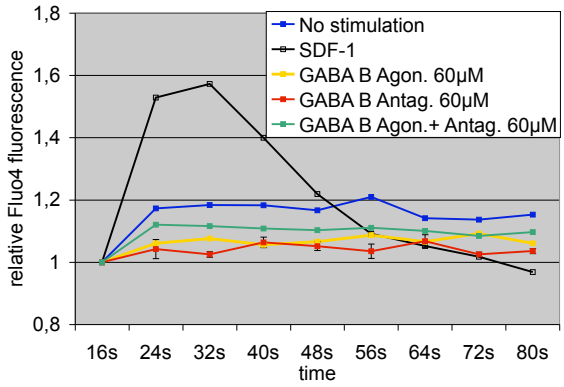
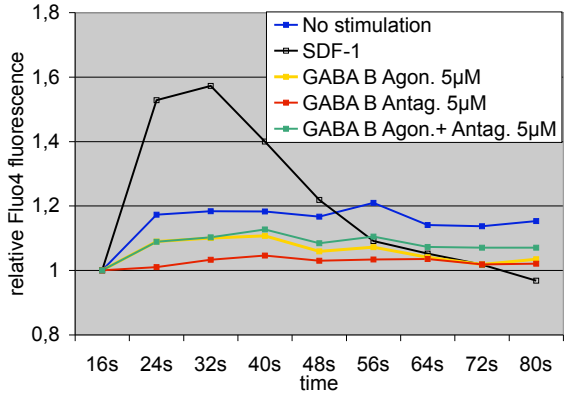


Figure 12.2: Example of measure by continuous flow cytometry. Highly enriched CD34+ cells from healthy volunteers were loaded with the the green fluorescent calcium indicator Fluo-4. After determining the baseline level of every sample during 16 seconds the samples were stimulated and measured. Time dependent changes of Fluo-4 fluorescence intensity were measured by continuous flow cytometry until the 80 seconds were over.

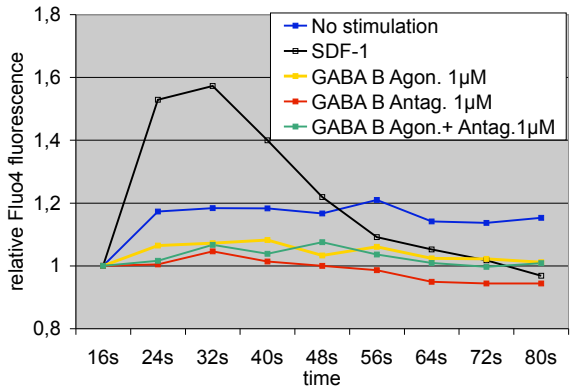
After these results, it was investigated wheter the GABA B receptor modulation influenced the intracellular calcium flux observed by SDF-1. Immediately before measuring, CD34+ cells were stimulated with both, SDF-1 and the agonist Baclofen and/or antagonist Hydroxisaclofen what did not significantly modify the SDF-1 induced calcium flux (Fig.12.4.a, page 88). Moreover, CD34+ cells were cultured in the presence of GABA B agonist or antagonist for 3 hours while cells were loaded with the calcium sensitive fluorescent dye Fluo-4. After addition of SDF-1, time dependent changes of mean fluorescence were measured and expressed as relative values. Following preincubation with Baclofen and Hydroxisaclofen the SDF-1 induced calcium flux was not modified (Fig.12.4.b, page 88b).



(a) Stimulation with 60μM concentration of agonist and/or antagonist of GABA B receptor

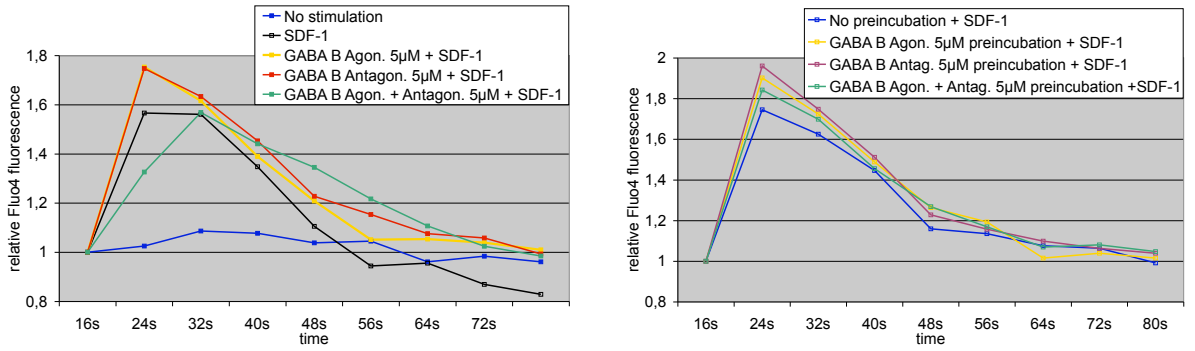


(b) Stimulation with 5μM concentration of agonist and/or antagonist of GABA B receptor



(c) Stimulation with 1μM concentration of agonist and/or antagonist of GABA B receptor

Figure 12.3: Effect of different concentrations of GABA B agonist and/or antagonist on the intracellular calcium flux. Highly enriched CD34+ cells from healthy donors were stimulated with agonists and/or antagonists of the GABA B receptor at different concentrations (60μM, 5μMand 1μM) and SDF-1 was used as a positive control. In every case, time dependent changes of Fluo-4 fluorescence intensity were measured by continuous flow cytometry. The fluorescence was analyzed in 8 s acquisition intervals and expressed as relative mean fluorescence compared to the baseline level. Results were based on three different experiments performed in duplicate.



(a) Measure immediately after the addition of GABA B agonist and or antagonist

(b) Measure following 3 hours of incubation with GABA B agonist and or antagonist

Figure 12.4: Effect of GABA B agonist and/or antagonist on the SDF-1 mediated intracellular calcium flux. CD34+ cells were stimulated with SDF-1 and, additionally, with 5μM of GABA B receptor agonist and/or antagonist, by pre-incubating for 3 hours or by addition at the same moment as the SDF-1. In every case, time dependent changes of Fluo-4 fluorescence intensity were measured by continuous flow cytometry. The fluorescence was analyzed in 8 s acquisition intervals and expressed as relative mean fluorescence compared to the baseline level. Results were based on three different experiments performed in duplicate.

In summary, no evidence could be found about the GABA-B receptor as a mediator for the calcium signaling in CD34+ cells.

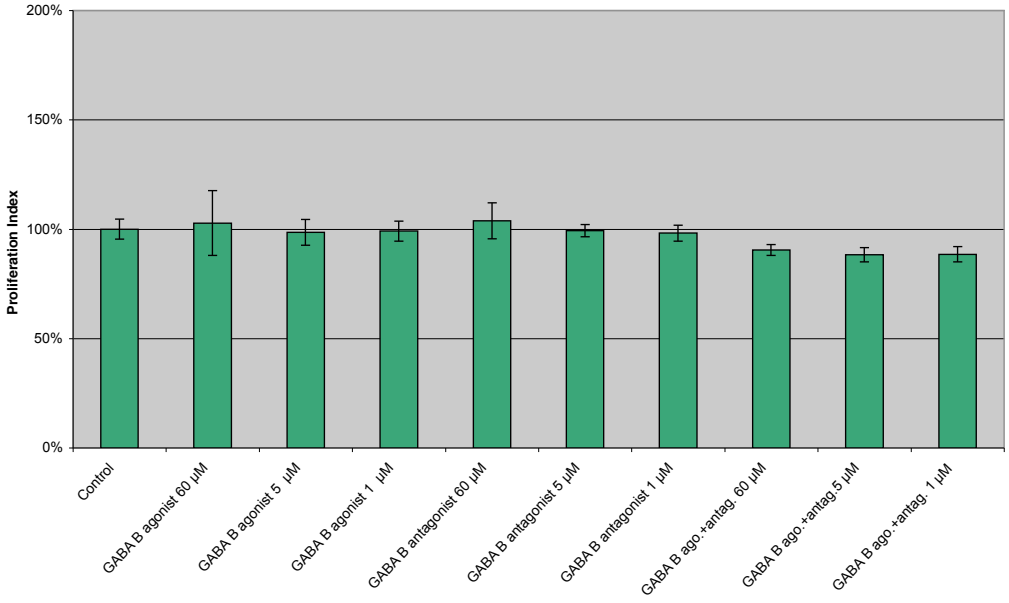
12.3. Proliferation and Clonogenic Growth

In order to asses if the expression of the GABA B receptor has a functional impact in the proliferation or in the clonogenic growth of the hematopoietic stem and progenitor cells, CD34+ cells from healthy donors were cultured in the presence or absence of agonist or antagonist of the receptor. All the experiments were performed in duplicate, incubating the cells with three different molar concentrations (60μM, 5μM and 1μM) of the agonist Baclofen or the antagonist Hydroxaclofen.

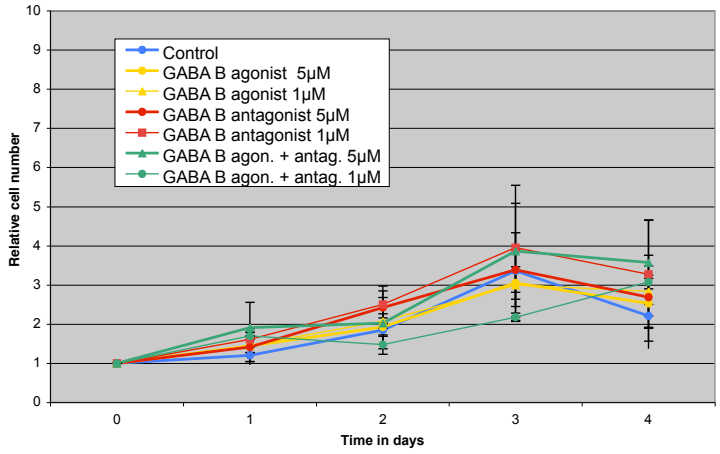
After 3 hours incubation no influence was observed by measuring the cellular proliferation using the 3[H]-Thymidin (Tritium-Thymidin) uptake assay (Fig.12.5.a, page 89).

This negative result was confirmed by determining the cell number with Trypan blue and a Hemocytometer along 4 days culture with GABA B agonist and antagonist. Baclofen and hydroxisaclofen did not influence the progeny of the cells compared to the not stimulated control (Fig. 12.5.b, page 89).

In order to investigate if the GABA B receptor modulation has an effect on the



(a) Tritium Thymidine incorporation measure.



(b) Measure of cell growth with Trypan blue and a Hemocytometer.

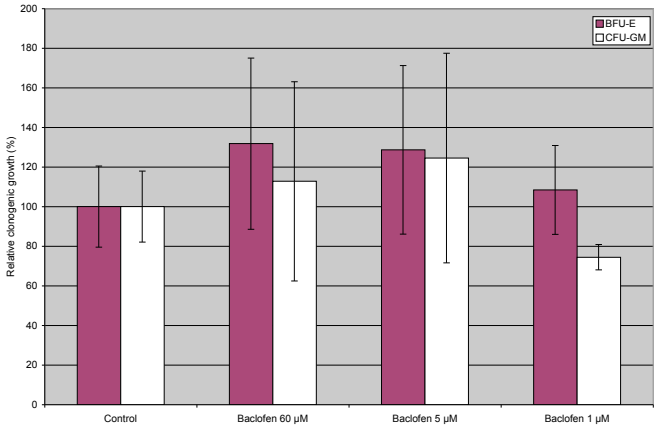
Figure 12.5: GABA B receptor modulation did not affect the proliferation of CD34+ cells. Highly enriched CD34+ cells from PB of three healthy donors were cultured with 60μM, 5μM and 1μM of GABA B receptor agonist and/or antagonist. (a) Proliferative capacity was studied by adding 1μCi of Tritium Thymidin to each well and measuring the amount of Tritium thymidine incorporated to DNA after incubation with the ligands. Mean values and standard deviations are shown. (b) Cell growth was measure with Trypan blue and a Hemocytometer. Proliferative capacity was determined daily and expressed by the (RCN= concentration of stimulated cells daily/ concentration of unstimulated cells at day 0). Each experiment was performed in duplicate. Mean values and standard deviations are shown.

clonogenic growth, CD34+ cells were cultured in the presence of GABA B agonist or antagonist in semisolid medium. After two weeks, no significant influence of GABA B receptor modulation on the formation of BFU-E and CFU-GM was found (Fig. 12.6, page 91).

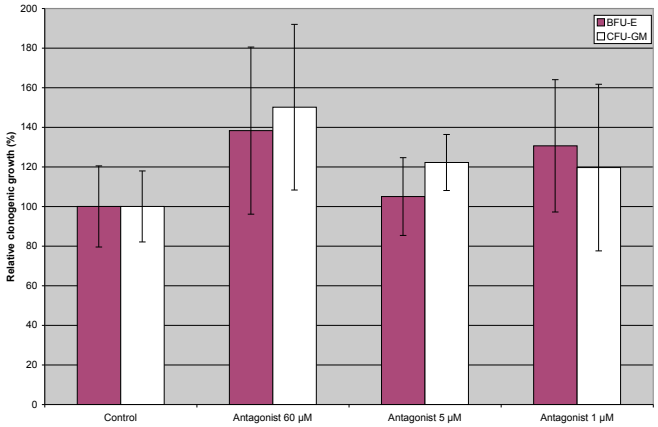
To conclude, it was assessed that the GABA B receptor modulation does not play a role in the proliferation or in the clonogenic growth of the CD34+ hematopoietic stem and progenitor cells.

