Effect of Chromatin Modeling by Histone Deacetylase-inhibitors (HDIs) on Hematopoietic Stem Cell (HSC) Fate

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1 Introduction

1.1 Normal Hematopoiesis

Hematopoiesis is the process by which mature blood cells of distinct lineages are produced from pluripotent hematopoietic stem cells (HSCs). Normal hematopoiesis depends on the presence of normal pluripotent HSCs, a bone marrow microenvironment and a complex system of highly regulated hematopoietic growth factors with overlapping lineage specificities (Smith 2003). The central feature of pluripotent HSCs is that they are capable of both self-renewal and differentiation into mature hematopoietic cells (Weissman 2000). Although single stem cells and their direct progeny may survive for long periods of time, there may be a dynamic process by which some stem cells cease functioning and are replaced by other stem cells that previously had been resting for a prolonged period of time in the G_0 phase of the cell cycle (Hao*, et al* 1996). Regardless of the situation, the purpose of the stem cells is accomplished, and normal individuals have stem cells that maintain hematopoiesis at a stable level throughout their lifetimes (Domen and Weissman 1999).

1.1.1 Hematopoietic Stem Cells (HSCs)

HSCs are the common ancestor of all types of blood cells (Kondo*, et al* 2003, Weissman 2000). While HSCs are primarily found in the bone marrow (BM), they are present in a variety of other tissues including peripheral blood (PB) and umbilical cord blood (CB), and are found at low numbers in the liver, spleen, and perhaps many organs (Holyoake*, et al* 1999, Mayani and Lansdorp 1998). Beginning with the groundbreaking experimental evidence, a population of clonogenic bone marrow (BM) cells was found to generate myeloerythroid colonies in the spleens of lethally irradiated hosts (McCulloch and Till 1960, Till and Mc 1961). These clonogenic cells in some cases gave rise to cells that also could be transferred to secondary hosts and there reconstitute all blood cell lineages (Siminovitch*, et al* 1963). These were proposed to be HSCs, or progenitor cells with the essential characteristic of self-renewal and differentiation potential for all types of blood cells (Osawa*, et al* 1996).

However, it has been indicated that spleen colony forming unit cells (CFU-S) could only achieve transient repopulation (8-12 days) of irradiated hosts and not a (long-term) regeneration of functional tissue, an essential characteristic of true stem cells. (Harrison 1980, Harrison and Astle 1997, Jones, *et al* 1989, van der Loo, *et al* 1994). The need for a practical assay that allowed the specific identification of primitive HSCs with long-term lymphomyeloid repopulating ability led to the development of competitive repopulation assay, which provides a relative measure of "repopulating activity," usually compared to a reference standard of normal bone marrow cells (Harrison 1980, Harrison, *et al* 1978). The development of clonal assays for all major hematopoietic lineages together with the availability of multiparameter fluorescence-activated cell sorting (FACS) has enabled the prospective purification of HSCs from mice and to highly enrich for HSCs from humans according to the cell-surface expression of specific molecules and their functional read-out *in vivo* and *in vitro* in stromal long-term colony-initiating assays (Baum, *et al* 1992).

HSCs can be divided into a long-term subset (LT-HSC), capable of indefinite self-renewal, and a commit to differentiation pass through a phase of being short-term HSC (ST-HSC) that self-renew for six to eight weeks, then advance to the multipotent progenitor (MPP) stage (Christensen and Weissman 2001, Morrison and Weissman 1994).

Several marker combinations, such as ($Lin^{neg/low}$, $Thy1.1^{low}$, c-Kit^{high}, Sca-1^{+}), (Lin^{-}, $Thy1.1^{low}$, Sca-1^{+}, rhodamine 123^{low}) or (Lin^{-}, $CD34^{-/int}$, c-Kit^{+}, Sca-1^{+}), have been used to isolate nearly pure mouse HSC populations. Similar marker combinations (Lin^{-}, $Thy1^{+}$, $CD34^{+}$, $CD38^{neg/low}$) (Morrison, *et al* 1995) are used to highly enrich human HSCs populations. Although there are some variations in the exact frequencies found, the different methods of isolation all indicate that HSC are rare cells. Using the ($Lin^{neg/low}$, $Thy1.1^{low}$, c-Kit^{high}, Sca-1^{+}) markers approximately 1 in 5,000 mouse bone marrow cells has long-term, multilineage, repopulating capability, i.e., is a LT-HSC, whereas approximately 1 in 1,000 has a more limited, short-term, multilineage repopulating capability, i.e., is a ST-HSC or a multipotent progenitor (MPP). Recently, the expression of the receptor tyrosine kinase Flk-2 has been identified as a reliable marker to discriminate between LT-HSC ($Thy1.1^{low}$, $Flk\text{-}2^{neg}$), ST-HSC ($Thy1.1^{low}$, Flk-2^{+}), and MPP ($Thy1.1^{-}$, Flk-2^{+}) in combination with the ($Lin^{neg/low}$, c-

Kit^{high}, and Sca-1^{+}) markers. Morphologically, HSC and MPP resemble lymphocytes (Christensen and Weissman 2001).

1.1.2 Early Lineage Hematopoietic Progenitor

MPPs self-renew for less than two weeks, but neither MPP nor ST-HSC are capable of dedifferentiating to long-term HSC (LT-HSC) under any circumstances yet tested (Morrison, *et al* 1997). These multipotent stem and/or progenitor cells then commit either to the myeloid or lymphoid lineage by differentiating to common myeloid progenitors (CMP) or common lymphoid progenitors (CLP) (Akashi, *et al* 2000, Hao, *et al* 2001).

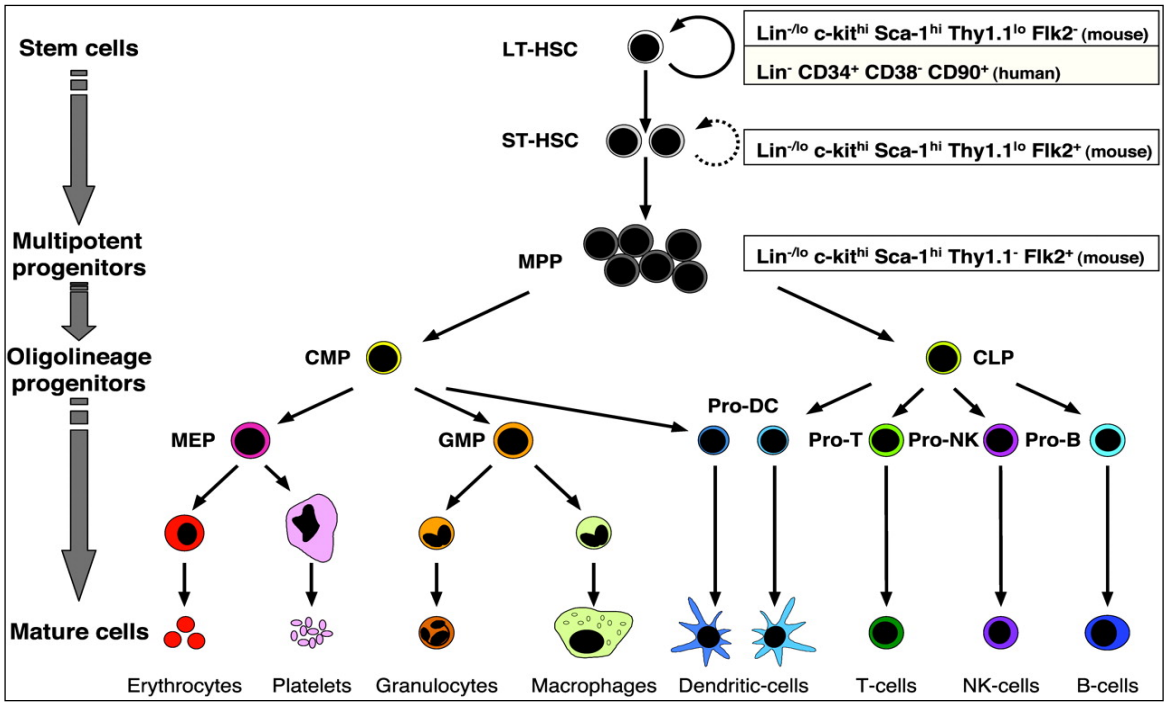

Fig.1. Hematopoietic and Stem/progenitor Cell Lineages (Passegue, *et al* 2003). HSCs can be divided into LT-HSCs with indefinite self-renewal or ST-HSCs with 6-8 weeks self-renewal. ST-HSCs differentiate into MPPs, which do not or briefly self-renew, and have the ability to differentiate into oligolineage-restricted progenitors that ultimately give rise to differentiated progeny. The CLPs give rise to T lymphocytes, B lymphocytes and natural killer (NK) cells. The CMPs give rise to Granulocyte/macrophage progenitors (GMPs), which then differentiate into monocytes/macrophages and granulocytes, and to megakaryotic/erythroid progenitors (MEPs), which produce megakaryocyte/platelets and erythrocytes. Both CMPs and CLPs can give rise to dendritic cells. All of these stem and progenitor populations are separable as pure populations by using cell surface markers.

Both CMPs and CLPs are clonal progenitors with little or no detectable self renewal capacity and limited differentiation. Although the progenitors cannot durably engraft, they nonetheless rescue lethally irradiated mice. CLPs give rise to progenitors of at least four classes of cells—the T, B, and natural killer (NK) lymphocyte lineages, and antigen presenting dendritic cells. CMPs give rise at least to granulocyte monocyte committed progenitors (GMPs) and megakaryocyte erythrocyte progenitors (MEPs) (Fig.1) (Morrison*, et al* 1997, Shizuru*, et al* 2005). Granulocytes and monocytes differentiate in a bone marrow under the influence of specific glucoproteins, so called colony stimulating factors (G-CSF, GM-CSF, M-CSF) whose structure and mechanism of action is not yet fully understood (Lotem and Sachs 1982). Besides them, there are several other growth and differentiation factors which play a role in a normal hematopoiesis like erythropoietin, thrombopoietin, IL-1, IL-3, IL-5, IL-6 as well as all-trans-retinoic acid (t-RA), which plays an important role in induction of the target genes in differentiation towards macrophages (Brach and Herrmann 1991, Collins 2002). However, in particular, t-RA delays the differentiation of primitive hematopoietic stem cells suggesting it has an effect on HSCs according to their differentation status (Collins 2002, Purton*, et al* 2000).

1.1.3 Regulation of HSC Fate

During or after cell division, the two daughter cells of a stem cell have to decide their fate. They can either choose to remain as HSC, commit to differentiation, or die by apoptosis. Alternatively, they can also stay in the bone marrow or migrate to the periphery. These processes are called HSC fate decisions and must be finely tuned to maintain a steady-state level of functional HSCs in the bone marrow and to constantly provide progenitors for the various hematopoietic lineages (Passegue*, et al* 2003). Expansion of HSCs by cell division and exit of a fraction of HSCs from quiescent state occurs under both steady state and conditions of mobilization.

The mechanisms that control whether a HSC self-renews or differentiates remain a mystery. However, many of the important players in this regulation have been identified. The signaling

pathways such as Wnt, *hoxb4* genes, Notch1, Sonic hedgehog (Shh) pathways have emerged as canditates for regulating self-renewal.

Besides transcriptional regulation and the stem cell niche that play a role in controlling stem cell fate, HSCs themselves have intrinsic characteristics or clocks such as the cyclin dependent kinase inhibitors (CDKI), which regulate the cell cycle with regard to the stage and, in part, determine the number of cell divisions of HSCs (Krause 2002).

Although the global gene expression profiles for HSCs have been recently described, very little is known about the dynamics of gene expression necessary for HSC fate decision. There is evidence that early HSCs express a promiscous set of transcription factors and open chromatin structure required to maintain their multipotentiality, which is progressively quenched as these cells progress down a particular pathway of differentation (Akashi*, et al* 2003).

