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Characterization Of The Proteins HPIP And VENTX2 As Novel Regulatory Proteins Of Human Hematopoiesis

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Submitted by
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Charakterisierung Von *HPIP* Und VENTX2 Als Neue Regulatorische Proteine Der Humanen Hämatopoese

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Dedicated to my parents Ilario and Teresa
to my brother Gabriele
and Frank my love

AIM OF THE STUDY

Hematopoiesis is the orderly process of the generation of mature blood cells from few committed progenitor cells, which in turn arise from a rare subset of hematopoietic stem cells. This process is governed by the interplay of a number of transcription factors that facilitate hematopoietic development and influence lineage commitment decisions. Several transcription factors of the homeobox and non-homeobox gene family, which are important in embryogenesis, are known to play a major role in hematopoietic development. *HOX* genes are highly expressed in early hematopoietic stem cell and in progenitor cells and down-regulated during differentiation. In parallel, *HOX* genes are implicated directly in leukemia, at the level of Hox-cofactors (Meis1 and PBX1) and *HOX* gene upstream regulators. Constitutive expression of *HOXB4* in human hematopoietic cells leads to an amplification of normal HSC. By knocking down the endogenous expression of *PBX1* in *HOXB4* overexpressing cells, HSC are 20 times more competitive in repopulating assays than these overexpressing *HOXB4* and PBX1. These findings underline the significant role of *PBX1* together with *HOX* genes for normal HSC development and for leukemogenesis. Therefore, genes that might interact with PBX1 are of particular interest for normal and as well for leukemic hematopoiesis.

Aim of the study was to define novel regulatory proteins of early human hematopoiesis. We first investigated the potential role of a novel human hematopoietic protein that interacts with the non-homeobox protein PBX1 (HPIP) in early hematopoiesis. In addition, we studied the impact of a novel ventralizing homeobox protein, the Human Vent-like Homeobox gene (*VENTX2*) on normal human hematopoietic development. We extended our analyses by determining the expression of both proteins in leukemic hematopoiesis by analyzing patient samples with different subtypes of acute myeloid leukemia (AML). As AML derives from a malignant stem cell we furthermore tested the efficacy of a new specific kinase inhibitor SU5614 on normal and leukemic stem cells from patients with AML, carrying an internal tandem duplications (ITD) or point mutation (D835) in the tyrosine kinase receptor FLT3.

1. INTRODUCTION

1.1. Hematopoiesis

Hematopoiesis is a process that involves the complex regulation of proliferation and differentiation of distinct blood cells to the functional components of the bone marrow and peripheral blood, including the erythroid, myeloid, lymphoid and platelet lineages that are produced from pluripotent hematopoietic stem cells (HSCs) [1] (Figure 1.1). In hematopoiesis the ancestral stem cell drives the production of a hierarchy of downstream cells comprised of multilineage and unilineage progenitors. This cascade is followed by increasingly mature cells more restricted in differentiation capacity, and eventually terminating with the production of fully differentiated functional blood cells. The key property that distinguishes pluripotent hematopoietic stem cells, from the immediate downstream progenitors which are also pluripotent, is their ability to self-renew. This means, that HSC have the potential to generate daughter cells with the exact stem cell properties of the parental cells. Normal hematopoietic development is noted for the delicate balance between self-renewal and differentiation. When the processes of self-renewal and differentiation become deregulated or uncoupled, despite the genetic control mechanism that are regulating hematopoiesis, mutation of crucial regulatory genes or a perturbation of the normal differentiation program with maturation arrest can occur. This can disrupt normal hematopoiesis and promote leukemogenesis, characterized by an accumulation of immature blast cells that fail to differentiate into functional cells [1]. Hematopoiesis, active throughout the human life, is a process involving extensive proliferation and differentiation of progenitor cells. These stem cells possess three important capacities: self renewal, proliferation and differentiation. The hematopoietic hierarchy can therefore be viewed as comprising three compartments stem cells, committed progenitor cells and proliferating cells of recognizable morphology each of greater size than the preceding (Figure 1.1). This intricate process takes place in a specialized hematopoietic microenvironment, which is localized in the bone marrow in the adult [2]. This hematopoietic microenvironment is of great importance in the maintenance of normal hematopoiesis [3].

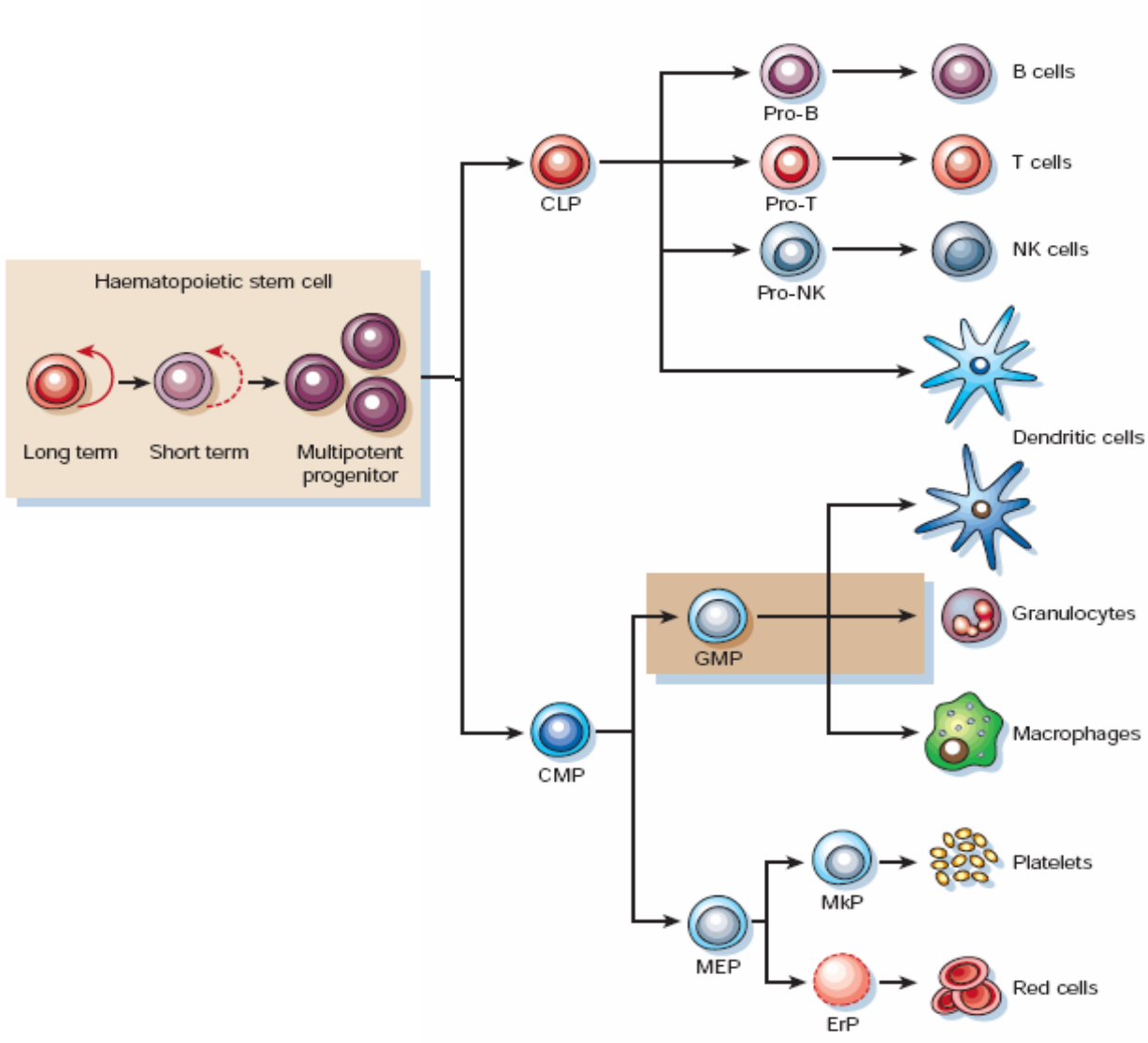


Figure 1.1. Development of hematopoietic stem cells. HSCs can be subdivided into long-term self renewing HSCs, short-term self renewing HSCs and multipotent progenitors (red arrows indicate selfrenewal). They give rise to common lymphoid progenitors (CLPs; the precursors of all lymphoid cells) and common myeloid progenitors (CMPs; the precursors of all myeloid cells). Both CMPs/GMPs (granulocyte macrophage precursors) and CLPs can give rise to all known mouse dendritic cells. MEP = macrophage erythroid progenitors; MkP = megakaryocyte precursor; ErP = erythrocyte precursor; NK = natural killer [4].

1.1.1. Hematopoietic Stem Cells

The existence of a pluripotential hematopoietic stem cell was first demonstrated in mice [5]. Mouse bone marrow cells injected into lethally irradiated syngenic mice were capable of forming discrete nodules in the spleens of the recipients 7 - 14 days after intravenous injection. These nodules consisted of erythroid, granulocytic, megakaryocytic and undifferentiated colonies and were later found to be derived from single cells called colony forming units spleen (CFU-S) [6]. The self renewal capacity of primary spleen colony cells could be confirmed by the generation of secondary spleen colonies. The equivalence of the mouse CFU-S cannot be demonstrated in humans but the presence of a similar pluripotent stem cell can be inferred from observations in clonal neoplastic transformations involving stem cells. In chronic myeloid leukemia, presence of the Philadephia chromosome can be shown in erythroid, granulocytic and megakaryocytic precursors. Isoenzyme analysis in patients heterozygous for the enzyme G6PD showed only one isoenzyme in cells of the above three lineages. These lines of evidence suggest that pluripotential hematopoietic stem cells exist in humans. Stem cells are normally not in active cycle and probably only a small proportion is involved in self-renewal. They cannot be morphologically identified and probably have the appearance of small lymphocytes. The progenies of stem cells may either self-renew or undergo a process called commitment and enter the progenitor cell compartment.

1.1.2. Hematopoietic progenitor cells

When a stem cell progeny undergoes commitment and therefore becomes a progenitor cell, it loses its ability to self-renew and is irreversibly committed to a single lineage of hematopoietic differentiation. Studies on progenitor cells became feasible by the development of clonogenic assays in semisolid culture media [7]. Briefly, in the presence of specific growth factors, called colony stimulating factors (CSF), these cells can be clonally cultured in agar or methylcellulose as discrete colonies (defined as aggregates of 50 or more cells). Analysis of cells of each colony confirmed their clonal nature [8]. Progenitor cells are therefore called colony forming units (CFU) or Colony-Forming Cells (CFC) and named after the types of colonies which they produce in culture (Table 1.1).

These progenitors do not have distinguished morphology and are probably blasts in appearance. Physiologically, they are in active cell cycle. Each is theoretically capable of proliferation and production of all differentiated cells of that particular hematopoietic lineage. Progenitor cells are more numerous than stem cells and represent the first amplification step in the formation of mature blood cells. Their proliferation is critically dependent on the presence of colony stimulating factors (CSF) and can be modified by other lymphokines and cellular products.

Colonies	Progenitor Cell
Granulocyte	G-CFU
Macrophage	M-CFU
Granulocyte/Macrophage	GM-CFU
Erythroid	E-BFU, E-CFU
Megakaryocyte	Meg-CFU
Eosinophil	Eo-CFU-
Granulocyte,Macrophage, Erythroid, Megakaryocyte	GEMM-CFU
Mast cell	Mast-CFU
Fibroblasts	F-CFU

Table 1.1. Progenitor cells and their corresponding colonies. Colony forming unit (CFC) granulocyte (G), macrophage (M), erythroid (E), megakaryocyte granulocyte (GM), granulocyte, macrophage, erythroid, megakaryocyte (GEMM).

1.2. Role of *HOX* and non-hox homeobox genes in hematopoiesis

Homeobox genes are a family of genes involved in regulatory development. These genes encode nuclear homeoproteins that act as transcription factors which play important roles in development as has been extensively documented in the fruit fly Drosophila melanogaster [9,10]. These proteins contain a highly conserved common 60-63 residues DNA-binding homeodomain (HD) that is capable of binding DNA as a monomer [11]. Although the homeobox genes were initially described as crucial for the correct anterior posterior patterning of the embryo in Drosophila and Caenorhabditis elegans as for vertebrates [12]. The

homeobox was subsequently been identified in a wide range of namly 100 mammalian proteins Stein et al. (1996)

1.2.1. Role of *HOX* genes in normal and leukemic hematopoiesis

HD encoding (homeobox-HB) genes are broadly divided into two classes. Class I includes clustered HB (HOX) genes, recognized for their role in anterioposterior patterning during embryogenesis, while the class II divergent HB (non-HOX) genes are dispersed throughout the genome. The *HOX* gene loci are believed to have arisen from gene duplication and consist of 13 paralogous groups organized into four clusters (A-D) on chromosomes 2, 7, 12 and 17, respectively. During embryonic development genes of the paralogous groups 1 to 13 are sequentially expressed 3′ to 5′ along the anteroposterior axis and are regulated in a temporal and spatial manner. *HOX* gene expression has been identified in normal hematopoietic tissues, including primary human $CD34^+$ stem/progenitor cells, and in leukemic samples [13,14]. Only expression of *HOX* genes from paralogous groups A, B and C has been detected in normal hematopoietic cells, while expression from all clusters has been demonstrated in leukemic cells. Expression of *HOXD* genes has been shown in a small number of leukemic cells, including *HOXD3* in the HEL erythroleukemic cell line [15] and *HOXD13*, which is fused to *NUP98* in AML [16,17]. Several studies imply that HOX proteins play a direct role in normal differentiation of hematopoietic cells, with some specificity in the lineages that they influence, and that leukemia may arise due to overexpression or perhaps absence of HD transcription factors [2]. The function of *HOX* genes in hematopoiesis has been investigated through knockout mouse models and enforced overexpression from retroviral vectors in hematopoietic stem cells from murine fetal liver and bone marrow and in human cord blood progenitors [2].

1.2.2. Non-*HOX* homeobox genes in normal and leukemic hematopoiesis

Homeobox genes are broadly classified into two subclasses: *HOX* and non-*HOX* homeobox (HB) genes. In contrast to the HOX family, the non-HOX family members are intermediate in size and contain five to nine members. Non-*HOX* HB genes like *HOX11* and members of the *CDX*, *HLX* and *PMX1* family are more restricted in their expression patterns. They are involved in organogenesis or differentiation of specific cell types. Deregulated

expression of non-*HOX* genes have been implicated in leukemogenesis. Ectopic expression of Cdx2 and Hox11 in murine bone marrow results in leukemia [18,19].

Several non-HOX HD proteins have been shown to function as cofactors for HOX proteins and are co-synthesized during embryonic development. These include the three-amino-acid-loop-extension (TALE) protein, PBX, MEIS and PREP1/KNOX1. They can be distinguished from other HD proteins by the inclusion of an additional three amino acid within the HD [2]. Overexpression of *MEIS1* was reported in 50 % of AML patients. Evidence from mouse models and human leukemic cells has indicated that inappropriate expression of TALE cofactors with certain HD proteins may contribute to leukemic transformation, while overexpression of TALE cofactors alone does not lead to disease development. The *PBX1* gene was originally identified at the breakpoint of the t(1;19) translocation found in 10 to 20 % of childhood pre-B-cell ALL [20]. PBX genes are widely expressed in fetal and adult tissues, although PBX1 transcripts are notably absent from lymphocytes [21]. Comparable to Meis1, the cofactor PBX1 enhanced the transforming capacity of the Hox genes Hoxb3 and Hoxb4 significantly in the transforming assay using RAT1 cells.

1.2.3. The homeodomain protein family PBX

PBX1 is a homeodomain protein that functions in complexes with other homeodomain-containing proteins to regulate gene expression during development and/or differentiation processes. PBX is a member of the PBC protein family. The human PBX1 protein was initially identified as the chromosome 1 participant of the t(1;19) translocation, which occurs in 25 % of pediatric pre-B cell acute lymphocytic leukemia which creates a chimeric gene designated *E2A-PBX1* [22]. The mechanism by which *E2A-PBX1* causes leukemia is still unclear. However, the structure of the fusion protein, in which the majority of PBX1, including the homeodomain, is fused to the transcriptional activation domain of E2A [23,24] suggests that the oncogenic properties of *E2A-PBX1* result from inappropriate regulation of target genes, of which the expression during hematopoiesis is normally regulated by wild-type PBX proteins [25,26]. *In vitro* and *in vivo* data strongly suggest that PBX functions in combination with heterologous homeodomain proteins, including class I HOX proteins. As HOX cofactors, PBC proteins improve *HOX* specificity due to the increased size of the cooperative binding site and the strength of DNA binding sites by different groups of HOX proteins [27,28]. In addition, cooperative DNA binding with PBC proteins may act to change the regulatory signal of HOX proteins, from repressors to activators [29]. PBX proteins appear to

function as part of large nucleoprotein complexes. The interactions within these complexes are probably decisive factors that allow the DNA binding proteins to discriminate among target regulatory elements. How these complexes are regulated during either early embryonic development or cellular differentiation of somatic cells to control gene expression is still unclear. Abramovich et al. (2000) speculated that the characterization of additional PBX-interacting proteins might shed light on the mechanism of PBX function, and specifically sought to identify novel cofactors or modifiers of *PBX1* [30]. Although the PBX homeodomain protein is thought to function as a transcription factor, its mechanism of action is still unknown.

1.2.4. The novel hematopoietic PBX-interacting protein (HPIP)

The identification of novel proteins which regulate human stem cells and early progenitor cell fate decisions is one of the major goals for experimental and clinical hematology. Recently, a novel human protein, the hematopoietic PBX-interacting protein (HPIP) that interacts with the homeobox gene and *HOX* co-factor PBX1 was identified [30]. The new hematopoietic PBX-interacting protein (HPIP) was identified by a yeast two-hybrid screen of a fetal-liver hematopoietic cDNA library using PBX1 as bait. *HPIP* cDNA encodes a novel protein of 731 amino acid residues containing no homology to any known protein [30], and has a calculated molecular mass of 80 kDa. *HPIP* is predicted to have a coiled-coil domain, suggesting that it interacts with other proteins. *HPIP* can bind to different members of the mammalian PBX family, inhibit the binding of PBX1/*HOX* complex to DNA and block the transcriptional activity of *E2A-PBX1*, an oncogene found in 25 % of pediatric pre-B cell acute lymphocytic leukemia [31]. The expression of *PBX1* and *HPIP* was observed by reverse transcription-PCR analysis of RNA obtained from bone marrow. *HPIP* expression was detected in $CD34^+$ fraction containing the hematopoietic progenitors and at lower levels in the $CD34^-$ mature cell population. The same pattern was found for *PBX1*, indicating that *HPIP* and *PBX1* are co-expressed in the same hematopoietic compartment [30]. The subcellular location shows that *HPIP* has a complex sub cellular distribution. It is largely bound to the cytoskeleton, but has the potential ability to shuttle between the nucleus and the cytoplasm by mechanisms involved in nuclear import and export signals [31].

1.2.5. The human VENT-like homeobox-2: VENTX2

The formation of hematopoietic stem cells during development occurs by a multistep process that begins with the induction of the ventral mesoderm. This mesoderm is patterned during gastrulation by a bone morphogenetic protein (BMP) signaling pathway that is mediated at least in part by members of the Mix and Vent families of homeobox transcription factors. Following gastrulation, a subset of ventral mesoderm is specified to become hematopoietic stem cells [32]. Members of the Xvent class of HB genes are essential for the patterning of ventral mesoderm and hematopoietic development in the Xenopus laevis embryo [33]. Recently, the mammalian homolog of the Xvent gene was cloned. The HD of this protein shares the highest homology with the HD of Xvent2B, which is a direct target of the bone morphogenetic protein 4 (BMP4) signaling pathway, making it an interesting candidate for involvement in the regulation of hematopoiesis or of hematopoietic stem cell maintenance [34]. The similarity between the HDs from *VENTX2* and the Xvent gene family place them into the same class of HD, as they have more than 60 % identity. As a Vent family member Moretti et al. (2001) demonstrated that it had ventralizing activity when injected in zebrafish embryos [35]. In addition, *VENTX2* expression was detected in an soybean agglutinin-negative (SBA-) population of bone marrow mononuclear cells (BMMNCs), contained 14.2 % CD34+ but not in the CD34- subset of BMMNCs that mainly consist of more differentiated hematopoietic precursor cells [35]. Analysis of a panel of hematopoietic cell lines representative for several lineages demonstrated that the *VENTX2* protein was present in an erythroid leukemia cell line (HEL) and not in several myeloid cell lines[35]. *VENTX2* is the first member of the Vent gene family described in mammals. *VENTX2* is localized to chromosome 10q 26.3 [35]. The *VENTX2* protein structure is show in figure 1.2.

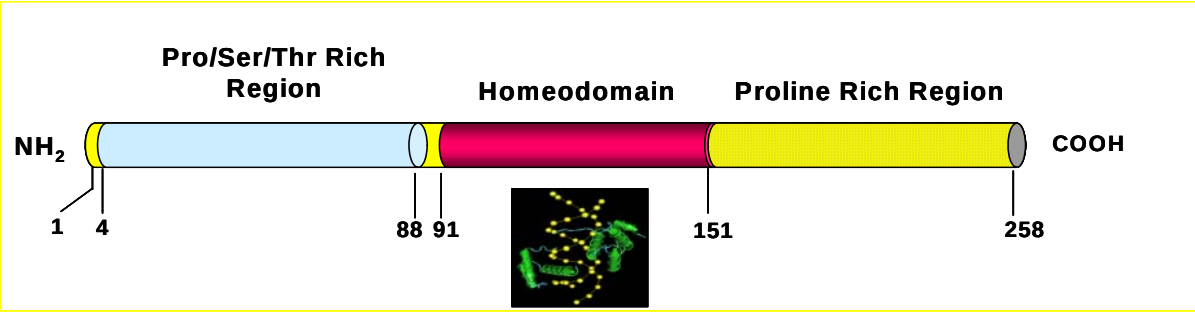


Figure 1.2. VENTX2 protein structure. VENTX2 encoded 3 exons and a length of 4.03 kb. VENTX2 contains a high percentage of serine and threonine residues at the amino terminus, a homeodomain (HD) between position 91 and 151, it forms a helix-turn-helix motif which is found in many eukaryotic transcription factors and an overall high frequency of proline residues on the carboxy terminus.

1.3. Leukemia

1.3.1. Acute Myeloid Leukemia

Acute myeloid leukemia (AML) arises from the clonal expansion of a malignant transformed hematopoietic stem cell (HSC) [36,37]. This malignancy is characterized by an accumulation of a large number of blasts (granulocyte or monocyte precursors) arrested at varying stages of differentiation in the blood or bone marrow. These malignant cells gradually replace and inhibit the growth and maturation of normal erythroid cells, monocytes and megakaryocytes. These deficiencies lead to the hallmark manifestation of this disease, namely, weakness, fatigue and pallor as a result of anemia, infection as a result of granulocytopenia, and hemorrhage as a result of thrombocytopenia. In addition, in order for a leukemic clone to become dominant, it must acquire a significant growth advantage over normal cells. Increased cell output could result from increased cell proliferation or from an increase in self-renewal potential of leukemogenic stem cells (LSC) with resultant expansion of the LSC pool [38].

AML belongs to a group of diseases, which have been classified into a number of subtypes, based on morphologies and cytochemistry of the AML cells. These subtypes are categorized, based on the stage at which normal differentiation is blocked in the leukemic blast [39].The most widely-accepted classification is designated by the French-American-British (FAB) system (Table 1.2) [40].