12.4. Migration

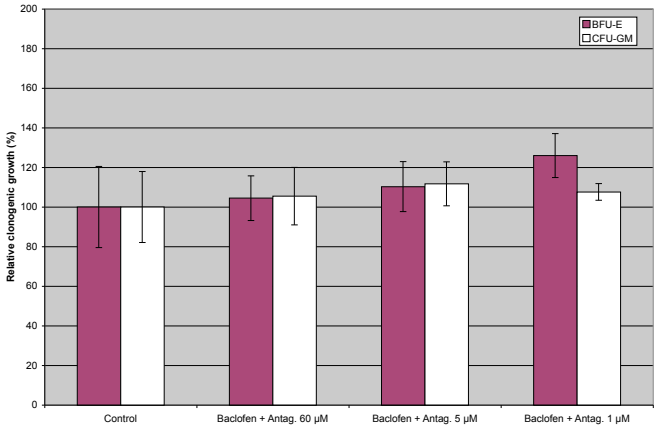
Recent studies have provided evidence for the expression of several 7-TMR (heptahelical membrane receptors) on immature hematopoietic progenitor cells, which potentially mediate chemotactic effects [114] [115]. The GABA B receptor belongs to the family of G protein-coupled 7-TMR.Even more, recently, it was reported that the GABA B receptor has an important modulatory role in the migration of cortical interneurons [116]. In this work, it was investigated wether the modulation of the GABA B receptor in human CD34+ stem and progenitor cells had an effect on the cellular migration by using an in vitro two-chamber migration assay. The membrane of a transwell plate was coated with fibronectin. Highly enriched CD34+ cells, stimulated with GABA B agonist or antagonist, were added to the upper chamber and the chemokine SDF-1 was added to the lower chamber. SDF-1 has been described to be involved in mobilization and homing of hematopoietic stem cells [117] [113]. After 8 hours, the transmigrated cells were collected and counted. Adding 250 ng/ml of SDF-1 significantly ($p<0.01$) increased the cellular migration, which was 1.9- fold higher than the not stimulated control, while incubation of the cells with 5μM of the GABA B agonist Baclofen or antagonist Hydroxisaclofen did not influence the cellular migration. Even more, no significant modification of the SDF-1 mediated migration could be observed by the GABA B receptor modulation (Fig. 12.7, page 92). Thus, an influence of the GABA B receptor modulation on migration of CD34+ stem and progenitor cells could not be observed.



(a) Stimulation with GABA B agonist Baclofen.



(b) Stimulation with GABA B antagonist.



(c) Stimulation with GABA B agonist and antagonist.

Figure 12.6: Clonogenic growth of CD34+ cells following modulation of GABA B receptor. The clonogenic growth of highly enriched CD34+ cells from PB of three healthy donors was examined by semisolid methylcellulose clonogenic assays incubating the cells with 60μM, 5μM and 1μM of GABA B receptor agonist and/or antagonist. BFU-E (red) and CFU-GM (white bars) colonies were counted after two weeks. Every experiment was performed in duplicate. Mean values and standard deviations are shown.

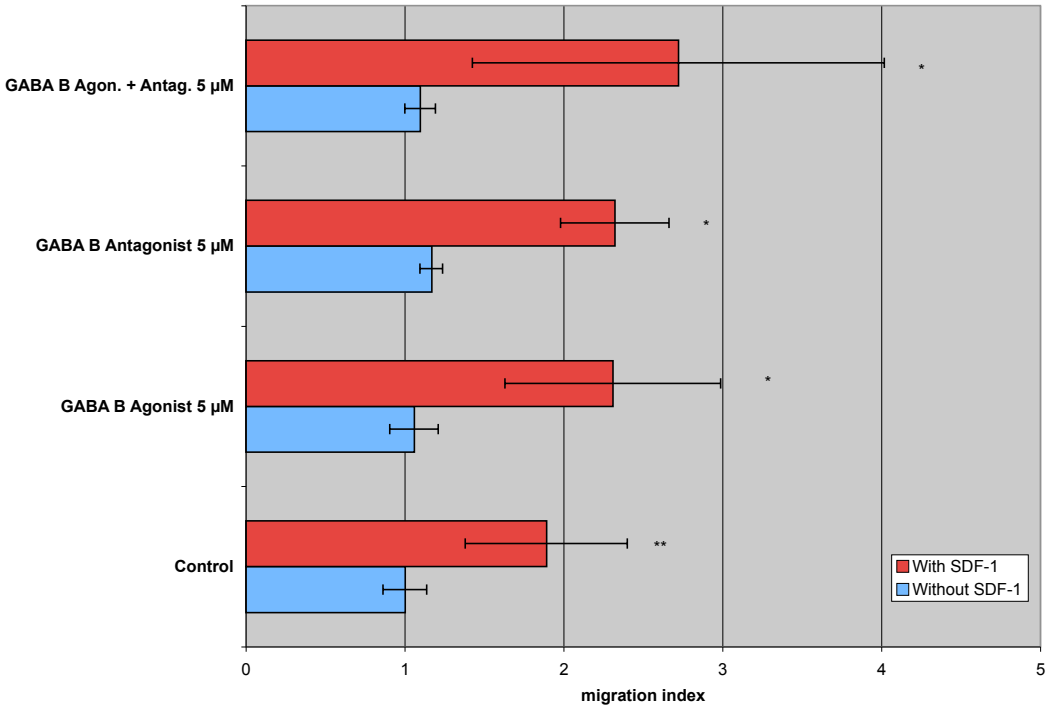


Figure 12.7: Effect of GABA B receptor modulation of CD34+ cells in vitro two-chamber Migration. In the upper chamber CD34+ cells were stimulated with different concentrations of GABA B agonist and/or antagonist. In the lower chamber the chemokine SDF-1 was added or not added. The migrated cells in the lower chamber were counted after 8 hours and expressed as relative values (migration index) referred to the control without SDF-1. The lines in each bar represent the standard deviation of 4 independent experiments which were sampled in duplicate. Statistically significant increases of SDF-1 mediated migration are indicated (**P < .01 and *P < .05)

Part IV.

Discussion

13. Gene expression signature of CD34+ cells from patients with CML in chronic phase

Investigating the molecular biology of stem cells can give novel insights into cancer research. The chronic myelogenous leukemia (CML) is one of the best-studied malignant stem cell disorder, which is characterized by a clonal expansion of hematopoietic progenitor cells.

In previous studies, large-scale gene expression analyses of leukemic cells from patients with Ph+ CML have been used to identify a genomic profile associated with response to therapy with the tyrosine kinase inhibitor imatinib [61] and have broadened the knowledge about alterations of gene expression in BCR-ABL positive cells in comparison with normal cells [62] [63]. An important limitation of this approach was that a very heterogenous population of CML cells was used, consisting of a broad spectrum of different precursors. This cell population was compared with fully differentiated normal control cells. Gene expression analyses were performed using BCR-ABL-positive mononuclear cells of patients with CML in chronic phase [63]. In another study, CD133 was used to enrich hematopoietic progenitor cells from patients with CML for gene expression analyses trying to identify molecular events associated with disease progression [62]. In our work, highly enriched CD34+ cells were used which still represent a heterogenous population containing stem and progenitor cells. However, CD34+ cells are closer to the cell of origin of the leukemic clone than mononuclear cells. Therefore, we examined highly enriched CD34+ cells from bone marrow (BM) of untreated patients with CML in first chronic phase using microarrays covering 8 746 genes to assess the molecular signature of hematopopietic stem and progenitor cells in CML.

Our gene expression analyses fulfilled the recently published consensus guidelines from three European leukemia networks, that is, I-BFM-SG, the German Competence

Network *Acute and Chronic Leukemias* and the European LeukemiaNet. The guidelines have been formulated for performing microarray experiments in leukemia, based on the exchange of knowledge and experience between the three networks. Critical steps of the method have been discussed and evaluated in different multi-center quality rounds within the three networks, including handling, processing and storage of the leukemic sample, purification of tumor cells, RNA extraction methods and quality control, normalization and biostatistical analysis. In our work, these items have been carefully performed according to the formulated guidelines. Samples were processed within two hours following bone marrow puncture, we used highly enriched CD34+ cells of a purity between 96% and 99%, RNA integrity was qualified by bioanalyzer and the data were statistically analyzed in cooperation with a bioinformatic institute.

Our study provides the first large-scale gene expression analysis of CD34+ stem and progenitor cells from patients with chronic myelogenous leukemia (CML) in chronic phase in comparison with the normal counterparts.

Comparing malignant BM CD34+ cells from patients with CML with normal BM CD34+ cells 1539 genes were significantly differentially expressed indicating a distinct molecular phenotype of chronic phase CML CD34+ cells. 1420 genes had a higher and 119 genes a lower expression in CML. A hierarchical cluster analysis showed the homogeneity of the samples and could clearly distinguish between normal and CML CD34+ cells. The data were corroborated by assessing the mRNA expression level of 8 genes by quantitative real-time reverse transcription-polymerase chain reaction (RT-PCR).

Comparing the expression profiles of sedentary leukemic CD34+ cells from BM with those of circulating CD34+ cells from peripheral blood (PB) of untreated patients with CML, no differentially expressed genes with sufficient significance were found. Interestingly, this is different to normal CD34+ cells, as other prior studies had observed distinct molecular phenotypes when examining normal CD34+ cells from BM and PB [118] [25] [119]. In those studies with normal CD34+ cells a significantly higher expression of genes governing cell-cycle progression was found in BM CD34+ cells in comparison to PB CD34+ cells. However, this result is in line with a previous report of our group, made with a smaller number of 1185 genes, which also assessed the molecular signature of hematopopietic stem and progenitor cells in CML [65] and with another recent work which determine the degree of correspondence of gene expression between BM and PB in acute myeloid leukaemia (AML) [120]. In both of them, the intraindividual comparison of cells from BM and PB showed no differen-

tially expressed genes. A possible explanation would be that the physiological switch from a more proliferating to a quiescent phenotype, which was found during egress of normal CD34+ cells from BM to PB, might be disturbed in chronic phase CML [65].

In the following, the expression data comparing normal CD34+ cells with CML CD34+ cells is discussed in detail by allocating genes into functional groups.

CML CD34+ cells showed a higher expression level of Genes involved in bcr-abl signaling

First, an up-regulation of genes encoding substrates of BCR-ABL was found. Several substrates of BCR-ABL as well as proteins of BCR-ABL signaling pathways, such as the Ras/mitogen-activated protein kinase (MAPK) and the phosphoinositide 3 (PI3) kinase pathways, were significantly up-regulated in primary CML CD34+ cells (Fig. 13.1, page 97).

For example, the BCR-ABL adapter proteins Grb2 and Crkl, as well as the signaling molecules STAT3, STAT4, STAT5, VAV3 and the Ras GTPase activating protein G3BP2 were higher expressed. Genes of the JNK MAPK pathway were higher expressed suggesting a functional role of this pathways in BCR-ABL-positive CD34+ cells as previously shown for BCR-ABL-positive cell lines [121] [122] [123]. The transcriptional target of MAPK pathways MYC was significantly up-regulated, which emphasizes the central role of MYC activation in BCR-ABL-induced transformation [124][125]. The heterogenous nuclear ribonucleoprotein K (HNRPK), which positively regulates MYC expression through ERK1/2 activation in blast crisis CML CD34+ cells [126], was also up-regulated in chronic phase CML CD34+ cells suggesting that HNRPK supports MAPK pathway-dependent activation of MYC. Out of the genes involved in the PI3 kinase pathway, besides components of the PI3 kinase itself, its substrate AKT and the downstream molecules NF-κB and BclXL were significantly up-regulated in CML CD34+ cells. Transcriptional activation of BclXL might be enhanced by increased expression of STAT5 in leukemic progenitors [127] [128]. Our data suggest that survival and proliferation depends on the PI3 kinase pathway not only in BCR-ABL-transformed murine cells and fibroblasts [129] [130] as described previously but also in primary CML CD34+ cells [131]. In summary, the upregulation of BCR-ABL signaling pathways in primary CML CD34+ cells indicates that increased signaling is not only based on phosphorylation but also on transcriptional activation of signaling molecules. Our data support the central role of the classical MAPK and the PI3 kinase pathways for BCR-ABL-induced transformation of CML CD34+ cells.