1.1.3.1 Wnt-signalling

Recent reports suggest that signaling through Wnt is a critical pathway utilized by LT-HSC in self-renewing divisions. Contact of HSCs with Wnt results in signaling events such that HSC enter the cell cycle with little differentiation out of the LT-HSC pool (Austin*, et al* 1997, Reya*, et al* 2003, Shizuru*, et al* 2005, Willert*, et al* 2003). Wnt proteins are intercellular signaling molecules that regulate development in several organisms (Cadigan and Nusse 1997). Such a signal activates a pathway from the surface receptor “frizzled” (Fzd) via a gene product called “dishevelled” (Dsh) to separate cytoplasmic β-catenin from a protein complex (APC/axin/GSK3_). In its phosphorphylated form, β-catenin is held in the cytoplasm within APC/axin/GSK3_/ β-catenin complex (Reya*, et al* 2001). The phosphorylation of β-catenin at its NH2 terminus by GSK3_ targets it for ubiquitination and degradation by proteasomes. GSK3_, one of the key components of the destruction complex, is known to be inhibited through phosphorylation on Ser9 by activated Akt and other kinases (Doble and Woodgett 2003, Jope and Johnson 2004) thereby coupling these kinases with GSK3_-dependent signalling pathway. When Wnt signals through Dsh, the complex dissociates and

unphosphorylated, stabilized β-catenin is free to translocate to the nucleus, where it binds to DNA binding proteins such as lymphoid enhancer factor (LEF) and T-cell factor (TCF), converting them from repressors to activators of gene transcription . Activation of Wnt-signalling in HSCs induces upregulation of genes such as *hoxb4* and *notch 1* (Reya*, et al* 2003), genes independently implicated in proliferation as well as self-renewal of HSC (Antonchuk*, et al* 2001, Antonchuk*, et al* 2002, Karanu*, et al* 2000).

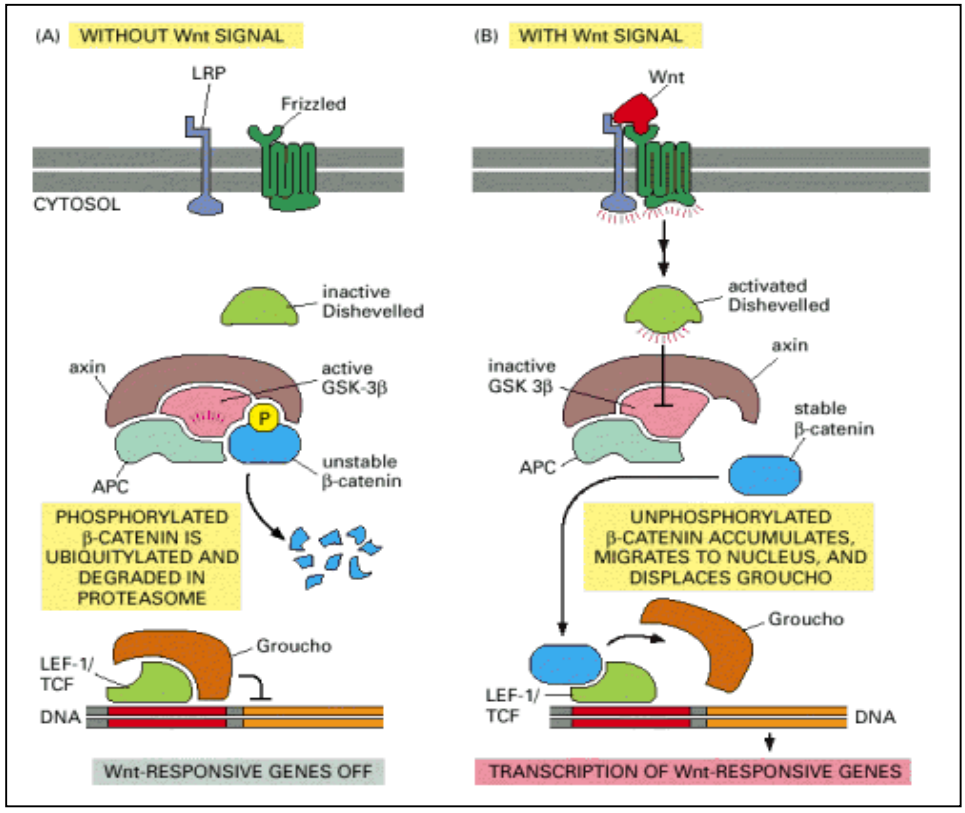

Fig.2. Wnt-signalling Pathway (Alberts, *et al* 2002). In the absence of Wnt-signalling (left panel), β-catenin is in a complex with axin, APC and GSK3-β, and gets phosphorylated and targeted for degradation In the presence of Wnt signalling (right panel), β-catenin is uncoupled from the degradation complex and translocates to the nucleus, where its binds Lef/Tcf transcription factors, thus activating target genes.

1.1.3.2 Homebox B4; *hoxb4* gene

The mammalian *Hox* homeobox gene family of transcription factors, consists of 39 members arranged in 4 clusters (A, B, C, and D), which were initially described as important regulators of pattern formation in a variety of embryonic tissues (van Oostveen, *et al* 1999). Several homeobox transcription factors, such as HoxB3 and HoxB4, have been implicated in regulation of hematopoiesis. In support of this, recent studies indicate that overexpression of HoxB4 strongly enhances hematopoietic stem cell regeneration. It was shown that long-term repopulating activity of murine bone marrow cells was increased to at least 10-fold when HoxB4 cDNA was over-expressed by retroviral infection in comparision to control stem cells infected with empty vector (Bjornsson, *et al* 2003, Sauvageau, *et al* 1995).

The potency of HoxB4 has been demonstrated as it enabled high-level ex vivo HSC expansion. Cultures of HoxB4-transduced HSCs achieved rapid, extensive, and highly polyclonal HSCs expansions, resulting more than 1,000-fold higher HSC content relative to controls. Besides, these HSCs retained full lympho-myeloid repopulating potential in mice and did not lead to any hematologic abnormalities in the animals (Antonchuk, *et al* 2002). Moreover, In another study, recombinant human TAT-HoxB4 protein carrying the protein transduction domain of the HIV transactivating protein (TAT) has been reported to expand hematopoietic stem cells by approximately 4- to 6-fold with normal *in vivo* differentiation potential as well as long-term repopulation capacity (Krosl, *et al* 2003).

In addition, it has been shown that human long-term culture-initiating cells and nonobese diabetic-severe combined immunodeficiency (NOD-SCID) mouse repopulating cells were expanded by more than 20- and 2.5-fold, respectively, over their input numbers when they were cultured on stromal cells genetically engineered to secrete HoxB4. Importantly, the HOXB4-expanded HSCs enhanced stem cell repopulating capacity *in vivo* as well as maintenance of pluripotentiality. (Amsellem, *et al* 2003).

1.1.3.3 Notch1 Signalling

Members of the Notch gene family encode large transmembrane proteins, and four Notch isoforms (Notch1_4) have been isolated from mammals (Robey 1997). Physiologic activation of Notch signaling is initiated by binding of Notch ligands, such as Jagged1, Jagged2 and Delta, that are also transmembrane proteins (Luo, *et al* 1997). Ligand binding leads to proteolytic cleavage that results in the transport of the intracellular domain of Notch to the nucleus, where Notch behaves as a transcriptional activator (Schroeter, *et al* 1998). A number of studies support a broad physiologic role of Notch in the regulation of hematopoiesis. Notch1 is expressed in bone marrow precursor cells as well as in peripheral blood T and B lymphocytes, monocytes, and neutrophils, whereas Notch ligands are expressed in bone marrow stromal cells, fetal liver, and thymus (Li, *et al* 1998). Notch activation promotes HSC self-renewal, or at least the maintenance of multipotentiality (Karanu, *et al* 2000, Varnum-Finney, *et al* 2000). A recent report has proposed a model in which Notch1 functions at multiple levels in the hematopoietic cascade with distinct outcomes dependent on the differentiation stage at which it is activated. In the primitive populations, Notch1 facilitates self-renewal of hematopoietic stem cells by impeding exit from the stem cell pool and influences lineage outcome on a very primitive level, affecting the relative abundance of lymphoid populations (Stier, *et al* 2002).

1.1.3.4 Sonic Hedgehog (Shh) Pathway

The vertebrate hedgehog family is represented by at least three members: Desert hedgehog (Dhh), Indian hedgehog (Ihh) and Sonic hedgehog (Shh). All encode secreted proteins implicated in cell-cell interactions. Shh is the most extensively characterized vertebrate member of this family and is involved in a wide variety of embryonic events (Johnson and Tabin 1995). Shh and its receptors, Ptc-1 and Smo, are expressed in highly purified populations of primitive human blood cells, whereas, Dhh is found in schwann and sertoli cell precursors and Ihh is expressed in gut and cartilage.

Addition of soluble Shh into HSC cultures results in an increase in the number of cells capable of repopulating function. Conversely, cytokine-induced differentation was inhibited by the addition of antibodies to hedgehog. These results indicate that Hedgehog proteins regulate the expansion of hematopoietic stem cells. It turns out that Hedgehog signaling acts upstream of bone morphogenetic protein-4 (BMP-4) to modulate BMP signaling and induce proliferation without differentiation in primitive blood cells (Bhardwaj*, et al* 2001, Zon 2001). Recently, the BMP signal has been shown to play a role in control of HSC number (Zhang*, et al* 2003).

1.1.3.5 Stage Specific Cell Cycle Regulation in Early Hematopoiesis

Although quiescence is a defining characteristic of hematopoietic stem cells, expansion of HSCs by cell division and exit of a fraction of them occurs under both steady state and conditions of mobilization. Cyclin dependent kinase inhibitors (CDKI) are natural candidates for proteins that oppose the cell cycle progression of HSCs. Just as there are several cyclin dependent kinases, there are several CDK inhibitors (CDKIs) including $p21^{cip\text{-}1/waf\text{-}1}$ (p21), $p27^{kip\text{-}1}$ (p27) and $p18^{INK4a}$.(p18). These CDKIs play distinct roles in the regulation of hematopoietic self-renewal and differentiation. For example, p21 acts to maintain a stem cell in a quiescent state of the cell cycle and limits the cell cycle entry of hematopoietic stem cells, while p27 and p18 more specifically inhibit cycling of committed progenitor cells (Cheng*, et al* 2000, Steinman 2002). On the other hand, p21 is higly expressed in replicating hematopoietic progenitor cells (Furukawa*, et al* 2000, Steinman*, et al* 1998). This bicameral role for p21, in which it restricts primitive cell cycling but promotes expansion of the progenitor cell pool may reflect its ability to serve as an assembly factor for cyclin-dependent complexes without inhibiting their activity (Cheng*, et al* 1999, Cheng*, et al* 2000, LaBaer*, et al* 1997). Moreover, recent studies imply that p21 possesses differentiation stage-specific effect in hematopoiesis (Stier*, et al* 2003).

1.1.3.6 Chromatin Modeling in Early Hematopoiesis

Local modeling of chromatin and dynamic changes in the nucleosomal packaging of DNA, key steps in the transcriptional activation of genes, must occur to allow transcriptional proteins contact with the DNA template and consequently affect proper cell function, differentiation and proliferation. Chromatin structure is an important factor in determining whether a particular gene is expressed or not. All of the human genome is packaged into chromatin, which is a dynamic macromolecular complex that consists of DNA, histones and non-histone proteins (Wu and Grunstein 2000). The nucleosomes are the fundamental units of chromatin structure, provide the first order and, at least in part, the higher-order packaging and compaction of the DNA. The nucleosome core particle consists of 146 bp of DNA wrapped around two molecules of each of the histones H2A, H2B, H3 and H4 (Luger and Richmond 1998) (Fig.3).

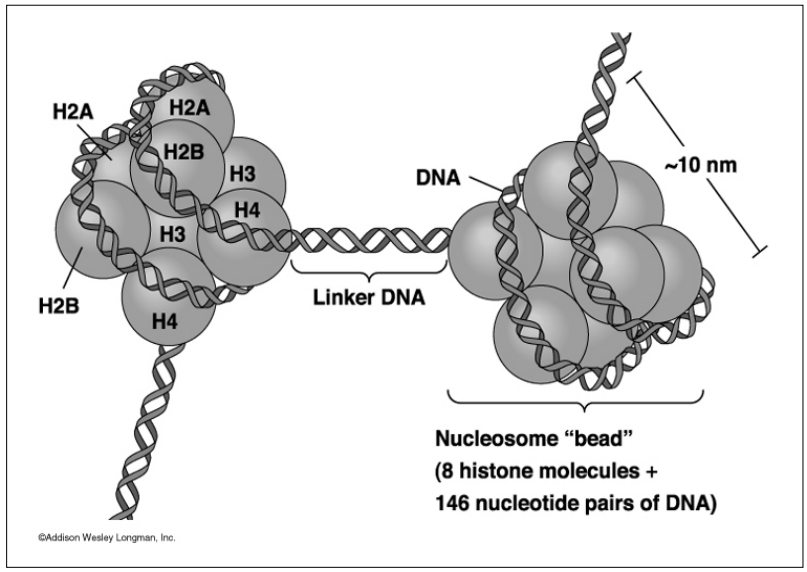

Fig.3. Nucleosome. Each nucleosome consists of eight histone molecules (two each of histones H2A, H2B, H3, and H4) associated with 146 nucleotide pairs of DNA and a stretch of linker DNA about 50 nucleotide pairs in length. The diameter of the nucleosome "bead," or core particle, is about 10 nm. Histone H1 (not shown) is thought to bind to the linker DNA and facilitate the packing of nucleosomes into 30-nm fibers. © 1999 by Addison Wesley Longman.