Fab subtype	Description	Comments
M0	Undifferentiated	Myeloperoxidase negative; myeloid markers positive
M1	Myeloblastic without maturation	Some evidence of granulocytic differentiation
M2	Myeloblastic with maturation	Maturation at or beyond the promyelocytic stage of differentiation can be divided into those with t(8;21), AML1-ETO fusion and those without
M3	Promyelocytic	APL; most cases have t(15;17) PML-RARα or another translocation involving RARα
M4	Myelomonocytic	
M4	Myelomonocytic	
M4Eo	Myelomonocytic with bone-marrow Eosinophilia	Characterized by inversion of chromosome 16 involving CBFβ, which normally forms a heterodimer with AML1
M5	Monocytic	
M6	Erythroleukeamia	
M7	Megakaryoblastic	GATA1 mutations in those associated with Down's syndrome

Table 1.2. French-American-British (FAB) classification of AML. Refers to a classification system of acute myeloid leukemia that is based on assessment of the stage of differentiation of blast. AML1 = Acute Myeloid Leukemia; APL = Acute promyelocytic Leukemia; PML = Promyelocytic Leukemia; RARα retinod-acid receptor-α. Table adapted from Daniel G Tenen at al. [40].

1.3.2. Epidemiology

Leukemias comprise 4-5 % of all the cancers among all the age groups. However, leukemia in elderly (> 60 years) age group comprises almost one tenth of the total leukemic patients. There is a male preponderance (1.4:1). All different types of leukemia are almost equal in frequency. The frequency is markedly variable among different age groups, as shown in Table 1.3 [41,42]. AML is seen in all ages, but more than half of the patients with AML are aged 60 years or above.

Type of leukemia	Age Group (Median age)	Incidence per 100,00/yr.	Sex ratio (M:F)
AML	<65 years	1.3	2.9:1.9
	>65 years	12.2	
CML	25-60 years (peak 40-50)	1.3	1.7:1.0
ALL	Children & Young adults (80 %)	1.3	M>F
	>70 years	small rise	M>F
CLL (30 %)	<50	1.0	
	>70	10.0	

Table 1.3. Incidence of Leukemias in different age groups. AML = Acute Myeloid Leukemia; CML = Chronic Myeloid Leukemia; CLL: Chronic Lymphoid Leukemia. Table adapted from [43].

1.3.3. Etiology and pathogenesis of acute leukemia

Acute Myeloid Leukemia (AML), like other cancers, is a progressive clonal disorder driven by mutations and is the most frequent leukemia in adults. Pathogenetically, AML arises from a malignant transformed clone and is a heterogenic disease, arising from a variety of pathophysiological mechanisms. The exact etiology is not known, but radiations, viruses, immunological factors, chemicals like benzene and toluene are associated with increased risk of leukemias. Despite advances in understanding the pathophysiology and treatment of AML,

the long term survival of patients <65 years old, is about 40 % [44]. The reasons for failure of therapy are e.g. multidrug resistance and therapy associated morbidity. Patients who have a so called myelodysplastic syndrome(MDS) have a high risk of developing AML which commonly occurs in the older age group (24 %) [45]. Secondary AML following chemotherapy, like alkylating reagents or radiotherapy, used for treatment of other malignancies, is also more common in older patients. These secondary AMLs are associated with chromosomal abnormalities like 5/del (5q) and or 7/del (7q) [45]. The cytogenetic analysis of leukemic blasts leads to the identification of various chromosomal, disease-specific clonal aberrations. Some of these aberrations correlate with morphological subtypes and response to therapy. Thus, cytogenetic and molecular analysis is an important part of the initial routine diagnostics and for disease monitoring in AML. A variety of other chromosomal abnormalities may occur in acute leukemia. Philadelphia chromosome (PH) designated as t(9;22) is present in more than 95 % of CML patients. There is increased incidence of (PH) chromosome in ALL patients of older age group and it is associated with poor prognosis [43]. Because of their importance for biology and prognosis, leukemia associated cytogenetics are an essential criteria within the WHO classification [46]. Although, the pathophysiology of AML is not fully understood, possible pathogenetic factors are shown in figure 1.3 [47].

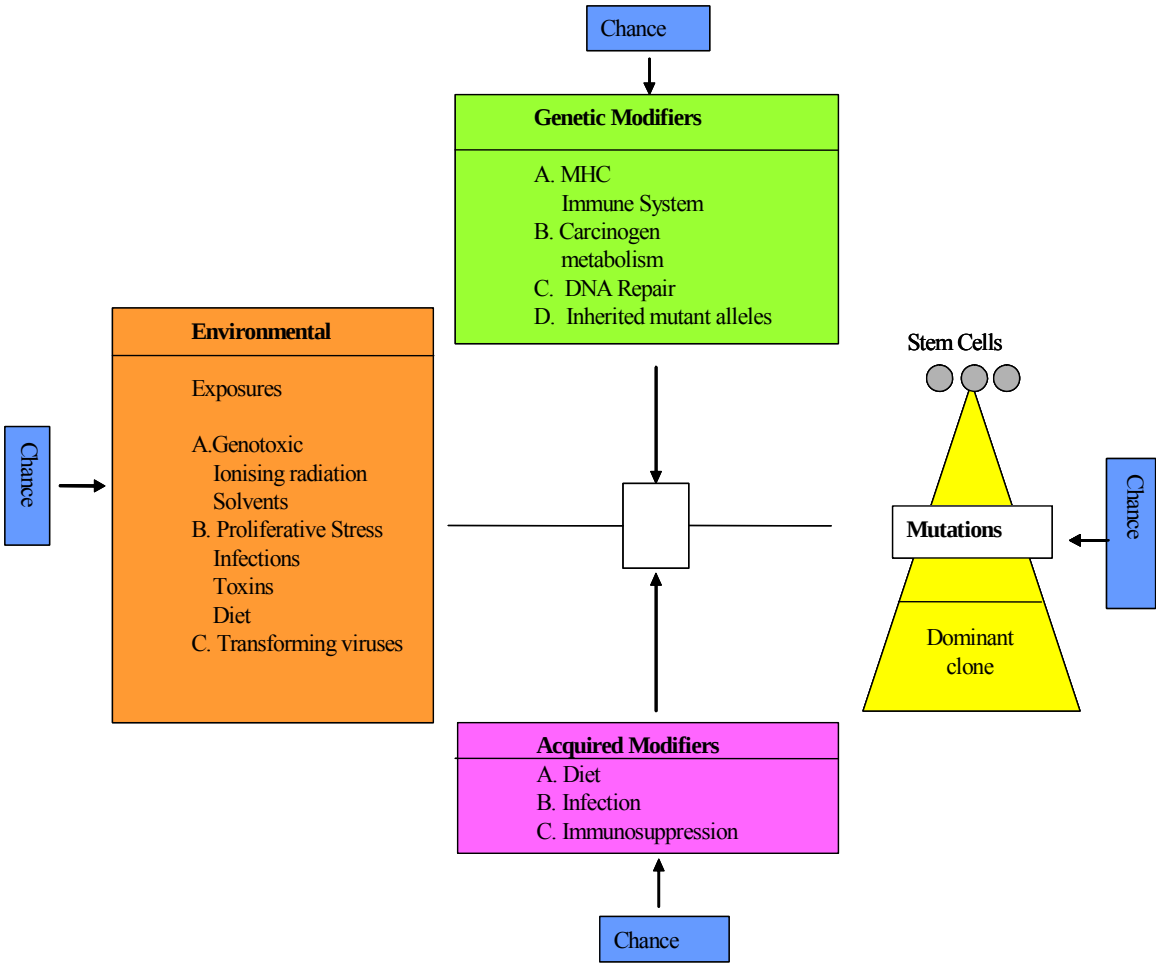


Figure.1.3. Major factors influencing etiology of cancer [47]

1.3.4. Hierarchy of leukemias

Several studies have shown that the hierarchy inside the hematopoietic compartment is maintained following leukemic transformation [48]. Some leukemia cells are capable of forming colonies in semi-solid media under addition of growth factors (AML colony-forming cells or AML-CFC) [49]. Additionally, progenitor cells have been found which are detectable in lower frequency than AML-CFC and which can sustain a malignant long-term hematopoiesis in stroma cell-dependent co-cultures or in liquid cultures upon addition of growth factors[50]. The immunophenotype of these cells initiated by long-term cultures (AML-LTC-IC) or short term cultures (AML-SC-IC) seems to be very similar to the normal LTC-IC [14,50,51]. AML-LTC-IC cells express CD34 in the absence of CD38 such as the corresponding cells in normal hematopoiesis. Therefore, among the leukemia population there are stages of clonogenic progenitor cells as well as LTC-IC which both, like the $CD34^+$ $CD38^-$ subpopulation, carry

leukemia-specific aberrations in the majority of patients [36,52-54]. They can also persist in the stage of morphologically complete remission [37]. Furthermore, using the mouse transplantation model with AML patient samples the stages of early re-populating stem cells are detectable. These early hematopoietic stem cells which are called competitive repopulating units (CRU) usually have the capability to reconstitute multilineage in lethally irradiated mice.

In the human system the repopulation occurs after sublethal irradiation of immune deficient Non-obese/LTSz-scid/scid (NOD/SCID) mice following intravenous injection of human bone marrow or cord blood stem cells [55-60].

In the leukemic population of patients with AML, these early AML inducing leukemic stem cells are detectable (so called AML NOD/SL-IC) and lead to the repopulation of mice, but do not yield a multilineage engraftment as seen with the normal CRU. Instead, they mostly repopulate the mice with blast cells similar to AML [59-64]. It could be shown that blast cells derived from patients with AML with an unfavorable karyotype engraft statistically better than blast cells with AML M3 or M2 [61]. The frequency of NOD/SCID leukemia-initiating cells (NOD/SL-IC) is variable from patient to patient and with 0.7 - 45 per 107 cells, about. 200- to 800-times lower than the frequency of AML-LTC-IC in the same samples. Immunophenotypically NOD/SL-IC are characterized by the following surface markers: $CD34^+$ $CD38^-$ [62,63], CD71- and HLA-DR- [65], Thy-1- [66]and c-kit [67]. Further on, it could be demonstrated that the morphologic/cytogenetic subtype is sustained in these mice. Using the NOD/SCID mouse model, it is therefore possible to identify earliest leukemic stem cells, to characterize them and to determine their frequency using the limiting dilution analyses [61,68]. The fact that these models are equally applicable for the detection and characterization of normal stem cells makes the comparative analysis of normal versus leukemic stem cells feasible (Figure 1.4).

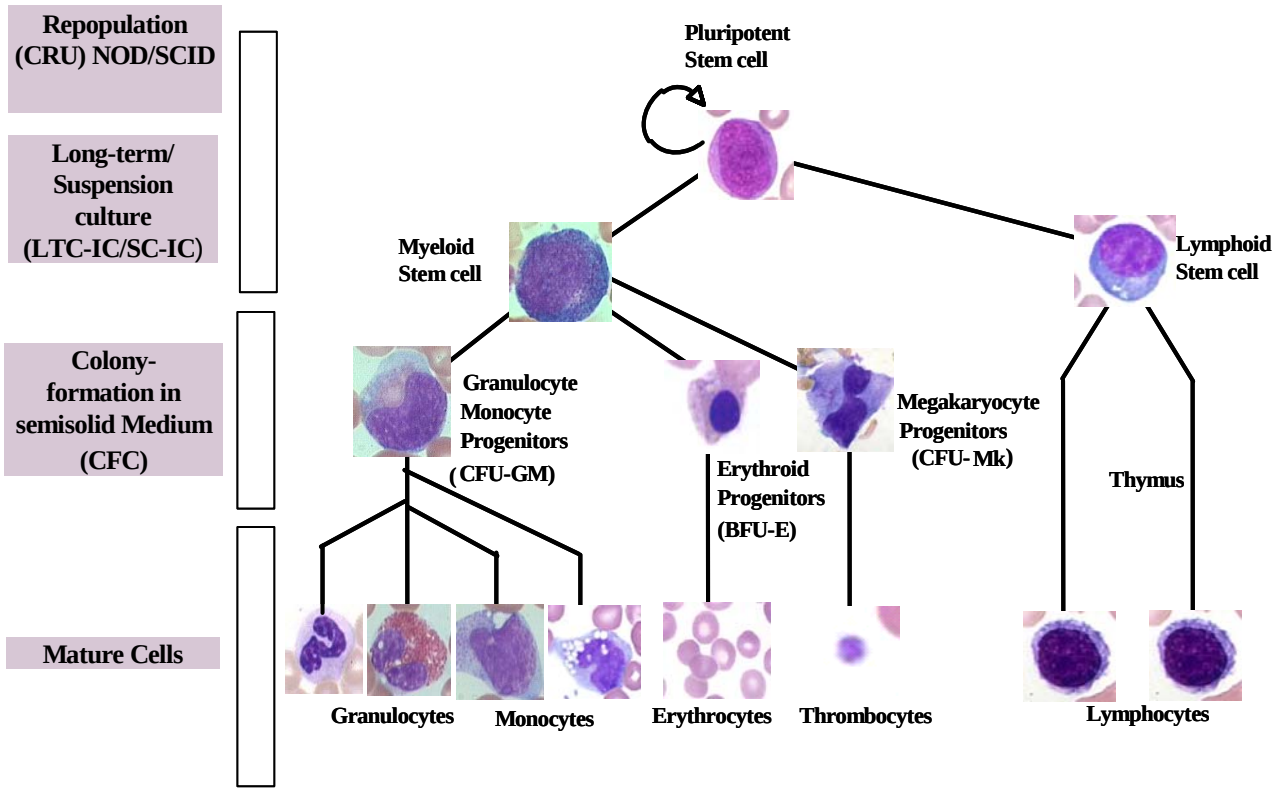


Figure 1.4. Hierarchy of hematopoiesis. The figure shows the *in vivo* and *in vitro* analyses which represent the individual levels of differentiation in the hematopoietic hierarchy.

1.4. AML-associated mutations

Cancers are known to arise from mutations or other acquired genetic changes. The genetic changes implicated in leukemias are most commonly chromosomal aberrations and receptor disregulation. Chromosomal aberrations contribute to a vast majority of cancers and the link between the occurrence of chromosomal aberrations and cancer has been very well established due to current advances in cytogenetic and molecular biology. Analyses of recurring chromosomal aberrations have led to the identification of numerous proto-oncogenes [69]. More than 80 % of myeloid leukemias are associated with at least one chromosomal rearrangement [70] and over 100 different chromosomal translocations have been cloned so far [71]. A few of the frequent chromosomal rearrangements are described below followed by a brief description of receptor internalization in AML (Table 1.4) [40].

Subtype	Medical Research Council MCR	MCR and mutation status
Favorable	t(15;17, Inv(16), t(8;21)	t(15;17), Inv(16), t(8;21) or C/EBPα mutation without *FLT3* ITD or adverse cytogenetics
Intermediate	All others	C/EBPα mutation with *FLT3* ITD or all others
Unfavourable	Del(5q)/-5, -7, 3q; complex karyotypic abnormalities	Del(5q)/-5, -7, 3q; complex karyotypic abnormalities

Table 1.4. Medical Research Council (MCR) classification of AML. AML = acute myeloid leukemia; C/EBPα, CCAAT/enhancer binding protein-α; ITD = internal tandem duplication [40].

1.4.1. Translocation t(8;21)

A common chromosomal translocation in acute myeloid leukemia (AML) involves the AML1 (acute myeloid leukemia 1, also called RUNX1, core binding factor protein (CBFa), and PEBP2aB) gene on chromosome 21 and the ETO (eight-twenty one, also called MTG8) gene on chromosome 8. This translocation generates an AML1-ETO fusion protein. AML1-ETO is a multifunctional protein exemplified by its role in regulating diverse cellular programs such as differentiation, proliferation, apoptosis, and self-renewal in various *in vitro* and *in vivo* models. It regulates the expression of both AML1 target and non-AML1 target genes via its interaction with various transcription regulators. Furthermore, additional mutations besides t(8;21) are necessary for the development of AML. t(8;21) is associated with 12 % of de novo AML cases and up to 40 % in the AML subtype M2 of the French–American-British classification. Moreover, it is also reported in a small portion of M0, M1, and M4 AML samples. Despite numerous studies on the function of AML1-ETO, the precise mechanism by which the fusion protein is involved in leukemia development is still not fully understood [72].

1.4.2. Inversion 16 inv(16) (p13;q22)

The inv(16) (p13;q22) translocation is found in more than 90 % of patients with AML M4Eo subtype, which has distinctive morphological abnormalities in the bone marrow

including myelomonocytic differentiation and an increase in atypical bone marrow eosinophils. The result of the fusion of the core-binding factor beta (CBFβ) gene and the myosin heavy polypeptide 1 (MYH11) gene is the inv(16)(p13;q22) and is thought to be a necessary genetic lesion in this form of leukemia. The most common fusion point occurs at CBFβ nucleotide (nt) 495 and MYH11 (nt) 1921; however, several rare variants have been described [73].

1.4.3. Translocation 11q23

Rearrangements of the MLL gene (also termed ALL1 or HRX) located at chromosome band 11q23 are commonly involved in adult and pediatric cases of primary acute leukemias and also found in cases of therapy-related secondary leukemias. These translocations lead to fusion genes generally resulting in novel chimeric proteins containing the amino terminus of MLL fused in-frame to one of about 30 distinct partner proteins. Abnormalities involving the MLL gene are observed in leukemias of either lymphoid or myeloid lineage derivation, as well as in poorly differentiated or biphenotypic leukemias. They are frequently seen in infant patients and patients with therapy-related secondary AML following treatment with inhibitors of topoisomerase II (epipodophyllotoxins). In the majority of cases, abnormalities involving the MLL gene are associated with a very poor prognostic outcome.

1.5. Signal transduction

Signal transduction pathways provide a mechanism for intracellular signals. Binding of ligands, such as e.g. G-CSF, GM-CSF or FLT3 ligand to their receptors causes intracellular reactions as proliferation, differentiation or apoptosis. As an example a simplified cartoon of the FLT3 signaling net work is shown in Figure 1.5.

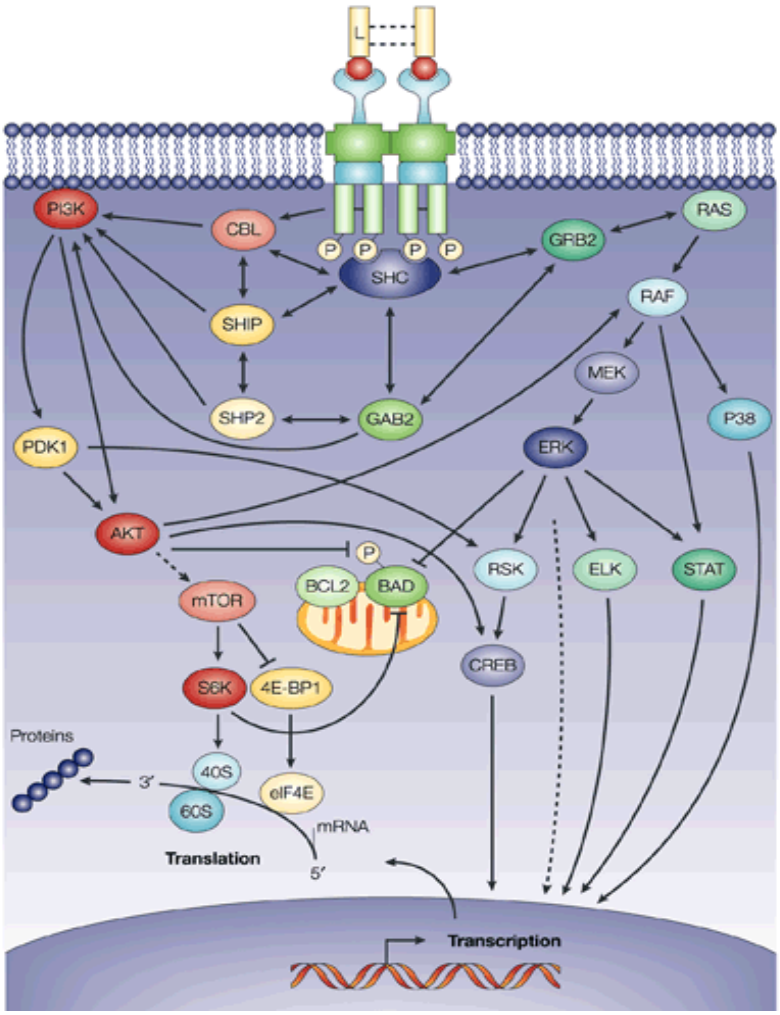


Figure 1.4. The FLT3 signaling cascade [1] Explanation see text

Although the FMS-like tyrosine kinase 3 (FLT3) signaling cascade has not been definitively characterized, this figure shows some of the complex associations and downstream effects that probably occur after activation of *FLT3*. Binding of FLT3 ligand (L)

to FLT3 triggers the PI3K (phosphatidylinositol 3-kinase) and RAS pathways, leading to increased cell proliferation and the inhibition of apoptosis. PI3K activity is probably regulated through various interactions between FLT3, SH2-containing sequence proteins (SHCs) and one or more other proteins, such as SH2-domain-containing inositol phosphatase (SHIP), SH2-domain-containing protein tyrosine phosphatase 2 (SHP2), CBL (a proto-oncogene) and GRB2-binding protein (GAB2). Activated PI3K stimulates downstream proteins such as 3-phosphoinositide-dependent protein kinase 1 (PDK1), protein kinase B (PKB/AKT) and the mammalian target of rapamycin (mTOR), which initiate the transcription and translation of crucial regulatory genes through the activation of p70 S6 kinase (S6K) and the inhibition of eukaryotic initiation factor 4E-binding protein (4E-BP1). In addition, PI3K activation blocks apoptosis through phosphorylation of the pro-apoptotic BCL2-family protein BAD (BCL2 antagonist of cell death). Activated *FLT3* also associates with GRB2 through SHC, so activating RAS. RAS activation stimulates downstream effectors such as RAF, MAPK/ERK kinases (MEKs), extracellular-signal-regulated kinase (ERK), and the 90-kDa ribosomal protein S6 kinase (RSK). These downstream effectors activate cyclic adenosine monophosphate-response element binding protein (CREB), ELK and signal transducer and activators of transcription (STATs), which lead to the transcription of genes involved in proliferation. Both pathways probably also interact with many other antiapoptotic and cell-cycle proteins, such as WAF1, KIP1 and BRCA1. Solid arrows depict direct associations between proteins, whereas dashed arrows depict associations that have been indicated indirectly [1].

1.5.1. Overview of normal RTK/RAS/MAP kinase pathway

Tyrosine kinases help to regulate cell growth, division and differentiation [74]. These proteins are generally divided into two major classes: proteins related to SRC proto-oncogene and transmembrane receptor tyrosine kinases (RTKs). Sub-class III RTXs (PDGFRA, PDGFRB, FMS, KIT and *FLT3*) are characterized by an extracellular ligand-binding region consisting of five immunoglobulin-like domains, a transmembrane and juxtamembrane (JM) and an intracellular domain with kinase activity RTXs are normally activated by receptor-specific ligand (L), which bind to the extracellular domains and promote phosphorylation of the intracellular tyrosine kinase domains (Figure 1.5) [75,76].

1.5.2. Receptor downregulation

Receptor downregulation is an important mechanism that regulates cell signaling by causing the internalization and degradation of the ligand or the entire ligand-receptor complex. Receptors that are defective for down regulation are oncogenic, furthermore, receptor overexpression is one of the most consistent features of cancerous cells. Overexpression of M-CSF and FMS is causally implicated in the development of the majority of uterine, ovarian and breast cancers. Down regulation of FMS is initiated by movement of the activation segment of the kinase domain as a result of ligand binding [77,78].

1.5.3. Abnormal signal transduction

Abnormalities, including mutations, aberrant expression and inappropriate methylation in genes that are involved in signal transduction pathways are common in AML. The precise cellular consequences of these genetic abnormalities are unknown, but functionally, these abnormalities probably promote proliferation, abate differentiation, and/or prevent apoptosis. Mutations in the *FLT3* gene are some of the most common events in AML [79].