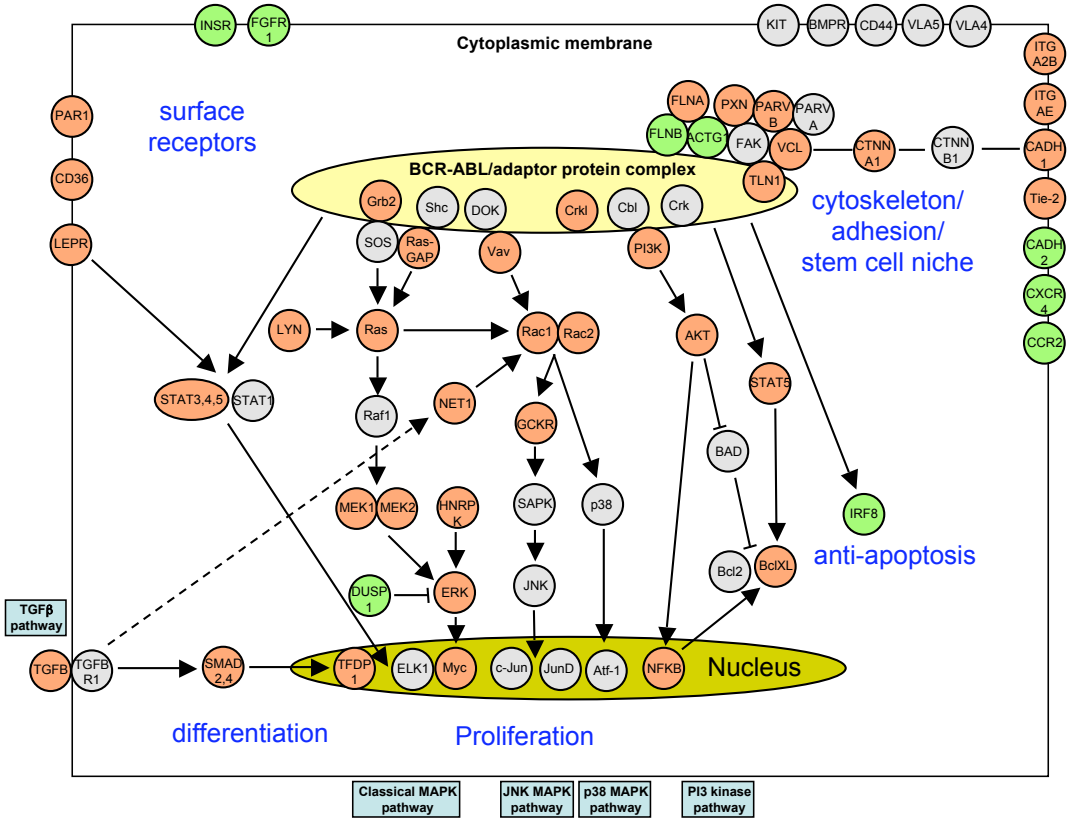


Figure 13.1: Differential expression of BCR-ABL substrates and genes involved in BCR-ABL-associated signaling pathways in primary CML CD34+ hematopoietic stem and progenitor cells. Genes that were covered by our array are displayed. Significantly up- and downregulated genes are indicated by red and green, respectively, and gray indicates no significantly differential expression. Arrows indicate activation of signaling proteins or transcriptional activation of genes and lines with crossbar indicate inhibition of signaling proteins.

CML CD34+ cells showed a higher expression level of proliferation-associated genes and down-regulation of the pro-apoptotic interferon regulatory factor 8

Next, we looked at functional endpoints of BCR-ABL-signaling such as proliferation, apoptosis and adhesion. In line with activation of mitogenic pathways, several genes encoding for cell cycle promoting proteins were up-regulated in BCR-ABL-positive CD34+ cells. Essential genes for initiation of DNA replication, and different DNA polymerases as well as the transcription factor Dp-1, which dimerizes with the E2F transcription factor and regulates the expression of various promoters involved in cell cycle in normal CD34+ cells [132] [133], were also up-regulated in CML progenitor cells. This finding is in line with the higher cell cycle activity of CML CD34+ cells [133]. With respect to apoptosis-related proteins we observed a heterogenous picture. On the one hand, the pro-apoptotic key proteins caspases 3, 6 and 10 and the FAS receptor were significantly up-regulated in BCR-ABL-positive CD34+ cells. On the other hand, the anti-apoptotic protein BclXL was also up-regulated in CML. Moreover, the interferon regulatory factor 8 (IRF8) was significantly down-regulated in primary CML CD34+ cells. IRF8 could induce apoptosis in bcr-abl-positive myeloid cells in mice [134] and IRF8 knock-out mice developed a myeloproliferative syndrome [135]. Recently it has been shown in bcr-abl-transformed murine cells that IRF8 antagonizes BCR-ABL by down-regulation of bcl-2 and could override imatinib resistance [136]. Thus, our data derived from CD34+ cells support the hypothesis that BCR-ABL-induced inhibition of apoptosis in primary human leukemic progenitor cells is mediated by down-regulation of IRF8.

CML CD34+ cells had an increased expression of focal adhesion proteins and a reduced expression of L-selectin and N-cadherin.

CML progenitor cells have a decreased capability to adhere to bone marrow stroma [56] [57] and are released from BM to peripheral blood to a larger extent in comparison to normal progenitor cells. The reasons for this phenomenon are not yet understood. Therefore, we especially looked on differential expression of genes involved in progenitor cell adhesion and migration. The BCR-ABL adapter protein Crkl which facilitates complex formation with focal adhesion proteins and is involved in regulation of cellular motility [137] was up-regulated in CML CD34+ cells. Among the Crkl-associated adhesion proteins vinculin, talin, parvin and filamin were also significantly up-regulated in CML (Fig. 13.1, page 97). Previous studies reported alter-

ations in integrin-mediated cell-adhesion of CML cells [57] [138]. Looking at changes in integrin expression we found only the integrins αE and α2b to be up-regulated. Therefore, there is no evidence for transcriptional activation of most of the integrins in CML CD34+ cells. However, BCR-ABL could alter integrin-mediated adhesion and signaling by modulation of the functional state rather than by the expression level of the integrin receptors. A finding along this line was the significantly higher expression of the SRC kinase LYN in CML CD34+ cells. LYN positively regulates CD34+ cell movement and lowers adhesion of CD34+ cells to stromal cells by inhibiting the ICAM-1-binding activity of β2 integrins [139]. Increased expression of LYN might therefore be involved in the release of CML progenitor cells into the peripheral blood. Additionally, over-expression of LYN was associated with imatinib resistance [140]. Thus, the up-regulation of LYN in CML CD34+ stem and progenitor cells provides the rational for using the combined ABL/SRC kinase inhibitor dasatinib even in therapy naive patients with CML. Interestingly, several proteins involved in regulation of the actin cytoskeleton were deregulated in CML CD34+ cells in comparison with normal cells. The BCR-ABL associated cytoskeletal protein, actin gamma 1, a component of f-actin, that regulates cell adhesion [141] [142] had a significantly lower expression level, whereas the regulators of the actin cytoskeleton, Rho GTPases Rac1 and Rac2, were significantly up-regulated in CML CD34+ cells [143]. Additional adhesion molecules were up-regulated and cadherin 2 (N-cadherin), a molecule involved in the interaction of the stem cell and its niche [144], was down-regulated in CML CD34+ cells. Finally, the chemokine receptors CXCR4 and CCR2, which are involved in stem cell and monocyte migration, respectively, were significantly down-regulated in CML CD34+ cells explaining previous findings of a reduced SDF-1 responsiveness of CML CD34+ cells [145][146]. Thus, our expression data reflect the heterogenous results from previous studies with respect to the adhesion defect of CML cells. In summary, our results suggest that the release of progenitor cells in CML into peripheral blood might be supported by a down-regulation of the chemokine receptors CXCR4 and CCR2, the adhesion molecules L-selectin and N-cadherin.

Upregulation of genes of the TGFβ signaling pathway

Activation of BCR-ABL-induced mitogenic signaling can be enhanced by up-regulation of growth factors and their receptors. Indeed, up-regulation of macrophage colony-stimulating factor, IL1β, IL7 and TGFβ1 as well as of the GM-CSF receptor was observed. Looking in detail at the TGFβ signaling pathway different genes were sig-

nificantly up-regulated in CML CD34+ cells. TGFβ1 generally suppresses stem cell proliferation and has inhibitory effects on cytokine-induced cell growth in chronic phase CML [147]. On the other hand, autocrine TGFβ stimulates early erythropoiesis and reactivates fetal hemoglobin synthesis in adult erythroid progenitor cells [148] [149]. Since erythropoiesis-associated genes and fetal globin genes were also up-regulated in CML progenitor cells (see below), we hypothesize that erythropoiesis and fetal hemoglobin synthesis is activated in chronic phase CML by an autocrine stimulation of the TGFβ pathway. Interestingly, the neuroepithelial cell transforming gene 1 (NET1) which is induced upon TGFβ stimulation [150] is also up-regulated in CML CD34+ cells. NET1 plays a central role in Rho GTPase (RAC1)-mediated reorganisation of the cytoskeleton [151] and activation of the alternative JNK MAPK pathway [152]. Therefore, TGFβ-induced NET1 up-regulation might enhance the MAPK-mediated mitogenic activation of CML CD34+ cells.

CML CD34+ cells showed an expression pattern characteristic for immature stem cells as well as megakaryocyte-erythrocyte progenitor cells

Further, we looked at genes involved in early hematopoiesis and differentiation. We found several genes associated with early hematopoietic stem cells and their self-renewal to be significantly up-regulated in CML CD34+ cells indicating a molecular phenotype of a more primitive hematopoietic progenitor cell. These genes include between others, the aldehyd dehygrogenase which is expressed in long-term reconstituting hematopoietic stem cells and considered to be involved in chemoresistance [125] [153] [154]. Thus, our gene expression data suggest a greater self-renewal capacity of leukemic CD34+ cells. Overexpression of HoxA9, MEIS1 and PBX3 might be relevant for the malignant transformation of CML CD34+ cells since it has recently been shown that Hox-MEIS-PBX complexes drive leukemogenesis in AML [155]. Looking at genes involved in differentiation we found that GATA1, associated with early myeloid differentiation, was significantly up-regulated and other genes as neutrophil elastase 2 (ELA2) which are expressed during later stages of myeloid differentiation, were down-regulated in CML CD34+ cells. Two of the central genes promoting lymphoid differentiation, GATA3 and IL-7 receptor α, as well as the B cell differentiation-associated genes terminal desoxynucleotidyltransferase, pre-B lymphocyte gene 1 (VPREB1), B-cell linker and BCL-6 were downregulated in CML CD34+ cells. On the other hand, erythropoiesis-associated genes such as erythropoietin receptor, Kruppel-like factor 1 and transferrin receptor were higher expressed in CML CD34+ cells. An

interesting finding was an up-regulation of fetal hemoglobin (Hb) components such as Hbγ1, Hbγ2 and Hbδ as well as Hbβ whereas the genes for adult hemoglobin chains Hbα1 and Hbα2 were not altered indicating an induction of fetal hemoglobin synthesis in CML progenitor cells. The re-activation of fetal hemoglobin synthesis in CML progenitors could be induced by an autocrine activation of the TGFβ signaling pathway (see above) [148] [149]. In summary, the gene expression signature of CML CD34+ cells indicates a molecular phenotype of early hematopoietic stem cells with preponderance for megakaryocyte-erythroid progenitors (MEP) rather than for granulocyte-macrophage progenitors (GMP). This result could be recently confirmed in further investigations of our group by subset analysis of CML CD34+ cells [156]. The proportion of early hematopoietic stem cells was significantly smaller in CML CD34+ cells compared to their normal counterparts. Within the lin-, CD38+ subfraction of CML CD34+ cells a greater proportion of MEP was observed in comparison with normal CD34+ cells whereas the proportions of GMP and the IL-3 receptor α-high progenitor population was significantly smaller in CML. The results also show that the expansion of malignant precursor cells in chronic phase CML does not occur on hematopoietic stem cell level. Additionally, the higher expression of stem cell-associated genes such as GATA2 or the Hox genes in CML CD34+ cells in combination with a smaller proportion of HSC suggests that those genes are transcriptionally activated in CML HSC and might result in an increased self-renewal capacity of those cells.

The receptor for leptin was up-regulated in CML CD34+ cells

The leptin receptor (LEPR) showed the greatest level of differential expression in CML CD34+ cells. Furthermore, in line with the microarray data, the higher expression of the leptin receptor in leukemic CD34+ cells compared to normal CD34+ cells was confirmed at the protein level by using fluorescence- activated cell sorter analysis. This receptor is a member of the cytokine receptor superfamily, which signals via activation of JAK2, STAT1, 3, and 5 proteins as well as MAPK pathways and plays an important role for several physiological and pathophysiological processes such as body mass control, metabolic pathways, reproduction, angiogenesis, bone remodelling and immunity [157]. Even more, leptin receptor has been related to leukemia [105][106] and breast cancer [104]. Since LEPR is up-regulated in primary CML CD34+ cells this receptor might activate proliferation and differentiation of leukemic progenitors and might therefore be a suitable target for novel therapeutic

approaches [158]. This background prompted us to deeply investigate this interesting receptor on CD34+ hematopoietic stem and progenitor cells. The results of these functional experiments are discussed in the next chapter of this work (*New therapeutical approaches in CML treatment: The lipid metabolism pathway*).