The linker histone H1 stabilizies the higher order folding by electrostatic neutralization of the linker DNA segment. In addition to their structural role, the histone proteins are also involved in regulation of gene expression. As the maintenance of health depends on the coordinated and tightly regulated expression of genetic information, this is a very important function of histones. Post-translational modifications of histone tails, such as acetylation, phosphorylation and methylation have emerged as common denominators in regulating several biological functions (Berger 2001a, Berger 2001b, Iizuka and Smith 2003).

Posttranslational modification of histones by acetylation is one of the best understood mechanism by which cells regulate chromatin structure (Davie 1998, Kouzarides 1999). Lysine residues in the N-terminal extensions of H2B, H3, and H4 that exposed on the exterior surface of nucleosomes are particularly accessible to acetylation. Acetylation of histones by histone acetyl transferase (HAT) activity disrupts nucleosomes by confering negative charge to the histone surface leading to breakage of the electrostatic attraction between histones and DNA and allows the DNA to become accessible to the transcriptional machinery; conversely, removal of the acetyl groups in other words, deacetylation by specific histone deacetylase (HDAC) enzymes allows the histones to bind more tightly to DNA and to maintain a transcriptionally repressed chromatin architecture. Both, HAT and HDAC activities can be recruited to target genes in complexes with sequence specific transcription factors and their coactivators or corepressors (Pazin and Kadonaga 1997, Redner*, et al* 1999).

In addition, HDAC activity is also an important component of epigenetic silencing by DNA methylation (Robertson 2001). DNA can be methylated at the C_5 position of the cytosine within CpG dinucleotides, by the DNA methyl transferases DNMT1, DNMT3A, DNMT3B These enzymes directly recruit HDACs, resulting in histone deacetylation and transcriptional repression. Moreover, methylated CpG islands are bound by methyl –CpG-binding proteins such as MeCP2, which can recruit HDACs and ATP-dependent chromatin modeling proteins to form a tightly condensed chromatin structure (Flaus and Owen-Hughes 2001, Johnstone 2002).

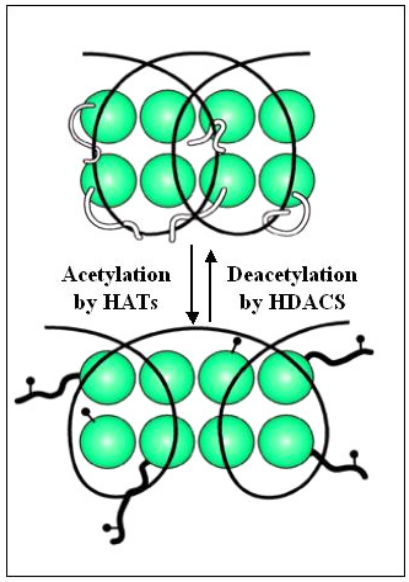

Fig.4. Cartoon Structure of Acetylation/Deacetylation of Histones (modified from Redner*, et al* 1999). The random-coiled tails of the histone octamer represents the nucleosome with histones acetylated (acetyl groups drawn as lollipop structures). The acetylated histone tails do not bind the DNA strands. This allows the DNA to assume a more open configuration that is accessible to the transcriptional machinery.

In addition, HDAC activity is also an important component of epigenetic silencing by DNA methylation (Robertson 2001). DNA can be methylated at the C_5 position of the cytosine within CpG dinucleotides, by the DNA methyl transferases DNMT1, DNMT3A, DNMT3B These enzymes directly recruit HDACs, resulting in histone deacetylation and transcriptional repression. Moreover, methylated CpG islands are bound by methyl –CpG-binding proteins such as MeCP2, which can recruit HDACs and ATP-dependent chromatin modeling proteins to form a tightly condensed chromatin structure (Flaus and Owen-Hughes 2001, Johnstone 2002).

Recently, it has been hypothesized that HSCs maintain a wide-open chromatin structure and express a promiscous set of transcription factors to maintain their multipotentiality which is progressively quenched as these cells progress down a particular pathway of differentation. According to gene expression profiles from murine HSCs, both nonhematopoietic and hematopoietic lineage-affiliated genes expressed at low level in HSCs and transcriptional accessibility for lineage-affiliated program is progressively decreased, whereas genes related to mature fuctions and structures become expressed as HSCs undergo differentiation (Akashi*, et al* 2003).

These results suggests the concept that mechanism that govern HSC fate decision are likely under the control of chromatin modeling programs that guide at least transcriptional accesssibility for each hematopoietic stage (Akashi*, et al* 2003). Recently, it has been demonstrated that the combination of chromatin modeling agents such as trichostatin (TSA) with 5aza 2'deoxycytidine (5azaD) which lead to histone acetylation and DNA demethylation, respectively, resulted in expansion of HSCs and repopulation of immunodeficient mice (Milhem*, et al* 2004). In addition, valproic acid (VPA; a potent HDAC inhibitor) has been reported to enhance the cytokine-induced expansion of HSCs at least *in vitro* (De Felice*, et al* 2005).

In line with the important function of chromatin modeling in normal hematopoiesis according to the recent studies, dysregulation of this dynamic process gives rise to transcriptional chaos and leukemic transformation. Dysregulation of chromatin modeling in acute myeloid leukemias (AMLs) and novel therapeutic approaches through differentiation-inducing agent like all-trans retinoic acid (t-RA), DNA methylation inhibitors and HDAC inhibitors will be further disscussed in the section about leukemia.

1.2 Leukemia

Leukemias are a heterogeneous group of malignancies of hematopoietic cells. They are monoclonal, meaning that leukemic phenotype arises as a consequence of malignant transformation of a single cell. According to the progress of disease, they are classified as chronic or acute; and with regard to the cell type affected as myeloid or lymphoid leukemias. Although leukemias are heterogeneous in terms of phenotypes, there are general mechanisms underlying leukemic transformation such as increased cell survival, proliferation capacity, self-renewal capacity and impaired differentiation.

1.2.1 Chronic Leukemias

1.2.1.1 Chronic Myeloid Leukemia (CML)

Chronic myelogenous leukaemia (CML) results from the neoplastic transformation of a haematopoietic stem cell. The initial chronic phase of this biphasic disease is characterized by a massive expansion of the granulocytic cell lineage, even though most, if not all, haematopoietic lineages can be produced from the CML stem cell. The median duration of the chronic phase is 3–4 years. Acquisition of additional genetic and/or epigenetic abnormalities causes the progression of CML from chronic phase to blast phase. This phase is characterized by a block of cell differentiation that results in the presence of 30% or more myeloid or lymphoid blast cells in peripheral blood or bone marrow, or the presence of extramedullary (spleen, liver or lymph nodes) infiltrates of blast cells (Ren 2005).

The cytogenetic hallmark of all phases of CML and a subset of cases of acute lymphoblastic leukemia (ALL) is a t(9;22)(q34;q11) translocation, which was first discovered as an abnormal, small chromosome, named the ‘Philadelphia chromosome’(Kurzrock*, et al* 1988). This translocation generates a *bcr–abl* fusion gene. Depending on the site of the breakpoint in *bcr*, various different fusion proteins are produced: p185 (185 kDa), p210 (210 kDa), or rarely p230. The p210 protein is seen in 95% of patients with CML and up to 20% of adult patients with de novo ALL; the p185 form is seen in approximately 10% of patients with ALL and in the majority of pediatric patients with Ph+ ALL (5% of all pediatric ALL cases). The

BCR–ABL fusion protein is required for the pathogenesis of CML, and the tyrosine-kinase activity of ABL is essential for BCR–ABL mediated transformation (Deininger*, et al* 2000).

Allogeneic stem-cell transplantation is the only known curative therapy for CML. However, most patients are not eligible for this therapy, because of advanced age or lack of a suitable stem-cell donor. Imatinib mesylate (Glivec, previously known as STI571 and CGP 57148) , is a rationally designed ABL specific tyrosine kinase inhibitor, has been shown to selectively induce apoptosis of BCR–ABL+ cells, and is remarkably successful in treating patients with CML except individuals with advanced phases (Druker 2002).

1.2.1.2 Chronic Lymphoblastic Leukemia (CLL)

Chronic lymphocytic leukemia (CLL) is a predominantly malignant clonal disorder of B lymphocytes. Apoptosis dysregulation is a major feature of CLL, and while no clear pattern has emerged, abnormal levels of Bcl-2 are common in CLL and Bcl-2 to Bax ratios are also commonly disturbed (Keating 1999). Clinical studies have delineated factors that are helpful in predicting prognosis and have provided data on promising new therapies for patients with this disease, including stem cell transplantation, monoclonal antibodies, and gene therapy (Wierda and Kipps 1999).

1.2.2 Acute Leukemias

Acute leukemias are characterized by the uncontrolled growth of the immature blood cell precursors. When untreated, patients have a life span of several weeks or months, because their normal blood cells become outnumbered by the leukemic ones (Hoelzer and Seipelt 1998).

It is important to distinguish between acute lymphoid (ALL) and acute myeloid leukemia (AML) because of the different therapeutic approaches applicable to these two malignancies.

They can be distinguished based on a variety of morphologic, cytochemical and biochemical features (Begemann et al., 1998).

1.2.2.1 Acute Lymphoblastic Leukemia (ALL)

ALL has its origin in a malignant transformation of a lymphoid precursor of either bone marrow, thymus or other lymphoid tissue. ALL is an aggressive disease with a poor prognosis which is further exacerbated by the presence of the Philadelphia chromosome (Ph+) — the cytogenetic substrate of the t(9;22) — or by the t(4;11) (Hoelzer, *et al* 2002)

With the currently applied chemotherapy regimens survival ranges between 0-10%, even though initial complete remission rates of 80% are comparable to those achieved in Ph negative patients. Allogeneic stem cell transplantation is the only treatment with curative potential, but treatment-related mortality and the relapse rate both remain high (Hoelzer and Gokbuget 2000).

1.2.2.2 Acute Myeloid Leukemia (AML)

Acute myeloid leukemia (AML) is a clonal malignancy characterized by a block in myeloid differentiation (Fialkow, *et al* 1987, McCulloch 1993). In the bone marrow and peripheral blood, AML leads to accumulation of proliferating hematopoietic precursors, which are not able to terminally differentiate and become functional blood cells. In this way space needed for normal hematopoietic processes is occupied by leukemic blasts, in a process which will finally be clinically manifested as leukemia (Hoelzer and Seipelt, 1998).

It is possible to distinguish between de novo, primary AML, and secondary AML which arises as a consequence of either myelodysplastic syndrome (MDS), myeloproliferative disease (MPD), treatment of neoplasia with cytotoxic substances such as alkylating agents (i.e Cyclophosphamid), Topoisomerease II blocking agents (such as Etoposide, for the treatment of ALL) or exposure to ionizing radiation (Pui, *et al* 1989, Scandura, *et al* 2002). There are several other factors which can provoke the onset of secondary leukemias, and among them

are exposure to benzene and different genetic abnormalities among them trisomy 21 and Fanconi's anemia etc (Ferti*, et al* 1996).

In 1976, a group of hematologists from France, America and England founded a morphologically and cytochemically based classification of myeloid and lymphoid leukemias, the FAB classification (Bennett*, et al* 1976). According to this classification, myeloid leukemias are divided according to the dominating cell type and its level of differentiation.