1.6. Expression of *FLT3* in normal and leukemic cells

Despite the many genetic checks and balances to regulate hematopoiesis, in place, mutations of crucial regulatory genes that disrupt normal hematopoiesis and promote leukemogenesis can occur. Receptor tyrosine kinases are a subclass of cell-surface growth-factor receptors with an intrinsic, ligand-controlled tyrosine-kinase activity. They regulate diverse functions in normal cells and have a crucial role in oncogenesis. The FMS-like tyrosine kinase 3 (*FLT3*) genes encodes a membrane-bound receptor tyrosine kinase (RTK) that has an important role in the normal development of stem cells and the immune system. *FLT3* is expressed in a variety of human and murine cell lines of both myeloid and B-lymphoid lineage [80,81]. In normal human bone marrow cells, expression of *FLT3* appears to be restricted to early myeloid and lymphoid progenitors [82], including CD34+ cells with high levels of CD117 (c-KIT) [82,83]. *FLT3* is not expressed in erythroid cells [84], megakaryocyte [85] or mast cells [86] (Figure 1.6). Many types of leukemic cells express *FLT3*. In most types, *FLT3* expression by the leukemic cell mirrors the expression of the normal cell counterpart (Figure

1.6). So, *FLT3* is expressed by myeloid and lymphoid progenitors, and by leukemic cells of 70 to 100 % of patients with acute myeloid leukemia (AML) [85,87]. *FLT3* is expressed in all FAB subtypes, which originate from primitive, multipotent colony-forming cells or myeloid-committed colony-forming unit cells [88] and in ALL deriving from immature, progenitor B cells [89]. B-cell chronic lymphocytic leukemia (CLL) cells do not express *FLT3* arising from a more mature lymphocytic population [90].

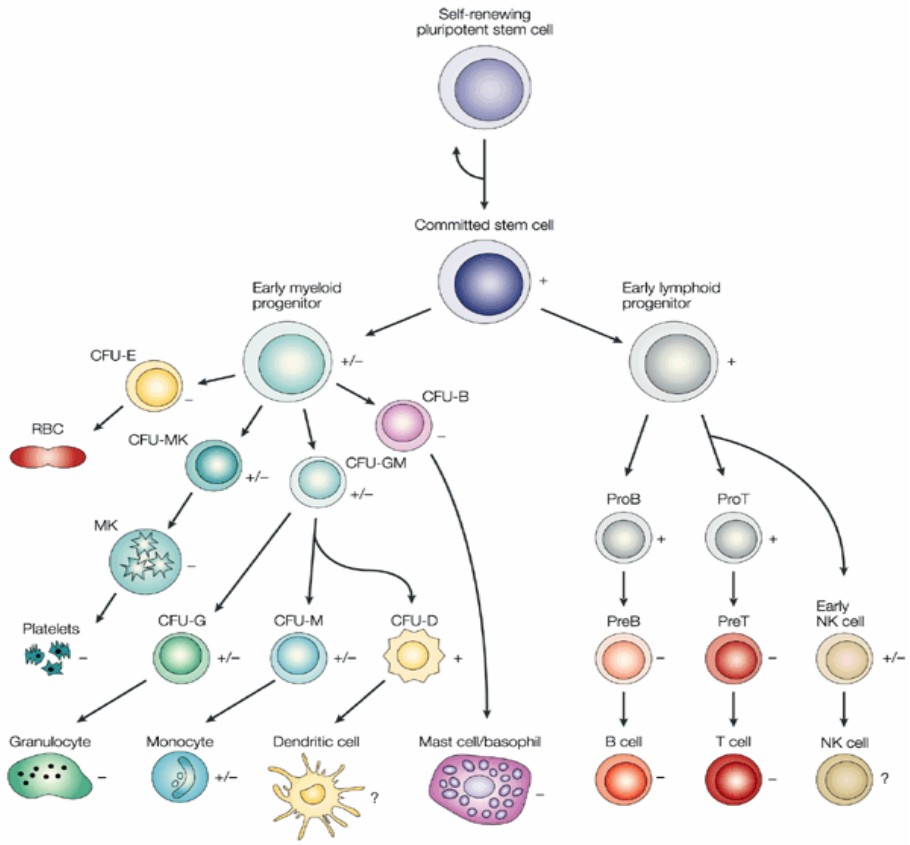


Figure 1.5. Expression of *FLT3* in normal hematopoiesis. The maturation and differentiation of cells during normal hematopoiesis is shown, indicating how expression of FMS-like tyrosine kinase 3 (*FLT3*) is linked to this process (shown as +, –, +/– or ? (unknown)). *FLT3* is mainly expressed by early myeloid and lymphoid progenitor cells, with some expression by the more mature monocytic lineage cells. Colony-forming units for the erythroid (E-CFU), megakaryocytic (MK-CFU), granulocytic–monocytic (GM-CFU), basophilic (B-CFU), granulocytic (G-CFU), monocytic (M-CFU), and dendritic (D-CFU) lineages are shown. NK cell, natural killer cell; RBC, red blood cell [1].

1.6.1. Structure of the FLT3 receptor

FLT3 (Fms-like tyrosine kinase 3), also known as FLK-2 (fetal liver kinase-2) and STK-1 (human stem cell kinase-1) [91] was cloned independently by two groups in 1991 [92]. The human *FLT3* gene is located on chromosome 13q12, shows an 85 % amino-acid sequence homology to the mouse Flt3 protein. *FLT3* encodes a receptor tyrosine kinase (RTK) of 993 amino acids in length [91,93]. The FLT3 receptor has the same general structure as four other tyrosine kinase receptors that comprise the type III RTK subfamily: c-FMS, the receptor for colony stimulating factor-1 (CSF-1), c-KIT, and both of the receptors for platelet-derived growth factor (PDGFRα and PDGFRβ). Each of these receptor molecules are characterized by membrane-bound receptors with five immunoglobulin-like extracellular domains, a transmembrane domain, a juxtamembrane domain and two intracellular tyrosine kinase domains (TDKs) linked by a kinase insert domain [1] (Figure 1.6).

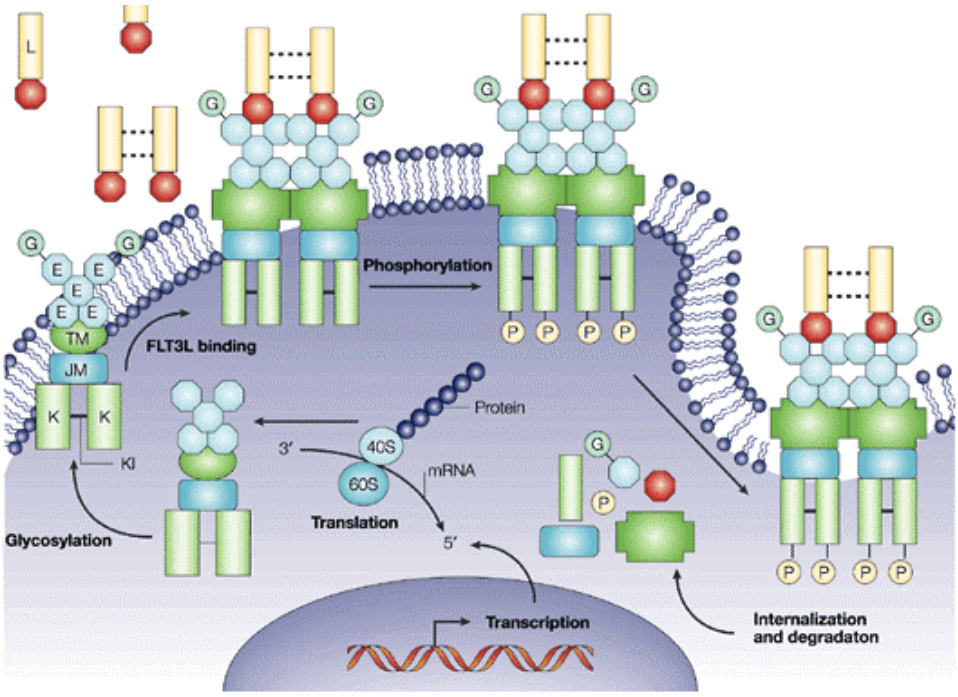


Figure 1.6. Processing, ligand-binding and internalization of FLT3. All members of the RTK subclass III family play an important role in proliferation, differentiation and survival of normal hematopoietic cells [1].

1.6.2. *FLT3* mutations in hematopoietic malignancies

The first "complementation group" of mutant genes in AML confers proliferative and/or survival advantage to hematopoietic progenitors, usually as a consequence of aberrant activation of signal transduction pathways. Nakao et al (1996) first described *FLT3* mutations in hematopoietic malignancies which stimulated a lot of research [94], characterizing the frequency and relevance of *FLT3* mutations [94,95]. The most common form of *FLT3* mutation is the internal tandem duplication (ITD) in exon 14 and 15 (previously known as exon 11 and 12) which occurs in 15-35 % of patients with AML [94,96,97] and 5-10 % of patients with myelodysplasia [95,98]. ITDs are formed when a fragment of the juxtamembrane-domain coding sequence is duplicated and insert in a direct head-to-tail orientation. The consequence of the ITD mutations is the constitutive activation of *FLT3* tyrosine kinase activity. The length mutation of the ITD varies from 3 to ≥ 400 base pairs, and the reading frame of the transcript is always preserved, either by a faithful in frame duplication or by insertion of nucleotides at the ITD junction to maintain the original reading frame [99].

The second most common type of *FLT3* mutation is a mis-sense point mutation in exon 20 (previously known as exon 17) of the tyrosine kinase domain (TKD). TKD mutations occur in patients with AML (5-10 %), MDS (2-5 %) and ALL (1-3 %) [100-102], and the most frequent nucleotide substitution (GAT-->TAT) changes an aspartic acid to tyrosine (D835Y). Other mutations in codon 835, such as deletions, have also been described, but these are far less common. In addition, point mutations, deletions and insertions in the codons surrounding codon 835 have also been found [101,103,104]. Similar to the ITDs, all TDK mutations maintain the same open reading frame (Figure 1.7).

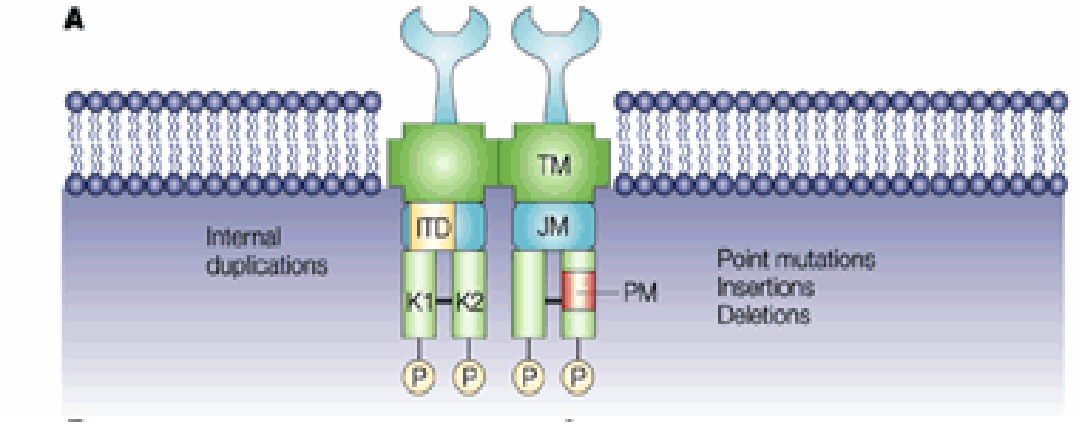


Figure 1.7. Structure and location of *FLT3* mutations. A | Internal tandem duplications (ITDs) occur in exons 14 or 15 of the juxtamembrane domain (JM), which lies directly between the transmembrane domain (TM) and the first tyrosine-kinase domain (K1). Point mutations (PM), insertions and deletions are found in exon 20 of K2 [1].

1.6.3. Biology of ITD mutations

In vitro transduction studies have shown that ITDs promote ligand-independent dimerization, autophosphorylation and constitutive activation of the receptor [105-107]. How ITDs promote constitutive activation is unknown. Experimental data indicate that mutations in the juxtamembrane domain probably eliminate the naturally occurring repressive regions of *FLT3*, which normally prevent dimerization without ligand stimulation. Various mutations (insertions, deletions or duplications) in several regions of the juxtamembrane domain cause constitutive activation of the receptor. In addition, *FLT3* engineered with an ITD, but not with a TDK region, dimerizes with wild-type receptor without the addition of ligand and can activate wild-type *FLT3* [108]. The constitutive activation induced by ITDs promotes ligand-independent proliferation and blocks myeloid differentiation of early hematopoietic cells in a mouse model system [109]. Recently, Kelly et al. (2000) found that ITDs induce a myeloproliferative-like disease in mouse bone marrow transplant models [110]. These experiments indicate that ITDs are sufficient to promote phenotypical changes associated with enhanced proliferation. But they are not sufficient to induce acute leukemia.

1.6.4. Biology of TDK point mutations

Mutations in the TDK region of *FLT3* also promote constitutive phosphorylation of the receptor and ligand-independent cell growth [102,103]. It is not known how TDK mutations activate the receptor or whether the activation of TDK-mutant *FLT3* more closely resembles that of wild-type *FLT3* or of ITD. There is ~ 80 % identity between the TDKs of FMS, KIT and *FLT3* [75], and TDK mutations have been found ion both FMS and KIT [111,112]. In TDK-mutated FMS and KIT, the tyrosine kinase activity is highly dependent on the type of amino-acid substitution in the TDK, such that some point mutations cause a marked increase in activity, whereas others inhibit activity [113,114]. These experiments are in sharp contrast to the mutational experiment with the juxtamembrane domain of *FLT3*, which shows that any

mutation in the juxtamembrane region causes an increase in activity [108]. On the basis of experimental data with other RTK subclass III receptors, TDK mutations probably increase the level of intrinsic tyrosine-kinase activity, rather than interfering with repressive domains and promoting dimerization [1].

1.7. Clinical relevance of ITD mutation

FLT3 mutations are of clinical importance [94,96,98,115-117]. ITDs have been strongly associated with leukocytosis, high blast count, normal cytogenetic, and the translocations t(15;17) and t(6;9). Recently, one study indicated that ITDs might also be associated with duplications and/or double strand DNA breaks in the breakpoint cluster of the MLL gene [118]. The prevalence of ITDs in patients with AML increases with age, ranging from 5-15 % in pediatric patients to 25-35 % in adults. Most studies in pediatric patients with AML have found that ITDs are strong, independent predictors of poor clinical outcome [116,117,119]. Studies in adult patients with AML are not as conclusive, but still show an overall trend for poor clinical outcome in patients with ITDs [96,99,120.]. Despite attempts to stratify patients with AML according to risk factors on the basis of their presenting clinical and laboratory characteristics, outcomes after chemotherapy for similar patients are highly variable. Perhaps not all ITDs are equal, although there are no data to support this hypothesis. Other possibilities are that other genetic abnormalities associated with ITDs confound the analyses, by making the outcome either worse or better.

ITDs have been associated with other known prognostic abnormalities such as t(15;17), t(6;9) and MLL mutations [101,118]. Some of these genetic abnormalities, such as t(15;17), are associated with a good prognosis, whereas others, such as t(6;9), are associated with poor clinical outcome [121]. Knowledge of how ITDs that interact with other cytogenetic abnormalities might provide a biological insight into leukemogenesis and clarify the uncertainties of risk stratification.

Two studies of patients with AML with t(15;17) found no significant impact of ITDs on clinical outcome, but there was a trend (not statistically significant) towards shorter disease-free survival and higher relapse rates in patients with a *FLT3* mutation [122,123]. In addition, both studies found a significant association between ITDs and leukocytosis, which indicates that the ITD has a proliferative biological effect in cases of AML with t(15;17). ITDs are mainly found in heterozygous state, but sometimes they are found in a single-copy,

homozygous state after loss of the normal *FLT3* allele. Some sub-populations of AML cells might have an ITD, whereas others contain only wild-type *FLT3*. For example, *FLT3* mutational status can change between diagnosis and relapse, with some patients developing a new or different *FLT3* mutation on relapse, whereas other patients lose their *FLT3* mutation completely [123,124]. If leukemia contained two clones with different *FLT3* mutation status (one with cells containing an ITD and one with wild-type *FLT3*), then patients with a higher percentage of clones with ITDs would have higher ITD wild-type ratios. If the cells with the ITD are more resistant to therapy, then patients with higher ITD wild-type ratios would have more likely a poor clinical outcome than patients having a lower ratio. It is not known how a high ITD: wild-type allelic ratio effects mRNA and protein expression of the mutant and wild-type alleles. It would be expected that those samples with a high ITD: wild-type allelic ratio would also have a higher level of ITD mRNA and of ITD protein expression. Recently, Libura et al. (2003) examined the relative mRNA expression of 31 patients with AML carrying ITDs compared to 100 patients with AML carrying wild-type *FLT3* [118]. Overall, the mean FLT3 mRNA expression of the ITD group was not significantly different from that of the wild-type group, but there was a wide range of *FLT3* expression within the patients with ITD and wild-type *FLT3*. As noted previously, wild-type *FLT3* expression is increased in high-risk patients with MLL translocations [125].

1.8. *FLT3* as a target for therapy in AML

FLT3 is the most commonly mutated gene in AML and confers a poor prognosis in most patients. As a consequence, there has been an intensive effort to develop selective inhibitors as therapeutic reagents.

After the success of Imatinib STI571 (Glivec; Novartis), a small-molecule tyrosine kinase inhibitor (TKI) used to treat CML [126], there has been a tremendous effort to discover other small-molecule inhibitors that could be efficacious for other malignancies. Given the high frequency of activating *FLT3* mutations in patients with AML, *FLT3* and its downstream pathway are attractive targets for directed inhibition. Preclinical studies in patients but also studies in cell culture and murine models of leukemia mediated by ITD, provide further support for this strategy [127,128].

In a pivotal set of preclinical experiments, STI571 was shown to suppress the proliferation of Bcr-Abl–expressing cells *in vitro* and *in vivo*. In colony-forming assays of

peripheral blood or bone marrow from patients with CML, STI571 caused a 92–98 % decrease in the number of Bcr-Abl colonies formed, with minimal inhibition of normal colony formation [129]. Cellular *in vivo* and human ex-vivo studies convinced that STI571 could be useful in diseases involving deregulated Abl PTK activity. The efficacy and specificity of STI571 has been confirmed and extended by several laboratories [130,131]. It was demonstrated that STI571 has activity against p185 Bcr-Abl and another activated Abl fusion protein, Tel-Abl [126,130,132].

Non-specific TKIs such as the tyrphostin A AG1296 and AG1295, block the constitutive activation of ITDs, as well as other proteins such as heat shock protein 90 (HSP90), thereby inhibiting the growth of cells that express ITDs [133,134]. These compounds are selective inhibitors of FLT3, KIT, and PDGFR. For example, the tyrphostin AG1296 inhibits the growth of Ba/F3 cells transformed by ITD and *FLT3* autophosphorylation and activation of downstream targets such as STAT5A/B [127]. A related compound, AG1295, also inhibits ITD mutants and has specific toxicity for primary AML blasts harboring ITD compared with cytosine arabinoside [135]. It has also been reported that Herbimycin A is a submicromolar inhibitor of *FLT3* and prevents leukemia progression in mice injected with 32D cells expressing ITD. Although none of these compounds would be suitable for consideration in clinical trials in humans, these experiments provide evidence that *FLT3* inhibition may be an effective approach to the subset of leukemias with activating mutations in *FLT3* and perhaps for AML associated with overexpression of the wild-type *FLT3*.

Several promising TKIs have been identified recently that might change the treatment of hematopoietic malignancies. CEP-701 and CEP-5214 (Cephalon) are two orally bioavailable TKIs derived from indolocarbazole [136,137]. Both compounds preferentially inhibit autophosphorylation of wild-type and mutant *FLT3*, and show limited inhibition of KIT, FMS and PDGFR. Levis et al. (2002) found that CEP-701 is cytotoxic to leukemia blasts from patients with AML *in vitro* and *in vivo*, and prolongs the survival of mice injected with BaF3/ITD cells [136]. A Phase I/II trial is now underway, testing CEP-701 in patients with refractory and relapsed AML with *FLT3* mutations, and preliminary results have been presented in key abstract form at the American Society of Hematology [138]. In eight patients, CEP-701 seemed to be relatively well tolerated, with nausea, fatigue and neutropaenia being the most commonly described side effects. CEP-701 dosage has now been titrated to 80 mg twice a day. At this early stage, it is difficult to discern the potential efficacy of CEP-701, but one patient did normalize his peripheral-blood counts, with <5 % blasts in his bone marrow, which indicates that this agent might be beneficial in some patients with AML.

The company Sugen has also identified several compounds (SU5416, SU5614 and SU11248) that block *FLT3* activation [139,140]. Unlike the compounds developed by Cephalon, these agents also inhibit other RTKs such as KIT, PDGFR and VEGFR [139,140]. After initial Phase I trials, Giles et al. recently examined SU5416 at a dose of 145 mg per m2 in 55 patients with refractory or relapsed AML regardless of *FLT3* status [141]. The main side effect included headaches (14 % of patients), dyspnoea (14 %), infusion-related reactions (11 %) and thrombotic episodes (7 %). Three patients (5 %) had a partial response. An early Phase I trial examined SU11248 in 32 patients with advanced AML. Again, *FLT3* mutations were not taken in account in this trial. SU11248 was relatively well tolerated, with fatigue being the main dose-limiting toxicity [142]. Approximately half of the patients had some form of haematological response as shown by a > 50 % reduction in the number of leukemia blasts.

MLN518 (otherwise known as CT53518 from Millenium) is a novel piperarazinyl quinazoline that inhibits the growth of ITD-transformed cells *in vitro* and *in vivo* [110]. Similar to the Sugen compounds, MLN518 also inhibits wild-type *FLT3*, PDGFRB and KIT. In a Phase I study including patients with relapsed or refractory AML, preliminary findings showed that two of six patients had < 50 % reduction in the number of bone blast. This study is currently in progress with additional patients [143].

Lastly, PKC412 (Novartis) is a benzoylstaurosporine initially developed as a VEGFR inhibitor that blocks the activity of wild-type and mutant *FLT3* [125,144]. On the basis of Phase I dosing studies in solid tumors, a Phase II trial started studying PKC412 at a dose of 75 mg three times a day in patients with relapsed or refractory AML with *FLT3* mutations [145]. All patients had a poor prognosis for myelosuppressive chemotherapy and had a poor performance status. Mild nausea was the most common side-effect in the first eight patients, but three of the eight patients discontinued use of the drug for reasons other than progression of disease (liver toxicity, fatal pulmonary toxicity and severe lethargy). It is unclear if these complications were related to drug administration. No partial or complete responses were obtained in this heavily pre-treated and debilitated patient population. Analysis of results is continuing, and additional data should be available in the near future. Of additional interest is the fact that PKC412 has been found to kill leukemia cells with MLL translocations that overexpress wild-type *FLT3* [125,146]. These findings indicate that PKC412 (and possibly other TKIs) might be effective for the treatment of a wide range of hematopoietic malignancies that overexpress *FLT3*, even if they do not have activating *FLT3* mutations. If similar experiments can validate these findings in other cell lines and primary AML samples, then the therapeutic

use of TKIs might broaden to include other malignancies, such as AML and B-cell ALL, or any hematopoietic disease that overexpresses *FLT3*.