Up-regulation of genes involved in fatty acid metabolism

We found several genes involved in fatty acid and lipid metabolism to be up-regulated in CML CD34+ cells. For example, the isoenzymes of the long-chain fatty acid coenzyme A ligases ACSL (acyl-CoA synthetase long-chain family member) 1,4 and 5, were higher expressed in leukemic CD34+ cells in comparison to normal cells. Since ACSL play a key role in lipid biosynthesis and are expressed in erythrocyte precursors [159] our expression data might reflect a higher proportion of erythroid progenitors in CML CD34+ cells. Furthermore, fatty acid synthase (FAS), which catalyzes the synthesis of long-chain saturated fatty acids, and hydroxyacyl-coenzyme A dehydrogenase alpha (HADHA), which promotes oxidation of fatty acids, were also up-regulated in CML. FAS was over-expressed in aggressive breast cancer and inhibition of fatty acid synthesis resulted in cell cycle arrest and apoptosis of tumor cell lines [160]. Thus, a pharmacological inhibitor of FAS as cerulenin could be useful for inhibition of leukemic cell growth in CML. In this work, we investigated the effect of fatty acid synthesis inhibition in CD34+ hematopoietic stem and progenitor cells. The results are discussed in the next chapter *New therapeutical approaches in CML treatment: The lipid metabolism pathway.*

Several genes, which have not been mentioned in the context of the previously addressed functional groups were also deregulated in CML CD34+ cells. As an example, interesting up-regulated genes were sorcin, which is related to drug resistance and poor prognosis in AML [161], and the candidate tumor suppressor gene DLC1 (deleted in liver cancer 1), which activates Rho GTPases and modulates the organization of the cytoskeleton [162]. Moreover, the tissue inhibitor of metalloproteinase 1 (TIMP1) was also up-regulated in CML CD34+ cells. TIMP1 mediates JAK2-, AKT- and PI3 kinase-dependent survival of erythroid cells and is up-regulated in leukemic blasts [163] [164]. Hence, TIMP1 over-expression might enhance activation of PI3 kinase-AKT pathway in CML progenitor cells. The selenoprotein P, which is involved in response to oxidative stress, was down-regulated in CML CD34+ cells. In addition, several genes involved in the ubiquitin-proteasome system were significantly

up-regulated. This supports a previous finding that BCR-ABL induces a proteasome-mediated degradation of inhibitory proteins [165].

14. New therapeutical approaches in CML treatment: The lipid metabolism pathway

In this work, we found several genes involved in energy expenditure and metabolical processes which were up-regulated comparing malignant CML CD34+ cells with normal CD34+ cells. Some of these genes had been already related to cancer formation. Considering the possibility that this kind of genes could be important for the pathophysiology of CML, we deeply investigated them by performing different functional *in vitro* experiments. From special interest were the genes involved in fatty acid metabolism and the leptin receptor, the gene that had showed the greatest level of differential expression in the array data.

Leptin is an adipocyte-specific hormone that regulates adipose-tissue mass [166] and in hematopoiesis it induces proliferation of hematopoietic cells and stimulates the differentiation of lineage-restricted precursors of the erythrocytic and myelopoietic lineages [167]. LEPR has also proliferative and anti-apoptotic activities in AML [105] and it had shown a high expression level in CML blast crisis [168] suggesting a pathophysiological role in leukemia. In addition, it has been described to increase the proliferation of AML colony-forming cells [105] and spontaneous AML blast proliferation [110]. However, when looking at the proliferation or differentiation of CD34+ CML cells, we could not assess any effect of the modulation of leptin receptor activity, concluding that this receptor is not a suitable target for antiproliferative therapies. Never the less, taking into account the higher expression the receptor not only at the transcriptional level but also at the protein level in CML CD34+ cells comparing to normal cells, we must suppose that LEPR plays an important role in the pathophysiology of the disease.Further studies will be necessary to elucidate the exact signaling pathways of this receptor in CML and other cancers.

Looking at genes involved in fatty acid and lipid metabolism, the proteins en-

coded by the ACSL genes are isoenzymes of the long-chain fatty-acid-coenzyme A ligase family, which play a key role in lipid biosynthesis and fatty acid degradation [169] and FAS gene, which catalyzes the synthesis of long-chain saturated fatty acids, were up-regulated in CML cells. Interestingly, the isoenzyme encoded by ACSL5 has markedly increased levels in malignant gliomas, so that this gene has been described to mediate fatty acid-induced glioma cell growth [103]. In addition, high levels of FAS expression have been observed in several cancers, including breast, prostate, colon and lung carcinoma compared with their respective normal tissue [107].

Here, we found that the upregulation of genes involved in fatty acid metabolism plays an active functional role in the differentiation and in the proliferation of malignant hematopoietic progenitor cells. Incubation of CD34+ CML cells with the fatty acid synthesis inhibitor cerulenin resulted in dose-dependent decreases in leukemic cell viability, as well as reduced clonogenic survival. Even more, comparing the effect of cerulenin on the clonogenic growth of leukemic CD34+ cells with the effect on normal cells from healthy donors, the inhibition of CFU-GM formation was stronger in leukemic than in normal samples. Cerulenin, a fungal metabolite, is known to be a specific inhibitor of fatty acid synthase, inducer of apoptosis in different wild-type p53 and mutant p53 tumor cell lines, whereas normal human keratinocytes and fibroblasts are resistant to the apoptotic effect [170]. In line with our results, cerulenin has been already used for the treatment of glioma tissue samples, resulted in time- and dose-dependent decreases in glioma cell viability, as well as reduced clonogenic survival [107].

Cerulenin has been shown to inhibit proliferation of leukemic cell lines HL60 [108] and K562 [109]. It was already reported that the FAS inhibitor is able to produce a rapid, profound inhibition of DNA replication and S phase progression in human cancer cells. Because S phase typically requires at least 6 h, this effect is consistent with inhibition of S phase progression rather than inhibition of the G,-S transition [160]. In line with these results in this work it was shown that cerulenin inhibits the proliferation of the cell line K562 and CD34+ CML cells. Interestingly, in comparison with normal CD34+ cells, this inhibition was not as strong as in leukemic cells. The differential effects of cerulenin in normal and malignant CD34+ cells suggest the therapeutic use of fatty acid synthesis inhibition in the treatment of human leukemia.

Despite dramatic short-term responses in vivo, the actual standard therapy with Imatinib presents still some weak points to be solved. Several studies suggest that the expansion of malignant cells in CML is not directly driven by the neoplastic stem cell

but by lineage-committed progenitor cells [171]. The treatment of patients with the tyrosine kinase inhibitor imatinib results in the persistence of BCR-ABL positive cells in most of the patients in complete cytogenetic response, suggesting that blocking of BCR-ABL induces an inhibition of progenitor cells while primitive leukemic stem cells are spared [172]. Definitive evidence of a primitive, quiescent, Philadelphia-positive stem cells subpopulation from patients with CML have been reported. This cells do not response to imatinib, and persist with both in vitro and in vivo stem cell properties [173] [174]. Since cerulenin is able to inhibit the proliferation of CD34+ hematopoietic stem and progenitor cells, it could be used as a supplementary therapy with imatinib in the treatment of CML. Whether cerulenin could also inhibit early HSC should be still evaluated in long-term culture initiating cell (LTC-IC) assays.

Even more, a substantial subset of human ovarian, endometrial, breast, colorectal, and prostatic cancers exhibit increased endogenous fatty acid biosynthesis and over-express certain enzymes in the pathway of fatty acid synthesis. Cell lines derived from these tumors use endogenously synthesized fatty acids for cellular functions, whereas normal cells and tissues appear to utilize dietary lipids preferentially [175]. Pizer et al. already showed that the difference in fatty acid biosynthesis between cancer and normal cells is an exploitable target for metabolic inhibitors in vitro and in vivo, such as cerulenin, which markedly reduces tumor cell fatty acid biosynthesis.

In summary, our data describe novel stem or progenitor cell-associated targets as genes involved in fatty acid methabolism, proposing that cerulenin could be useful for the supplementation of therapy with imatinib in the treatment of the chronic myeloid leukemia.

15. Functional role of GABA B receptor on hematopoietic stem and progenitor cells

Although adult hematopoietic stem cells from bone marrow have long been recognized as capable of developing into blood and immune cells, it was also reported that, under certain conditions, the same stem cells could also develop into cells that have many of the characteristics of neurons what could open novel therapeutic avenues in the treatment of various degenerative diseases [176].

Recent studies have demonstrated a potential molecular interrelation of neuronal and hematopoietic signaling mechanisms, supporting the model of a possible neuronal regulation of immature hematopoietic progenitors. Several investigators have described partly overlapping genetic programs of hematopoietic and neuropoietic cells in mice [22] [81] and, interestingly, the detection of a potential human neurohematopoietic stem cell population has been reported [82].

Since our group showed that the GABA B receptor mRNA as well as surface protein were expressed in human CD34+ hematopoietic stem and progenitor cells [83], GABA could be viewed as a mediator between neuronal and hematopoietic cells. Even more, we found that the GABA B receptor was upregulated in CD34+ cells from patients with CML in chronic phase in comparison to normal CD34+ cells [65], indicating a higher expression of the GABA B receptor in immature, developmentally early hematopoietic cells or in malignant cells, contrarily to its undetectable expression in fully differentiated blood cells.

GABA is involved in early events during neuronal development, playing an essential role in the synaptic integration of newly generated neurons in the adult brain [177]. In the last years several studies have related this neuromediator with the phenomena of plasticity, transdifferentiation and neurogenesis. Bone marrow stromal cells express the GABA receptor when transplanted into brain that has been subjected

to cerebral infarction, contributing to neural tissue regeneration through migrating towards the periinfarct area and acquiring the neuron-specific receptor function [178]. Bone marrow derived murine adult progenitor cells have been described to differentiate to cells with biochemical, morphological, and electrophysiological characteristics of midbrain GABA-ergic neurons [179]. The role of the GABA B receptor in human CD34+ hematopoietic stem and progenitor cells has not been described so far.

Within the nervous system the GABA B receptor is classified as a metabotropic seven transmembrane receptor which is G(i)/G(o) protein-coupled effecting on adenylate cyclase (AC) activity [96]. AC is an enzyme which catalyzes the conversion of ATP to cyclic 3',5' -adenosine monophosphate (cAMP) which activates cAMP - dependent kinases eliciting a wide array of metabolic and functional processes [111] [112]. In this work it could be shown that a modulation of the GABA B receptor specifically modified the intracellular concentration of cAMP in CD34+ cells. Higher concentrations of baclofen activate the intracellular production of cAMP while small concentrations resulted in an inhibition. Within the nervous system, baclofen and other GABA B agonists have been already described to produce very mixed effects on adenylate cyclase activity and cAMP production [96] [180] [181] [181] [182] [183]. Our findings indicate that the GABA B receptor is actively signaling in human CD34+ cells and that it is coupled to G proteins. These observations are in line with a prior study of our group, which described the coupling to G proteins of CRH and orexin, other neuroreceptors that have been found to be expressed in CD34+ cells hematopoietic stem and progenitor cells as well [83].

Numerous neurotransmitters, hormones, chemokines, local mediators, and sensory stimuli exert their effects on cells by binding to heptahelical membrane receptors (7-TMR) coupled to heterotrimeric G proteins. These highly specialized transducers can modulate the activity of multiple signaling pathways leading to diverse biological responses [84]. Recent studies have provided evidence for expression of several 7-TMR on immature hematopoietic progenitor cells, which potentially mediate chemotactic effects as chemokine receptors (e.g., CXCR4, receptor for stromal cell-derived factor-1) [114], or receptors for lipid mediators (e.g., the cysteinyl leukotriene receptor cysLT1) [115]. The signal transduction events that result in a chemotactic response after ligand binding are well characterized in 7-TMR coupled to pertussis toxin-sensitive Gi proteins [184]. Activation of Gi proteins results in a rapid release of intracellular free calcium and changes in the cytoskeleton, which includes polymerization of monomeric actin. These effects eventually lead to migration or chemotaxis

of hematopoietic stem and progenitor cells [185].

Within the nervous system GABA has been shown to stimulate intracellular Ca2+ mediated chemotactic responses in embryonic cortical cells [186]. During embryogenesis, neuroblasts proliferate within germinal zones, then migrate to their final positions. Although many neurons migrate along radial glial fibers, evidence suggests that GABA and other environmental factors, also influence neuroblast movement. In vitro assays of migration showed that the GABA B agonist baclofen stimulates motility of these cells via Gi/Go GTP binding proteins, partially attenuated by using GABA receptor antagonists [187]. Recent interesting studies showed the important modulatory role of the GABA B receptor in the migration of cortical interneurons in vivo [116].

This background prompted us to study the role of the 7-TMR GABA-B receptor on the intracellular calcium mobilization and on the cellular migration of hematopoietic stem and progenitor cells. Surprisingly, we could detect neither calcium fluxes nor in vitro transedothelial migration of highly enriched CD34+ hematopoietic progenitor cells in response to GABA B agonist or antagonist at different concentrations. Contrarily, it could be assessed that CD34+ hematopoietic progenitor cells clearly respond to SDF-1 with mobilization of intracellular calcium as an initial event of Gi protein-mediated signaling. Even more, in vitro migration of CD34+ progenitor cells was increased by a gradient of SDF-1, confirming the observations of prior reports which described that this also occur *in vivo* and contribute to bone marrow homing of hematopoietic stem cells [113]. It has been estimated that 1% of the mammalian genome codes for seven-transmembrane receptors (7-TMR), but, in line with our results, not all of these cell surface molecules induce chemotactic responses after ligand activation [188].