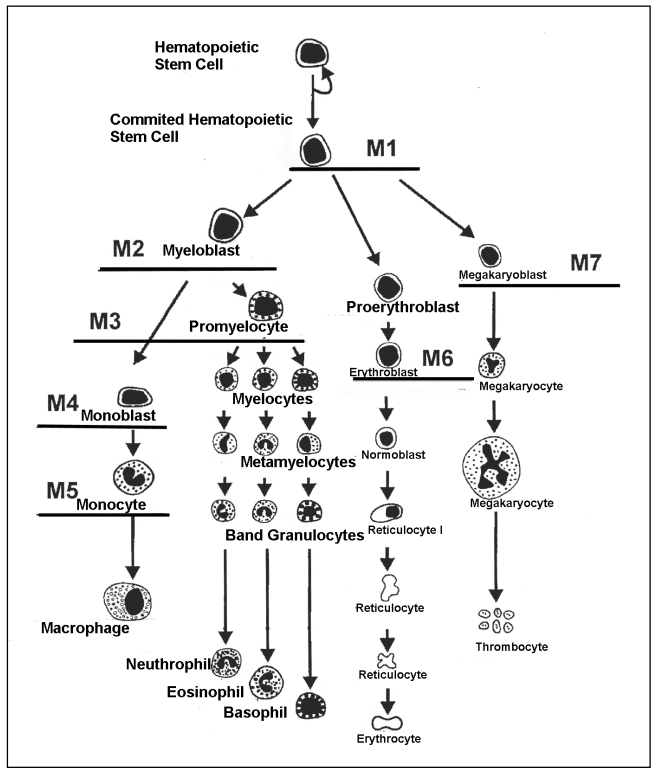

Figure 5: Morphology and differentiation level in AML

M1: Acute myeloblastic without maturation

M2: Acute myeloblastic with maturation

M3: Acute promyelocytic leukemia (APL)

M4: Acute myelomonocytic leukemia

M5: Acute monoblastic leukemia

M6: Acute erythroleukemia

M7: Acute megakaryoblastic leukemia

In addition to the FAB classification, specific chromosomal rearrangements/translocations have been linked to distinct subtypes of acute myeloid leukemia or associated with particular stages of disease progression or prognostic outcomes (Harris*, et al* 1999).

1.3 Molecular Pathogenesis of AML

1.3.1 Structural Themes

More than half of newly diagnosed cases of AML display detectable and usually single cytogenetic abnormalities (Mrozek*, et al* 2001). The vast majority AML-associated genetic aberrations are typified by balanced chromosomal translocations. Among the most common gene rearrangements in AML are those involving the transcription factor complex Core Binding Factor (CBF) and the nuclear hormone receptor retinoic acid receptor alpha (RAR_) which account ~25% and ~10% of AML, respectively (He*, et al* 1999, Scandura*, et al* 2002). Though less common then the CBF and RARα fusions, several other genes are recurrently involved in leukemia associated translocations such as the MLL (Mixed Lineage Leukemia gene) gene fusion which, though common in infant leukemias, occurs in less than 3% of adult AMLs (Grimwade*, et al* 2001).

These structural rearrangements frequently involve the in-frame fusion of transcription factors or regulators whose altered functions can interfere with regulatory cascades that have been demonstrated to be critical for controlling the growth, differentiation and survival of normal blood cell precursors (Look, 1997). This supports the widely held view that AML occurs principally through transcriptional dysregulation. The abnormal regulation of transcriptional networks occurs through common mechanisms that include alterations of specific subnuclear compartments, dysregulation of chromatin modeling and recruitment of aberrant co-repressor complexes (Alcalay*, et al* 2001, Minucci and Pelicci 1999).

1.3.1.1 CBF Leukemias (AML1 and CBFβ)

CBF is a heterodimeric protein consisting of an AML1 subunit that directly contacts DNA (_ subunit), and a CBFβ subunit, which facilitates DNA binding. The normal functions of both subunits are critical to hematopoietic development as mice lacking either gene fail to develop definitive hematopoiesis (Okuda*, et al* 1996).

The heterodimeric complex activates transcription of target genes involving in myeloid differentation (i.e. IL-3, GM-CSF, M-CSF etc.) (Hiebert*, et al* 1996, Meyers*, et al* 1995). Both AML1 and CBFβ genes are targets of chromosomal translocations in leukemia.

In one of the common translocation in AML, the N-terminal part of AML1 is fused to C-terminal portion of ETO (eight twenty one) as a consequence of the t(8;21) translocation found predominantly in the AML FAB subtype M2 (Miyoshi*, et al* 1991). ETO is a member of a family of nuclear proteins which appears to be involved in the regulation of transcription (Scandura*, et al* 2002). It interacts through coiled-coil regions (Minucci 2000) with N-CoR (nuclear receptor corepressor) and recruits HDACs. (Gelmetti*, et al* 1998, Wang*, et al* 1998a).

Although the AML1/ETO fusion protein retains the ability to bind AML1-regulated target sequences, it does not activate transcription, but instead dominantly represses AML1-mediated activation (Lowenberg*, et al* 1999, Meyers*, et al* 1995). Transcriptional repression appears to be mediated through the direct interaction of ETO with the nuclear corepressor and recruitment of HDACs (Wang*, et al* 1998a) (Fig.6).

Moreover, AML1 has been found to be the target of the t(12;21) translocation in pediatric ALL (Lowenberg*, et al* 1999). Here, almost the entire AML1 protein fused to the N-terminal part of TEL, which acts as a transcription repressor (Chakrabarti and Nucifora 1999). TEL/AML1 binds DNA via the runt homology domain (RHD) of AML1, redirecting the repressor functions of TEL to AML1 targets. Similar to ETO, TEL directly binds corepressor molecules and HDACs with its dimerization region and therefore represses AML1 target genes (Fenrick*, et al* 1999, Romana*, et al* 1995).

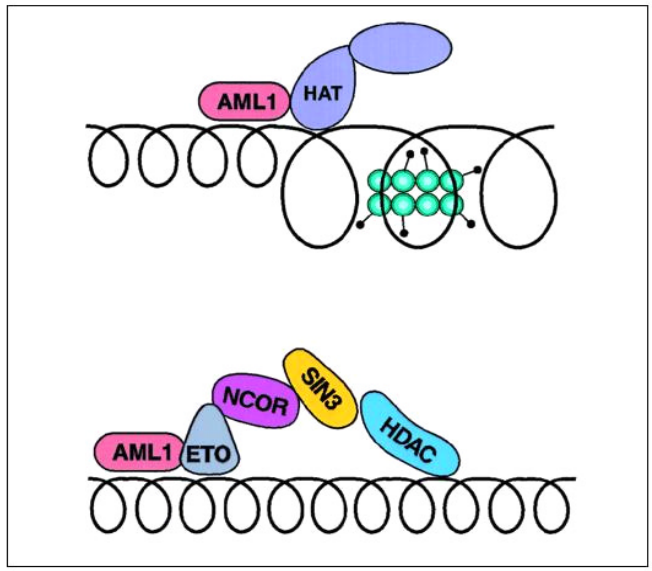

Fig 6. AML1-ETO fusion protein (Redner, *et al* 1999). The association of the AML1 transcriptional activator with a coactivator complex; indicates the binding of a corepressor to the AML-ETO fusion protein. NCOR: nuclear receptor corepressor, HDAC: histone deacetylase, HAT: histone acetyl transferase, SIN3: Mammalian SIN3 repressor protein.

The inv(16) and the t(16;16) mutations are mainly (but not only) seen in patients with FAB subtype M4Eo AML. In these chromosomal rearrangements, the CBFß subunit of the core binding-factor complex fuses to the smooth-muscle myosin heavy-chain (SMMHC) gene MYH11 which is not known to play a role in normal hematopoiesis (Liu, *et al* 1993). In the resulting in CBFß -MYH11 chimeric product, the N-terminal portion of CBFß, including its AML1-interaction domain, is fused in-frame to a variable amount of the C-terminal domain of MYH11 resulting in repression of AML1-mediated transcriptional activation either through cryptic repressor domain of SMMHC portion of the fusion protein or by sequestering AML1 into functionally inactive complexes within the cytoplasm (Kanno, *et al* 1998).

1.3.1.2 APL

Nearly all cases of APL are associated with the t(15,17) translocation which results in a fusion of RAR_ to the PML (promyelocytic leukemia) nuclear protein that localizes in distinct matrix-associated structures known as nuclear bodies or PML oncogenic domains (PODs). PML regulates senescence and apoptosis and functions as a growth suppressor (Dyck*, et al* 1994, Pandolfi 2001, Zhong*, et al* 2000).

The retinoic acid receptor (RAR) is a ligand dependent transcriptional regulator that is important for myeloid differentiation. It forms heterodimers with retinoic X receptors (RXR) which in the absence of the retinoic acid (RA) ligand interact with a large ubiquitous nuclear protein (N-CoR). N-CoR mediates transcriptional repression through its interaction with other proteins including mSin3A and HDAC (Heinzel*, et al* 1997, Horlein*, et al* 1995). The addition of the retinoic acid ligand normally converts RAR-RXR from a transcriptional repressor to a transcriptional activator (Collins 1998).

Fusion of PML to RAR_ results in enhanced interaction with the co-repressor complex N-CoR, mSin3A and HDACs through PML coiled-coil regions by oligomerization (Minucci*, et al* 2000). Only at pharmacological levels of ligand does PML/RARα release N-CoR, recruit a coactivator complex, and allow histone acetylation and chromatin modeling to proceed. Thus, for PML/RARα-expressing APL cells, pharmacological levels of RA are needed for differentiation-inducing therapy (Grignani*, et al* 1998, Lin*, et al* 1998). However, rare variants of APL expressing the t(11;17) PLZF/RARα fusion are not sensitive to RA–induced differentation. This result has been explained by demonstrating that PLZF itself is a transcriptional repressor that provides the fusion protein with a second binding domain for N-CoR, mSin3A, HDAC co-repressor complex (Grignani*, et al* 1998, Ruthardt*, et al* 1997).

In addition to aberrant recruitment of HDAC complexes, it has been recently reported that PML/RARα fusion proteins can induce gene hypermethylation and silencing by aberrant recruitment of DNA methyltranferases (DNMT1 and DNMT3a) to target promoters (i.e. RARß2) in APL blasts, NB4 or U937 cells expressing PML/ RARα. (Di Croce*, et al* 2002, Robertson 2001).

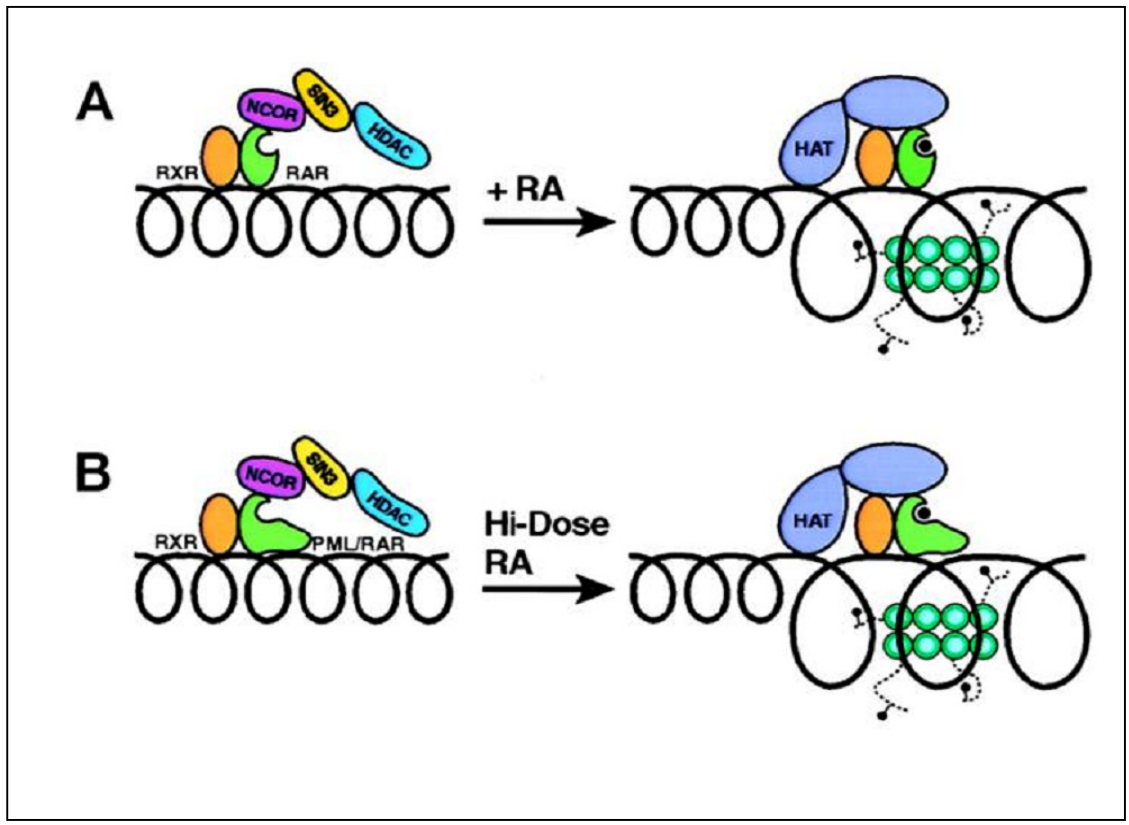

Fig 7. PML/RAR_ Fusion Protein in Acute Promyelocytic Leukemia (modified from Redner*, et al* 1999). (A) The interactions of the RXR/RAR_ heterodimer with an N-CoR/Sin3/HDAC1 complex. Upon binding retinoid acid, the RXR/RAR_ heterodimer releases the corepressor complex and binds a coactivator complex with histone acetylase activity. (B) The analogous interactions of the RXR/PML-RAR_ heterodimer with the corepressor complex. Release of the corepressor complex occurs only in the presence of pharmacological levels of retinoic acid. RXR: retinoic X receptors, RAR_: retinoic acid receptor alpha, N-CoR: nuclear receptor corepressor, HDAC: histone deacetylase, HAT: histone acetyl transferase, SIN3: Mammalian SIN3 repressor protein.