2. MATERIALS

2.1. Reagents, cytokines and antibodies

Reagents	Company
Acetic acid	Sigma-Aldrich, Taufkirchen, Germany
Agar	Sigma-Aldrich, Taufkirchen, Germany
Agarose	Sigma-Aldrich, Taufkirchen, Germany
BIT	Stem Cell Technologies, Vancouver, BC, Canada
Bromphenolblue	Sigma-Aldrich, Taufkirchen, Germany
BSA	
Calcium Chloride	Sigma-Aldrich, Taufkirchen, Germany
Ciprobay 400	Bayer
DMSO	Sigma-Aldrich, Taufkirchen, Germany
DMEM	PAN Biotech, Aidenbach, Germany
Ethanol	Sigma-Aldrich, Taufkirchen, Germany
Ethidium Bromide	Sigma-Aldrich, Taufkirchen, Germany
Fetal Bovine Serum	PAN Biotech, Aidenbach, Germany
Formaldehyde	Sigma-Aldrich, Taufkirchen, Germany
Geneticin	GIBCO, Invitrogen Corporation,Karlsruhe,Germany
HEPES	GIBCO, Invitrogen Corporation,Karlsruhe,Germany
HLTM (Myelocult H5100)	Stem Cell Technologies, Vancouver, BC, Canada
Hydrocortisone (solucortef)	Stem Cell Technologies, Vancouver, BC, Canada
IMDM	GIBCO, Invitrogen Corporation,Karlsruhe,Germany
Isopropanol	Sigma-Aldrich, Taufkirchen, Germany
LDL	Stem Cell Technologies, Vancouver, BC, Canada
L-Glutamine	
Methanol	Sigma-Aldrich, Taufkirchen, Germany
Methilcellulose H4330	Stem Cell Technologies, Vancouver, BC, Canada
Methilcellulose H4334	Stem Cell Technologies, Vancouver, BC, Canada
Pancoll	PAN Biotech, Aidenbach, Germany
PBS	PAN Biotech, Aidenbach, Germany
Penicillin/Streptomycin	GIBCO, Invitrogen Corporation,Karlsruhe,Germany

RPMI	PAN Biotech, Aidenbach, Germany
SDS	Sigma-Aldrich, Taufkirchen, Germany
Sodium Chloride	Sigma-Aldrich, Taufkirchen, Germany
Trypan blue	GIBCO, Invitrogen Corporation,Karlsruhe,Germany
Trypsin/EDTA	GIBCO, Invitrogen Corporation,Karlsruhe,Germany
β- Mercaptoethanol	Sigma-Aldrich, Taufkirchen, Germany
Cytofix (Cell fixation reagent)	BD Pharmingen

Cytokine	
SF Steel factor	ImmunoTools Friesoythe; Germany
GM-CSF	Tebu-bio, Frankfurt, Germany
G-CSF	Stem Cell Technologies
Flt-3-ligand	PAN Biotech GmbH, Aidenbach, Germany
IL6	Tebu-bio, Frankfurt, Germany
IL3	ImmunoTools Friesoythe; Germany

Antibody	Company
CD45PE	BD Pharmingen,Heidelberg, Germany
CD34 PE	BD Pharmingen,Heidelberg, Germany
CD38 APC	BD Pharmingen,Heidelberg, Germany
CD15PE	BD Pharmingen,Heidelberg, Germany
CD19 APC	BD Pharmingen,Heidelberg, Germany
CD33PE	BD Pharmingen,Heidelberg, Germany
CD36 APC	BD Pharmingen,Heidelberg, Germany
CD135 PE	BD Pharmingen,Heidelberg, Germany
CD71APC	BD Pharmingen,Heidelberg, Germany
CD41APC	BD Pharmingen,Heidelberg, Germany
CDF123PE	BD Pharmingen,Heidelberg, Germany
CD25APC	BD Pharmingen,Heidelberg, Germany
CD11bPE	BD Pharmingen,Heidelberg, Germany

CD40APC	BD Pharmingen,Heidelberg, Germany

Reagent Kits	Company
MACS CD34 Cell Isolation Kit	Miltenyi Biotec GmbH, Bergisch Gladbach, Germany
Qiaquick gel extraction kit	Qiagen, Hilden, Germany

Standards, ladders	Company
1Kb Plus DNA ladder	Invitrogen
100 bp DNA	NEB, Frankfurt, Germany

Drug	Company
Inhibitor SU5614	Calbiochem-Novabiochem, Bad Soden, Germany

2.2. Cell lines, bacteria and biological material

Mammalian Cell Lines
Phoenix Ampho Packaging cell line (Stanford University, Medical Centre, USA)
PG13 mouse embryonic fibroblast r packaging cell line (ATCC)
K562 erythroleukemia cell line (ATCC)
SLSL-J-IL3-neo-murine fibroblast (Terry Fox Laboratory, Vancouver, Canada)
SL/SL- J-SF-tkneo J-IL-3-hytk-murine fibroblast (Terry Fox Laboratory, Vancouver, Canada)
M2-10B4 j-GCSF-tkneo j-IL-3-hytk- murine fibroblast (Terry Fox Laboratory, Vancouver, Canada)

Bacteria
Eschereschia coli DH5α
Eschereschia coli BL-21

Biological Materials	Company

Peripheral blood (PB) AML patients
Bone marrow (BM) AML patints
Cord blood (CB) healthy donors

2.3. Material for *in vivo* mice experiments

In Vivo	
NOD/LtSz-scid/scid (NOD/SCID) mice	Animal House Facility at the GSF Institute for Experimental Hematology

Mice related reagents and equipement	
Avertin Solution	Sigma-Aldrich, St. Louis, MO
Formalin soloution	Sigma –Aldrich, St. Louis, MO
Sterile Syringes	BD Bioscience, Palo Alto, CA
Kendall Monoject 3 ml syringes	Tyco Healthcare, UK
Sterile needels 0,5 x 25mm and 0.55 x 0.25	BD Microlance, Drogheda, Ireland
Sterile needles for methylcellulose	Stem Cell Technologies, Vancouver, Canada
Ammonium Chloride solution	Stem Cell Technologie, Vancouver, Canada
Heparinized capillaries Microcuvette CB 300	Sarsted, Numbrech, Germany

2.4. Software and machines

Softwares	Company
CellQuest version 3.1f	Becton Dickinson Immunocytometry Systemss

Machines	Company
FACS vantage	Becton Dickinson FACScan
FACS vantage	Becton Dickinson FACSort flow cytometer
137Cs source	

3. METHODS

3.1. Biological materials

Umbilical cord blood (CB) was obtained from mothers undergoing cesarean delivery of normal, full-term infants and collected in heparin coated syringes. Approved institutional procedures were followed to obtain informed consent of the mothers. Frozen CD34+ cord blood or bone marrow (BM) cells from healthy donors were buying by (CellSystems, St. Katharinen, Germany). Peripheral blood or bone marrow was obtained from 12 patients with newly diagnosed AML after informed consent and with the approval of the Clinical Research Ethics Board of the LMU University of Munich.

3.2. Chemical material: SU5614

The PTK inhibitor SU5614 was obtained from Calbiochem (Calbiochem-Novabiochem, Bad Soden, Germany), dissolved in dimethyl sulfoxide (DMSO) at stock concentrations of 10 mM and 50 mM, aliquoted, and stored at -20 °C. Final concentrations of DMSO in growth medium did not exceed 0.1 %. 1x 106cell/ml frozen mononuclear PB or BM cells from AML patients and normal CD34+ enriched BM cells (98 % CD34 positive) were incubated in IMDM with 20 % FCS, at 1x106 cells/ml with 50 ng/ml G-CSF with or without 1–10 μM SU5614. After 24 hours incubation cells were harvested, adherent cells trypsinized, washed twice in IMDM with 20 % FCS and viability measured by trypan blue dye exclusion prior to plating in the various assays. Equal fractions of the cells recovered from cultures with or without the PTK inhibitor were assayed without regard to any change in cell numbers during the 24 hours culture period. Assays for AML-colony forming cell (CFC) long term initiating cells (LTC-IC) and suspension culture (SC) were performed.

3.3. Virus producer packaging cell lines

Phoenix Ampho cells cultured in DMEM with 10% fetal bovine serum were plated at 2.5 x 106 cells per 10 cm plate one day before transfection. Medium was changed 4 hours prior to transfection. In a 5 ml tube 20 μg of plasmid DNA, 62.5 μl of 2M CaCl2 and dH2O to make up to 500 μl were mixed together. 500 μl of 2x HBS was added drop wise to form a

precipitate within the next few minutes. This mixture was then added to the cells drop wise. After about 12 hours, the medium was replaced with fresh medium to collect virus particles. The virus containing medium (VCM) was collected after 24 hours, filtered trough 0.45 μm filter, supplemented with protamine sulphate to give a final concentration of 5 μg/ml, and layered on PG13 packaging cells for viral infection. After repeated infections (3-5 times), PG13 cells were allowed to express the YFP contained in the plasmid vector for a period of about 48 hours & then YFP expressing cells were sorted out using FACS and cultured for up to 2 weeks. From these YFP+ PG13 cells, single cells were sorted into 96 well plates, expanded & viral production was titered using K562 cells. Individual YFP+ PG13 clones were tested & the clone that was producing the highest viral titer was identified & used for infecting umbilical cord blood derived hematopoietic cells.

3.4. Feeders and Co-cultures

For the normal cord blood as well as Bone Marrow long term culture initiating cells (LTC-ICs), a mixture of M2-10B4-J-GCSF-tkneo-J-IL3-hytk fibroblasts and SL/SL-J-SF-tkneo-J-IL3-hytk fibroblasts were used. M2-10B4 cells are a cloned line of mouse bone marrow origin engineered to produce G-CSF and IL-3 (190 and 4 ng /ml respectively) and the SlSl fibroblasts are a cell line originally established from Sl/Sl mouse embryos engineered to produce high levels of soluble Steel factor with or without production of the transmembrane form of SF (60 and 4 ng / ml respectively). The M2-10B and the Sl/Sl cells were maintained by plating at a concentration of 1-2 x105 cells per 10 cm tissue culture dish (Corning) in RPMI, 10 % FCS, 0,4 mg/ml G418 and 0,06 mg/ml hygromicin and DMEM, 15 % FCS, 0,8 mg/ml G418 and 0,125 mg/ml hygromicin respectively. The mixing of the two feeders was done at a 1:1 ratio following irradiation.

All murine feeders were irradiated with 8000 Rads (80 Gy) before being cocultured with human hematopoietic cells and placed on 35 mm tissue culture dishes (Corning) pre-coated with collagen solution (StemCell Technolgies) form a film that help the adherence of fibroblast cell lines used in the LTC-IC assay.

For the AML LTC-ICs, SLSL-J-IL3-neo-only fibroblasts originally obtained from Sl/S1 mouse embryos and engineered to produce human IL-3 at a concentration of 16,5 ng /ml. They were maintained by plating at a concentration of 1-2 x105 cells per 10cm tissue culture dish (Corning) in DMEM supplemented with 15 % FCS and 0.8 mg/ml G418 (Gibco).

3.5. Retroviral constructs

HPIP cDNA was already cloned in pMSCV-IRES-GFP cassette (provided by Carolina Abramovich) [30,31]. As a control, the MSCV vector carrying only the IRES green fluorescent protein cassette (GFP virus) was used.

VENTX2 cDNA was provided by Paul Moretti (Hanson Centre for Cancer Research Institute of edical and Veterinary Science, Adelaide). VENTX2 cDNA was sub-cloned in pMSCV-IRES-GFP vector. As a control, the MSCV vector carrying only the IRES green fluorescent protein cassette (GFP virus) was used. High-titer, helper-free recombinant retrovirus was generated by first transfecting the amphotropic Phoenix cell line and subsequentlyto to make the stable virus producing cell line PG13 cells were transduced. High-titer producer clones were isolated for each virus and analysis of GFP expression was tested in K562 cells).

3.6. Protein expression

Protein expression of the VENTX2 was documented by Western blotting using standard procedures. Total cellular protein was extracted from PG13-VENTX2 and PG13-MIG packaging cells line by using RIPA lysis buffer. Whole cell lysate were separated on 12 % SDS-page gel and transferred to nitrocellulose membrane. Membranes were probed with an anti-VENTX2 polyclonal antibody (kindly provided by Richard J. D.'Andrea, Departement of Medicine Adelaide, Australia) [35]. The membrane was reprobed with secondary goat anti-rabbit immunoglobulin labeled with horseradish peroxidase. Proteins were visualized using an ECL plus kit, according to manufacturer's recommendations.

3.7. Purification of human CB $CD34^+$ cells

Umbilical cord blood was collected in heparinised syringes according to institutional guidelines following normal full-term deliveries. Informed consent was obtained in all cases. Mononuclear cells (MNC) were separated using density gradient centrifugation. Fresh umbilical cord blood, not older than 12 hours, was diluted with 2 volumes of PBS and layered over Pancoll. Usually 35 ml of diluted blood was layered over 15 ml Pancoll in a 50 ml conical tube. This was centrifuged at 400x g for 30 minutes at 20°C in a swinging-bucket rotor without brakes. The upper layer was aspirated and discarded, leaving the interphase

undisturbed. The interphase containing MNC such as lymphocytes, monocytes and thrombocytes was then transferred to a new 50 ml tube, washed twice with large volumes of PBS, and then counted before labeling with magnetic bead or fluorochrome conjugated antibodies.

$CD34^+$ cell purification was conducted using MACS CD34 Cell Isolation Kit that uses positive selection method. Cells were resuspended in a volume of 300 µl per 108 cells, blocked with 100 µl of FcR Blocking Reagent and labeled with 100 µl of CD34 Microbeads. When working with higher cell number, all the reagent volumes & the total volume was scaled up accordingly. This was followed by incubation for 30 minutes at 4-8°C. Cells were then washed twice by adding 10x the labeling volume of buffer and centrifuged at 300 x g for 15 minutes. The resultant cell pellet was then resuspended in 500 µl of MACS buffer and loaded into MS Column mounted on magnetic separator. The negative cells were allowed to pass through and the column was washed at least three times with 2 ml buffer. The column was then removed from the separator, placed on a collection tube, loaded with fresh buffer, and the magnetically labeled cells flushed out using the plunger. The magnetic separation was usually repeated to get a purity of more than 95%. Purified cells were then frozen in FBS with 10% DMSO and thawed when needed for pre-stimulation and transduction.

$CD34^+$ cell enrichment was done either by MACS or by FACS. For separation by FACS, MNCs were thawed from frozen stocks or prepared freshly from UCB and labeled using anti CD34-PE antibody (100 µL per 108 cells), for 30 minutes on ice. Labeled cells were then washed twice with PBS, resuspended in FACS buffer and sorted. The sorted cells with purity above 95 % were used for 48 hour pre-stimulation followed by transduction.

3.8. Transduction of Human cord blood Cells

Enriched $CD34^+$ cord blood (CB) cells were thawed and count. 2×10^6/ml cells were pre-stimulated for 48 hours in Iscoves modified Dulbecco medium (IMDM) containing a serum substitute (BIT, Stem Cell Technologies, Vancouver, BC, Canada) 10^{-4}M mercaptoethanol (Sigma), and 40 µg/ml low-density lipoproteins (Sigma) supplemented with the following recombinant human cytokines: 100 ng/ml Flt-3 ligand (L; PAN Biotech GmbH, Aidenbach, Germany), 100 ng/ml Steel factor (SCF; ImmunoTools Friesoythe; Germany), 20 ng/ml interleukin-3 (IL-3; ImmunoTools), 20 ng/ml IL-6 (Tebu-bio, Frankfurt, Germany) and 20 ng/ml granulocyte colony-stimulating factor (G-CSF; Stem Cell Technologies). After 48

hours cells were resuspended in filtered virus-containing medium (VCM) supplemented with the same cytokines combination and protamine sulfate (5 μg/ml) on tissue culture dishes, preloaded twice with VCM, each time for 1 hour (Corning, Acton, MA). This procedure was repeated on the next 2 consecutive days for a total of 3 infections. For *in vitro* studies, aliquots of these cells were transferred to fresh serum-free medium plus the same additives and cytokines and then incubated for an additional 48 hours prior to being stained with PE-labeled anti-CD34 antibody (Becton Dickinson, San Jose, CA) and isolation of the $YFP^+/GFP^+/ CD34^+$ cells on a FACS Vantage (Becton Dickinson) sorter. For *in vivo* studies, transduced cells were injected into non obese diabetic/severe combined immunodeficiency (NOD/SCID) mice immediately after transduction (< 6 hours after the last exposure to fresh VCM) without pre-selection.

3.9. Liquid culture of Transduced Cord Blood Cells

For *in vitro* liquid expansion assays, transduced cord blood $CD34^+$*VENTX2*/GFP^+ cells and $CD34^+$ MIG/GFP+ cells were placed in cytokine-supplemented serum-free medium containing 10^{-4}M 2-Mercaptoethanol, (Gibco) 20 % BIT (StemCell). For Cord blood transduced cells 100 ng/ml each of Flt-3 ligand and SF and 20ng/ml each of IL-3, IL-6 and G-CSF was added. Half-media change was performed every week and morphology of cells present in cultures at various time points was determined by performing cytospin. For this step 1x 105 cells were fixed on slide and stained with Wright-Giemsa. Every week 1x 10^4 cells were plated in CFC assay after half-media change. In addition, separate aliquots were taken and incubated for 30 minutes on ice with a mouse isotype-matched control antibody (Becton Dickinson) and antibodies against human antigens and analyzed by FACS for the expression of different lineages.

3.10. Suspension culture initiating cells (SC-IC) assay for AML cells

AML cells or normal BM cells were incubated at a concentration of 1x 10^6 cells/ml in serum free media containing 10^{-4}M 2-Mercaptoethanol, 2mM glutamine and a cocktail of growth factors in IMDM with 20 % BIT (StemCell). For AML cells 20ng/ml each of IL-3, IL-6, G-CSF, and GM-CSF and 50ng/ml SF was used and for normal marrow cells 100 ng/ml each of Flt-3 ligand and SF and 20ng/ml each of IL-3, IL-6 and G-CSF was added along with

40 µg/ml low density lipoproteins. After 6 wks with weekly half medium changes, cells were harvested by trypsinization and placed into AML-CFC methylcellulose medium or normal CFC (Methocult H4330, Stem Cell Technology) supplemented with 3 U/ml Epo, 50 ng/ml SF and 20 ng/ml each of IL-3, GM-CSF, G-CSF (Amgen, Thousand Oaks, CA) and IL-6 (Cangene, Mississauga, Ontario). Granulopoietic, erythroid and mixed colonies were detected and counted after 16 days of incubation at 37°C.

3.11. *In vitro* progenitors assay for normal and AML cells: Colony-Forming Cells (CFC)

Assays for *in vitro* colony-forming cells (CFCs) for tranduced $CD34^+$ CB cells were carried out in methylcellulose cultures (GF H4434; 0.92 % methylcellulose, 30 % FCS, 2mM L-glutamine, 10^{-4}M 2-mercaptoethanol, 1 % BSA in IMDM; Stemcell Technologies) supplemented with 50 ng/ml human SF, 20 ng/ml each of human IL-3, IL-6, GM-CSF (Novartis) and G-CSF and 3 U/ml erythropoietin (Stemcell Technologies). Secondary CFC assays were performed by replating aliquots of cells obtained by harvesting primary CFC dishes.

CFC assay for AML-CFC were performed plating 1 to 2 $x10^5$ cells/ml in methylcellulose medium (GF H4430; Stemcell Technologies) supplemented with 3 U/ml human erythropoietin (Epo, StemCell), 10 ng/ml GM-CSF, 10 ng/ml IL-3, 50 ng/ml Steel factor (SF) (Terry Fox Laboratory) and 50 ng/ml Flt-3 ligand (all Tebu-bio GmbH, Offenbach, Germany). Cultures were scored after 14 days for the presence of clusters (4-20 cells) and colonies (more than 20 cells).

3.12. Long term culture-initiating cells (LTC-IC) assay for AML and Normal Bone Marrow cells

Six-week long-term culture-initiating cell (LTC-IC) assays were carried out using AML cells co-cultured in Myelocult H5100 LTC media (CellSystems) supplemented with 10^6M solucortef (Sigma-Aldrich, Taufkirchen, Germany) with a supportive feeder layer of murine fibroblast cell lines (Sl/Sl-J-IL3) irradiated at 80Gy and then seeded into new collagen-coated tissue culture dishes at 2.2 $x10^5$cells/dish. Sl/Sl fibroblasts consist of Sl/Sl mouse embryos. Sl/Sl-J-IL3 fibroblasts were obtained by transduction of Sl/Sl fibroblast with

a human IL3 cDNA containing retrovirus. These cells produce bioactive human IL3 at a concentration of 16ng/ml as determined by the ability of their growth medium to stimulate 3H-thymidine incorporation into Mo7e cells, an IL3-dependent cell line Otsuka et al (1991). LTC-IC was incubated at 37°C in 5 % CO2 and received weekly one half-media changes. In LTCs which were supplemented with SF of FL, the weekly half-medium change contained the cytokine of interest at twice the final concentration (50 ng/ml). After 6 weeks both, adherent and non adherent cells were trypsinized, pooled and plated in methylcellulose as described above. The number of CFC per million cells initially plated in LTC could then be calculated as follows:

$$\frac{\#\text{CFC/dish MeCell}}{\#\text{cell plated in MeCell}} \times \frac{\#\text{cells/LTC at harvest}}{\#\text{cells plated in LTC at time 0}} \times 10 = \text{CFC/}10^6\text{ cells plated at time 0}$$

In this way CFC output could be standardized to allow comparison of different culture condition. To detect normal LTC-IC human BM cells were plated onto preformed mix feeder layers of irradiated Sl/Sl j-SF-tkneo j-IL-3-hytk and M2-10B4 j-GCSF-tkneo j-IL-3-hytk cell lines genetically engineered to produce SF, G-CSF and IL-3, with weekly half-media changes. After 6 weeks adherent and non adherent cells were harvested and assessed for their AML-CFC or CFC has described above.

3.13. Limiting Dilution LTC-IC assay

M2-10B4 G-CSF / IL-3 and Sl/Sl SF / IL-3 cells (1:1 mixture) were established in 96-well flat-bottom culture plates at a density of 1,25 x 10^4 cells. On the day of assay, HLTM was removed using multi-channel pipette or with sterile tips and discarded. The test cells were added to the wells in 0,2 ml of HLTM with solucortef. The number of cells seeded per well is indicated in table 3,3. LTC-IC cultures were incubated at 37°C in humidified incubator (>95%) with 5 % CO2 in air for six weeks. For weekly half media exchanges, one half of the medium and cells were removed and replaced with HLTM for five weeks. To harvest the LTC-IC, the HLTM and non-adherent cells were removed from wells and place into individual 12x75 mm sterile tubes using a pipette and sterile tips. Single wells were harvested at a time to avoid cross contamination of samples. Wells were rinsed once with 0,2 ml PBS and added to tube. 0,1 ml Trypsin-EDTA was added to each well and incubated for 3 to 5 minutes and examined for detached cells. Once the adherent cells are detached, the wells are washed with more PBS and the medium collected in the appropriated tube. The wells are

finally washed with 0,2 ml IMDM containing 2 % FBS and transferred to the appropriate tube. The tubes were centrifuged at 1200 rpm for 10 minutes and the supernatant removed without disturbing cell pellet. Approximately 0,1 ml of medium was left along with the cell pellet and vortexed. To this 1 ml of Methocult (H4435) methylcellulose medium was added and vortexed again. Each tube (contents of one well) was plated individually into 35 mm petri dish with 1 ml syringe (without needles attached). Several dishes (6-8) were placed in a 15 cm petri-dish along with an additional 60 mm open dish containing 5 ml sterile water to maintain humidity. The dishes are incubated at 37°C in humidified incubator (>95 %) with 5% CO2 in air for 12 to 16 days. Colonies were counted and a well scored as positive if one or more E-BFU, GM-CFU or G-CFU GEMM-CFU were detected or scored as negative if no colonies were present. The LTC-IC frequency in the test cell population was calculated from the proportion of negative wells (no CFC present) and the method of maximum likelihood. Statistical analysis was performed using L-Calc™ software for limiting dilution analyses.