For example, expression of the 7-TMR somatostatin receptor type 2 has been demonstrated in hematopoietic progenitor cells in a functionally active form. Somatostatin and its analog octreotide are neuropeptides which have been shown to induce chemotaxis and other G protein-mediated effects [189]. In cells of the nervous system, somatostatin also induced rapid fluxes of intracellular free calcium dependent on activation of other cell surface receptors [190]. However, octreotide did not induce significant intracellular calcium mobilization in circulating mobilized CD34+ cells when compared to equimolar doses of SDF-1 [185]. The same occurred with the G protein-coupled 7-TMR cannabinoid cb2 receptor, whose role in the hematopoietic system could not been exactly defined, particularly its potential effect on hematopoietic stem

cell migration [185].

On the other hand, the GABA B agonist baclofen has not only been described to increase the migration but also the proliferation of neuronal progenitors providing evidence for a functional role of GABA B receptor in oligodendrocyte development [191]. The role of this receptor in the proliferation or development of hematopoietic progenitor cells has not been described so far. In this work, it was assessed that the GABA B receptor modulation does not influence the proliferation or the clonogenic growth of the CD34+ hematopoietic stem and progenitor cells. This result is in line with further investigations of our group which have shown that the GABA B receptor is not involved in the proliferation or differentiation of leukemic CD34+ cells (Butterweck et al., unpublished results).

In summary, after a variate functional study of the GABA B receptor on CD34+ hematopoietic progenitor cells, it was found that the effects on receptor modulation on this cells are different from the ones on neuronal progenitors. It could be speculated that the signaling pathways of this receptor are distinct in the hematopoietic and nervous systems, or, following the theory of plasticity, that cells develop a functionally activation of the GABA B receptor while neuronal differentiation. To support this theory it must be remarked that the CD34+ HSCs represent a heterogeneous population of cells at many different stages of functional differentiation, from pluripotent stem cells to lineage-restricted progenitor cells. Most of the CD34+ cells are progenitors for myeloid and lymphoid lineages, but a subpopulation, defined by the expression of the c-kit receptor and lack of expression of CD38 and hematopoietic lineage markers, is considered to be composed of pluripotent HSCs that can give rise to all hematopoietic lineages [192] and other types of cells as neurons [176]. At some point during differentiation, these pluripotent HSCs become committed to particular lineages and lose their capacity to function as stem or progenitor cells. In both directions of differentiation, hematopoietic or neuronal progenitor cells have shown a loss of the CD34+ phenotype [193]. While following hematopoietic development the cells loose the expression of the GABA B receptor, in neuronal progenitor cells GABA B has been shown to play a fundamental role. A recent interesting study reported that implanted CD34+ HSCs into lesions of the developing spinal cord in the chicken, exhibit neuronal cytoarchitecture and GABA+ synaptic terminals, developing a loss of CD34 expression [194]. In these *in vivo* experiments, the neuronal microenvironment [194], specially the presence of astrocytes [195], stimulated substantial proportions of human HSCs to differentiate into full-fledged neurons with consecutive activation

of the GABA B signalling pathways. In addition, special transcription factors, as Scl [196], have been described to play a crucial role in the development of GABA-ergic neurons.

Thus, our findings may represent the behavior of the population of HSCs with a preprogrammed propensity toward hematopoietic differentiation with lost of the functionality of the GABA B receptor, while other recent studies which implies a neuronal cell transdifferentiation, may show the subpopulation of HSC with a preprogrammed propensity toward neuronal differentiation or, even more, a partial realization of a neurogenic potential in all human HSCs.

Further studies will be necessary to elucidate the exact signaling pathways of GABA B receptor and other newly described neuromediator receptors in human CD34+ cells. Still, a better understanding of the molecular framework underlying transdifferentiation and plasticity will be a prerequisite for a purposeful use of the entire therapeutic potential of hematopoietic stem cells.

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Bibliography

[1] Kirchstein R, Skirboll LR (2001) Stem Cells: Scientific Progress and Future Research Directions. National Institute of Health, U.S. Department of Health and Human Services.

[2] Amit M, Carpenter MK, Inokuma MS, Chiu CP, Harris CP, et al. (2000) Clonally derived human embryonic stem cell lines maintain pluripotency and proliferative potential for prolonged periods of culture. Dev Biol 227:271–278.

[3] Haas R, Kronenwett R (2005) Fragen und Antworten zur hämatopoetischen Stamzellen. Köln: Deutscher Ärzte Verlag.

[4] Weissman IL (2000) Stem cells: units of development, units of regeneration, and units in evolution. Cell 100:157–168.

[5] Osawa M, Hanada K, Hamada H, Nakauchi H (1996) Long-term lymphohematopoietic reconstitution by a single CD34-low/negative hematopoietic stem cell. Science 273:242–245.

[6] Marshak D, Gardner R, Gottlieb D (2001) Stem cell biology. New York: Cold Spring Harbor Laboratory Press.

[7] Harley CB, Vaziri H, Counter CM, Allsopp RC (1992) The telomere hypothesis of cellular aging. Exp Gerontol 27:375–382.

[8] Steidl U, Kronenwett R, Martin S, Haas R (2003) Molecular biology of hematopoietic stem cells. Vitam Horm 66:1–28.

[9] Akashi K, Traver D, Kondo M, Weissman IL (1999) Lymphoid development from hematopoietic stem cells. Int J Hematol 69:217–226.

[10] Akashi K, Kondo M, Cheshier S, Shizuru J, Gandy K, et al. (1999) Lymphoid development from stem cells and the common lymphocyte progenitors. Cold Spring Harb Symp Quant Biol 64:1–12.

[11] Akashi K, Traver D, Miyamoto T, Weissman IL (2000) A clonogenic common myeloid progenitor that gives rise to all myeloid lineages. Nature 404:193–197.

[12] Civin CI, Strauss LC, Brovall C, Fackler MJ, Schwartz JF, et al. (1984) Antigenic analysis of hematopoiesis. a hematopoietic progenitor cell surface antigen defined by a monoclonal antibody raised against KG-1a cells. J Immunol 133:157–165.

[13] Lanza F, Healy L, Sutherland DR (2001) Structural and functional features of the CD34 antigen: an update. J Biol Regul Homeost Agents 15:1–13.

[14] Fina L, Molgaard HV, Robertson D, Bradley NJ, Monaghan P, et al. (1990) Expression of the CD34 gene in vascular endothelial cells. Blood 75:2417–2426.

[15] Civin CI, Gore SD (1993) Antigenic analysis of hematopoiesis: a review. J Hematother 2:137–144.

[16] Sutherland HJ, Eaves CJ, Eaves AC, Dragowska W, Lansdorp PM (1989) Characterization and partial purification of human marrow cells capable of initiating long-term hematopoiesis in vitro. Blood 74:1563–1570.

[17] Terstappen LW, Huang S, Safford M, Lansdorp PM, Loken MR (1991) Sequential generations of hematopoietic colonies derived from single nonlineage-committed CD34+CD38- progenitor cells. Blood 77:1218–1227.

[18] Huang S, Terstappen LW (1994) Lymphoid and myeloid differentiation of single human CD34+, hla-dr+, CD38- hematopoietic stem cells. Blood 83:1515–1526.

[19] Ramalho-Santos M, Yoon S, Matsuzaki Y, Mulligan RC, Melton DA (2002) "stemness": transcriptional profiling of embryonic and adult stem cells. Science 298:597–600.

[20] Ivanova NB, Dimos JT, Schaniel C, Hackney JA, Moore KA, et al. (2002) A stem cell molecular signature. Science 298:601–604.

[21] Phillips RL, Ernst RE, Brunk B, Ivanova N, Mahan MA, et al. (2000) The genetic program of hematopoietic stem cells. Science 288:1635–1640.

[22] Terskikh AV, Easterday MC, Li L, Hood L, Kornblum HI, et al. (2001) From hematopoiesis to neuropoiesis: evidence of overlapping genetic programs. Proc Natl Acad Sci U S A 98:7934–7939.

[23] Park IK, He Y, Lin F, Laerum OD, Tian Q, et al. (2002) Differential gene expression profiling of adult murine hematopoietic stem cells. Blood 99:488–498.

[24] Terskikh AV, Miyamoto T, Chang C, Diatchenko L, Weissman IL (2003) Gene expression analysis of purified hematopoietic stem cells and committed progenitors. Blood 102:94–101.

[25] Steidl U, Kronenwett R, Rohr UP, Fenk R, Kliszewski S, et al. (2002) Gene expression profiling identifies significant differences between the molecular phenotypes of bone marrow-derived and circulating human cd34+ hematopoietic stem cells. Blood 99:2037–2044.

[26] Archuleta TD, Devetten MP, Armitage JO (2004) Hematopoietic stem cell transplantation in hematologic malignancy. Panminerva Med 46:61–74.

[27] Baron F, Storb R (2004) Allogeneic hematopoietic cell transplantation as treatment for hematological malignancies: a review. Springer Semin Immunopathol 26:71–94.

[28] Baron F, Storb R, Little MT (2003) Hematopoietic cell transplantation: five decades of progress. Arch Med Res 34:528–544.

[29] Voso MT, Hohaus S, Moos M, Pfoersich M, Cremer FW, et al. (1999) Autografting with CD34+ peripheral blood stem cells: retained engraftment capability and reduced tumour cell content. Br J Haematol 104:382–391.

[30] Koerbling M, Doerken B, Ho AD, Pezzutto A, Hunstein W, et al. (1986) Autologous transplantation of blood-derived hemopoietic stem cells after myeloablative therapy in a patient with burkitt's lymphoma. Blood 67:529–532.

[31] Haas R, Hohaus S, Goldschmidt H, Moehle R, Witt B, et al. (1994) High-dose therapy and autografting with peripheral blood stem cells in malignant lymphoma: granulocyte-macrophage colony-stimulating factor for stem cell mobilization. Semin Oncol 21:19–24.

[32] Haas R, Moos M, Moehle R, Doehner H, Witt B, et al. (1996) High-dose therapy with peripheral blood progenitor cell transplantation in low-grade nonhodgkin's lymphoma. Bone Marrow Transplant 17:149–155.

[33] Haas R, Murea S, Goldschmidt H, Doehner H, Moos M, et al. (1995) High-dose therapy with peripheral blood progenitor cell support in patients with non-hodgkin's lymphoma. Stem Cells 13 Suppl 3:28–35.

[34] Kobbe G, Schneider P, Aivado M, Zohren F, Schubert D, et al. (2002) Reliable engraftment, low toxicity, and durable remissions following allogeneic blood stem cell transplantation with minimal conditioning. Exp Hematol 30:1346–1353.

[35] Passegue E, Jamieson CHM, Ailles LE, Weissman IL (2003) Normal and leukemic hematopoiesis: are leukemias a stem cell disorder or a reacquisition of stem cell characteristics? Proc Natl Acad Sci U S A 100 Suppl 1:11842–11849.

[36] Reya T, Morrison SJ, Clarke MF, Weissman IL (2001) Stem cells, cancer, and cancer stem cells. Nature 414:105–111.

[37] Faderl S, Talpaz M, Estrov Z, O'Brien S, Kurzrock R, et al. (1999) The biology of chronic myeloid leukemia. N Engl J Med 341:164–172.

[38] Lagasse E, Connors H, Al-Dhalimy M, Reitsma M, Dohse M, et al. (2000) Purified hematopoietic stem cells can differentiate into hepatocytes in vivo. Nat Med 6:1229–1234.

[39] Orlic D, Kajstura J, Chimenti S, Jakoniuk I, Anderson SM, et al. (2001) Bone marrow cells regenerate infarcted myocardium. Nature 410:701–705.

[40] Orlic D, Kajstura J, Chimenti S, Bodine DM, Leri A, et al. (2001) Transplanted adult bone marrow cells repair myocardial infarcts in mice. Ann N Y Acad Sci 938:221–9; discussion 229–30.

[41] Hess DC, Hill WD, Martin-Studdard A, Carroll J, Brailer J, et al. (2002) Bone marrow as a source of endothelial cells and neun-expressing cells after stroke. Stroke 33:1362–1368.

[42] Mezey E, Chandross KJ, Harta G, Maki RA, McKercher SR (2000) Turning blood into brain: cells bearing neuronal antigens generated in vivo from bone marrow. Science 290:1779–1782.

[43] Terada N, Hamazaki T, Oka M, Hoki M, Mastalerz DM, et al. (2002) Bone marrow cells adopt the phenotype of other cells by spontaneous cell fusion. Nature 416:542–545.

[44] Ying QL, Nichols J, Evans EP, Smith AG (2002) Changing potency by spontaneous fusion. Nature 416:545–548.

[45] Wagers AJ, Sherwood RI, Christensen JL, Weissman IL (2002) Little evidence for developmental plasticity of adult hematopoietic stem cells. Science 297:2256–2259.

[46] Sawyers CL (1999) Chronic myeloid leukemia. N Engl J Med 340:1330–1340.