1.3.2 Functional Themes

1.3.2.1 Effect on Differentiation

A hallmark of AML is the aberrant differentiation with the accumulation of blasts resembling a specific level of granulocytic maturation. Concerning the vast collection of AML-associated fusion proteins, one of the two components of each fusion protein that directly interfere with the hematopoietic differentiation program is generally a transcription factor (AML1, CBFß, and RARα). On the other hand, the other partner is more variable in function, but is often involved in the control of cell survival and apoptosis such as the nuclear structure protein PML. Moreover, AML-associated fusion proteins have been shown to affect hematopoietic differentiation in a variety of experimental models, and the specific stage of myeloid maturation arrest appears to be directly dependent on the nature of the fusion protein (Meyers, *et al* 1995, Passegue, *et al* 2003, Scandura, *et al* 2002).

1.3.2.2 Effect on Apoptosis

The Bcl-2 family members, such as Bcl-2, Bcl-xl, Mcl-1, and A1, function as cell death antagonists against a wide array of apoptotic stimuli, whereas their binding partners, like Bax, Bad, and Bak, promote apoptosis. The fusion protein AML1/ETO was shown to directly up-regulate expression of Bcl-2 by binding to its promoter elements. Activation of the Bcl-2 promoter is the first demonstration of transcriptional activation by AML1/ETO, in contrast to its previously identified repressor activity.

In fact, cells from most human AML have been found to express Bcl-2 at much higher levels than their normal counterparts (Passegue, *et al* 2003). A consistent story has emerged from studies of the APL-associated fusion partners in transgenic mice (Pandolfi 2001). Within nuclear bodies, PML colocalizes and physically interacts with P53 and Daxx, both mediators of apoptotic stimuli. PML acts as a transcriptional coactivator of P53 and as such can participate in the activation of P53 dependent apoptosis (Guo, *et al* 2000). PML/RARα disrupts nuclear body structure and is a dominant negative inhibitor of PML function, thus its not surprising that hematopoietic progenitors from PML/RARα transgenic mice are resistant to both P53-dependent apoptotic stimuli (Wang, *et al* 1998b). Similarly, Fas mediated

apoptosis is abrogated because the interaction between PML and Daxx is also disrupted (Zhong*, et al* 2000).

1.3.2.3 Effect on Self-renewal

Acute myelogenous leukemia (AML) is typically a disease of stem/progenitor cell origin. Interestingly, the leukemic stem cell (LSC) shares many characteristics with normal hematopoietic stem cells (HSCs) including the ability to self-renew and a predominantly G_0 cell-cycle status (Bonnet and Dick 1997, Dick 2005, Guzman*, et al* 2002). Leukemic cells absolutely require self-renewal capability to propagate the disease (Passegue*, et al* 2003). Wnt signaling is an evolutionarily conserved signal transduction pathway that governs cell fate decisions during embryogenesis and on the stem cell level. Wnt signaling has been implicated in the self-renewal and proliferation of hematopoietic progenitor cells (Austin*, et al* 1997, Reya 2003, Reya*, et al* 2003) .

Recently, the Wnt signaling pathway has been shown to be involved in human leukemia development. _-catenin (plakoglobin) is closely related to β-catenin and is able to transform cells, whereas β-catenin exhibits a transforming capacity only when it is constitutively stabilized by mutation. It has been demonstrated that the AML associated translocation products (AATPs) PML/RARα, AML1/ETO, PLZF/RARα activate Wnt signaling by upregulating _ -catenin, which contributes to leukemogenesis by increasing the self-renewal of early hematopoietic stem cells (Muller-Tidow*, et al* 2004, Zheng*, et al* 2004).

1.4 Novel Therapeutic Approaches in AML

Progress in acute myeloid leukemia conventional therapy and supportive care over the past three decades has led to gradual improvement in the overall results, especially in adults up to age 55-60. However, treatment outcome in elderly patients, which represent the vast majority of AML, has remained disappointing despite intensive induction chemotherapy with complete remission rates from 30% to 50%.

In elderly patients, disease-free survival (DFS) typically is less than 10% at 3 years, with an overall median survival of less than 12 month (Copplestone, *et al* 1989, Rowe, *et al* 2004). In AML, different genetic alterations result in common patterns of deregulated gene expression, leading to blockage of differentiation and favoring myeloid leukemogenesis. The limitations and toxicity of conventional therapy aimed at killing the proliferating leukemic cells and the underlying biology of AML have prompted the development of novel therapeutic concepts based on the use of differentiation-inducing agents.

1.4.1 All-trans Retinoid acid (t-RA)

All-trans Retinoid acid (tretinoin: t-RA) is one of the naturally occurring vitamin A derivatives (Miller 1998). t-RA is a very potent promoter of growth and controller of differentiation in many organ systems (Lotan, *et al* 1980), including hematopoiesis, by inducing differentiation of promyelocytes into mature granulocytes in normal hemotopoiesis (Douer and Koeffler 1982) as well as in acute premyelocytic leukemia expressing *PML*/RARα fusion proteins (Huang, *et al* 1988). Therapy of APL with t-RA is well established. Combined data published from Chinese, French, American and Japanese sources indicate a mean complete remission response of 85% (Warrell, *et al* 1998). When this remission is consolidated with chemotherapy, it leads to the long-term survival of APL patients in more than 75% (Soignet, *et al* 1997). Although all other subtypes of AMLs express RARs, they are not sensitive to retinoic acid action on differentiation (Fenaux and Degos 1997).

1.4.2 Demethylating Agents (DNA Methylation Inhibitors)

5-azacytidine (5-Aza-C), and its deoxy version 5-aza-2'-deoxycytidine (5-Aza-dC) are nucleoside analogues that inhibit DNA methyltransferases (DNMTs), which are involved in methylating DNA, and lead to activation of silent genes, decondensation of chromatin, differentiation in vitro (Maio, *et al* 2003) . PML-RARα-induced repression of RARß2 (RA receptor) which is considered to be a putative tumor suppressor was only partially released by either the DNA methylamine inhibitor 5-Aza-dC or the HDAC inhibitor trichostatin (TSA) but was completely released by simultaneous treatment. These data revealed that PML-

RARα-induced transcriptional repression and differentiation blockage was due to the temporally recruitment of both histone deacetylases and DNA methyltranferases (Di Croce*, et al* 2002). A synergistic effect of HDAC inhibitors and 5-Aza-C in restoring gene expression has been observed both in hematological and non-hematological malignancies (Cameron*, et al* 1999, Klisovic*, et al* 2003).

1.4.3 HDAC Inhibitors (HDIs)

Various classes of compounds (hydroxamic acids, short-chain fatty acids, cyclic tetrapeptides, cyclic depsipeptides and phenylene diamines) are known to be potent inhibitors of HDAC activity (Johnstone, 2002). HDAC inhibitors prevent deacetylation resulting in a net increase in acetylation of core histones, a release of chromatin folding thereby facilitating access of transcription factors to DNA and activation of gene expression (Tse*, et al* 1998). As single agents HDIs can induce a diverse array of leukemias to express differentiation characteristics and stop proliferating. Inhibition of the cell cycle is a necessary event in cellular differentiation, and the cytostatic effects of HDIs are important for their anti-cancer activities. Analysis of the cell cycle of tumour cells as well as in cell line models of acute leukemia that have been treated with HDIs indicate that almost all HDAC inhibitors activate CKDI p21 and the cells often arrest in G1, but sometimes accumulate in what seems to be the G2 phase of the cycle (Gurvich*, et al* 2004, Kramer*, et al* 2001, Marks*, et al* 2000, Romanski*, et al* 2004).

Butyrates, short-chain fatty acid-based (SCFA) substances, were the first HDIs to be identified (Candido, 1978 cell) and successfully employed in experimental cancer therapy (Gore and Carducci 2000). Unfortunately, butyrates require high concentrations in order to achieve inhibitory effects and are highly unspecific. Other compounds such as the hydroxamic acid based HDI TSA, have more powerful and have higher inhibitor specifity (Grunstein 1997, Marks*, et al* 2000).

APL has become the paradigm for the application of HDAC inhibitors. It has been shown that either TSA or phenyl butyrate in combination with t-RA induce differentiation in some PML-RARα as well as PLZF-RARα- expressing APL cells that do not respond to RA alone. (Lin*, et al* 1998, Warrell*, et al* 1998). Aside from APL, HDAC inhibitors (TSA or phenylbutyrate)

were reported to have a potential role in the treatment of *AML1-ETO* AML (Wang*, et al* 1999). Recently, Ferrara et al have shown that in AMLs, regardless of their underlying genetic alteration, the retinoic acid signaling pathway is constitutively repressed through a histone deacetylase (HDAC)-dependent mechanism and that inhibition of HDAC activities restores the retinoic acid-differentiation response. These results indicate that repression of the retinoic acid signaling pathway is a general pathogenetic event in AMLs and that HDACs are common targets for AMLs, highlighting the possibility of transcriptional/differentiation therapy also in AMLs other than APL (Ferrara*, et al* 2001).

1.4.3.1 Valproic Acid (VPA)

VPA (2-propylpentanoic acid) is a short-chain branched fatty acid with favorable pharmacokinetic properties that has been used for decades in the treatment of epilepsy and bipolar disorder (Johannessen 2000, Tunnicliff 1999). Recent data suggest that this drug, in addition to its other known classical actions, can modulate the epigenome by inhibiting histone deacetylases (HDACs) thus triggering an increase in gene expression. This inhibition is most likely due to binding of VPA to the catalytic center of HDACs and thereby preventing access to the physiological substrate, e.g. acetylated lysine residues in histone proteins (Gottlicher*, et al* 2001, Gurvich*, et al* 2004).

It has been shown that VPA and its analogs inhibit multiple but not all HDACs with a characteristic order of potency *in vitro* (Gurvich*, et al* 2004). Furthermore, VPA is the first therapeutic agent to erase DNA methylation patterns in a replication-independent manner by stimulating the accessibility of a demethylase enzyme (Detich*, et al* 2003).

Similar to more widely studied HDIs, VPA is under clinical evaluation due to its potent differentiation effect on carcinoma cells, transformed hematopoietic progenitor cells and leukemic blasts from AML patients (Gottlicher*, et al* 2001, Kuendgen*, et al* 2004). In a previous clinical study (Bug et al., 2005b), the favorable effects of the combination treatment with VPA/t-RA in patients with advanced acute myeloid leukemia were reported to result in blast cell reduction and hypergranulocytosis (>10^5 cells/L; ref. Bug *et al*. 2005b).

Furthermore, it was possible to distinguish malignant from normal hematopoiesis in one patient, by the presence of the isochromosome (17)(q10) in the leukemic blasts.

The analysis revealed that whereas the $CD34^+$ progenitor cells contained residual isochromosome (17)(q10), all granulocytes had a normal karyotype, suggesting dominance of normal hematopoiesis over the malignant clone most likely due to an enhancement of nonleukemic myelopoiesis and the suppression of malignant hematopoiesis rather than enforced differentiation of the leukemic blast. Recently, VPA has been reported to enhance cytokine effect on the maintenance and expansion of HSCs at least *in vitro* consistent with the hypothesis that VPA influences normal hematopoiesis (De Felice*, et al* 2005).

1.4.3.2 LAQ-824

The clinical candidate LAQ-824 is a hydroxamate-based HDAC inhibitor. *In vitro*, LAQ-824 inhibits HDAC enzyme activity with an IC 10 nM, inhibits and tumor cell growth at submicromolar concentrations. Moreover, it induces apoptosis in several types of leukemia including acute myeloid leukemia, acute lymphoblastic leukemia and chronic myeloid leukemia cells. In addition, LAQ824 has significant activity *in vivo*, as demonstrated by its ability to prolong the survival of mice injected with a myeloid leukemia cell line (Romanski*, et al* 2004, Weisberg*, et al* 2004).