3.14. Screening for the LM and the mutations at codon 835/836 in the *FLT3* gene

Analysis of Asp835/836 mutations was performed using the restriction fragment gene length polymorphism at codon 835/836 exactly as described previously [102]. After amplification of a 114-bp fragment from exon 20 using gDNA, polymerase chain reaction (PCR) products were digested by EcoRV and separated on an 8% polyacrylamide gel. Undigested bands were cut out from the gel, purified with a Qiaquick gel extraction kit (Qiagen, Hilden, Germany), and directly sequenced on a DNA sequencer (ABI PRISM 310 Genetic Analyzer; Perkin Elmer; obtained from Applied Biosystems, Weiterstadt, Germany) using a Big Dye terminator cycle sequencing kit (Applied Biosystems, Darmstadt, Germany). Screening for *FLT3*LM was performed as described by Nakao et al.(1996) [94].

3.15. NOD/SCID Mice

NOD/LtSz-scid/scid (NOD/SCID) mice were bred and maintained in the animal facility of the GSF (Haematologikum) in microisolator cages containing autoclaved food and water. Seven to eight week old NOD/SCID mice were sublethally irradiated with 275 cGy from a 137Cs source one day prior injection. For transplantation, transduced cells were washed, counted, resuspended in PBS and injected into the lateral tail vein of irradiated mice

(300-400µL/mouse). Bone marrow cell aspirates were performed 3, 6 and 10 weeks after transplantation following anesthesia with Avertin (2,2,2 – Tribromoethanol, Sigma Aldrich , St. Louis, MO) 11 µl/g mouse injected i.p. Cells were stored in Alpha MEM with 50% FCS and used for FACS staining. After 6-18 weeks mice were sacrificed by CO2 inhalation. The cells from both tibiae and femurs of each mouse were collected for additional analyses. The absolute number of cells in the marrow of each mouse was calculated assuming that the contents of both femurs and both tibiae represent 25% of the total marrow.

3.15.1 Analysis of mice

Cells harvested from bone marrow of NON/SCID mice were harvested, resuspended in 7 % ammonium chloride (Stem Cell Technologie, Vancouver, Canada) and placed on ice for 20 minutes to lyse red blood cells. Afterwards, cells were washed again and resuspended in Hanks balanced salt solution (Stem Cell Technologie, Vancouver, Canada). Cells were washed and stained with anti-human CD45-PE (Becton Dickinson). A proportion of the cells were incubated 30 minutes on ice with a mouse IgG1 isotype control (Becton Dickinson Immunocytometry Systems, San Jose, CA) to evaluate nonspecific immunofluorescence. The remaining cells were incubated with fluoresceinated anti-CD45 a human-specific pan-leukocyte marker to detect human cells. The percentage of $CD45^+$ cells was determined after excluding 99.9 % of nonviable (propidium iodide [PI]) cells and at least 99.9 % of cells labeled with isotype control antibodies. To determine lineage differentiation and multilineage engraftment, cells were stained for 30 minutes at 4°C, with the antihuman CD45-PE.
phycoerythrin (PE;Becton Dickinson) and antihuman CD71-APC antibodies (OKT9) to quantitate the total number of human cells present ($CD45^+/71^+$), with antihuman CD34 8G12-Cy5 and antihuman CD19-PE to quantitate the number of human B cells present, and with antihuman CD15-PE (Becton Dickinson) to quantitate the number of human myeloid cells present. Additional antibodies used for the detection of erythroid positive cells fraction were the antihuman GlyA-PE and antihuman CD36-APC (Becton Dickinson), and antihuman CD38- PE (Becton Dickinson). Expression of basophiles was detected using CD25-APC (IL-2Rα), antihuman CD40-APC and Cd11b-PE and antihuman CD41a-PE (Becton Dickinson).

Multilineage engraftment was considered if there were >5 $CD34^+CD19^+$ human cells and > 5 $CD45^+/CD15^+$ human cells per $2x10^4$ viable cells analyzed (Holyoake TL, Nicolini FE, Eaves CJ. Functional differences between transplantable human hematopoietic stem cells from fetal liver, cord blood, and adult marrow. Exp Hematol. 1999;27:1418-1427).

3.16. Flow Cytometry and Cell Sorting

To determine the gene transfer efficiencies of cultured CD34 cord blood cells after transduction, aliquots of cells were stained with anti-human CD34-Cy5, washed twice with PBS and stained with propidium iodide 2μg/mL to exclude non-viable cells. Subsequently, cells were analyzed using a FACS Calibur (Becton Dickinson) with Cellquest software (Macintosh, Cupertino,CA). 20.000 events were acquired to determine the proportion of positive cells present; positive cells were defined as those exhibiting a level of fluorescence exceeding 99.98% of that obtained with isotype-control antibodies labeled with the same fluorochromes. Green fluorescent protein (GFP) positive cells were detected by their increased fluorescence intensity within the fluorescence 1 channel. Gene transfer efficiencies were calculated by dividing the number of GFP^+CD34^+ cells by the total number of $CD34^+$ cells.

3.17 Statistical analysis

Data were statistically tested using Student's *t*-test for dependent or independent samples (software STATISTICA 5.1, StatSoft Inc, Tulsa, USA). Differences with p-values < 0.05 were considered statistically significant.

4. RESULTS

4.1 The human non-homeobox hematopoietic PBX interacting protein HPIP

4.1.1 Efficient retroviral gene transfer of HPIP in normal human hematopoietic progenitor cells

The complete cDNA of the PBX interacting protein HPIP was cloned into the bicistronic vector with an IRES - GFP cassette based on the murine stem cell virus (MSCV) viral backbone (*HPIP* - virus). The MSCV –IRES – GFP vector was used as a control (GFP virus)(Fig.1a). High titer clonal viral producer cells were generated from the GALV pseudotyped PG13 packaging cell line for both viruses. Full-length proviral integration and expression was confirmed by Southern blot, use RT – PCR and Western blot analysis (Fig. 1b). The mean transduction efficiency was 61% (55 – 67%) and 35% (29 – 41%) in primary CB cells with the GFP and *HPIP* - GFP virus, respectively (n=9). We did not see differences in the percentage of $CD34^+$ cells in the transduced GFP^+ fraction between both experimental arms.

4.1.2 HPIP decrease proliferation of hematopoietic progenitors in vitro

As an initial test to determine whether *HPIP* would affect the proliferative capacity of human hematopoietic progenitor cells, 1 x 10^4 cells highly purified $CD34^+$/*HPIP*-GFP^+ and $CD34^+$/GFP^+ progenitor populations were cultured in serum free medium supplemented with cytokines for 4 weeks. Constitutive expression of *HPIP* decreased viable cell numbers at every time point from week 1 to 4 with a mean 2.1fold (+ 0.3) decreased cell count ($p<0.05$)(n=5): *HPIP* expressing cells expanded 803 fold (± 395) after 2 weeks of culture compared to a 1638 old (± 614) expansion within the GFP control arm (p=0,03). After 3 weeks of in vitro culture on average a 1155fold (± 695) expansion was obtained in the HPIP compared to a 3675 fold (± 1184) expansion in the GFP control arm (p=0,04).

After 4 weeks the the proliferative disadvantage for human progenitors overexpressing

HPIP was still demonstrable, although to a lesser extent (3106fold for *HPIP* versus 4637 fold expansion for GFP (±2327 fold and ± 1946fold respectively). Thus, over time constitutive expression impaired cell expansion *in vitro* by > 50 % (57.7 + 4.7) with up to 70.4 % week 3 (Figure 4.1).

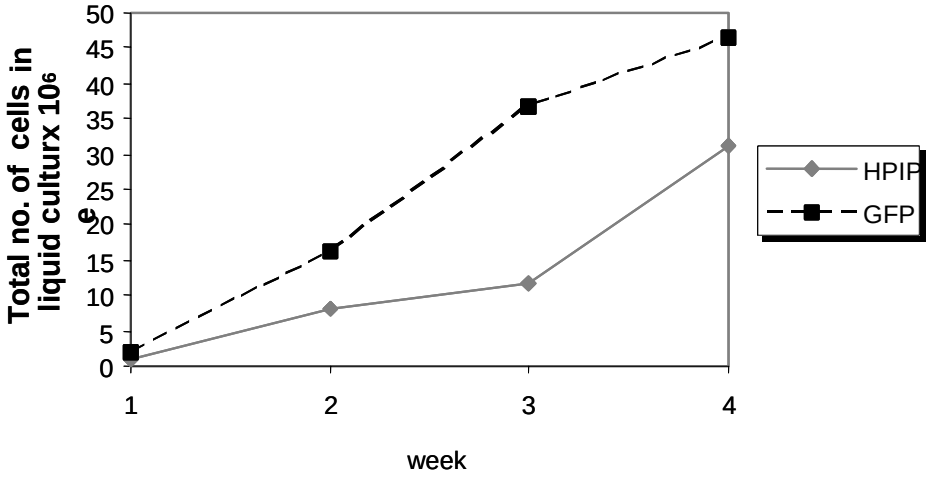


Figure 4.1. Expansion kinetics for human progenitor cells in liquid expansion experiments. *HPIP*-GFP and GFP transduced $CD34^+$ cells were cultured in serum free medium supplemented with cytokines for 4 weeks. Expression of *HPIP* was determined at every time point from week 1 to 4.

4.1.3 Expression of HPIP decreases the frequency and proliferative capacity of clonogenic progenitors in short-term in vitro assays.

In a second step we wanted to assess whether *HPIP* would decrease the number of clonogenic progenitors during short-term *in vitro* expansion. When the frequency of clonogenic progenitors was determined after 7 days of *in vitro* culture in serum free medium supplemented with cytokines *HPIP* expression was associated with a 24 % lower frequency of CFC compared to the control (26 CFC/1000 cells ± 7 and 34/1000 cells ± 10, respectively)(n=4). When the absolute numbers of clonogenic progenitors were calculated for this time point *HPIP* expression decreased the CFC number 5.7 fold (range 0.8 – 15, n=4). In order to test the impact of *HPIP* expression on clonogenic progenitors in a different assay

system, *HPIP*-GFP and GFP – infected $CD34^+$ progenitor cells were plated into methylcellulose and colony formation was evaluated after 14 days: *HPIP* expressing progenitor cells formed 209 (+ 16.6) colonies / 1000 cells initially plated versus 222 (+ 27.9) colonies in the control (n=6). However, the lineage distribution of colonies differed significantly with more erythroid colonies in the *HPIP* transduced cord blood cells vs. the GFP control (34 BFU/CFU-E / 1000 cells initially plated, [19 – 50] in *HPIP*+, vs. 18 BFU/CFU-E / 1000 cells initially plated, [5 – 34] in the GFP control arm, $p < 0{,}00003$). In contrast, no significant differences were observed in the formation of GM-CFU or GEMM-CFU colonies. When aliquots of primary methylcellulose dishes were re-plated *HPIP* expression decreased the number of secondary colonies 8fold (range 2-12 fold) with a mean number of secondary colonies of 2.2×10^3 colonies / 1000 cells initially plated versus 18.8 x 103 colonies in the control (n=4). The mean reduction of secondary colony formation was 79 % (+ 9.4) ranging from 51 % up to 92 % ($p < 0.004$).

4.1.4 HPIP increases the frequency of primitive progenitor cells in long-term in vitro cultures.

As we had seen that *HPIP* expression had a major impact on progenitor behavior *in vitro* we wanted to test the influence of *HPIP* expression on the maintenance of human progenitors in long-term cultures. First we determined the frequency and the yield of clonogenic progenitors in serum free liquid culture supplemented with cytokines plating aliquots of cells after 1-6 weeks, weekly into methylcellulose. Intriguingly, *HPIP* expression induced a 3.9 fold and a 3.4fold increase in the CFC frequency after 3 and 6 weeks, respectively. The mean frequency was 48.1 (20-102) CFC / 1000 plated cells with *HPIP* versus 12.4 (6-16) CFC / 1000 plated cells in the control (n=3) at week 3 and 7.2 (1.5-16) versus 2.2 (0.5 – 4.5) at week 6 (n=4)(Figure 4.2).

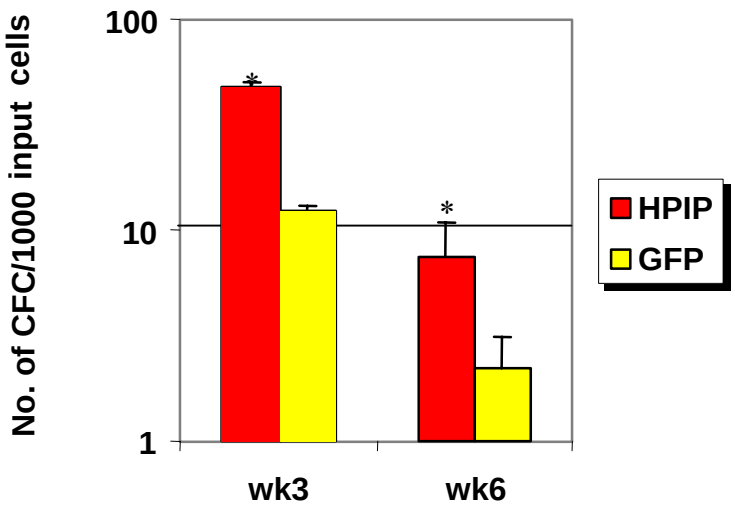
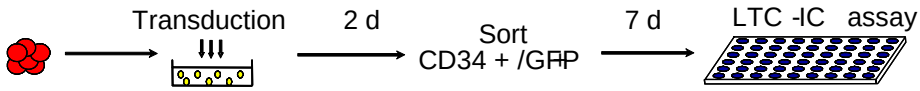
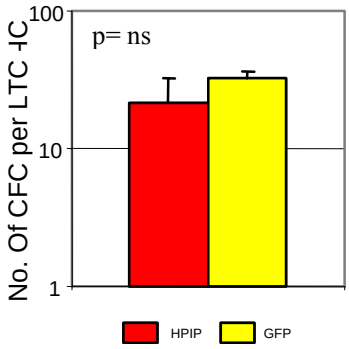


Figure 4.2 Colony Forming Cells (CFC) assy. CFCs colonies were scored after four and six weeks and the increase of clonogenic progenitors was determined in cells transduced with HPIP vs. the empty control. Frequency and refer to the number of CFCs per 1000 cells plated initially and yield indicates the total number of CFCs generated per arm was analyzed with Poisson statistic *p<0.02.

As *HPIP* transduced cord blood cells were able to increase the yield of CFC compared to the control we next asked whether *HPIP* expression would also have an effect of human stem cell pool size. Thus, we analyzed the impact of *HPIP* at the level of the long-term culture initiation cell (LTC-IC), the *in vitro* equivalent of repopulating stem cells. Highly purified $CD34^{+}GFP^{+}$ and $CD34^{+}$ *HPIP*-GFP^{+} cells from 5 independent cord blood samples were cultured for 7 days in serum free medium supplemented with cytokines and transferred into long term culture conditions by plating on murine stromal fibroblasts engineered to produce human SF, IL-3, and G-CSF under LTC-IC conditions. Two of the 5 assays were performed as limiting dilution LTC-IC assays, to calculate the LTC-IC frequency and the CFC output per LTC-IC under *HPIP*-GFP versus GFP expression. Using limiting dilution settings in two experiments and calculating the LTC-IC frequency for all 5 independent analyses referred to the cell equivalent after 7d of liquid culture extended and enhanced expression of *HPIP* induced a median 4.8 fold increase ranging from 2 to over 140 fold ($p < 0.02$)(Figure 4.3).





Experiment	LTC-IC Frequency/10E^6 GFP	LTC-IC Frequency /10^6 HPIP/GFP	Fold increase
1	14,1	35,7	2,5
2	10,9	23,1	2,1
3	2,7	48,7	18,2
4	156,5	755,7	4,8
5	19,8	2893	146

HPIP induced a median 4.8 fold increase ranging from 2 to over 140 fold ($p<0.02$)

Figure 4.3. Limiting dilution assay (LDA-LTC-IC). After pre-cultivation in liquid culture CD34$^+$*HPIP*$^+$GFP$^+$ and CD34$^+$GFP$^+$ transduced cord blood cells were placed on a monolayer of feeder cells in 96 well plates. Cultures were maintained with a weekly half-media change. After six weeks each well was harvested and cells were plated into methylcellulose. After two weeks the number of growing colonies was scored. The frequency of LTC-IC was determined as described in the text. $p<0.02$.

When the total yield of CFC per 1x10^4 initially plated cells was calculated *HPIP* increased the CFC output over 12 fold with a mean of 69 CFC (± 41) versus 5.6 CFC (± 2.3). This increase associated with constitutive *HPIP* expression was confirmed when the number of LTC-IC derived CFC was calculated for the 3 bulk LTC-IC assays: *HPIP* increased the CFC output over 13 fold with a mean of 2.66x 10^4 versus 0.195 x 10^4 per 10^6 cells for *HPIP* versus GFP, respectively ($p<0.04$) (Figure 4.4).

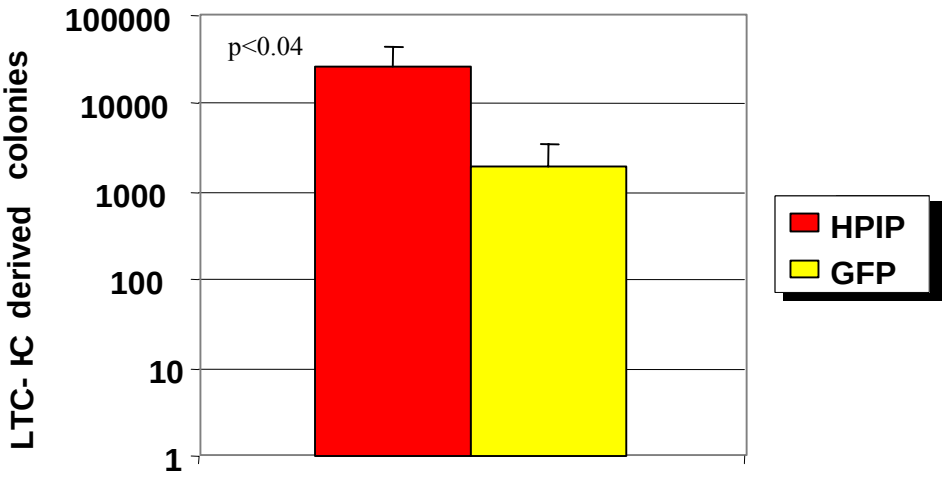


Figure 4.4. The constitutive expression of *HPIP* enhances the frequency of bulk LTC-IC.

To determine, whether this increase was mainly due to the increase of the LTC-IC frequency or caused by a higher production of CFC per LTC-IC, the CFC output per LTC-IC was calculated. We could detect a somewhat lower output of CFC/LTC-IC for the *HPIP* transduced cells with 22 vs 33 CFC per LTC-IC for *HPIP* and GFP, respectively (Figure 4.5).

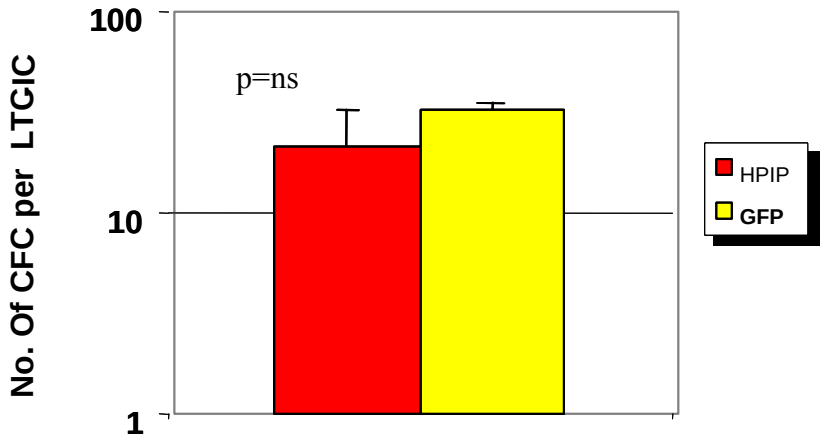


Figure 4.5 Constitutive expression of *HPIP* decreases LTC-IC derived CFC. P=ns, not significant.

4.1.5 The constitutive expression of HPIP enhances the proportion and number of engrafted human myeloid cells in NOD/SCID mice

As we had seen that *HPIP* affected lineage differentiation and expanded human progenitor cells *in vitro* we extended our studies *in vivo* using the xenograft NOD/SCID mouse model. In order to determine whether gene transfer of *HPIP* into repopulating stem cells would be efficient and allow reasonable levels of long-term engraftment, cohorts of sublethally irradiated NOD/SCID mice were injected with the CB progeny of a day 0 input number between of 8.3 x 10^4 - 14 x 10^4 $CD34^+$ cells per mouse immediately post-infection without any pre-selection of $CD34^+$ GFP^+ cells in two independent experiments. Engraftment with human transduced or non-transduced cells was first assessed by FACS analysis of bone marrow cells obtained by femoral bone marrow aspirate at week 3, 6, and at 14 weeks when the mice were sacrificed.

Already at week 3 the majority of mice in both experimental arms engrafted with human transduced cells (4/4 mice and 2/4 mice (>0.1 % $CD45^+/GFP^+$) in the *HPIP* and GFP arm control, respectively). Human engraftment was stable and at week 6 7/8 control mice and all *HPIP* mice (12/12) were engrafted with human lymphoid and myeloid cells and none of the animals of both experimental groups lost lymphomyeloid engraftment at week 14 (5/7 mice in the control group and 12/12 in the *HPIP*).

The engraftment level of transduced human cells at week 14 ranged from 0.2 – 18.05 % and from 0.1 – 9.3 % GFP^+CD45^+ cells for GFP and the *HPIP* group, respectively. Both experimental groups showed lymphoid predominance of engrafted human cells with lymphoid/myeloid ratios > 1 as previously reported. Thus, we achieved reasonable long-term lymphomyeloid engraftment in both experimental groups, indicating efficient gene transfer into long-term repopulating stem cells. Furthermore, constitutive expression of *HPIP* was permissive for lymphoid/myeloid differentiation *in vivo*.

When a more refined analysis of the proportion of human transduced B cells ($GFP^+/CD19^+/CD45^+$) and myeloid cells ($GFP^+/CD15^+/CD45^+$) was performed there was a trend towards a higher proportion of transduced human myeloid cells at the early time point of week 3 ($p<0.06$). At week 6 *HPIP* induced a significant increase in the proportion of engrafted myeloid cells (24 % vs 10 % in the control; $p<0.005$) and a significant decrease of engrafted lymphoid cells (35 % vs. 58 % in the control; $p<0.04$) within the transduced compartment compared to the GFP control group (Figure 4.6).