[47] Fialkow PJ, Gartler SM, Yoshida A (1967) Clonal origin of chronic myelocytic leukemia in man. Proc Natl Acad Sci U S A 58:1468–1471.

[48] Vardiman JW (2003) The new World Health Organization classification of myeloid neoplasm: Q&A with James W. Vardiman, MD. Clin Adv Hematol Oncol 1:18, 21.

[49] Faderl S, Talpaz M, Estrov Z, Kantarjian HM (1999) Chronic myelogenous leukemia: biology and therapy. Ann Intern Med 131:207–219.

[50] Savage DG, Szydlo RM, Goldman JM (1997) Clinical features at diagnosis in 430 patients with chronic myeloid leukaemia seen at a referral centre over a 16-year period. Br J Haematol 96:111–116.

[51] Kantarjian HM, Dixon D, Keating MJ, Talpaz M, Walters RS, et al. (1988) Characteristics of accelerated disease in chronic myelogenous leukemia. Cancer 61:1441–1446.

[52] Sokal JE, Baccarani M, Russo D, Tura S (1988) Staging and prognosis in chronic myelogenous leukemia. Semin Hematol 25:49–61.

[53] Groffen J, Stephenson JR, Heisterkamp N, de Klein A, Bartram CR, et al. (1984) Philadelphia chromosomal breakpoints are clustered within a limited region, bcr, on chromosome 22. Cell 36:93–99.

[54] Deininger MW, Goldman JM, Melo JV (2000) The molecular biology of chronic myeloid leukemia. Blood 96:3343–3356.

[55] Bedi A, Zehnbauer BA, Barber JP, Sharkis SJ, Jones RJ (1994) Inhibition of apoptosis by BCR-ABL in chronic myeloid leukemia. Blood 83:2038–2044.

[56] Gordon MY, Dowding CR, Riley GP, Goldman JM, Greaves MF (1987) Altered adhesive interactions with marrow stroma of haematopoietic progenitor cells in chronic myeloid leukaemia. Nature 328:342–344.

[57] Salesse S, Verfaillie CM (2002) Mechanisms underlying abnormal trafficking and expansion of malignant progenitors in cml: Bcr/abl-induced defects in integrin function in cml. Oncogene 21:8605–8611.

[58] Deininger MWN, Druker BJ (2003) Specific targeted therapy of chronic myelogenous leukemia with imatinib. Pharmacol Rev 55:401–423.

[59] Talpaz M, Shah NP, Kantarjian H, Donato N, Nicoll J, et al. (2006) Dasatinib in imatinib-resistant Philadelphia chromosome-positive leukemias. N Engl J Med 354:2531–2541.

[60] Kantarjian HM, Giles F, Quintas-Cardama A, Cortes J (2007) Important therapeutic targets in chronic myelogenous leukemia. Clin Cancer Res 13:1089–1097.

[61] McLean LA, Gathmann I, Capdeville R, Polymeropoulos MH, Dressman M (2004) Pharmacogenomic analysis of cytogenetic response in chronic myeloid leukemia patients treated with imatinib. Clin Cancer Res 10:155–165.

[62] Ohmine K, Ota J, Ueda M, Ueno S, Yoshida K, et al. (2001) Characterization of stage progression in chronic myeloid leukemia by dna microarray with purified hematopoietic stem cells. Oncogene 20:8249–8257.

[63] Nowicki MO, Pawlowski P, Fischer T, Hess G, Pawlowski T, et al. (2003) Chronic myelogenous leukemia molecular signature. Oncogene 22:3952–3963.

[64] Szaniszlo P, Wang N, Sinha M, Reece LM, Hook JWV, et al. (2004) Getting the right cells to the array: Gene expression microarray analysis of cell mixtures and sorted cells. Cytometry A 59:191–202.

[65] Kronenwett R, Butterweck U, Steidl U, Kliszewski S, Neumann F, et al. (2005) Distinct molecular phenotype of malignant CD34(+) hematopoietic stem and progenitor cells in chronic myelogenous leukemia. Oncogene 24:5313–5324.

[66] Frank O, Brors B, Fabarius A, Li L, Haak M, et al. (2006) Gene expression signature of primary imatinib-resistant chronic myeloid leukemia patients. Leukemia 20:1400–1407.

[67] Neumann F, Teutsch N, Kliszewski S, Bork S, Steidl U, et al. (2005) Gene expression profiling of Philadelphia chromosome (Ph)-negative CD34+ hematopoietic stem and progenitor cells of patients with Ph-positive CML in major molecular remission during therapy with imatinib. Leukemia 19:458–460.

[68] Villuendas R, Steegmann JL, Pollan M, Tracey L, Granda A, et al. (2006) Identification of genes involved in imatinib resistance in CML: a gene-expression profiling approach. Leukemia 20:1047–1054.

[69] Yong ASM, Szydlo RM, Goldman JM, Apperley JF, Melo JV (2006) Molecular profiling of CD34+ cells identifies low expression of CD7, along with high expression of proteinase 3 or elastase, as predictors of longer survival in patients with cml. Blood 107:205–212.

[70] Radich JP, Dai H, Mao M, Oehler V, Schelter J, et al. (2006) Gene expression changes associated with progression and response in chronic myeloid leukemia. Proc Natl Acad Sci U S A 103:2794–2799.

[71] Zheng C, Li L, Haak M, Brors B, Frank O, et al. (2006) Gene expression profiling of CD34+ cells identifies a molecular signature of chronic myeloid leukemia blast crisis. Leukemia 20:1028–1034.

[72] Calvo W (1968) The innervation of the bone marrow in laboratory animals. Am J Anat 123:315–328.

[73] DePace DM, Webber RH (1975) Electrostimulation and morphologic study of the nerves to the bone marrow of the albino rat. Acta Anat (Basel) 93:1–18.

[74] Artico M, Bosco S, Cavallotti C, Agostinelli E, Giuliani-Piccari G, et al. (2002) Noradrenergic and cholinergic innervation of the bone marrow. Int J Mol Med 10:77–80.

[75] Bonilla S, Alarcon P, Villaverde R, Aparicio P, Silva A, et al. (2002) Haematopoietic progenitor cells from adult bone marrow differentiate into cells that express oligodendroglial antigens in the neonatal mouse brain. Eur J Neurosci 15:575–582.

[76] Mahmood A, Lu D, Yi L, Chen JL, Chopp M (2001) Intracranial bone marrow transplantation after traumatic brain injury improving functional outcome in adult rats. J Neurosurg 94:589–595.

[77] Maestroni GJ (1998) Catecholaminergic regulation of hematopoiesis in mice. Blood 92:2971; author reply 2972–2971; author reply 2973.

[78] Benestad HB, Strom-Gundersen I, Iversen PO, Haug E, Nja A (1998) No neuronal regulation of murine bone marrow function. Blood 91:1280–1287.

[79] Miyan JA, Broome CS, Whetton AD (1998) Neural regulation of bone marrow. Blood 92:2971–2973.

[80] Iversen PO, Hjeltnes N, Holm B, Flatebo T, Strom-Gundersen I, et al. (2000) Depressed immunity and impaired proliferation of hematopoietic progenitor cells in patients with complete spinal cord injury. Blood 96:2081–2083.

[81] Goolsby J, Marty MC, Heletz D, Chiappelli J, Tashko G, et al. (2003) Hematopoietic progenitors express neural genes. Proc Natl Acad Sci U S A 100:14926–14931.

[82] Shih CC, Weng Y, Mamelak A, LeBon T, Hu MC, et al. (2001) Identification of a candidate human neurohematopoietic stem-cell population. Blood 98:2412–2422.

[83] Steidl U, Bork S, Schaub S, Selbach O, Seres J, et al. (2004) Primary human CD34+ hematopoietic stem and progenitor cells express functionally active receptors of neuromediators. Blood 104:81–88.

[84] Cabrera-Vera TM, Vanhauwe J, Thomas TO, Medkova M, Preininger A, et al. (2003) Insights into G protein structure, function, and regulation. Endocr Rev 24:765–781.

[85] Kroeze WK, Sheffler DJ, Roth BL (2003) G-protein-coupled receptors at a glance. J Cell Sci 116:4867–4869.

[86] Hamm HE (1998) The many faces of G protein signaling. J Biol Chem 273:669–672.

[87] Ballesteros JA, Jensen AD, Liapakis G, Rasmussen SG, Shi L, et al. (2001) Activation of the beta 2-adrenergic receptor involves disruption of an ionic lock between the cytoplasmic ends of transmembrane segments 3 and 6. J Biol Chem 276:29171–29177.

[88] Shapiro DA, Kristiansen K, Weiner DM, Kroeze WK, Roth BL (2002) Evidence for a model of agonist-induced activation of 5-hydroxytryptamine 2a serotonin receptors that involves the disruption of a strong ionic interaction between helices 3 and 6. J Biol Chem 277:11441–11449.

[89] Marinissen MJ, Gutkind JS (2001) G-protein-coupled receptors and signaling networks: emerging paradigms. Trends Pharmacol Sci 22:368–376.

[90] Gilman AG (1987) G proteins: transducers of receptor-generated signals. Annu Rev Biochem 56:615–649.

[91] Hall RA, Lefkowitz RJ (2002) Regulation of G protein-coupled receptor signaling by scaffold proteins. Circ Res 91:672–680.

[92] Bockaert J, Marin P, Dumuis A, Fagni L (2003) The 'magic tail' of G protein-coupled receptors: an anchorage for functional protein networks. FEBS Lett 546:65–72.

[93] Kandel, Schwartz, Jessell (2000) Principles of Neural Science. New York: McGraw-Hill, 4th edition.

[94] Purves, Augustine, Fitzpatrick, Katz, LaMantia, et al. (2001) Neuroscience. Sinauer Associates, Inc., second edition edition.

[95] Austin (2004) Fundamentals of the nervous system and nervous tissue. Pearson Education, Inc., Benjamin Cummings Human Anatomy & Physiology, sixth edition edition.

[96] Bowery NG, Enna SJ (2000) gamma-aminobutyric acid(b) receptors: first of the functional metabotropic heterodimers. J Pharmacol Exp Ther 292:2–7.

[97] Bowery NG, Bettler B, Froestl W, Gallagher JP, Marshall F, et al. (2002) International union of pharmacology. xxxiii. mammalian gamma-aminobutyric acid(b) receptors: structure and function. Pharmacol Rev 54:247–264.

[98] Kaupmann K, Huggel K, Heid J, Flor PJ, Bischoff S, et al. (1997) Expression cloning of GABA(B) receptors uncovers similarity to metabotropic glutamate receptors. Nature 386:239–246.

[99] Couve A, Filippov AK, Connolly CN, Bettler B, Brown DA, et al. (1998) Intracellular retention of recombinant GABAB receptors. J Biol Chem 273:26361–26367.

[100] Calver AR, Robbins MJ, Cosio C, Rice SQ, Babbs AJ, et al. (2001) The c-terminal domains of the GABA(B) receptor subunits mediate intracellular trafficking but are not required for receptor signaling. J Neurosci 21:1203–1210.

[101] Calver AR, Davies CH, Pangalos M (2002) GABA(B) receptors: from monogamy to promiscuity. Neurosignals 11:299–314.

[102] Neumann F, Herold C, Hildebrandt B, Kobbe G, Aivado M, et al. (2003) Quantitative real-time reverse-transcription polymerase chain reaction for diagnosis of bcr-abl positive leukemias and molecular monitoring following allogeneic stem cell transplantation. Eur J Haematol 70:1–10.

[103] Yamashita Y, Kumabe T, Cho YY, Watanabe M, Kawagishi J, et al. (2000) Fatty acid induced glioma cell growth is mediated by the acyl-CoA synthetase 5 gene located on chromosome 10q25.1-q25.2, a region frequently deleted in malignant gliomas. Oncogene 19:5919–5925.

[104] O'brien SN, Welter BH, Price TM (1999) Presence of leptin in breast cell lines and breast tumors. Biochem Biophys Res Commun 259:695–698.

[105] Konopleva M, Mikhail A, Estrov Z, Zhao S, Harris D, et al. (1999) Expression and function of leptin receptor isoforms in myeloid leukemia and myelodysplastic syndromes: proliferative and anti-apoptotic activities. Blood 93:1668–1676.

[106] Bruserud O, sheng Huang T, Glenjen N, Gjertsen BT, Foss B (2002) Leptin in human acute myelogenous leukemia: studies of in vivo levels and in vitro effects on native functional leukemia blasts. Haematologica 87:584–595.

[107] Zhao W, Kridel S, Thorburn A, Kooshki M, Little J, et al. (2006) Fatty acid synthase: a novel target for antiglioma therapy. Br J Cancer 95:869–878.

[108] Pizer ES, Wood FD, Pasternack GR, Kuhajda FP (1996) Fatty acid synthase (FAS): a target for cytotoxic antimetabolites in hl60 promyelocytic leukemia cells. Cancer Res 56:745–751.

[109] Chen B, Huang P, Dai Z, Lin G (2000) [proliferation inhibition and apoptosis induction of k562 cells by fatty acid synthase inhibitor–cerulenin]. Zhonghua Xue Ye Xue Za Zhi 21:244–246.