1.5 Purpose of the Study

Acute myeloid leukemia (AML) is a clonal malignancy characterized by a block in myeloid differentiation. The rapid expansion of immature blasts in the bone marrow and the peripheral blood progressively replaces the normal hematopoiesis, leading to evident hematopoietic insufficiency. The vast majority of AML-patients are over 60 years old and experience a median survival below 1 year even if treated with intensive chemotherapy (Rowe, *et al* 2004, (Copplestone*, et al* 1989). It has been shown that the induction of the leukemic phenotype in acute myeloid leukemia is strictly related to aberrant chromatin modeling, a major mechanism of transcriptional control (Grignani*, et al* 1998, Minucci and Pelicci 1999) Therefore, the profound toxicity, limited efficacy of current chemotherapy strategies and biology of AML has prompted the development of alternative treatment strategies, such as a differentiation-

inducing therapy through histone deacetylase inhibitors (HDIs). HDIs have been shown to promote differentiation regardless of the primary genetic lesion due to transcription regulation by modification of chromatin structure either alone or in combination with differentiating agents such as all-*trans* retinoic acid (Ferrara*, et al* 2001, Gottlicher*, et al* 2001).

The potent HDI valproic acid (VPA), safely used for over two decades in the therapy of epilepsy and bipolar disorders (Johannessen 2000, Tunnicliff 1999) is now under clinical evaluation due to its potent differentiation effect on carcinoma cells, transformed hematopoietic progenitor cells and leukemic blasts from AML patients (Gottlicher*, et al* 2001, Kuendgen*, et al* 2004). On the other hand, in a previous clinical study (Bug et al., 2005b), favorable effects of the combination treatment with t-RA/VPA in patients with advanced acute myeloid leukemia were reported most likely due to an enhancement of nonleukemic myelopoiesis and the suppression of malignant hematopoiesis rather than enforced differentiation of the leukemic blast. (Bug *et al*. 2005b).

Moreover, It has been recently hypothesized that an open chromatin structure is required for HSCs multipotentiality and the lineage potential is hierarchically controlled during early hematopoiesis most likely through the control of chromatin modeling program (Akashi*, et al* 2003). Consistent with this hypothesis, a combination of DNA demethylating agent and trichostatin A (TSA) resulted in a significant expansion of primitive HSCs capable of repopulating immunodeficient mice (De Felice*, et al* 2005, Milhem*, et al* 2004). Moreover, VPA has been demonstrated to enhance early acting cytokine effect on the maintenance and expansion of primitive human HSCs at least in vitro.

The aim of the present study was to investigate the effect of chromatin modeling through VPA on HSC fate in order to evaluate the potential for clinical usage of HDIs in acute myeloid leukemia (AML) therapy.

2 Materials

2.1 Instruments

Bacteria culture

Shaker inkubator (*New Brunswick Scientific*, Edison NJ, USA)

Petri, culture and cryotubes (*Greiner*, Heidelberg)

Centrifuges

Centrifuge 5415D (*Eppendorf*, Hamburg) and EBA12 (*Hettich*, Tuttlingen)

Centrifuge J2-MC with rotor JA 20 and JA 10 (*Beckman Instruments*, Munich)

Centrifuge Rotina 48 RS with rotor 4394 (*Hettich*, Tuttlingen)

Cool centrifuge Z320K (*Hermle*, Gosheim)

Megafuge 1.0 with rotor BS4402/A (*Heraeus*, Hanau)

Sorvall RC-5B refrigerated superspeed centrifuge and Biofuge 13R (*Heraeus*, Hanau)

Table centrifuge 5415C (*Eppendorf Gerätebau*, Hamburg)

Chemiluminescence Detection

Filme X-omat AR und BioMax (*Eastman-Kodak*, Rochester, USA)

FACS (Fluorescence-activated cell sorting)

FACScan (*Beckton Dickinson*, Heidelberg)

Gel electrophoresis system

DNA-subcell and Mini-Subcell System (*Gibco,* Betheseida, USA)

Geldryer modell 583 (*BioRad*, Munich)

Mini-PROTEN®II system and Power PAC 2000 (*BioRad,* Munich)

Irradiation of mice

Betatron 500A (*Siemens*, Munich)

Magnetic cell separation (MACS)

„MiniMACS separation unit“, „MidiMACS separation unit“ and „MACS multistand“(*Miltenyni Biotech,* Bergisch Gladbach)

Microscope

SZ40 Binocular (*Olympus,* Munich)

Inversed microscope (*Zeiss,* Oberkochen)

PCR

PCR-instrument and Thermal Cycler (*Perkin Elmer Cetus*, Norwalk, CT, USA)

Photos from CFU-S

Digital camera DSC- P72 *(Sony*, NJ, USA)

SZ40 Binocular (*Olympus*, Munich)

Spectrohotometry

DNA-Photometer GeneQuant (*Pharmacia*, Uppsala, Sweden)

Gene Quant II and Ultraspec® 2000 (*Pharmacia Biotech*, Freiburg)

Protein transfer cell

Trans-Blot® SD Semi-dry Transfercell (*BioRad,* Munich)

Real time PCR

ABI PRISM 7700 (*PE Biosystem,* Weiterstadt)

2.2 Chemicals

Acetic acid glacial, Borax, BrdU, bromphenol blue, BSA, $CaCl_2$, coumaric acid, DEPC, DMSO, EDTA, ethidiumbromide, eosin, Ficoll, glucose, glycerol, HEPES, H_2O_2, isobutanol, methylen blue, NaH_2PO_4, Na_2HPO_4, NaN_3, neutral buffered formalin, Ponceau S, propedium iodide, SDS, TRIS, Triton X-100 und Tween20, xylencyanol (*Sigma*, Deisenhofen)

Luminol (*Fluka*, Deisenhofen)

Ammonium persulphate, β-Mercapto ethyl alcohol, chloroform, ethyl acetate, isopropanol, $MgSO_4$, sodium acetate, sodium citrate, paraformaldehyde and phenole (*Merck*, Darmstadt)

Ethanol, formaldehyde, glycine, HCL, potassium acetate, potassium chloride, methanol, NaCl, Sodium deoxycholate, NaOH, phenol und X-Gal (*Roth GmbH*, Karlsruhe)

2.3 Antibodies

Primary

„Human CD34 progenitor cell isolation kit " (*Miltenyi Biotech*, Bergisch Gladbach)

„Mouse lineage panel" (*Pharmingen*, San Diego, CA, USA)

„Sca-1 multisort kit" (*Miltenyi Biotech*, Bergisch Gladbach)

CD11b-Fluorescein-Isothiocyanat (FITC), CD14-Phycoerythrin (PE) and CD15-PE (*Beckton Dickinson*, Heidelberg)

CD33-FITC (*Immunotech*, Marseille, France)

Anti BrdU (FITC)-conjugated (*Becton Dickinson*, Heidelberg, Germany)

Anti- mouse CD45.2/Ly-5.2 FITC, anti-mouse CD45.1/Ly-5.1 PE congugated (*Becton Dickinson*, Heidelberg)

Monoclonal Rat $IgG_{2a,\kappa}$ Immunglobulin, Isotypkontrolle, R-Phycoerythrin conjugated,

Monoclonal Rat $IgG_{2b,\kappa}$ Immunglobulin, Isotypkontrolle, R-Phycoerythrin conjugated,

Monoclonal Rat anti-mouse Sca-1 $IgG_{2a,\kappa}$ Antibody, R-Phycoerythrin conjugated,

Monoclonal Rat anti-mouse c-Kit $IgG_{2a,\kappa}$ Antibody, R-Phycoerythrin conjugated

Monoclonal Rat anti-mouse Gr-1 $IgG_{2b,\kappa}$ Antibody, R-Phycoerythrin conjugated,

Monoclonal Rat anti-mouse Mac-1 $IgG_{2b,\kappa}$ Antibody, R-Phycoerythrin conjugated,

Monoclonal Rat anti-mouse CD45R/B220 $IgG_{2a,\kappa}$ Antibody, R-Phycoerythrin conjugated,

Monoclonal Hamster anti-mouse CD3ε IgG Gruppe 1, κ Antibody, R-Phycoerythrin conjugated

Monoclonal Rat anti-mouse Ter-119 $IgG_{2b,\kappa}$ Antibody, R-Phycoerythrin conjugated

(all from *BD Biosciences*, San José, CA, USA)

Polyclonal rabbit-anti Akt IgG-Antibody, (α-Akt),

Polyclonal rabbit-anti Phospho-Akt (ser473) IgG-Antibody, (α-PAkt),

And -anti B-catenin IgG-Antibody, (α-B-catenin),

(all from *Cell signalling,* Beverly, MA, USA)

Monoclonal mouse-anti-B-tubulin antibody, (α-B-tubulin) (*Merck*, Darmstadt)

Monoclonal mouse-anti-GSK3B antibody, (α-GSK3B),

Monoclonal mouse-anti-Ser9-Phosho-GSK3B antibody, (α-P- GSK-3B) (both from *Santa Cruz Biotechnology*, Santa Cruz, CA, USA)

Monoclonal mouse-anti-HoxB4 antibody, (α-HoxB4), (*University of Iowa*, Iowa City, IA, USA).

Secondary

POD conjugated anti-rabbit, anti-mouse, and anti-goat IgG (*Dako*, Glostrup, Denmark)

2.4 Materials for Mouse Experiment

2.4.1 Mouse Strain

C57BL/6J (Ly5.2) and congenic C57BL/6.SJL-Ly5.1 female mice (*Charles River*, Sulzfeld).

2.4.2 Other Materials

Neo-Kodan *(Schülke & Mayr*, Norderstedt)

Forene (isoflurane) (*Abbott GmbH*, Wiesbaden)

Neomycin (Sigma, Deisenhofen)

27 GA _ Kanülen (Microlance 3) (*Becton Dickinson*, Franklin Lakes, NJ USA)

Titriplex III, 1,1% (*Sigma,* Deisenhofen)

Scalpel blade (*Radiometer*, Kopenhagen, Denmark)

2.5 Special Materials, Reagents and Cell Lines for Cell Culture

Special materials and reagents

DMEM- and RPMI-Medium, FCS, L-Glutamat, Penicillin/Streptomycin and Trypsin/EDTA, trypan blue (*Gibco BRL*, Paisley, Scotland)

X-VIVO 10 medium, (*BioWhittaker,* Verviers, Belgium)

9 % NaCl-solution(*Braun*, Melsungen)

Cell culture flasks, plates, cryotube TM vials (*Nunc*, Roskilde, Denmark)

MACS „LS-separation columns", MACS „pre separation filter" and „Sca-1 multisort kit" (*Miltenyi Biotech*, Bergisch Gladbach)

0,2 and 0,45 µm inject filter (Millex-HA) (*Millipore*, Cork, Ireland)

„FACS Lysing solution" (*BD Biosciences*, San José, CA, USA)

Methylcellulose-based semi-solid medium (Methocult GF M3434, Methocult GFH4434) (*StemCell Technologies*, Vancouver, BC, Canada)

70 µm and 40µm cell strainer filter (*BD Labware*, Franklin Lakes, NJ, and USA)

Cell lines

KG-1: human acute myeloid leukemia, established from the bone marrow of a 59-year-old man with erythroleukemia that developed into acute myeloid leukemia (AML) in 1977 (Koeffler&Golde, Science 200: 1153-1154 (1978).

TF-1: human erythroleukemia, established from bone marrow of a 35-year-old man with erythroleukemia in 1987 (Kitamura et al., Blood 73: 375-380 (1989).

Cell stimulators

All-trans retionic acid (t-RA) and Valproic acid (VPA) (*Sigma*, Deisenhofen), LAQ824 (*Novartis*, Basel, Switzerland)

Cytokines

Murine IL-3 (Interleukin 3), murine IL-6 (Interleukin 6), murine SCF (stem cell factor) (*Cell Concepts*, Umkirch)

Human IL-3 (10 ng/ml), TPO (20 ng/ml), SCF, FL (50 ng/ml each), EPO (3 U/ml)
G-CSF,GM-CSF (*R&D*, Wiesbaden)

2.6 Materials for Molecular Biology

Microbiological Materials

Bacto-Trypton, yeast extrakt and Bacto-Agar (*Biotest*, Dreieich)

Gelatine, Maltose and milk powder *(Difco*, Detroit, MI, USA)

Ampicillin, kanamycin, streptomycin (*Sigma*, Deisenhofen)

Bacterial strain

E. coli – HB101: sup E44, hsd s20, ($r^-_Bm^-_B$)rec A13 ara-14, pro A2, lacY1, galK2, rps L20 xyl-5mtl-1 *(Invitrogen*, Karlsruhe)

Vectors

pCDNA3 (CMV-Pro, lacP, lacZ, f1(-) ori, SV 40 ori, Neo, TK poly(A), Col E1 ori) (*Stratagene*, La Jolla, CA, USA)

pCR2.1 (lacZ', Amp, Col E1, f1 ori, M13rev/T7-Pro) (*Invitrogen*, Karlsruhe)

Enzymes, Kits for Real Time PCR

Qiagen Plasmid Mini Preparations kit (*Qiagen*, Düsseldorf)

Superscript II RT

Platinium qPCR Supermix-UDG

Rox reference dye

„TA-Cloning-Kit"
(all from *Stratagene*, Mannheim)

Restrictions endonucleases (*New England Biolabs*, Frankfurt; *Gibco BRL*, Bethesda, NY, USA),

T4-Ligase and DNase I (*Gibco BRL*, Bethesda, NY, USA)

Rnase A (*Sigma*, Deisenhofen)

3 Methods

3.1 Handling of Animals

C57BL/6J (Ly5.2) or congenic C57BL/6.SJL-5.1 female mice from 8 to 12 weeks of age were obtained from *Charles River* (Sulzfeld, Germany). Mice were bred at the stabulary of the Clinic of J.W-Goethe University. All animals were housed (6 mice per cage) in specific pathogen-free conditions and maintained on acidified drinking water (PH 3) or drinking water with neomycin sulphate (1 mg/ml) for 7 d prior to and during the experiment for competitive repopulating assay or colony formation unit spleen (CFU-S) assay experiments in order to minimize radiation-induced mortality in lethally irradiated mice.