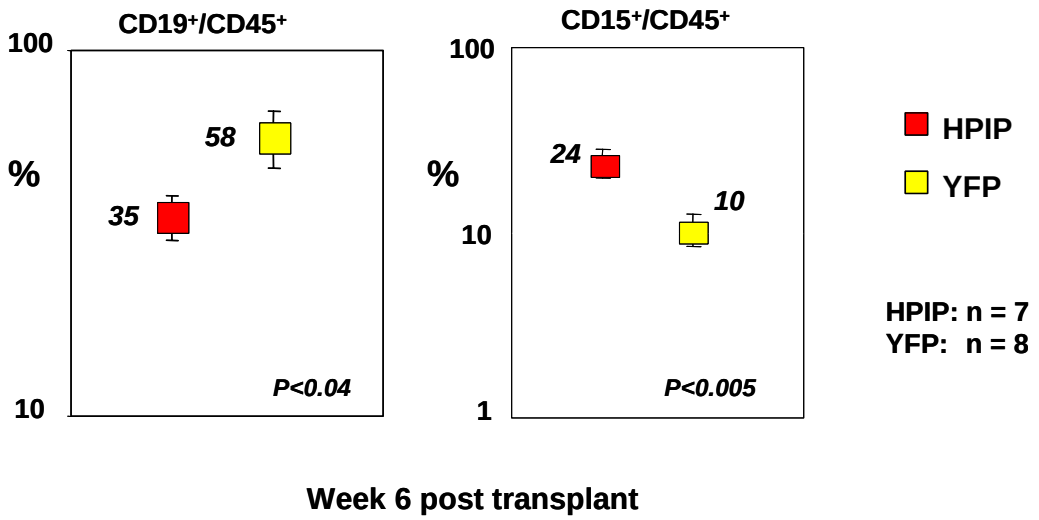


Figure 4.6 *HPIP* enhances the proportion of human myeloid cells in Transplanted NOD/SCID mice. Six weeks post transplantation bone marrow aspiration was performed on *HPIP*-GFP and GFP transduced NOD/SCID mice. Cells were detected for lineage engraftment by flow cytometry.

At the late time point of 14 weeks post transplantion the difference in the proportion of human transduced B cells was stable with 33 % versus 56 % $GFP^+/CD19^+$ cells in the *HPIP* and the GFP group, respectively (p=0.03). When the total number of transduced lymphoid and myeloid cells was calculated for both experimental groups, there was a 20 % increase for human myeloid cells and a 20% decrease in human lymphoid cells within the transduced compartment for *HPIP* compared to the control (ns). The effect of *HPIP* on B cells was further confirmed when the proportion of mature $CD20^+$ B cells was quantified: at the early time point week 3 mice of the *HPIP* group had significant decreased percentage of $CD20^+$ cells in the transduced compartment compared to the control (3.8 % versus 42.2 % in the control; $p<0.004$); at week 6 and week 14 *HPIP* decreased the proportion of $GFP^+/CD20^+$ cells by 34 % and 33 % respectively. When the absolute numbers of transduced CD20 positive cells were calculated for week 14 the *HPIP* mice had a mean of 3.5×10^5 cells (+ 1.2) versus 14.6×10^5 cells (+ 9.8)(ns).

Most intriguingly, constitutive expression of *HPIP* increased the proportion and absolute cell number of early hematopoietic progenitor cells characterized by expression of the CD34 antigen: *HPIP* expression increased the proportion of $CD34^+/GFP^+$ cells by 83 % compared to the control (2.9% ± 0.9 vs. 1.6 % ± 0.7). In addition, the absolute number of this

immature cell population was increased by 117 % per mouse (0.48 x 10^5 ± 0.3 cells and 1.04 x 10^5 ± 0.4 per mouse for the control and the *HPIP* group, respectively).

4.1.6 cDNA microarray analysis from AML samples and normal bone marrow from healthy donors.

In order to investigate the malignant transformation of cells it is important to first understand the mechanisms of normal hematopoiesis. Thus, fresh bone marrow samples from healthy volunteers as well as bone marrow samples from thoroughly characterized patients with newly diagnosed and untreated AML as defined by the World Health Organization classification were used and processed immediately as described by Schoch et al 2002 [147] to perform comparative microarray analysis.

Samples were analyzed by cytomorphology, cytochemistry, immunophenotyping, cytogenetic, and molecular genetics in all cases and were characterized by specific chromosomal aberrations or molecular genetic alterations from the leukemia diagnostics laboratory, Klinikum Großhadern, Munich. The studies were done in accordance with the rules of the local internal review board and the tenets of the revised Helsinki protocol

Using cDNA microarrays, we have first evidence that *HPIP* is highly expressed in AML patients' bone marrow with specific cytogenetic aberrations (FLT3 length mutation and acute promyelocytic leukemia AML M3 and M3v) as compared to bone marrow from healthy donors as well as compared to other cytogenetic subtypes (MLLPTD, complex, AMLM2 and M4Eo). These data point to a potential role of *HPIP* in AML(Figure 4.7).

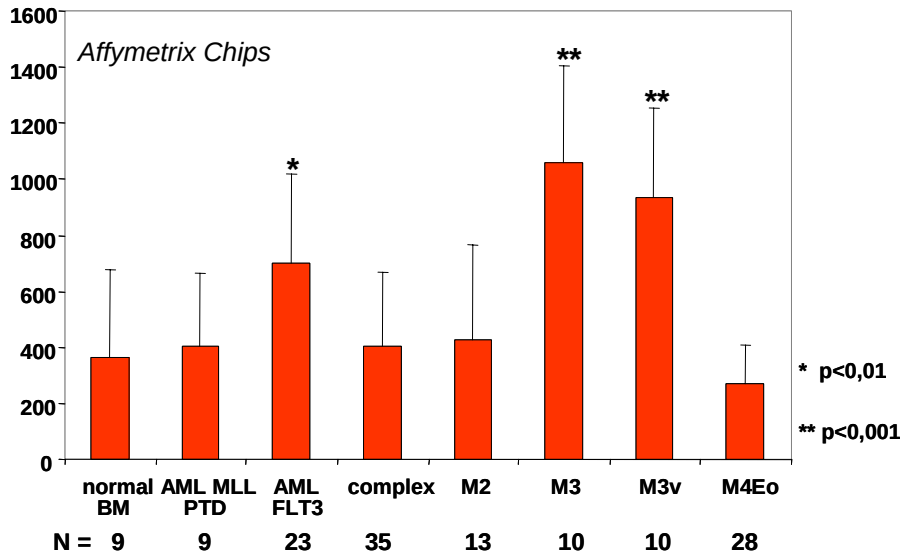


Figure 4.7 Level of expression of *HPIP* in cytogenetic subgroup of patients with AML. Bone marrow samples from patients with newly diagnosed and untreated AML and fresh bone marrow samples from healthy volunteers were analyzed. Expression of *HPIP* was found to be significantly high express in AML samples from patient carrying different cytogenetic aberrations (N=9). Significance was determined by Student's t-test and is indicated above the respective bars. *P < 0.01, **P < 0.001, indicate statistically significant differences from the normal bone marrow used as control.

4.2. *VENTX2* as a stem cell relevant gene

4.2.1. Efficiency of retroviral gene transfer

In attempt to characterize the functional relevance of *VENTX2* expression in hematopoietic development we retrovirally transduced human cord blood cells with either *VENTX2* or the empty GFP control vector (see Methods 2.5). The mean transduction efficiency was 24.07 % ± 11.13 % for *VENTX2* and 52.68 % ± 21.45 % for the empty vector control (GFP), respectively ($p<0.001$) (Figure 4.8).

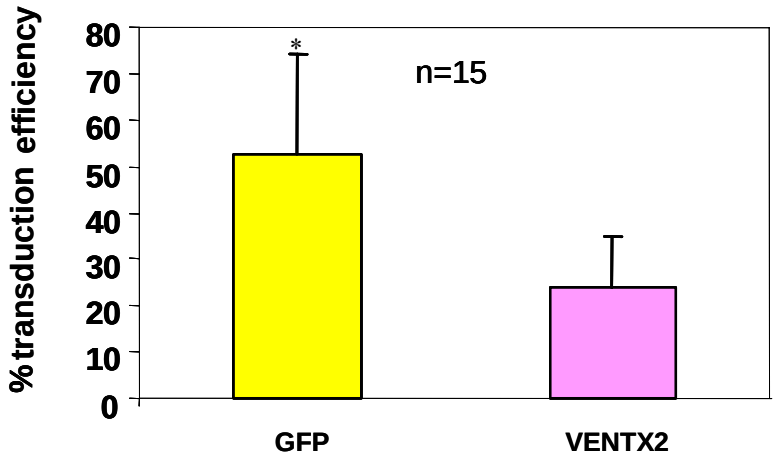


Figure 4.8. Efficiency of retroviral transduction in human hematopoietic CD34+ cord blood cells. Cord blood cells were analyzed seven days after start of transduction (n=15). $p<0.001$

4.2.2. Western Blot Analysis of VENTX2 protein

To confirm the expression of *VENTX2* we used polyclonal antisera against the N-terminal region of *VENTX2* and carried out western blot analysis of cell lysates from PG13-*VENTX2* and PG13-GFP. The antisera detected the expressed 28-kDa VENTX2 protein in PG13-*VENTX2* cells, which was not detectable in the PG13-GFP control (Figure 4.9).

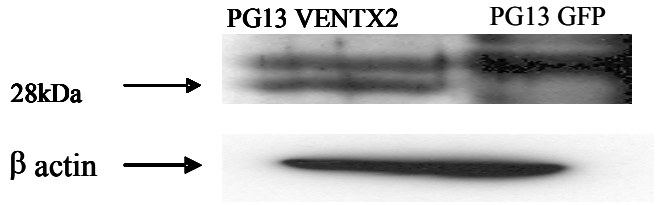


Figure 4.9 Western Blot analysis of the VENTX2 protein. The VENTX2 protein was detected with VENTX2 antisera in PG13 cells after retroviral infection.

4.2.3. VENTX2 inhibits the formation of erythroid colonies and significantly increases the production of myeloid colonies in clonogenic progenitor cell assays.

To detect the effect of *VENTX2* expression on clonogenic progenitor cells, we sorted $CD34^+$ cord blood (CB) cells transduced with *VENTX2*/GFP^+ or GFP^+ and plated them in methylcellulose. After 14 days of culture GEMM-CFU (Colony Forming Unit-Granulocyte, Erythrocyte, Macrophage and Monocyte) as well as lineage committed precursors GM-CFU, M, BFU-E CFU-E (Colony Forming Unit-Granulocyte-Macrophage, Monocyte) and Burst-Forming Unit-Erythroid (BFU-E) were detected. The total number of CFCs was reduced in the *VENTX2* transduced cells as compared to the control. This was due to an almost complete reduction of erythroid colony formation generated by *VENTX2*-transduced cells ($p<0.05$) leading to a 4.25 fold decrease compared to the GFP control cells, *VENTX2* 14,8, GFP 63, respectively (Fig. 4.10).

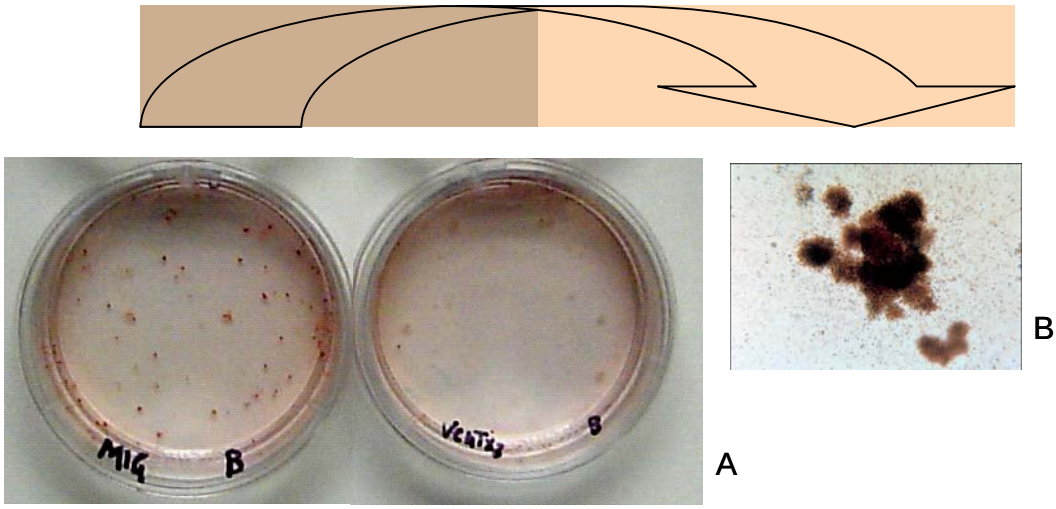


Figure 4.10 CFC assay. (A) Macroscopic picture showing the difference in growth of erythroid colonies in the GFP control compared to *VENTX2* transduced CB cells (B). Microscopic picture (10x) from a single BFU-E colony.

In contrast to the effect of *VENTX2* on erythroid differentiation, we observed a significant increase of myeloid colonies when cord blood cells transduced with *VENTX2* were analyzed and compared to the GFP control: the production of granulocyte colonies (G-CFU) was significant.

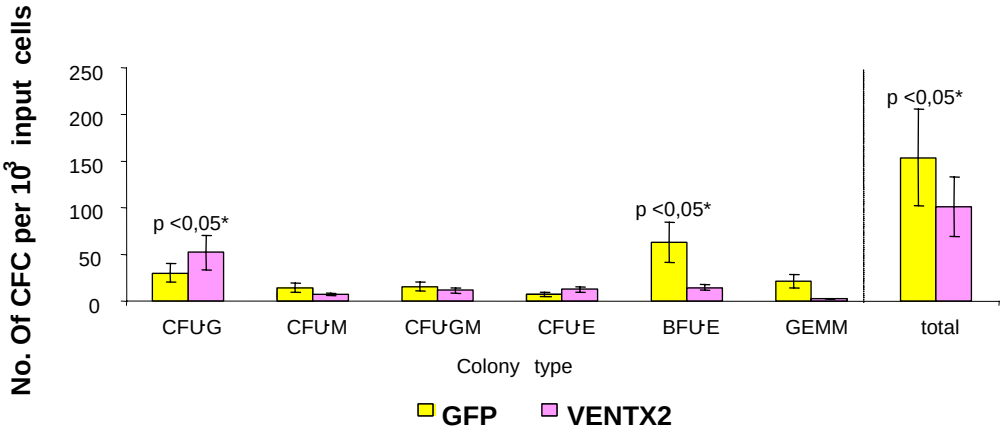


Figure 4.11. Primary CFC assay. Mean number (± SEM) of colonies formed after 14 days: $CD34^{+}$*VENTX2*/GFP^{+} compared to $CD34^{+}GFP^{+}$ transduced cells (n=9).

To test the proliferative potential of the clonogenic progenitor cells we performed secondary CFC assays by re-plating cells from primary CFC assays in methylcellulose (n=6). *VENTX2* transduced cells did not increase the total number of 2nd CFCs as compared to the control (n=6; *VENTX2* mean = 126,8 colonies, GFP mean =131,1 colonies/1000 input cells plated in the primary CFC; p=ns). However, we observed a trend for an increase in the number of myeloid colonies generated from *VENTX2* transduced cord blood cells as compared to the control (Figure 4.12): The number of G-CFU colonies (*VENTX2* 15,1 ± 6,35 versus GFP 11 ± 4,51) as well as the number of M-CFU colonies per 1000 input cells in primary CFC assays (*VENTX2* 73,3 ± 30,21 versus GFP 36,9 ± 15,10) generated by *VENTX2* transduced cells were increased by 1,4 fold and 2 fold, respectively, as compared to the control. Interestingly, we could again observe a decrease of erythroid colonies (BFU-E) by 15,5 fold as compared to the control (*VENTX2* 1,6 ± 0,67 versus GFP 25,3 ± 10,35).

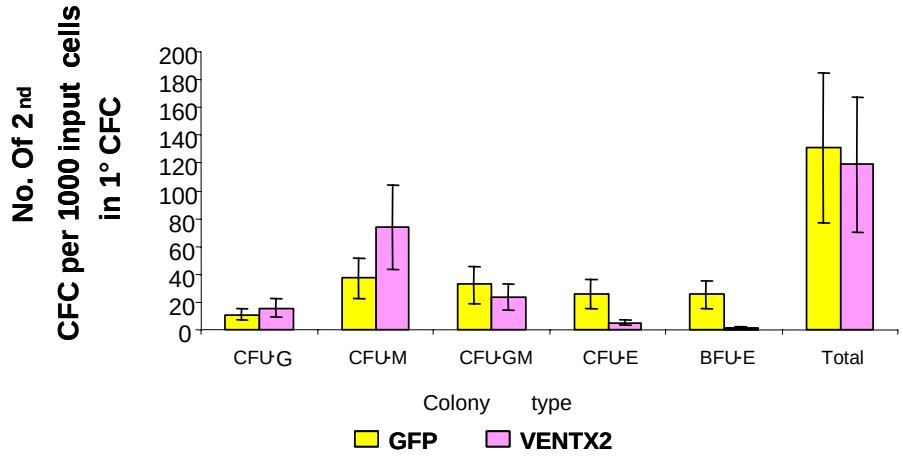


Figure 4.12. Secondary CFC assays. Cells from primary methylcellulose dishes were harvested on day 14, washed twice and resuspended in IMDM 2% FCS. These cells were immediately replated in secondary methylcellulose plates and scored after further 14 days of culture (n=6); p=ns). Bars represent the mean (± SEM) number of 2nd CFC from six independent experiments.

4.2.4. The constitutive expression of VENTX2 does not change the number and differentiative potential of primitive hematopoietic progenitor cells

As a test to determine whether *VENTX2* would affect the proliferative capacity of human hematopoietic progenitor cells, 2 x 10^4 cells highly purified $CD34^+$/*VENTX2*-GFP^+ and $CD34^+/GFP^+$ progenitor populations were cultured in serum free medium supplemented with cytokines for 4 weeks. There was no significant difference in the total number of cells generated after 4 weeks of *in vitro* expansion culture between *VENTX2* transduced cord blood cells and the GFP control (*VENTX2* $1{,}2x10^6 \pm 5{,}6x10^5$ vs. GFP $1{,}3x10^6 \pm 5{,}8x10^5$, n=5). In addition, the clonogenic potential of cells from liquid culture was tested by plating aliquots of cells each week in methylcellulose assays. The number and distribution of all colony types did not change significantly over a time of 4 weeks.

4.2.5. Limiting dilution assay

The Limiting Dilution Analysis (LDA-LTC-IC) was used to determine the frequency of Long-Term-Culture-Initiating Cells (LTC-IC) as well as the average number of colony-forming cells (CFC) produced per LTC-IC. When the effect of *VENTX2* on the frequency of LTC-IC was determined by limiting dilution assays (n=2), no major differences were detected between the *VENTX2* transduced cells and the control arm (453 LTC-IC vs. 801 LTC-IC per 1x 106 cells, respectively; p = n.s.) (Figure 4.13).

	VENTX2	GFP
1° LDA	1/1791	1/1747
2° LDA	1/2860	1/957

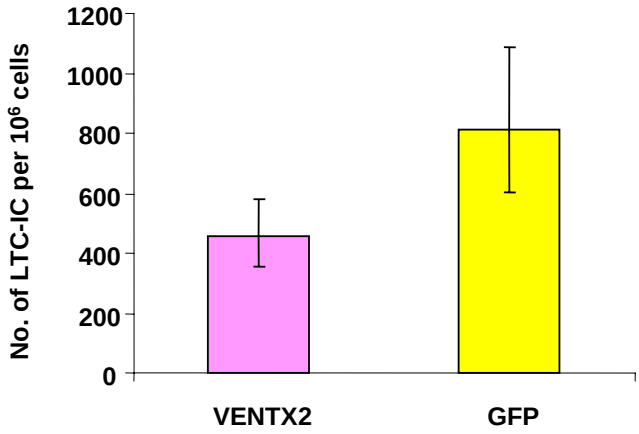


Figure 4.13. Limiting dilution long term-initiating cells (LDA-LTC-IC) assay. The frequency of LDA-LTC-IC/10^6 cells was calculated for *VENTX2* and the GFP empty vector control as described in Material and Methods. p= ns (not significant)

Furthermore, the number of colonies generated per LTC-IC did not significantly differ between the two arms (175.5 ± 13 CFC/LTC-IC for *VENTX2* and 170 ± 18 CFC/LTC-IC for the control)(p=ns). CFCs were counted and classified according to the morphology. No major differences were observed in the number of GEMM-CFU (*VENTX2* 26 ± 25.1, MIG 27,33 ± 25,5) GM-CFC, M-CFU and G-CFU and BFU/CFU-E colonies (Figure 4.14).

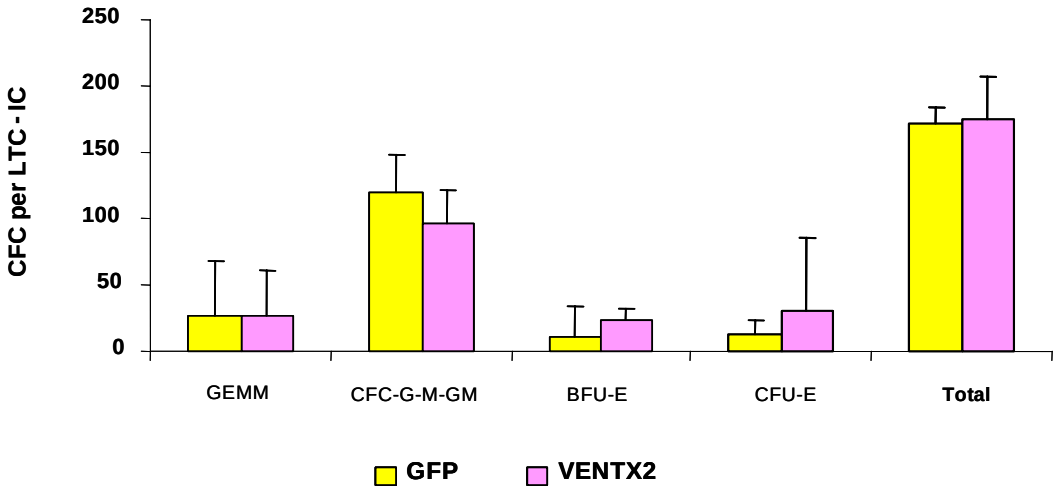


Figure 4.14. LTC-IC derived CFC. Colonies derived from LTC-IC limiting dilution analyses classified according to their morphology. Bars represent mean (±SEM) frequency from two independent experiments. p=ns (not singnificant) (n=2).

4.2.6. Human SCID repopulating cells (SRC)

Assays to enable the measurement of human repopulating cells have been developed using murine xenograft models. Human cells are transplanted intravenously (i.v.) into sub-lethally irradiated mice such as the non-obese diabetic/severe combined immunodeficient (NOD/SCID) mouse. Six to nine weeks later, the percentage of engrafted human cells $CD45^+$ in the bone marrow is assessed by flow cytometry. The cell responsible for initiating engraftment in the NOD/SCID mouse is termed the SCID-Repopulating Cell (SRC), and occurs at low frequencies in the order of one in $9.3x\ 10^5$ mononuclear cord blood cells. Engraftment with human cells was first assessed by FACS analysis of bone marrow cells. 5/5 mice transplanted with *VENTX2* and 9/9 mice transplanted with GFP control transduced cord blood cells showed lympho-myeloid engraftment over a period of 9 weeks, indicating that sufficient transduced long-term repopulating stem cells have been transplanted. In both arms the ratio of lympho-myeloid engraftment was 3 for *VENTX2* and 5 for GFP transplanted animals.

To test the impact of *VENTX2* on hematopoietic differentiation *in vivo*, lymphoid ($CD19^+$), myeloid ($CD15/CD33^+$), erythroid (Glycophorin $A/CD36^+$) and megakaryocytic ($CD41^+$) engraftment was analyzed.

VENTX2 induced a 2,9 fold increase in the proportion of granulocytic cells ($CD15^+$ mature myeloid cells) within the GFP-positive compartment (n=7) compared to the control (n=9) (6.4 ± 1,5 % vs. 2,2 ± 0,5 %, respectively; p<0,01) (Figure 4.15).