[110] Tabe Y, Konopleva M, Munsell MF, Marini FC, Zompetta C, et al. (2004) Pml-raralpha is associated with leptin-receptor induction: the role of mesenchymal stem cell-derived adipocytes in apl cell survival. Blood 103:1815–1822.

[111] Simonds WF (1999) G protein regulation of adenylate cyclase. Trends Pharmacol Sci 20:66–73.

[112] Francis SH, Corbin JD (1999) Cyclic nucleotide-dependent protein kinases: intracellular receptors for camp and cgmp action. Crit Rev Clin Lab Sci 36:275–328.

[113] Aiuti A, Webb IJ, Bleul C, Springer T, Gutierrez-Ramos JC (1997) The chemokine SDF-1 is a chemoattractant for human CD34+ hematopoietic progenitor cells and provides a new mechanism to explain the mobilization of cd34+ progenitors to peripheral blood. J Exp Med 185:111–120.

[114] Moehle R, Bautz F, Rafii S, Moore MA, Brugger W, et al. (1998) The chemokine receptor CXCR-4 is expressed on CD34+ hematopoietic progenitors and leukemic cells and mediates transendothelial migration induced by stromal cell-derived factor-1. Blood 91:4523–4530.

[115] Bautz F, Denzlinger C, Kanz L, Moehle R (2001) Chemotaxis and transendothelial migration of CD34(+) hematopoietic progenitor cells induced by the inflammatory mediator leukotriene d4 are mediated by the 7-transmembrane receptor cyslt1. Blood 97:3433–3440.

[116] Lopez-Bendito G, Lujan R, Shigemoto R, Ganter P, Paulsen O, et al. (2003) Blockade of GABA(B) receptors alters the tangential migration of cortical neurons. Cereb Cortex 13:932–942.

[117] Naiyer AJ, Jo DY, Ahn J, Mohle R, Peichev M, et al. (1999) Stromal derived factor-1-induced chemokinesis of cord blood CD34(+) cells (long-term culture-initiating cells) through endothelial cells is mediated by e-selectin. Blood 94:4011–4019.

[118] Graf L, Heimfeld S, Torok-Storb B (2001) Comparison of gene expression in CD34+ cells from bone marrow and g-csf-mobilized peripheral blood by high-density oligonucleotide array analysis. Biol Blood Marrow Transplant 7:486–494.

[119] Ng YY, van Kessel B, Lokhorst HM, Baert MRM, van den Burg CMM, et al. (2004) Gene-expression profiling of CD34+ cells from various hematopoietic stem-cell sources reveals functional differences in stem-cell activity. J Leukoc Biol 75:314–323.

[120] Sakhinia E, Farahangpour M, Tholouli E, Yin JAL, Hoyland JA, et al. (2006) Comparison of gene-expression profiles in parallel bone marrow and peripheral blood samples in acute myeloid leukaemia by real-time polymerase chain reaction. J Clin Pathol 59:1059–1065.

[121] Cortez D, Reuther G, Pendergast AM (1997) The Bcr-Abl tyrosine kinase activates mitogenic signaling pathways and stimulates g1-to-s phase transition in hematopoietic cells. Oncogene 15:2333–2342.

[122] Raitano AB, Halpern JR, Hambuch TM, Sawyers CL (1995) The Bcr-Abl leukemia oncogene activates Jun kinase and requires Jun for transformation. Proc Natl Acad Sci U S A 92:11746–11750.

[123] Shi CS, Tuscano JM, Witte ON, Kehrl JH (1999) GCKR links the Bcr-Abl oncogene and Ras to the stress-activated protein kinase pathway. Blood 93:1338–1345.

[124] Sawyers CL, Callahan W, Witte ON (1992) Dominant negative MYC blocks transformation by ABL oncogenes. Cell 70:901–910.

[125] Zhu J, Emerson SG (2002) Hematopoietic cytokines, transcription factors and lineage commitment. Oncogene 21:3295–3313.

[126] Notari M, Neviani P, Santhanam R, Blaser BW, Chang JS, et al. (2006) A mapk/hnrpk pathway controls BCR/ABL oncogenic potential by regulating myc mrna translation. Blood 107:2507–2516.

[127] Horita M, Andreu EJ, Benito A, Arbona C, Sanz C, et al. (2000) Blockade of the Bcr-Abl kinase activity induces apoptosis of chronic myelogenous leukemia cells by suppressing signal transducer and activator of transcription 5-dependent expression of bcl-xl. J Exp Med 191:977–984.

[128] Sillaber C, Gesbert F, Frank DA, Sattler M, Griffin JD (2000) STAT5 activation contributes to growth and viability in Bcr/Abl-transformed cells. Blood 95:2118–2125.

[129] Skorski T, Bellacosa A, Nieborowska-Skorska M, Majewski M, Martinez R, et al. (1997) Transformation of hematopoietic cells by BCR/ABL requires activation of a pi-3k/akt-dependent pathway. EMBO J 16:6151–6161.

[130] Skorski T, Kanakaraj P, Nieborowska-Skorska M, Ratajczak MZ, Wen SC, et al. (1995) Phosphatidylinositol-3 kinase activity is regulated by BCR/ABL and is required for the growth of philadelphia chromosome-positive cells. Blood 86:726–736.

[131] Andreu EJ, Lledo E, Poch E, Ivorra C, Albero MP, et al. (2005) BCR-ABL induces the expression of skp2 through the pi3k pathway to promote p27kip1 degradation and proliferation of chronic myelogenous leukemia cells. Cancer Res 65:3264–3272.

[132] Williams CD, Linch DC, Watts MJ, Thomas NS (1997) Characterization of cell cycle status and E2F complexes in mobilized cd34+ cells before and after cytokine stimulation. Blood 90:194–203.

[133] Wu CL, Classon M, Dyson N, Harlow E (1996) Expression of dominant-negative mutant DP-1 blocks cell cycle progression in g1. Mol Cell Biol 16:3698–3706.

[134] Hao SX, Ren R (2000) Expression of interferon consensus sequence binding protein (icsbp) is downregulated in bcr-abl-induced murine chronic myelogenous leukemia-like disease, and forced coexpression of icsbp inhibits bcr-abl-induced myeloproliferative disorder. Mol Cell Biol 20:1149–1161.

[135] Holtschke T, Löhler J, Kanno Y, Fehr T, Giese N, et al. (1996) Immunodeficiency and chronic myelogenous leukemia-like syndrome in mice with a targeted mutation of the icsbp gene. Cell 87:307–317.

[136] Burchert A, Cai D, Hofbauer LC, Samuelsson MKR, Slater EP, et al. (2004) Interferon consensus sequence binding protein (icsbp; irf-8) antagonizes bcr/abl and down-regulates bcl-2. Blood 103:3480–3489.

[137] Uemura N, Griffin JD (1999) The adapter protein Crkl links Cbl to C3G after integrin ligation and enhances cell migration. J Biol Chem 274:37525–37532.

[138] Bazzoni G, Carlesso N, Griffin JD, Hemler ME (1996) Bcr/Abl expression stimulates integrin function in hematopoietic cell lines. J Clin Invest 98:521–528.

[139] Nakata Y, Tomkowicz B, Gewirtz AM, Ptasznik A (2006) Integrin inhibition through lyn-dependent cross talk from CXCR4 chemokine receptors in normal human cd34+ marrow cells. Blood 107:4234–4239.

[140] Donato NJ, Wu JY, Stapley J, Gallick G, Lin H, et al. (2003) BCR-ABL independence and lyn kinase overexpression in chronic myelogenous leukemia cells selected for resistance to sti571. Blood 101:690–698.

[141] McWhirter JR, Galasso DL, Wang JY (1993) A coiled-coil oligomerization domain of bcr is essential for the transforming function of bcr-abl oncoproteins. Mol Cell Biol 13:7587–7595.

[142] Wertheim JA, Perera SA, Hammer DA, Ren R, Boettiger D, et al. (2003) Localization of BCR-ABL to F-actin regulates cell adhesion but does not attenuate cml development. Blood 102:2220–2228.

[143] Tapon N, Hall A (1997) Rho, Rac and Cdc42 GTPases regulate the organization of the actin cytoskeleton. Curr Opin Cell Biol 9:86–92.

[144] Adams GB, Scadden DT (2006) The hematopoietic stem cell in its place. Nat Immunol 7:333–337.

[145] Dürig J, Rosenthal C, Elmaagacli A, Heyworth C, Halfmeyer K, et al. (2000) Biological effects of stroma-derived factor-1 alpha on normal and cml cd34+ haemopoietic cells. Leukemia 14:1652–1660.

[146] Peled A, Hardan I, Trakhtenbrot L, Gur E, Magid M, et al. (2002) Immature leukemic CD34+CXCR4+ cells from CML patients have lower integrin-dependent migration and adhesion in response to the chemokine sdf-1. Stem Cells 20:259–266.

[147] Murohashi I, Endho K, Nishida S, Yoshida S, Jinnai I, et al. (1995) Differential effects of tgf-beta 1 on normal and leukemic human hematopoietic cell proliferation. Exp Hematol 23:970–977.

[148] Bhanu NV, Trice TA, Lee YT, Gantt NM, Oneal P, et al. (2005) A sustained and pancellular reversal of gamma-globin gene silencing in adult human erythroid precursor cells. Blood 105:387–393.

[149] Bohmer RM, Campbell TA, Bianchi DW (2000) Selectively increased growth of fetal hemoglobin-expressing adult erythroid progenitors after brief treatment of early progenitors with transforming growth factor beta. Blood 95:2967–2974.

[150] Shen X, Li J, Hu PP, Waddell D, Zhang J, et al. (2001) The activity of guanine exchange factor NET1 is essential for transforming growth factor-beta-mediated stress fiber formation. J Biol Chem 276:15362–15368.

[151] Qin H, Carr HS, Wu X, Muallem D, Tran NH, et al. (2005) Characterization of the biochemical and transforming properties of the neuroepithelial transforming protein 1. J Biol Chem 280:7603–7613.

[152] Alberts AS, Treisman R (1998) Activation of rhoa and sapk/jnk signalling pathways by the rhoa-specific exchange factor mnet1. EMBO J 17:4075–4085.

[153] Pineault N, Helgason CD, Lawrence HJ, Humphries RK (2002) Differential expression of Hox, Meis1, and Pbx1 genes in primitive cells throughout murine hematopoietic ontogeny. Exp Hematol 30:49–57.

[154] Storms RW, Trujillo AP, Springer JB, Shah L, Colvin OM, et al. (1999) Isolation of primitive human hematopoietic progenitors on the basis of aldehyde dehydrogenase activity. Proc Natl Acad Sci U S A 96:9118–9123.

[155] Wang GG, Pasillas MP, Kamps MP (2006) Persistent transactivation by meis1 replaces hox function in myeloid leukemogenesis models: evidence for co-occupancy of meis1-pbx and hox-pbx complexes on promoters of leukemia-associated genes. Mol Cell Biol 26:3902–3916.

[156] Diaz-Blanco E, Bruns I, Neumann F, Fischer JC, Graef T, et al. (2007) Molecular signature of CD34(+) hematopoietic stem and progenitor cells of patients with cml in chronic phase. Leukemia 21:494–504.

[157] Fruehbeck G (2006) Intracellular signalling pathways activated by leptin. Biochem J 393:7–20.

[158] Paraskevas KI, Liapis CD, Mikhailidis DP (2006) Leptin: a promising therapeutic target with pleiotropic action besides body weight regulation. Curr Drug Targets 7:761–771.

[159] Malhotra KT, Malhotra K, Lubin BH, Kuypers FA (1999) Identification and molecular characterization of acyl-CoA synthetase in human erythrocytes and erythroid precursors. Biochem J 344 Pt 1:135–143.

[160] Pizer ES, Chrest FJ, DiGiuseppe JA, Han WF (1998) Pharmacological inhibitors of mammalian fatty acid synthase suppress dna replication and induce apoptosis in tumor cell lines. Cancer Res 58:4611–4615.

[161] Li G, Tan Y, Yang C, Zhao C, Zhao H, et al. (2002) Expression and clinical implication of soluble resistance-associated calcium-binding protein gene and multidrug resistance gene in leukemia. Zhonghua Zhong Liu Za Zhi 24:370–374.

[162] Durkin ME, Avner MR, Huh CG, Yuan BZ, Thorgeirsson SS, et al. (2005) Dlc-1, a Rho GTPase-activating protein with tumor suppressor function, is essential for embryonic development. FEBS Lett 579:1191–1196.

[163] Janowska-Wieczorek A, Marquez LA, Matsuzaki A, Hashmi HR, Larratt LM, et al. (1999) Expression of matrix metalloproteinases (mmp-2 and -9) and tissue inhibitors of metalloproteinases (timp-1 and -2) in acute myelogenous leukaemia blasts: comparison with normal bone marrow cells. Br J Haematol 105:402–411.

[164] Lambert E, Boudot C, Kadri Z, Soula-Rothhut M, Sowa ML, et al. (2003) Tissue inhibitor of metalloproteinases-1 signalling pathway leading to erythroid cell survival. Biochem J 372:767–774.

[165] Dai Z, Quackenbush RC, Courtney KD, Grove M, Cortez D, et al. (1998) Oncogenic abl and src tyrosine kinases elicit the ubiquitin-dependent degradation of target proteins through a ras-independent pathway. Genes Dev 12:1415–1424.