3.2 Enrichment of Human $CD34^+$ HSCs

Umbilical cord blood (CB) was collected immediately after delivery in a sterile tube containing 5,000 I.E. heparin. Informed consent of the mother was obtained. Adult bone marrow (BM) samples were obtained from healthy volunteer donors (J.W. Goethe University). Mononuclear cells (MNC) were isolated from cord blood samples or adult bone marrow by Ficoll/Hypaque centrifugation (2000 rpm, 20 min). Isolated MNC were washed twice with MACS buffer (1100 rpm, 7 min). The $CD34^+$ selection was performed with the Miltenyi Mini MACS column according to the manufacturer's instructions. Obtained MNC were resuspended at a density of $1x10^8$ cells in 300 µl MACS buffer containing a human IgG to block the Fc receptors and an anti-CD34 antibody modified with a hapten and incubated for 15 min at 4 °C . Cells were washed with MACS buffer and incubated for another 15 min at 4 °C with an anti-hapten mouse monoclonal antibody conjugated to colloidal superparamagnetic beads (*Miltenyi Biotec*).

After washing once with MACS buffer, labeled cells were applied to magnetic columns (MS column, *Miltenyi Biotec*), unbound cells washed out, and $CD34^+$ cells eluted from the column with MACS buffer. To improve the purity of the $CD34^+$ cells, a second purification cycle was performed using a smaller column (VS column, *Miltenyi Biotec*).

3.3 Suspension Cultures of Human $CD34^+$ HSCs

5 x 10^3 $CD34^+$ cells/ml were seeded in 12-well-plates using X-VIVO 10 plus 1 % BSA and 1 % glutamine supplemented with recombinant human IL-3 (10 ng/ml), TPO (20 ng/ml), SCF, and FL (50 ng/ml each) and were exposed to valproic acid (VPA) with increasing concentration (30-150 µg/mL) for 7 d in liquid culture and incubated at 37 °C in humidified atmosphere of 5 % CO_2. Without medium exchange or cytokine readdition, cells were harvested after 7 d counted and assayed for CD34 and CD14 expression by FACS.

3.4 Flow Cytometry of Human HSCs

For fluorescence-activated cell sorter (FACS) analysis, cultured CB and BM CD 34^+ cells for 10 d in methylcellulose and adult BM CD 34^+ for 7 d in liquid culture were collected and washed twice with PBS containing 1 % FCS and 0.1 % NaN_3 (1200 rpm, 5 min). 5 µl of fluorescein isothiocyanate (FITC)-conjugated antibody were added, followed by incubation for 15 min at 4 °C. After washing, the cells were fixed in 300 µl PBS with 2 % formaldehyde. The antibodies were labeled as follows: FITC-conjugated CD34, CD14 for $CD34^+$ adult bone marrow and CB cells. As negative control FITC-conjugated anti-huIgG_1 was used. 5000 to 10000 events were counted. Analysis was performed at a FACScan using CellQuest and PC-Lysis software.

3.5 Colony Assay of Human HSCs

Cells were suspended at 5000 cells/ml on day 0 into 35-mm culture dishes containing semi-solid methylcellulose medium supplemented with 30 % FBS, SCF (50 ng/mL), GM-CSF (10 ng/mL), IL-3 (10 ng/mL) and EPO (3 U/mL) (Methocult GFH4434, *Stem Cell Technologies*) and were exposed to VPA with increasing concentrations (30-150 µg/mL) and incubated at 37 °C for 10 d in humidified atmosphere of 5 % CO_2. Each assay was plated in triplicates. After 10 d of incubation, colonies were derived from methylcellulose, washed twice with PBS and stained for the expression of CD34 and CD 14 surface markers for FACS analysis.

3.6 Enrichment of Murine Sca1$^+$/lin$^-$ HSCs

Sca1$^+$/lin$^-$ HSCs were isolated from female C57BL/6J (Ly.5.2) or congenic C57BL/6.SJL-5.1 mice from 8 to 12 weeks of age (*Charles River*). Mice were exposed to isoflouran 10-15 sec, then sacrified by cervical dislocation. Before the separation of femora and tibiae from the animal, legs area was sterilized by Neo-kodan. The femora and the tibiae were separeted and all muscle tissue was removed from samples. BM was harvested from femora and tibiae by flushing the bones with a 1 ml syringe and 26-gauge needle with PBS and 2 % penicillin/streptomycin solution into 50 ml Falcon tube. Before mononuclear cell (MNC) derivation, BM cells were passed through a 70 µm and 40 µm cell strainer filter, respectively. BM cells were centrifuged at 2000 rpm for 20 minutes on Ficoll-Hypaque to obtain MNC. Enriched MNC cells were washed twice with MACS buffer (phosphate-buffered saline (PBS) containing 0.5 % bovine serum albumin (BSA) 0.1 % EDTA (Titriplex III, *Sigma*, Deisenhofen) and 1 % Penicillin/Streptomycin after Ficoll. Sca1$^+$/lin$^-$ cells were purified by immunomagnetic beads using the magnetic-activated cell separation (MACS) cell separation columns (*Miltenyi Biotec*) according to the manufacturer's instruction.

For lineage depletion (lin$^-$cells), enriched and washed mononuclear cells were resuspended at a density of 2 x 10^8 cells per ml in MACS buffer containing monoclonal antibodies specific for murine T lymphocytes (CD3), B lymphocytes (B220), macrophages (Mac-1; CD11b), granulocytes (Gr-1) and erythrocytes (TER-119) After 15 min of incubation at 4 °C, the cells were washed (1000 rpm for 7 min), resuspended in MACS buffer at a concentration of 2 x 10^8 cells/ml together with streptavidin coatet MicroBeads (100 µl for 2 x 10^8 cells). After 15 min of incubation at 4 °C, cells were centrifuged and labeled cells were removed by using streptavidin-loaded MACS cell separation columns. Then selected and centrifuged Lin- cells were stained with biotinylated anti-Ly6A/E (Sca-1, rat IgG2a, clone E31-161.7) following a 15 min incubation and centrifugation. Labeled cells were applied to magnetic columns (MS column, *Miltenyi Biotec*) and unbound cells washed out. Sca1+ cells were eluted from the column with MACS buffer.

3.7 Colony forming unit assays (CFU) and Replating efficiency of murine HSCs

After a 2 d stimulation in DMEM medium with 1 % P/S, 1 % glutamine, 10 %FCS mIL-3 (20 ng/mL), mIL-6 (20 ng/mL), and mSCF (100 ng/mL). $Sca1^+/lin^-$ cells from Ly5.2 female mice were counted and plated at 5000 cells/ml into 35-mm culture dishes containing methylcellulose-based semi-solid medium (Methocult GF M3434, *StemCell Technologies,* Vancouver, BC, Canada) and incubated at 37°C for 10 d in humidified atmosphere of 5 % CO_2 for colony formation assay. Each assay was plated in triplicates. On day 10 after plating, the colony number was recorded and colony photos were taken under the microscope. After washing out twice with PBS from methylcellulose, cells were either stained for the determination of c-kit, Sca-1, Gr-1, and Mac-1 surface marker expression by fluorescence-activated cell sorting analysis as explained before (FACS, *Becton Dickinson*) or plated again (5000 cells/35-mm culture dish) in methylcellulose determining replating efficiency by serial plating until the end of colony formation.

3.8 Day 12 spleen colony-forming unit assay (CFU-S D12 assay)

1000 Ly5.1 $Sca1^+/lin^-$ cells were cultured in the presence of VPA alone or in combination with t-RA (VPA: 150 µg/mL -/+ t-RA: 1 µmol/l) supplemented with DMEM medium, 1 % P/S, 1 % glutamine, 10 %FCS mIL-3 (20 ng/mL), mIL-6 (20 ng/mL) and mSCF (100 ng/mL) and incubated at 37°C for 2 d in humidified atmosphere of 5 % CO_2. After 2 d, all cells that grew from 1000 Ly5.1 $Sca1^+/lin^-$ cells were washed twice with PBS (1200 rpm, 5 min.). Then cells were resuspended in 150 µl PBS per mouse for all groups and injected retroorbitally with a 1 mL syringe with a 25-gauge needle into lethally irradiated (10 Gy; 5 Gy for the following 2 d) Ly 5.2 recepient mice (n=7). Moreover, one group was also included as a control, consisting of 3 mice that received experimental treatment and radiation, but not hematopoietic stem cells (nt: not transplanted mice). Mice were kept under sterile conditions with 4 animals per cage and maintained drinking water with neomycin sulphate (1 mg/ml) for 1 week before and during the experiment. The cages were inspected daily. 12 d later, transplanted animals were sacrified by cervical dislocation (a protocol approved by the local animal-care committee).

The spleens were removed through a left lateral incision and immediately fixed in Bouin's fixative for 5 min, then transferred to 10 % neutral buffered formalin. The spleen colonies were enumerated under the binocular microscope and the spleen colony pictures were taken by digital camera (*Sony*, NJ, USA) after fixation. For fluorescence-activated cell sorter (FACS) analysis of CFU-S assay, 3 spleen samples from different treatment groups (control, t-RA, VPA) were passed through 40 µm cell strainer filters separately with 3 ml PBS and washed once with PBS (1200 rpm, 5 min). Then spleen cells were resuspended in 100 µl PBS and incubated with 1 µl of the phycoerythrin (PE)- conjugated monoclonal antibodies specific for murine macrophages (Mac-1; CD11b), granulocytes (Gr-1) and stem cell marker (c-Kit, Sca1) for 15 min at 4 °C. After washing with PBS once (1200 rpm, 5 min), the cells were fixed in 300 µl PBS with 2 % formaldehyde. As negative control PE- conjugated anti-muIgG_1 was used. 5000 to 10000 events were counted. Analysis was performed at a FACScan using CellQuest and PC-Lysis software.

3.9 Competitive Repopulation Assay

1000 Ly5.1 $Sca1^+/lin^-$ cells were deposited into 96-well plates supplemented with DMEM medium, 1 % P/S, 1 % glutamine, 10 % FCS mIL-3 (20 ng/mL), mIL-6 (20 ng/mL), and mSCF (100 ng/mL) and incubated at 37 °C for 2 d in humidified atmosphere of 5 % CO_2. All cells that grew in culture under each culture condition were washed twice with PBS (1200 rpm, 5 min) and injected with a 1 ml syringe with a 25-gauge needle retroorbitally into lethally irradiated (10 Gy) Ly5.2 female recipients mice of 8 to 12 weeks together with 1 x 10^5 normal Ly5.2 bone marrow cells.

Mice (6 animals per cage) were kept under sterile conditions and maintained drinking water with neomycin sulfate (1 mg/ml) 1 week before and during the experiment. The cages were inspected daily. 12 weeks later, the femora and the tibiae were separeted and all muscle tissue was removed from the samples. BM cells were harvested as explained before and stained with conjugated monoclonal antibodies specific for Ly5.2 and Ly5.1 or mouse IgG2a (all from *PharMingen*, San Diego, CA, USA) for 30 min at 4 °C for fluorescence-activated cell sorting analysis.