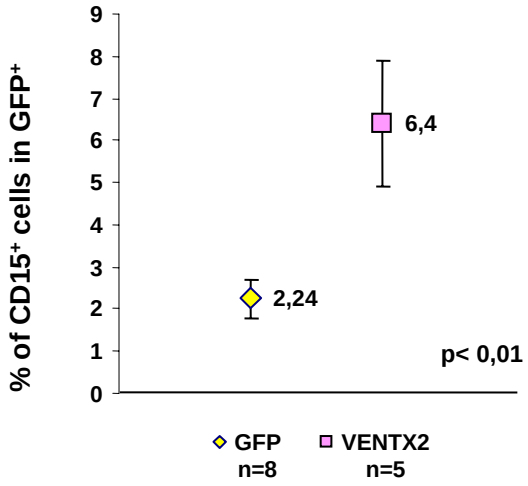


Figure 4.15. Engraftment of mature human cells. Percent of $CD15^+$ cells within the transduced compartment of *VENTX2* and GFP mice. p>0.01.

In addition, constitutive expression of *VENTX2* in human cord blood cells induced an 8-fold decrease in the total number of $CD34^+CD38^+$ transduced cells (*VENTX2* 3,5 x 10^5 (± 1,8 x 10^5 $CD34^+CD38^+$ in GFP, GFP 3 x 10^6 ± 1,1 x 10^6 $CD34^+CD38^+$ cells in GFP, p<0,05). In contrast, the number of $CD38^+$ cells within the transduced compartment was significantly increased by 7 fold, when cord blood cells were transduced with *VENTX2* as compared to the GFP control: *VENTX2* 3x10^7 ± 1x107 $CD38^+$ cells in GFP vs. 1,4 x 10^6 ± 1,1 x 10^6 GFP control cells, p<0.02.

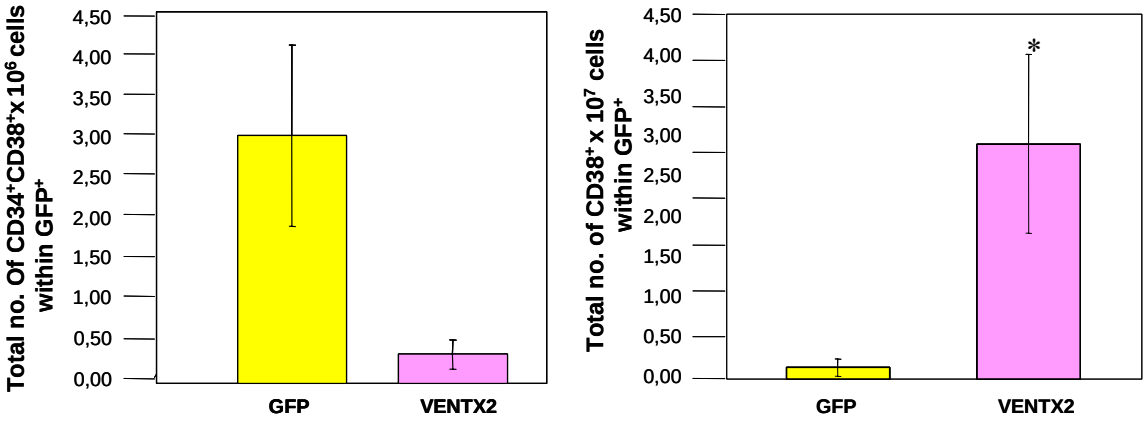


Figure 4.16. Number of CD38 positive cells in NOD-SCID mice. After six weeks NOD-SCID mice were sacrificed and the number of human CD38 positive cell was determined. *p<0.02

4.3. SU5614 as a stem cell therapeutic agent

4.3.1. Cytogenetic profile, classification and characterization of patient samples

To test, whether the protein tyrosine kinase inhibitor (PTKI) SU5614 targets leukemic stem cells and spares their normal counterparts, we first analyzed the toxicity profile on leukemic cells of patients with acute myeloid leukemia (AML). For this purpose, we used *in vitro* assays representing different stages of hematopoietic development.

Three groups of patient samples (n=4 per group) were analyzed with non-mutated wild-type FLT3 (WT) receptor, with internal tandem duplication *FLT3* (ITD) also called length mutations (LM) as well as point mutations in the second tyrosine kinase domain at codons 835, 836, 841 or 842 (TDK) (Table 4.1). Acute myeloid leukemia was confirmed by cytogenetic analysis performed on bone marrow of patients at initial diagnosis. Classification and diagnosis of AML patients were based on the criteria of the French-American-British (FAB) group. Patient samples were screened by flow cytometry for the presence of the cell surface molecules CD117 (c-KIT) and CD135 (FLT3 receptor). Interestingly, FACS analysis revealed a high expression of c-KIT (CD117) in 9 of 12 patients. AML was confirmed by the percentage of blasts in the bone marrow (BM) and white blood cells (WBC). All data are summarized in the table 4.1.

Patient	Cytogenetics	Age	FAB	WBC (x109/ml)	% blasts in BM or PB	FLT3 status	% of $CD117^+$	% of $CD135^+$
1	46,XX, Nras+	68	M1	284	95	WT	33	80
2	47,XX,I(21),+i(21), c-KIT-, Nras-	67	M2	41	60	WT	41	10
3	46,XY, c-KIT-, Nras-	46	M4	105	90	WT	32	87
4	47,XY,+13 48,XY,+13,+13,c-KIT-	71	M1	160	90	WT	64	96
5	46,XY, c-KIT-, MLL dupl-	47	M1	66,4		ITD	48	40
6	46,XX, Nras-	55	M4	8,2	90	ITD	23	11
7	46,XX, c-KIT-	73	M5b	206	90	ITD	2	51
8	46,XX,t(11;16),c-KIT-, Nras-	42	M4	75	90	ITD	10	12
9	47,XX,+14, c-KIT-, Nras-	68	M5a	94	95	D835H	8	93
10	46,XX, c-KIT-, Nras-	85	M5b	268	95	D835H	15	45
11	46,XY, c-KIT-, Nras-	48	M2	9	80	Del835	8	3
12	46,XX, c-KIT-, Nras-	38	M4	10,6	90	D835Y/ITD	37	54

Table 4.1. Patient Characteristics. Patient samples were divided into three different groups based on the mutations of the FLT3 receptor. The standard methods for establishing the diagnosis of acute leukemias are cytomorphology and cytochemistry in combination with immunophenotyping. For this purpose leukemic cells were stained for CD117 (c-KIT) antigens and CD135 (FLT3 receptor). FAB = French-American-British group; WBC: white blood cell; BM: bone marrow; PB = peripheral blood; WT = wild-type; ITD = internal tandem duplication; D835H = point mutation at codon 835.

4.3.2. Preparation of cells used for toxicological studies with SU5614

1x 10^6 AML bone marrow cells from patient samples were pre-incubated for 24 hours with or without (control) 10 µM SU5614 and 50 ng/ml of granulocyte colony-stimulating

factor. It was earlier reported by Spiekermann et al (2003) that the inhibitor induces apoptosis at a concentration of 10 μM in transduced myeloid leukemic cell lines [148]. After 24 hours, cells pre-incubated in liquid culture with and without (control) SU5614 were removed, washed and the viability was measured by trypan blue.

We observed that 1 out of 4 (1/4) patient samples with non-mutated *FLT3* (WT), 1/4 patient samples with ITD, and 2/4 patient samples with TKD ($p<0.05$) responded with a greater than 50 % reduction of leukemic cell number (Table 4.2). However, equal fractions of cells pre-incubated with or without (control) SU5614 recovered from cultures, were assayed without regard to changes in the cell number.

Patient	Cytogenetics	% of Blasts in Bone Marrow (BM)	FLT3 status	% of CD117+	% of CD135+	% Toxicity of SU5614 after 24 hrs pre-incubation*
1	46,XX, Nras+	95	WT	33	80	4
2	47,XX,I(21),+i(21), c-KIT-, Nras-	60	WT	41	10	0
3	46,XY, c-KIT-, Nras-	90	WT	32	87	9
4	47,XY,+13 48,XY,+13, +13, c-KIT-	90	WT	64	96	54
5	46,XY, c-KIT-, MLL dupl-	90	ITD	48	40	39
6	46,XX, Nras-	90	ITD	23	11	20
7	46,XX, c-KIT-	90	ITD	2	51	74
8	46,XX,t(11;16), c-KIT-, Nras-	90	ITD	10	12	0
9	47,XX,+14, c-KIT-, Nras-	95	D835H	8	93	17
10	46,XX, c-KIT-, Nras-	96	D835H	15	45	13
11	46,XY, c-KIT-, Nras-	80	Del835	8	3	83
12	46,XX, c-KIT-, Nras-	90	D835Y/ITD	37	54	73

Figure 4.2. Toxicity of SU5614 after 24 hours pre-incubation. Adherent and non adherent cells were harvested after 24 hours. The reduction of living cells between the control arm vs. SU5614 arm was calculated starting at day 0 and after 24 hours.*Reduction of cell number compare to the control after 24hrs pre-incubation with the inhibitor.

4.3.3. Toxicity of SU5614 on clonogenic progenitors tested with the Clonogenic forming cells (CFC) assay

After 24 hours pre-incubation with or without (control) 10 μM of SU5614, 1 to 2x 10^5 patient cells were plated in methylcellulose and CFC assays were performed. Colonies were scored after 14 days for the presence of clusters (4 to 20 cells) and colonies (more than 20 cells). The toxicity profile of SU5614 was analyzed on the level of clonogenic progenitors (CFC). 2/4 patient samples with WT, 3/3 patient samples with ITD ($p<0.004$) and 2/2 patient samples with TKD responded to therapy with an up to 100 % reduction in the number of leukemic clonogenic progenitors as compared to the untreated AML cells. The percentage of killing (toxicity) of SU5614 on AML cells for each different cytogenetic group is calculated in comparison to the cells pre-incubated without SU5614 (control). It was not possible to detect colony growth in some methylcellulose dishes (Table 4.3). The response of leukemic CFC to SU5614 could not be predicted from the expression level of FLT3 on the bulk leukemic population as determined by the percentage of CD135 positive cells (Table 4.3). Response to SU5614 was also not correlated with the presence of activating mutations or surface expression of c-KIT, an RTK receptor also targeted by the SU5614 compound.

Patient	Cytogenetics	% of CD117+	% of CD135+	FLT3 status	* % Toxicity of SU5614 on CFC
1	46,XX, Nras+	33	80	WT	0
2	47,XX,I(21),+i(21), c-KIT-, Nras-	41	10	WT	0
3	46,XY, c-KIT-, Nras-	32	87	WT	93
4	47,XY,+13 48,XY,+13, +13, c-KIT-	64	96	WT	26
5	46,XY, c-KIT-, MLL dupl-	48	40	ITD	98
6	46,XX, Nras-	23	11	ITD	77
7	46,XX, c-KIT-	2	51	ITD	77
8	46,XX,t(11;16), c-KIT-, Nras-	10	12	ITD	n.g
9	47,XX,+14,c-KIT-, Nras-	8	93	D835H	32
10	46,XX, c-KIT-, Nras-	15	45	D835H	100
11	46,XY, c-KIT-, Nras-	8	3	Del835	n.g
12	46,XX, c-KIT-, Nras-	37	54	D835Y/ITD	n.g

Table 4.3. Toxicity of SU5614 on clonogenic progenitor cells. The effect of SU5614 on leukemic clonogenic progenitor cells was analyzed in the CFC assay with or without SU5614. Colonies were scored after two weeks.* % toxicity of SU5614 on AML progenitors CFC is calculated in numbers of outgrowing colonies in the untreated arm (control) vs. SU5614 treated AML cells.

4.3.4. Toxicity of SU5614 on Long Term-Initiating Cells (LTC-IC) and on Suspension Culture-Initiating Cells (SC-IC)

AML patient sample cells previously pre-incubated with or without (control) 10 µM SU5614 were maintained sex weeks in Long-Term Culture-Initiating Cell (LTC-IC) assays. Half-medium change was performed every week as described in chapter 2.12 (Material and Methods). After six weeks cells were harvested and assessed for their AML-CFC assay.

On the level of more immature hematopoietic stem cell candidates, LTC-IC assays revealed a response of more than 50 % cell killing in 3/4 ($p<0.03$) patient samples with WT, 3/4 ($p<0.001$) patient samples with ITD and 2/4 patient samples with TDK.

Furthermore, to the toxicity of SU5614 on the level of long term initiating cells, cells after pre-incubation with and without (control) 10 µM SU5614 inhibitor were detected by Suspension Culture Initiating Cell (SC-IC). On the level of long term initiating cells 2/4

patient samples with ITD, and 4/4 patient samples with WT responded to the therapy with SU5614. Only in one sample with TDK mutations, SU5614 reduced leukemic HSC. For some samples we could not detect any growth in the control arm and thereby the samples were not considered (n.g.) (Table 4.4).

Patient	Cytogenetics	FAB	FLT3 status	% kill of LTC-IC	% of kill of SC-IC
1	46,XX, Nras+	M1	WT	100	100
2	47,XX,I(21),+i(21),c-KIT-, Nras-	M2	WT	100	69
3	46,XY, c-KIT-, Nras-	M4	WT	25	100
4	47,XY,+13 48,XY,+13,+13, c-KIT	M1	WT	59	89
5	46,XY, c-KIT-, MLL dupl-	M1	ITD	100	100
6	46,XX, Nras-	M4	ITD	100	n.g
7	46,XX, c-KIT-	M5b	ITD	0	n.g
8	46,XX,t(11;16), c-KIT-, Nras-	M4	ITD	69	100
9	47,XX,+14, c-KIT-, Nras-	M5a	D835H	0	n.g
10	46,XX, c-KIT-, Nras-	M5b	D835H	100	100
11	46,XY, c-KIT-, Nras-	M2	Del835	83	n.g
12	46,XX, c-KIT-, Nras-	M4	D835Y/ITD	14	n.g

Table 4.4. Toxicity of SU5614 on Long Term Initiating Cells (LTC-IC) and Suspencion culture-initiating cells (SC-IC). After six weeks in LTC-IC and SC-IC assay cells were plated in methylcellulose. After two weeks the methylcellulose dishes were scored, colonies were counted and toxicity was calculated as described in Material and Methods. In some control arms no growth was detected (n.g). For this reason it was not possible to calculate the percentage of toxicity of the compound for five samples analyzed with the SC-IC assay.

4.3.5. Toxicity of SU5614 on CFC, LTC-IC as well as on SC-IC in normal bone marrow cells

As the inhibitor eliminates leukemic HSC from AML patient samples with non-mutated *FLT3* we were further interested to analyze its effect on hematopoietic stem cell candidates from healthy donors. As a control $CD34^+$ bone marrow stem cells from healthy donors (NBM) were incubated with 1 µM and 10 µM of SU5614 and toxicity was tested for

the different stages in the hematopoietic progenitor hierarchy. Depending on the dose of SU5614 we observed a considerable toxicity on the level of clonogenic progenitor cells on normal bone marrow from healthy donors. At the concentration of 10 µM SU5614 had considerable toxicity, killing with a mean of 67.5 % (s.d ± 30.6) of the total of CFC and 40.5 % (s.d ± 32.4) when 1 µM of the drug were applied (n=3) (Figure 4.17).

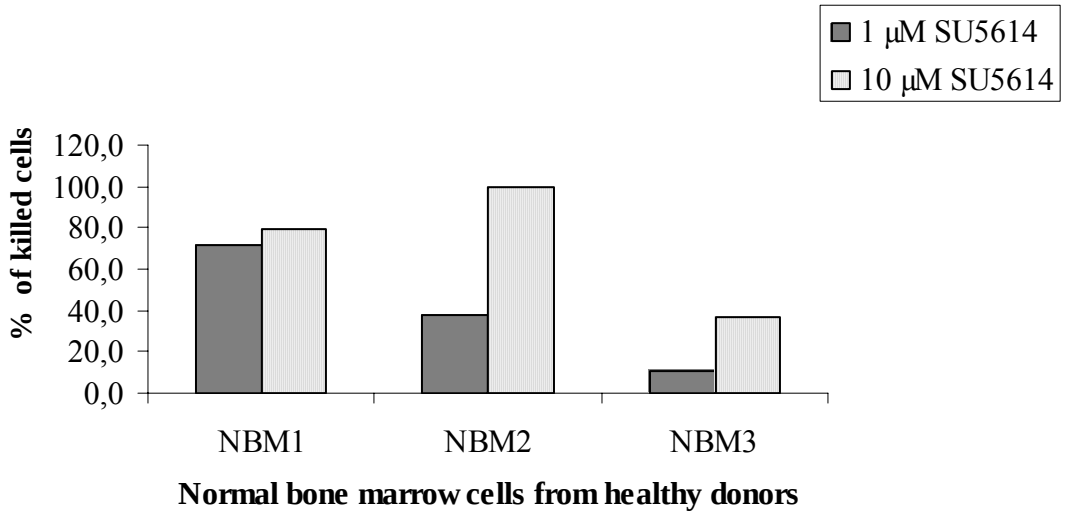


Figure 4.17. Toxicity of SU5614 on normal bone marrow (NBM) cells. Normal bone marrow cells were incubated with 1 µM and 10 µM of the RTKI SU5614. After pre-incubation with the inhibitor, the drug was washed away and cells were put in methylcellulose. After two weeks, the growth of colonies was scored in both arms and the toxicity was determined.

In addition, we performed assays to detect the toxicity on the *in vitro* equivalent of normal long-term repopulating stem cells, the LTC-IC and on suspension culture initiating cells, SC-IC, of human BM cells. Based on our previous results of SU5614 on leukemic cells, we pre-incubated normal bone marrow cells with 10 µM of the inhibitor. Surprisingly, we observed that the compound efficiently eliminated human LTC-IC (78 - 86 %) analyzed in the long term initiating cell assay (n=2). In addition, the SC-IC assay (n=1) showed a reduction of 100 % on the suspension cells analyzed after 24 hours pre-incubation (Table 4.5).

% CD34+ enriched BM (<98%)	Arms	No. of LTC-IC derived colonies /104 CD34+ cells	% of cells reduction
NBM1	Control	187	
	SU5614	26	86
NBM2	Control	259	
	SU5614	56	78

% CD34+ enriched BM (<98%)	Arms	No. of SC-IC derived colonies /104 CD34+ cells	% of cells reduction
NBM1	Control		
	SU5614	3202	100

Table 4.5 Toxicity of SU5614 on normal bone marrow (NBM) cells. Toxicity of SU5614 was tested on normal bone marrow cells from healthy donors. Assays to detect normal LTC-IC human BM cells and normal SC-IC human BM cells were performed plating the cells after six weeks of long term liquid culture and short term liquid culture in methylcellulose. Colonies were scored after two weeks.

5. DISCUSSION

5.1. The hematopoietic PBX interacting protein HPIP plays a role in early stem cell development

The identification of novel proteins which regulate human stem cell and early progenitor cell fate decisions is a major goal in experimental and clinical hematology. The molecular network controlling stem cell fate decisions is largely unknown, multiple studies have attributed a key role in this developmental process to transcription factors. Homeobox (HOX) genes are characterized as 'master genes' of early hematopoietic development. *HOX* genes are highly expressed in early hematopoietic stem and progenitor cells and down-regulated during differentiation. In parallel, Hox genes are implicated directly in human leukemia, at the level of Hox-cofactors (*MEIS1* and *PBX1*) and *HOX* gene upstream regulators (*MLL*, *CDX2*) (Lawrence, Sauvageau et al. 1996; Buske and Humphries 2000). Among these HOX proteins, the significance of the interaction with Pbx and Meis partners for oncogenic function has been addressed for *HOXB3, HOXB4*, *HOXB7*, and *HOXA9*, as well as for the fusion genes *NUP98-HOXA9* and *NUP98-HOXD13* [149-152]. The interaction between *PBX1* and *HOX* genes is also important for normal hematopoietic differentiation. Buske et al. (2002) could show that the constitutive expression of *HOXB4* in human hematopoietic progenitor cells leads to an amplification of normal HSC with a normal differentiation program [153]. By knocking down the endogenous expression of *PBX1* (PBX1,K.D.) in *HOXB4* overexpressing cells, Krosel et al. (2003) were able to generate HSCs that are >20-times more competitive in repopulating assays than those that overexpress *HOXB4* and *PBX1* [154]. They also showed that the *in vivo* expansion of *HOXB4-PBX1*(K.D.) expressing HSCs regenerated normal stem cell pools and did not lead to HSC levels above those detected in unmanipulated mice [154]. Together, these findings underline the significant role of *PBX1* together with *HOX* genes for normal HSC development and for leukemogenesis. Therefore, genes that might interact with PBX1 are of particular interest for normal as well as leukemic hematopoiesis.

Recently, the novel human protein HPIP, which interacts with PBX1 and is highly expressed in human $CD34^+$ progenitor cells, has been characterized [30,31]. *HPIP* can bind all members of the PBX family. Interestingly, *HPIP* seems to inhibit the transcriptional activation of the E2A-PBX1 fusion protein [30,31]. The *E2A/PBX1* fusion gene is the result of a reciprocal translocation t(1;19)(q23;p13). This translocation occurs only rarely in adults with

ALL but accounts for 25–30 % of pediatric ALL cases with a pre-B immunophenotype and has been associated with a relatively poor outcome with traditional combination chemotherapy [30,31,155]. We analyzed the role of *HPIP* in human hematopoiesis by retroviral gene transfer into human cord blood cells.

We could demonstrate that the constitutive expression of *HPIP* in human hematopoietic stem cells has an impact on self renewal and differentiation. It induces a significant increase in the frequency of clonogenic (CFC) (figure 4.2) as well as long-term culture initiating cells (LTC-IC), the *in vitro* equivalent of hematopoietic stem cells (Table 4.3). This effect was comparable with the stem cell amplificatory effect of HOXB4 in the human system as recently shown by Buske et al. (2002) *in vitro* and *in vivo* in the NOD/SCID mouse model [153]. Notably, besides its effect on maintenance of primitive hematopoietic progenitor cells, constitutive expression of *HPIP* did not block terminal hematopoietic differentiation. In none of the animals tested and in none of the *in vitro* assay systems we could observe a leukemic transformation. Interstingly, microarray data could prove high level of *HPIP* expression in bone marrow of AML patients, but further studies have to show the functional relevance of these findings. Our data characterize *HPIP* as a novel regulatory protein of early human hematopoietic development

5.2. The Vent gene family member *VENTX2* plays a role in myeloid development

As we could determine the stem cell amplificatory effect of *HPIP*, we were further interested to identify new potential stem cell regulatory proteins. Recent data indicate that a variety of regulatory molecules, active in early development, may also play a role in the maintenance of hematopoietic stem cells with repopulating activity. One such regulatory protein is the VENTX2 protein which belongs to family of Xvent genes. The X. laevis Vent (Xvent) genes constitute a multigene family encoding homeodomain (HD) proteins with very similar DNA-binding domains [156]. Xvent genes can be divided into two subfamilies on the basis of sequence homology outside of the HD. Each subfamily member contains almost identical HD sequences and has more than 80 % identity over the remainder of the protein sequence. Xvent1, Xvent1B, and PV1 [156,157] are members of the Xvent1 HD subfamily and contain little sequence homology outside of the HD to the Xvent2 subfamily members Xvent2, Xvent2B, Vox1 (also known as Xbr1b), Vox15, Xom, and Xbr1a [156-158]. Members of the Xvent class of HB genes are essential for the patterning of ventral mesoderm and

hematopoietic development in the X. laevis embryo [33]. Ectopic expression of all Xvent genes leads to ventralization of X. laevis embryos[156,159]. The recently described zebrafish ventral homeobox (vox) and ventral expressed homeobox (vent) genes show a high degree of sequence homology to the Xvent family within the HD and have similar expression patterns. These genes are also potent ventralizing factors in both zebrafish and X. laevis embryos. Many HB sequences have been isolated from an immature population of human hematopoietic progenitor cells [160]. The *VENTX2* HD had 65 % identity to the HD in X. laevis Xvent2B [156]. *VENTX2* is to our knowledge the first member of the Vent gene family of diverged HB genes described in mammals. The Vent-like homeobox genes were shown to be involved in early developmental processes and the ventralizing activity of Vent downstream of the morphogen BMP4, which has potent ventralizing activity and is involved in specifying the dorsal-ventral axis during early embryogenesis [161,162].