[166] Auwerx J, Staels B (1998) Leptin. Lancet 351:737–742.

[167] Gainsford T, Willson TA, Metcalf D, Handman E, McFarlane C, et al. (1996) Leptin can induce proliferation, differentiation, and functional activation of hemopoietic cells. Proc Natl Acad Sci U S A 93:14564–14568.

[168] Nakao T, Hino M, Yamane T, Nishizawa Y, Morii H, et al. (1998) Expression of the leptin receptor in human leukaemic blast cells. Br J Haematol 102:740–745. Leptin.

[169] Oikawa E, Iijima H, Suzuki T, Sasano H, Sato H, et al. (1998) A novel acyl-CoA synthetase, ACS5, expressed in intestinal epithelial cells and proliferating preadipocytes. J Biochem (Tokyo) 124:679–685.

[170] Heiligtag SJ, Bredehorst R, David KA (2002) Key role of mitochondria in cerulenin-mediated apoptosis. Cell Death Differ 9:1017–1025.

[171] Marley SB, Gordon MY (2005) Chronic myeloid leukaemia: stem cell derived but progenitor cell driven. Clin Sci (Lond) 109:13–25.

[172] Bhatia R, Holtz M, Niu N, Gray R, Snyder DS, et al. (2003) Persistence of malignant hematopoietic progenitors in chronic myelogenous leukemia patients in complete cytogenetic remission following imatinib mesylate treatment. Blood 101:4701–4707.

[173] Holyoake T, Jiang X, Eaves C, Eaves A (1999) Isolation of a highly quiescent subpopulation of primitive leukemic cells in chronic myeloid leukemia. Blood 94:2056–2064.

[174] Graham SM, J¿rgensen HG, Allan E, Pearson C, Alcorn MJ, et al. (2002) Primitive, quiescent, Philadelphia-positive stem cells from patients with chronic myeloid leukemia are insensitive to sti571 in vitro. Blood 99:319–325.

[175] Pizer ES, Wood FD, Heine HS, Romantsev FE, Pasternack GR, et al. (1996) Inhibition of fatty acid synthesis delays disease progression in a xenograft model of ovarian cancer. Cancer Res 56:1189–1193.

[176] Mezey E, Chandross KJ (2000) Bone marrow: a possible alternative source of cells in the adult nervous system. Eur J Pharmacol 405:297–302.

[177] Ge S, Goh ELK, Sailor KA, Kitabatake Y, li Ming G, et al. (2006) GABA regulates synaptic integration of newly generated neurons in the adult brain. Nature 439:589–593.

[178] Shichinohe H, Kuroda S, Yano S, Ohnishi T, Tamagami H, et al. (2006) Improved expression of gamma-aminobutyric acid receptor in mice with cerebral infarct and transplanted bone marrow stromal cells: an autoradiographic and histologic analysis. J Nucl Med 47:486–491.

[179] Jiang Y, Henderson D, Blackstad M, Chen A, Miller RF, et al. (2003) Neuroectodermal differentiation from mouse multipotent adult progenitor cells. Proc Natl Acad Sci U S A 100 Suppl 1:11854–11860.

[180] Oset-Gasque MJ, Parramon M, Gonzalez MP (1993) GABAB receptors modulate catecholamine secretion in chromaffin cells by a mechanism involving cyclic amp formation. Br J Pharmacol 110:1586–1592.

[181] Robichon A, Tinette S, Courtial C, Pelletier F (2004) Simultaneous stimulation of GABA and beta adrenergic receptors stabilizes isotypes of activated adenylyl cyclase heterocomplex. BMC Cell Biol 5:25.

[182] Uezono Y, Ueda Y, Ueno S, Shibuya I, Yanagihara N, et al. (1997) Enhancement by baclofen of the Gs-coupled receptor-mediated cAMP production in xenopus oocytes expressing rat brain cortex poly (a)+ rna: a role of g-protein beta gamma subunits. Biochem Biophys Res Commun 241:476–480.

[183] Steiner D, Avidor-Reiss T, Schallmach E, Butovsky E, Lev N, et al. (2005) Regulation of adenylate cyclase type VIII splice variants by acute and chronic gi/o-coupled receptor activation. Biochem J 386:341–348.

[184] Murphy PM (1994) The molecular biology of leukocyte chemoattractant receptors. Annu Rev Immunol 12:593–633.

[185] Moehle R, Bautz F, Denzlinger C, Kanz L (2001) Transendothelial migration of hematopoietic progenitor cells. role of chemotactic factors. Ann N Y Acad Sci 938:26–34; discussion 34–5.

[186] Behar TN, Li YX, Tran HT, Ma W, Dunlap V, et al. (1996) GABA stimulates chemotaxis and chemokinesis of embryonic cortical neurons via calcium-dependent mechanisms. J Neurosci 16:1808–1818.

[187] Behar TN, Schaffner AE, Tran HT, Barker JL (1995) GABA-induced motility of spinal neuroblasts develops along a ventrodorsal gradient and can be mimicked by agonists of gabaa and gabab receptors. J Neurosci Res 42:97–108.

[188] Morris AJ, Malbon CC (1999) Physiological regulation of G protein-linked signaling. Physiol Rev 79:1373–1430.

[189] Oomen SPMA, van Hennik PB, Antonissen C, Lichtenauer-Kaligis EGR, Hofland LJ, et al. (2002) Somatostatin is a selective chemoattractant for primitive (CD34(+)) hematopoietic progenitor cells. Exp Hematol 30:116–125.

[190] Connor M, Yeo A, Henderson G (1997) Neuropeptide y y2 receptor and somatostatin sst2 receptor coupling to mobilization of intracellular calcium in sh-sy5y human neuroblastoma cells. Br J Pharmacol 120:455–463.

[191] Luyt K, Slade TP, Dorward JJ, Durant CF, Wu Y, et al. (2007) Developing oligodendrocytes express functional GABA(B) receptors that stimulate cell proliferation and migration. J Neurochem 100:822–840.

[192] Ikehara S (2000) Pluripotent hemopoietic stem cells in mice and humans. Proc Soc Exp Biol Med 223:149–155.

[193] Hermann A, Maisel M, Liebau S, Gerlach M, Kleger A, et al. (2006) Mesodermal cell types induce neurogenesis from adult human hippocampal progenitor cells. J Neurochem 98:629–640.

[194] Sigurjonsson OE, Perreault MC, Egeland T, Glover JC (2005) Adult human hematopoietic stem cells produce neurons efficiently in the regenerating chicken embryo spinal cord. Proc Natl Acad Sci U S A 102:5227–5232.

[195] Reali C, Scintu F, Pillai R, Cabras S, Argiolu F, et al. (2006) Differentiation of human adult CD34+ stem cells into cells with a neural phenotype: role of astrocytes. Exp Neurol 197:399–406.

[196] Bradley CK, Takano EA, Hall MA, Goethert JR, Harvey AR, et al. (2006) The essential haematopoietic transcription factor scl is also critical for neuronal development. Eur J Neurosci 23:1677–1689.

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- *„Molecular signature of CD34+ hematopoietic stem and progenitor cells of patients with CML in chronic phase."*
  ***Diaz-Blanco E**, Bruns I, Neumann F, Fischer JC, Graef T, Rosskopf M, Brors B, Pechtel S, Bork S, Koch A, Baer A, Rohr U, Kobbe G, von Haeseler A, Gattermann N, Haas R, Kronenwett R. (2007)*
  Leukemia 21:494-504
  (Impact Factor: 6.612)

- *„Absence of the JAK2 mutation V617 in CD34+ hematopoietic stem and progenitor cells from patients with BCR-ABL-positive CML in chronic phase and blast crises."*
  *Kronenwett R, Gräf T, Neumann F, Pechtel S, Steidl U, **Diaz Blanco E**, Haas R. (2006)*
  Leukemia Research Volume 30, Issue 10:1323-1324

- *„Distinct molecular phenotype of malignant CD34+ hematopoietic stem and progenitor cells in chronic myelogenous leukemia.“*

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- *„Functional role of the G-protein coupled GABA-B receptor in CD34+ hematopoietic stem and progenitor cells“*

  Gemeinsame Jahrestagung der deutschen, österreichischen und schweizerischen Gesellschaften für Hämatologie und Onkologie (Hannover, 2005)

- *„Expression and functional activity of G protein-coupled receptors in primary human CD34+ hematopoietic stem and progenitor cells“*

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Zusammenfassung

Die Hämatopoese wird durch ein Gleichgewicht zwischen Selbsterneuerung, Differenzierung und Migration von mehr oder weniger reifen hämatopoetischen Stamm- und Progenitorzellen reguliert. Die Schlüsselfaktoren der Blutbildung zu verstehen, stellt die Grundlage für verschiedene klinische und wissenschaftliche Anwendungen von Blutstammzellen dar.

Das aus der Stammzellbiologie gewonnene Wissen kann auch neue Einblicke in die Krebsforschung eröffnen. Die am besten charakterisierte Stammzellerkrankung ist die chronische myeloische Leukämie (CML), die durch die spezifische chromosomale t(9;22)-Translokation gekennzeichnet ist. Diese Translokation resultiert in der Expression des BCR-ABL-Fusionsonkogens und in der Expansion von hämatopoetischen Stamm- und Progenitorzellen.

In dieser Arbeit erstellten wir eine molekulare Signatur von hoch angereicherten CD34+ hämatopoetischen Stamm- und Progenitorzellen aus dem Knochenmark. Mittels Microarrays (8746 Gene) wurden CD34+ Zellen von unbehandelten Patienten mit CML in der chronischen Phase mit normalen CD34+ Zellen verglichen. Die Expressionsdaten zeigten verschiedene BCR-ABL-induzierte Effekte in primären CML Vorläuferzellen, wie beispielsweise die transkriptionelle Aktivierung des klassischen MAPK-Signalweges und des PI3 Kinase/AKT-Signalweges, sowie die Herunterregulation des proapoptotischen Gens IRF8. Außerdem waren Gene, wie HoxA9 und MEIS1, die mit hämatopoetischen Stammzellen (HSC) und Leukämogenese assoziiert sind, transkriptionell aktiviert. Darüber hinaus konnten einige differentiell exprimierte Transkripte identifiziert werden, die bisher noch nicht für die CML beschrieben worden waren und somit von therapeutischer Relevanz sein könnten. Hierzu gehörten hochregulierte Gene des TGFβ-Signalwegs, fötale Hämoglobin-Gene, Sorcin, TIMP1, NET 1 und das herunterregulierte Selenoprotein P.

Die Hochregulation des Leptinrezeptors und von Genen, die in die Fettsäuresynthese involviert sind, lässt auf eine wichtige Rolle des Lipidstoffwechsels in der Pathophysiologie dieser Krankheit schließen. Die funktionelle Analyse der Kandidatengene zeigte, dass die Hochregulation von Genen des Lipidstoffwechsels eine aktive Rolle in der Differenzierung und der Proliferation von malignen hämatopoetischen Progenitorzellen spielt. Die Inkubation der CML CD34+ Zellen mit dem Fettsäuresynthese-Inhibitor Cerulenin resultierte in einer Dosisabhängigen Abnahme der Viabilität der leukämischen Zellen. Interessanterweise war die Wachstumshemmung in leukämischen Zellen verglichen mit dem Effekt auf normale CD34+ Zellen wesentlich stärker ausgeprägt. Dieser Befund eröffnet neue Möglichkeiten für die Behandlung der CML.

Neuere Untersuchungen konnten zeigen, dass hämatopoetische Stammzellen die Kapazität haben, in ein weitaus größeres Spektrum an Geweben zu differenzieren, als früher für möglich gehalten wurde. Darüber hinaus haben Studien erst kürzlich eine potentielle molekulare Interaktion von neuronalen und hämatopoetischen Signalwegen aufdecken können. Eine frühere Publikation unserer Arbeitsgruppe konnte die Expression von neurobiologischen Genen in menschlichen CD34+ hämatopoetischen Stamm- und Progenitorzellen zeigen. Dazu gehörte unter anderem der GABA B Rezeptor, der bis dato dem Nervensystem zugeschrieben wurde. In der vorliegenden Arbeit wurde die funktionelle Rolle dieses Rezeptors auf CD34+ Zellen untersucht. Hierbei konnten wir zeigen, dass der GABA B Rezeptor in hämatopoetischen Stamm- und Progenitorzellen funktionell aktiv ist und die intrazelluläre cAMP-Konzentration beeinflusst. In weiteren funktionellen Tests beeinflusste die Modulation des GABA B Rezeptors weder die Proliferation, das klonogene Wachstum, noch die Migration von CD34+ Zellen. Unsere Daten haben gezeigt, dass im Laufe der Differenzierung der hämatopoetischen Stammzelle die Expression des GABA B Rezeptors verloren geht. Dieser Befund weist auf eine Funktion in der frühen Hämatopoese hin.

Zusammenfassend lässt sich sagen, dass in dieser Arbeit eine molekulare und funktionelle Untersuchung von malignen und normalen CD34+ Zellen durchgeführt wurde, die neue Einblicke in die Biologie von hämatopoetischen Stamm- und Progenitorzellen eröffnet.