Therefore the aim of the project was to evaluate the expression of *VENTX2* in different lineages of the hematopoietic compartment and to test the function of the human homologue *VENTX2* in hematopoietic development.

To examine the impact of *VENTX2* expression on hematopoietic progenitors we first tested the clonogenic potential of $CD34^{+}$*VENTX2*$^{+}$ transduced cord blood cells. At the level of clonogenic progenitors *VENTX2* induced myeloid and blocked erythroid differentiation with a significant 1.7 fold increase in the number of G-CFU and a 4,25 fold decrease in the number of erythroid colonies without any change of the total number of colonies produced (Figure 4.10). In addition, we did not only observe a shift in the differentiation program towards the myeloid lineage, but also an increased proliferative activity of clonogenic progentitors when colonies from primary methylcellulose were replated (Figure 4.11).

To test, whether the constitutive expression of *VENTX2* would also have an impact on the most primitive hematopoietic progenitors, we performed long-term culture assays and transplanted NOD/SCID mice. We could not detect a change of the frequency of LTC-IC's between *VENTX2* transduced cells and the control arm (Figure 4.13). Furthermore, the number of colonies generated per LTC-IC did not significantly differ between the two arms. Similarly, the frequency of NOD/SCID repopulating cells was not changed, when cord blood cells transduced with *VENTX2* were transplanted and compared to the control.

However, when NOD/SCID mice transplanted with human cord blood cells constitutively expressing *VENTX2* were analyzed over time, we saw a decrease in the number of $CD34^{+}CD38^{-}$ primitive cells as compared to those animals that received control cells. In contrast, we detected a 7 fold increase in the absolute number of $CD38^{+}$ cells and a 3 fold

increase in the number of $CD15^+$ myeloid cells indicating again an impact of *VENTX2* on more mature hematopoietic myeloid progenitor cells (Figure 4.15).

Preliminary RT-PCRs data could detect *VENTX2* expression in CD15, CD19 and CD33 cells highly purified by FACS from umbilical cord blood Expression of *VENTX2* was detected in B cells - as well as differentiated myeloid cells indicating that *VENTX2* is expressed in multiple hematopoietic lineages. In addition, *VENTX2* expression was detected in $CD34^+$, $CD38^+$ and $CD34^-CD38^+$ cord blood cells. Furthermore, we did not found *VENTX2* expression within the compartment of early $CD34^+CD38^-$ cord blood cells. (data not shown). In summary, we could identify *VENTX2* as a new hematopoietic regulatory gene that mainly plays a role in more mature myeloid hematopoietic development. Using our model systems, we could not detect any malignant transformation caused by the constitutive expression of *VENTX2*.

5.3. SU5614 as a stem cells therapeutic agent

It has been shown that acute myeloid leukemia results from the malignant transformation of an early hematopoietic progenitor cell. Primitive normal and leukemic hematopoietic progenitors share many cell surface phenotypic characteristics such as the expression of CD34 with the lack of CD38 and other lineage markers. However, some differences between these cells have been identified that allow them to be separated by flow cytometry (Feuring-Buske et al Blood). It seems possible that such unique characteristics of AML progenitors could be targeted for therapeutic purposes. The use of agents such as kinase inhibitors in hematological malignancies offers the exciting possibility of treating patients based on the molecular pathology of diseases. Acute myeloid leukemia (AML) is a malignant disorder of hematopoietic cells characterized by an accumulation of highly proliferative blasts blocked in differentiation at various stages [40]. It is known that mutations in the receptor tyrosine kinase *FLT3* occur in 30% of AML patients and in a subset of acute lymphoblastic leukemia [1,163]. Activating mutations in *FLT3* are most frequently internal tandem duplications (ITD), also called length mutation (LM), within the juxtamembrane coding sequence of the receptor occurring in 20 - 27 % of patients with AML [97,98] and a point mutation at residue 835 (Asp835) within the second kinase domain occurring in about 7 % of patients with AML [101,102]. Due to this high frequency of mutations *FLT3* is the most commonly mutated gene in AML. ITD and point mutation spontaneously activate tyrosine kinase activity and induce factor independent proliferation of hematopoietic cell lines. Recently, a number of small

molecule protein tyrosine kinase (PTK) inhibitors targeting constitutively activated *FLT3* were developed. Inhibition of *FLT3* kinase activity using small molecule inhibitors induces apoptosis in cell lines and prolongs survival of mice expressing mutant *FLT3* in their bone marrow cells [136,164]. In our study the correlation between drug sensitivity *in vitro* and the mutation status of the *FLT3* receptor gene was evaluated on blast cells from patient with AML. We used SU5614, a member of the rapidly growing family of PTKI, an indolinone compound that inhibits the tyrosine kinase activity of the *FLT3* receptor. It was predicted to target leukemic stem and progenitor cells while sparing their normal counterparts.

To test, whether these characteristics are met by SU5614, we first analyzed the anti-leukemic activity of the compound on the leukemic cell population of patients with AML. Three groups of patients (n=4 per group) were analyzed with non-mutated wild-type *FLT3* (WT), internal tandem duplication ITD, as well as point mutations in the *FLT3* kinase domain (D835H, D835H, D835Y, del835). After 24 hours of pre-incubation with the inhibitor in liquid culture, patient samples with non-mutated *FLT3* with ITD and with point mutations responded with a greater than 50 % reduction of leukemic cell number (Table 4.3). On the level of clonogenic progenitors 2/4 patient samples with WT, 3/3 with ITD and 2/2 patient samples with point mutations responded to therapy with an up to 100 % reduction of the number of leukemic clonogenic progenitors (Table 4.3).

Importantly, on the level of leukemic stem cells, the compound achieved a > 50 % cell death in 3/4 and 4/4 patient samples with WT *FLT3*, as determined by LTC-IC assay and SC-IC assay, respectively (Table 4.4). In the group of ITD positive 3/4 and 2/2 patient samples showed a more than 50 % cell kill in the LTC-IC and SC-IC assay, respectively. For samples with the D835 mutations SU5614 reduced leukemic stem cells by more than 50 % in 2/4 samples as determined by the LTC-IC assay (Table 4.4). These data indicate that the RTK inhibitor is highly active in the majority of cases in reducing the size of the leukemic bulk population, the number of leukemic clonogenic progenitors and most importantly, the number of leukemic stem cells. However, sensitivity to SU5614 was very heterogeneous, ranging from total eradication of leukemic cells to non-response. Furthermore, the response of both clonogenic progenitor cells as well as leukemic stem cells to SU5614 could not be predicted from the expression level of *FLT3* on the bulk leukemic population as determined by the percentage of CD135 positive cells (Table 4.1). This may reflect different expression profiles for *FLT3* functionally distinct progenitor populations or for other stem cell-relevant tyrosine kinase that are also targeted by the inhibitor. Response to SU5614 RTKI did also not correlate

with the presence of activating mutations or surface expression of c-KIT, an RTK also targeted by the compound SU5614.

The RTK family includes KIT (CD117; c-KIT, stem cell factor), the receptors for macrophage colony-stimulating factor (M-CSF) and platelet-derived growth factor (PDGF), which all show a high sequence homology. Because of this high homology SU5614 is not only *FLT3* specific but also inhibits c-KIT and VEGFR [148]. None of the patient samples analyzed showed an activating mutation of c-KIT (Table 4.2). However, FACS analysis revealed a high expression of c-KIT (CD117) on 9/12 patients. The combined inhibitory effect of SU5614 on *FLT3* as well as c-KIT might be responsible for the observed response. Furthermore, CD34+ bone marrow stem cells from healthy donors were incubated with and without (control) SU5614 and toxicity was tested for the different stages in the hematopoietic progenitor hierarchy. On the level of normal clonogenic progenitors SU5614 had considerable toxicity also using different concentration of the drug. The cell killing leads to a mean reduction of 40.5 % (s.d ± 30.6) of the cells at 1 μM and 67.5 % (s.d ± 32.4) at 10 μM (Figure 4.17). In addition, the compound efficiently eliminated normal human stem cells analyzed in the LTC-IC assay (n=2) or the SC-IC assay (n=1) at 10 μM with a range between 78 – 100 % after 24 hours incubation (Table 4.6). Mice deficient for two RTKs, such as c-kit and flk2 demonstrate a more severe phenotype characterized by large overall decreases in hematopoietic cell numbers, further reductions in the relative frequencies of lymphoid progenitors, and a postnatal lethality. These observations suggest a role of flk2 in multipotent stem cells and lymphoid differentiation [165]. Our data indicate that SU5614 is a potent drug for the elimination of leukemic stem cell candidates from patients with ITD+, D835+ AML as well as those with non-mutated FTL3. However, SU5614, targeting *FLT3*, VEGFR-2 and Kit, is not sparing the normal stem cell compartment. These data suggest that PTKI's with activity against different receptor tyrosine kinases have a considerable inhibitory effect on normal human progenitor cells which might have clinical relevance in the treatment of patients with AML or MDS with compromised normal hematopoietic capacity.

6. SUMMARY

The hallmark of hematopoietic stem cells (HSC) is their ability of self-renewal and differentiation into multiple hematopoietic cell lineages. Although the molecular network controlling stem cell fate decisions is largely unknown, multiple studies have attributed a key role to transcription factors in this developmental process. In this context the family of homeobox genes was characterized as 'master genes' of this early hematopoietic development. The identification of new genes involved in normal and leukemic hematopoiesis and the development of therapies against deregulated processes in hematopoiesis are the major goals in experimental and clinical hematology. . The identification of new genes involved in normal and leukemic hematopoiesis and the development of therapies against deregulated processes in hematopoiesis are the major goals in experimental and clinical hematology. Therefore, the focus of this thesis was the characterization of two novel putative regulatory proteins of early human hematopoiesis, the hematopoietic PBX-interacting protein (HPIP) and the human Vent-like Homeobox gene (*VENTX2*) and to investigate to the activity of the FLT3 protein kinase inhibitor SU5614 on leukemic blast from AML patient samples.

Using complex in vitro assays we analyzed the impact of constitutive expression of *HPIP* and *VENTX2* on stem cell and early human hematopoietic development. To detect clonal progenitor cells primary and secondary colony-forming-unit (CFC) assays were performed. In addition the in vitro equivalent of HSC long-term culture initiating cells were detected with the (LTC-IC) assay.

We were able to show that the constitutive expression of HPIP can rapidly lead to increased numbers of cells detected on the level of committed clonogenic progenitor cells and LTC-ICs. In addition, the production of CFC per LTC-IC is markedly enhanced when cord blood (CB) cells are transduced with HPIP as compared to the control. Notably, besides its effect on maintenance of primitive hematopoietic progenitor cells, constitutive expression of HPIP did not block terminal hematopoietic differentiation. Additional we could show that the constitutive expression of *HPIP* leads to an increase of myeloid cells in transplanted NOD/SCID mice. These data characterize *HPIP* as a novel regulator of the early human hematopoietic stem cell, demonstrating that its constitutive expression has a notable impact on self renewal and differentiation of human hematopoietic stem cells.

In vitro and in vivo analyses shed light on the understanding to the function of the homeobox gene *VENTX2*. On the level of the most primitive hematopoietic progenitors we could not observe a significant increase in the frequency of HSCs. Furthermore, the number

of colonies generated per LTC-IC did not significantly differ between the VENTX2 arm and the control arm. A strong effect was obtained on the level of clonogenic progenitor cells. *VENTX2* increased the production of myeloid cells 1.7-fold in comparison to the control. Secondary replating assay confirmed the amplificatory effect of VENTX2 transduced cells in the number of secondary G-CFU indicating that *VENTX2* promote myeloid lineage differentiation. Interestingly, on the level of clonogenic progenitors *VENTX2* expression resulted in a significantly decreased growth of erythroid colonies by 4.2-fold compared to the control suggesting that constitutive expression of *VENTX2* may inhibit early erythroid differentiation. This inhibition did not occur on the level of primitive hematopoietic cells detected by Limiting Dilution LTC-IC assay where *VENTX2* increased within a 2.2 fold compared to the control.

The observation that *VENTX2* overexpression drives $CD34^+$ to differentiate into myeloid lineage was additional proved by in vivo experiments. In NOD/SCID mice VENTX2 induced a 3-fold increase in the proportion of $CD15^+$ mature myeloid cells within the GFP-positive compartment compared to the control. A 7-fold increase was observed in the total of $CD38^+$ GFP^+ cells in comparison to the MIG mice control ($p<0.01$). Furthermore, *VENTX2* transduced cells increased the proportion of $CD34^-CD38^+$ cells 1.2 fold compared to the control suggesting that *VENTX2* expands the compartment of more differentiated progenitors in vitro as well as in the NOD/SCID mouse model. This implicates a amplificatory effect of the *VENTX2* expression on differentiated progenitor cells. All together, these data characterize VENTX2 as a novel regulatory protein in human hematopoiesis adding information about the role of non-clustered homeobox genes in early blood development.

In an additional project of this thesis, we tested the specificity of the FLT3 protein kinase inhibitor SU5614 on normal and leukemic blasts from patients with acute myeloid leukemia. Here we could demonstrate the efficiency of the compound to eliminate the leukemic stem cell in AML patient samples with mutated as well as non mutated FLT3 receptor. However, our data also point to a considerable toxicity on normal HSC, which should be taken in account in the management of patients with compromised normal hematopoiesis.

7. ZUSAMMENFASSUNG

Das Merkmal hämatopoetischer Stammzellen ist ihre Fähigkeit sich selbst zu erneuern und in verschiedene hämatopoetische Zelllinien zu differenzieren. Obwohl das molekulare Netzwerk, welches die Entscheidung über das Schicksal der Stammzellen kontrolliert, noch größtenteils unbekannt ist, wurden in verschiedenen Studien diversen Transkriptionsfaktoren eine Schlüsselrolle in diesem Entwicklungsprozess zugeschrieben. In diesem Zusammenhang wurde die Familie der Homeoboxgene als eine Klasse von „Mastergenen" in der frühen Hämatopoese charakterisiert. Das Hauptziel der experimentellen und klinischen Hämatologie ist die Identifizierung neuer Gene, welche eine entscheidene Rolle in der normalen und leukemischen Hämatopoese spielen, sowie die Entwicklung von Therapien gegen deregulierte Prozesse in der Hämatopoese.

Das Ziel dieser Doktorarbeit war es die Rolle der Gene *HPIP* (hämatopoetisches PBX-interagierendes Protein) und *VENTX2* (humanes *Vent*-ähnliche Homeoboxgen 2) in der normalen und leukemischen Hämatopoese zu klären. Des Weiteren wurde die Wirkung des spezifischen FLT3 Proteinkinaseinhibitors SU5614 an leukämischen Blasten von AML Patientenproben untersucht.

Mit Hilfe von anspruchsvollen *in vitro* Methoden haben wir die einzelnen Stadien der Zelldifferenzierung hämatopoetischer Stammzellen in Abhängigkeit der Expression von *HPIP* und *VENTX2* analysiert. Zur Detektion von klonalen Vorläuferzellen wurden primäre und sekundäre Kolonie-formende Zellassays (CFCs) durchgeführt, die Detektion von *in vitro* Equivalenten hämatopoetischer Stammzellen erfolgte mittels Langzeit-Kultur-initiierenden Zellassays (LTC-ICs). Desweiteren wurden *in vivo* NOD/SCID Mausmodelle etabliert.

Wir waren in der Lage zu zeigen, dass die konstitutive Expression von HPIP zu einer raschen Zunahme an klonalen Vorläuferzellen und LTC-ICs führt. Zusätzlich konnten wir belegen, dass nach Transduktion von Nabelschnurblutzellen mit *HPIP* die Produktion der CFCs pro LTC-IC deutlich zunimmt. Es ist bemerkenswert, dass die konstitutive Expression von *HPIP*, neben den Effekten auf die Aufrechterhaltung primitiver hämatopoetischer Vorläuferzellen, die terminale hämatopoetische Differenzierung nicht blockiert. Außerdem konnten wir zeigen, dass die konstitutive Expression von *HPIP* zu einer Zunahme an myeloiden Zellen in transplantierten NOD/SCID Mäusen führt. Diese Daten charakterisieren *HPIP* als einen neuen Regulator der frühen humanen hämatopoetischen Stammzelle. Seine konstitutive Expression in humanen hämatopoetischen Stammzellen hat einen deutlichen Einfluss auf die Selbsterneuerung und Differenzierung dieser Zellen.

In vitro und *in vivo* Untersuchungen im Rahmen dieser Arbeit haben zum Verständnis der Funktion des Homeoboxgens *VENTX2* beigetragen. Auf dem Level der primitivsten hämatopoetischen Vorläuferzellen konnten wir nach Expression von *VENTX2* keine signifikante Zunahme an hämatopoetischen Stammzellen im Vergleich zur Kontrolle erkennen. Außerdem zeigt sich in der Anzahl der generierter LTC-IC nach *VENTX2* Expression kein signifikanter Unterschied im Vergleich zur Kontrolle. Einen deutlichen Effekt konnten wir auf dem Level der klonalen Vorläuferzellen beobachten. Die *VENTX2* Expression erhöhte die Produktion myeloider Zellen im Vergleich zur Kontrolle um das 1,7-fache. Sekundäre Wiederausplattierungs-Experimente bestätigten den amplifizierenden Effekt der *VENTX2* Expression auf die myeloide Blutzelllliniendifferenzierung. Des Weiteren stellten wir fest, dass bei CFCs das Wachstum erythroider Kolonien signifikant um das 4,95-fache erniedrigt ist, woraus man schließen kann, dass eine konstitutive Expression von *VENTX2* möglicherweise die Differenzierung von erythroiden Vorläuferzellen (BFU-E) inhibitiert. Diese Inhibition trat nicht auf dem Level primitiver hämatopoetischen Zelle auf. Die *VENTX2* Expression führte zu einer 2,2 fachen Zunahme von LTC-ICs im Vergleich zur Kontrolle.

Die Feststellung, dass die *VENTX2* Überexpression Stammzellen zur Differenzierung in Richtung der myeloide Blutzelllinie treibt, wurde zusätzlich durch *in vivo* Experimente bestätigt. In NOD/SCID Mäusen induzierte die *VENTX2* Überexpression eine dreifache Zunahme an reifen, myeloiden $CD15^+$ Zellen innerhalb des GFP-exprimierenden Anteils an Blutzellen. Es konnte insgesamt eine siebenfache Zunahme an $CD38^+$ GFP^+ Zellen im Vergleich zu MIG transformierten Kontrollmäusen festgestellt werden ($p<0.01$). Des Weiteren erhöhen *VENTX2* transduzierte Zellen den Anteil an $CD34^-CD38^+$ Zellen um das 1,2-fache im Vergleich zur Kontrolle. Dies lässt vermuten, dass die *VENTX2* Expression den Anteil an differenzierten Vorläuferzellen sowohl *in vitro* als auch in dem NOD/SCID-Mausmodell erhöht. Dies legt einen amplifikatorischen Effekt der *VENTX2* Expression in differenzierten Vorläuferzellen nahe. Zusammenfassend kann man sagen, dass die Ergebnisse dieser Doktorarbeit *VENTX2* als ein neues regulatorisches Protein in der humanen Hämatopoese charakterisieren und neue Informationen über die Rolle von nicht-geclusterten Homeoboxgenen in der frühen Blutzellentwicklung geben.

In einem weiteren Projekt dieser Doktorarbeit haben wir schließlich den spezifischen FLT3 Proteinkinaseinhibitor SU5614 auf normalen und leukemischen Blasten von Patienten mit akuter myeloider Leukemie (AML) getestet. Hierbei konnten wir die Effizienz des Inhibitors zur Eliminierung leukemischer Stammzellen von AML Patienten, sowohl mit als auch ohne mutierten FLT3 Rezeptor, nachweisen. Dennoch muss gesagt werden, dass unsere

Daten auf eine nicht unerhebliche Toxizität des Inhibitors auf normale HSC hinweisen, die man bei der Behandlung von Patienten mit eingeschränkter normaler Hämatopoese mit diesem Inhibitor unbedingt in Betracht ziehen muss.

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10. ABBREVIATIONS

µL	Microliter
µM	Micromola
ALL	Acute lymphoid leukemia
AML	Acute myeloid leukemia
BFU-E	
BM	Bone marrow
CB	Cord blood
CD	Cluster of Differentiation
CFC	Colony forming cell
CSF	Colony stimulating factor
DMSO	Dimethylsulfoxid
DNA	Desoxyribonucleic acid
EPO	Erytropoietin
FAB	French American British
FACS	Fluoresence Activated Cell Sorting
FISH	Flurecence in situ hybridisation
FLT3	FMS-like tyrosine kinase 3
G-CSF	Granulocite-Colony stimulating factor
GM-CSF	Gramulocite-Macrophage-Colony stimulating factor
HB	Homeobox gene
HD	Homeodomain
IL	interlulukin
ITD	Internal tandem duplication
LDA-LTC-IC	Limiting dilution assay LTC-IC
LM	Lenght mutation
LTC-IC	Long-term culture-initiating cell
LTR	Long terminal repeat
M-CSF	Macrophage-Colony stimulating factor
MIG	MSCV-IRES-EGFP
MNC	Mononuclear cell

MSCV	Murine Stem Cell Virus
NBM	Normal bone marrow
NOD-SCID mice	Non Obese Deficent-Sever Compromise Immuno Deficent Mice
PB	Peripheral blood
PCR	Polymerase Chain Reaction
PI	Propidium iodide
PTK	Phospho tyrosine kinase
PTK	Protein Tyrosine Kinase
PTKI	Protein Tyrosine Kinase Inhibitor
RNA	Ribonucleic acid
S-CFU-S	Spleen-Colony forming unit
SC-IC	Suspension culture- initiating cell
SEM	Standard error of the mean
t	translocation
TDK	Tyrosine kinase domain
TM	Transmembrane domain
VCM	Virus containing medium
WBC	White blood cell
WT	Wild type
YFP	Yellow Fluorescent Protein

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Publications

Arseni N, Ahmed F, Hiddeman W, Buske C, Feuring-Buske M. Effects of the protein tyrosine kinase inhibitor, SU5614, on leukemic and normal steam cell. **Haematologica 2005; 90(11)**

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Konstantin Petropoulos, Farid Ahmed, Christina Schessl, **Natalia Arseni**, Aniruddha Deshpande, Wolfgang Hiddemann, Rudolf Grosschedl, Michaela Feuring-Buske, Christian Buske. Constitutive Expression of the 'Lymphoid Enhancer Factor 1' (Lef-1) Perturbs Early Hematopoietic Development and Induces a Lethal Myeloproliferative Syndrome in a Subset of Transplanted Mice. **Blood Nov.(2005)Abstract (644)**

Conferences attended

44th American Society of Hematology annual meeting (ASH), **Philedelphia, USA Dec. 2002. Poster presentation**: SU5614 leukemic cells from patient with acute myeloid leukemia (AML)

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Annual Meeting of the German, Austrian and Swiss Societies for Hematology and Oncology, (DGHO), **Basel, Schweiz Oct. 2003. Poster presentation**: "Leukemic stem cells from patients with acute myeloid leukemia with and without activating FLT3 mutations are highly sensitive to the protein tyrosine kinase inhibitor SU5614

Third Harvard-Munich Workshop on AML, **Herrsching, Jun 2004. Oral presentation:** *VENTX2*, a Vent-like homeobox gene, is a novel candidate for a hematopoietic regulatory protein

7th American Society of Hematology annual meeting (ASH), **Atlanta ,USA, Dec 2005. Poster presentation:** The Vent-like Homeobox Gene VENTX2 is a Novel Candidate for a Human Hematopoietic Regulatory Protein