3.10 Cell Cycle Analysis

5000 $Sca1^+/lin^-$ cells were exposed to different histone deacetylase inhibitors (HDIs) alone or in combination with t-RA(VPA: 150 µg/ml, Laq 824: 0,01 µmol/l -/+ t-RA: 1 µmol/l) in the presence of DMEM medium,1 % P/S, 1 % glutamine, 10 %FCS mIL-3 (20 ng/ml), mIL-6 (20 ng/ml), and mSCF (100 ng/ml) and incubated in semi-solid medium at 37 °C for 7 d in humidified atmosphere of 5 % CO_2. After 7 d, cells washed in phosphate-buffered saline (PBS), and fixed with 70 % ethanol at -20 °C.

Afterwards, cells were resuspended in PBS containing propidium iodide (PI; 50 µg/ml), RNAse (5 µg/ml; Sigma) and incubated at 37 °C for 30 min and immediately evaluated by FACS. For the bromodeoxyuridine (BrdU) incorporation, 2 x 10^6 cells were incubated with 10 µM BrdU (Sigma) at 37 °C for 25 min.

Cells were fixed by ice-cold 70 % ethanol, denatured by 4N HCL, stained with a fluorescein isothiocyanate (FITC)-conjugated antibody against BrdU (*Becton Dickinson*, Heidelberg) and with PI (5 µg/ml) and then analyzed by FACS

3.11 Cell culture

Cell culture of Eukaryotic cells was performed under sterile conditions (laminar air flow hood). All plastic materials and solutions were autoclaved before usage (30 min at 121 °C). Cells were grown in culture flasks in a humidified atmosphere at 37 °C and by 5 % CO_2.

TF-1 cells were cultivated in RPMI 1640 and supplemented with 10 % FBS, 2 mM L-glutamine, 40 µg/ml penicillin and 40 µg/ml streptomycin, whereas KG-1 cells were cultivated in X-VIVO 10 (BioWhittaker) supplemented with 1 % BSA and 1 % glutamine (200 mM, *GIBCO BRL*). Cultures were maintained at 0.2-1.0 x 10^6 cells/ml; splitted 1:2 to 1:4 every 2-3 d and sowed out at about 1 x 10^6 cells/ml.

Cell number and viability were controlled by trypan blue exclusion. 40 µl cell suspension was mixed with 10 µl of 0.4 % trypan blue in 0.9 % NaCl. Only unstained cells were considered viable and their number was calculated as follows:

Cell N° [10^6/ml]= N° of counted cells X dilution

N° of quadrants counted

Freezing:

For storing in liquid nitrogen, approximately 1 x 10^7 cells were collected at 1200 x g for 5 min and washed with PBS. After centrifugation, the cells were resuspended in 0.7 ml of RPMI containing 20 % FCS, transferred into cryovials, and mixed with dropwise added 0.7 ml of RPMI containing 10 % DMSO. This suspension was immediately placed at –80 °C and after 3-5 h stored in a liquid nitrogen tank.

Thawing:

Cryopreserved cells were taken out of liquid nitrogen, thawed rapidly in a 37 °C incubator and resuspended in 5 ml of the culture medium. After one washing step (1200 x g, 5 min, RT) to remove residual DMSO, cells were transferred into culture flasks and resuspended with fresh medium.

3.12 Western Blotting

3.12.1 Lysis of Cells (Sambrook et al., 1989)

SDS lysis buffer: 0,1 % (w/v) SDS
1 % (w/v)Triton X-100
1 % (w/v) Sodium deoxycholat in 1xTBS
to 100 ml sterile water

Cells of each experiment group were washed twice with PBS (2000 x g for 10 min at 4 °C), the supernatant was discarded and the cell pellet was resuspended with 60 °C hot lysis buffer (100 µl per million cells) on ice. After 10 min of incubation on ice, lysates were exposed to benzonase at RT for 5 min. After lysates were cleared, cells were vortexed and stored at -20 °C before usage.

3.12.2 Determination of Protein Concentration

Protein concentration was measured according to Bradford (1976) using commercially available BioRad Kit. The standard curve was set by using 1:10 diluted BSA (0.05-0.4 mg/ml) and protein samples compared to it by measuring the absorbance at λ=620 nm.

3.12.3 SDS-Polyacrylamide Gel Electrophoresis (SDS-PAGE)

SDS-PAGE electrophoresis (Laemmli, 1970) is used to separate proteins prior to their analysis.

Sample buffer (2 X)	32 mM	Tris-HCl pH6.8
	10 % (v/v)	glycerol
	1 % (w/v)	SDS
	5 % (v/v)	2-mercaptoethanol
Running buffer	25 mM	Tris
	192 mM	glycine
	0.1 %(w/v)	SDS

Mini Protean II® (8.5 cm X 6.5 cm) apparatus was assembled by using two glass plates and side spacers. The resolving gel was mixed in acrylamide-bisacrylamide concentration that matches protein weight (Sambrook et. al, 1989), and poured between the two glasses. Isobutanol saturated with ddH_2O was laid over to ensure a flat surface and prevent from trapping of air bubbles.

After the gel polimerization, isobutanol was washed out with water and stacking gel layed over. When the stacking gel was polimerized, samples were prepared (80 µg of protein) with sample buffer (25 µl of end volume), boiled for 3 min and loaded to the gel.

Since SDS denaturates proteins, their net migration through the gel is determined not by the electrical charge but by molecular weight. Therefore stained protein standards (*BioRad*, Novex) were always used for maching the positions of examined proteins.

The gel was run for 1 h at a constant voltage of 150 V. After electrophoresis, the gel was stained with Comassie-blue (0.1 % Coomassie Brilliant Blue, 10 %(w/v) acetic acid, 30 % (w/v) methanol) for visualising separated proteins.

3.12.4 Transfer of Proteins onto a Nitrocellulose Membrane (Western Blotting)

Following the gel electrophoresis, proteins were transferred to 0.2 µm nitrocellulose membranes (*BioRad*, Munich) in a process called Western blotting. This transfer was carried out between two horizontal metal plates (Kyhse-Andersen, 1984) in a SemiDry System (BioRad).

Transfer buffer	62.5 mM	Tris-base, pH 7.4
	20 % (v/v)	methanol

The membrane was soaked in transfer buffer for 10 min, placed on top of the 3MM Whatman® paper immersed in the same buffer and the gel was layed over. This was covered by another sheet of wet 3MM Whatman® paper, all dimensions being slightly wider then the gel. Transfer was carried out in excess of blotting buffer for 1 h by electric density of 0.3 mA/cm^2 and a 300-400 mA current.

Ponceau-S solution	5 g Ponceau-S was ressolved in 1 ml phosphoric acid and ddH_2O added to 100 ml.

Following Western blotting, membranes were usually stained with Ponceau-S for 5 minutes to visualise protein bands. The ponceau was removed by washing with TBST, and membrane stored by 4°C for further analysis.

3.12.5 Immunodetection of Specific Proteins

Proteins immobilized on a membrane were identified by using highly specific antibodies. The membrane was first incubated in TBS-T containing 5% (w/v) Carnation milk in order to saturate nonspecific protein binding sites, and after one hour (or overnight incubation by 4°C on a rocking platform) washed three times for 5 minutes in TBS-T.

Primary antibody was diluted to appropriate concentration (1:200-1:10000) in a blocking solution or TBS-T alone and membrane incubated for 3 hours to overnight.
Incubation was followed by three washing steps in TBS-T and secondary antibody added (pig-anti-rabbit-couppled to POD IgG, 1:2000, or as recommended). After one hour, membrane was again repeatedly washed in TBS-T to remove unbound secondary antibody.

ECL I:	2.5mM	Luminol
	0.4ml	p-coumaric acid (90mM in DMSO)
	10mL	1M TrisHCl pH 8.5
	to 100ml	with dd H_2O

ECL II:	64µl	30% H_2O
	10ml	1M TrisHCl pH 8.5
	to 100 ml	with dd H_2O

Signals were detected in the dark by rinsing the membrane shortly in water and then incubating with ECL (enhanced chemoluminescence system) I and II solutions in 1:1 ratio for one minute.
Membranes were exposed to X-OMAT film for 30 to 5 min depending on the intensity of the signal detected and antibody-bound proteins visualized.

Stripping buffer	62.5	mM	Tris-HCl, pH8.0
	100	mM	2-mercaproethanol
	2% (w/v)		SDS

Blots were occasionaly stripped and reprobed with different antibodies. This was performed by incubating the membrane in stripping buffer at 55°C for 1 hour, followed by extensive washing in TBS-T. When 2-mercaptoethanol was no longer detectable, membrane was covered with blocking solution and incubation procedures with primary and secondary antibody repeated.

3.13 Quantitative Real Time Polymerase Chain Reaction RT-PCR

3.13.1 Isolation of Total RNA from Human Cells

During the isolation of the RNA from the eukaryotic cells $CD34^{+}$, TF-1cells, sterile and disposable plasticware were used, all reagents prepared with DEPC-H_2O, and several other precautions were taken in order to prevent the activity of RNases. Isolation of RNA from the eukaryotic cells were performed with the RNAeasykit (Qiagen according to the supplied protocol. $1x10^6$-$3x10^8$ cells were collected in a 50-ml falcon by centrifugation (1500 rpm, 10 minutes). The cells were washed twice with sterile PBS and supernatant removed. 350µl RLT buffer (RNAeasykit, Qiagen, Hilden) together with B-mercaptoethanol were added directly to the cells and vortexed. Then, 350µl fresh 70% EtOH (with DEPC-Wasser) were also added to cells together with RLT+B-MET and carefully mixed. 700µl mixed solution were given to seperation column and centrifuged at 10 000 rpm, 1min. After collection tube were emptied, separation column were washed with 500 µl RPE-buffer twice. Separation column was transferred to eppendorf tubes centrifuged and dried. Following centrifugation, 30 µl DEPC water were given to column. After incubation of 1 min, column were centrifuged at 10 000, 1 min and another 30 µl DEPC water was added to column. After centrifugation, dissolved RNA in 60 µl DEPC were collected and stored at -80°C.

3.13.1.2 Quantitation of RNA

The concentration of RNA is determined by Agilent 2100 bioanalyzer. RNA samples were thawed on ice and diluted 1/10 (100-400 ng/µl) with nuclease free water.1µl RNA dispensed in to PCR tubes, denaturated at 70°C at least two minutes and was spinned. At the same time RNA ladder aliquoted as 1µl in to nuclease free microcentrifuge tubes and heated at 70°C for 2 minutes. After RNA ladder is aliquoted and placed on ice, 9 µl gel-dye mixes loaded into each of the well of the new chip. 5µl RNA 6000 Nano marker (Green), 1µl denaturated RNA samples and 1µl denaturated RNA ladder were also pipetted into each well. The chip gently placed on the vortex and vortexed for 1 minute. After vortexing, chip were placed receptacle

and runned in the bioanalyzer. Individual electrograms were analyzed for quantitation and quantification of RNA.

3.13.2 One Step Reverese-Transcriptase Polymerase Chain Reaction (RT-PCR)

After the measurement of the concentration of RNA by bioanalyzer, RT-PCR was performed by Superscript one step RT-PCR (*Invitrogen*, Karlsruhe)

RT-PCR reaction mix:

25 µl 2x reaction buffer (a buffer containing 0.4 mM of each dNTP, 2.4 mM MgSO4)

1 µg RNA

0.5µM HoxB4fwd primer (2.5µl)

0.5 µM HoxB4rev primer (2.5µl)

1 µl RT/Taq mix (2x reaction buffer, 5mM and 50mM Magnesium Sulfate)

Ad 50µl with DEPC-H_2O

Water control was also performed for RT-PCR reaction as the same reaction mix with the exception of RNA. The reactions were set up on ice in 0.5ml PCR tubes, and PCR performed in Thermal Cycler 480 (Perkin Elmer Biosystems, Weiterstadt). Following primer pairs were used:

HOXB4 (corresponding to 1093-bp)

Human HOXB4 Fw: 5′-CCT GGA TGC GCA AAG AAG TTC A -3′
Human HOXB4 Rev: 5′-AAT TCC TTC TCC AGC TCC AAG A -3′

Invitrogen Life technologies,
One step RT-PCR performed during the following steps:

1.	cDNA synthesis:	50°C	30 min
2.	pre-denaturation :	94°C	2 min
3.	denaturation:	94 °C	45 sec
4.	anneal:	50 °C	45 sec
5.	extension:	72°C	45 sec
6.	final extension:	72°C	7 min

Reactions were controlled by gel electrophoresis (Agarose gel 2%).

3.13.3 Cloning of PCR product

3.13.3.1 Ligation

DNA fragments were ligated with linearized, dephosphorylated vectors in order to obtain different plasmid constructs. This reaction was performed in presence of T4 DNA ligase, which catalyzes formation of phosphodiester bonds between adjacent 3'-OH and 5'-P termini of the DNA. Ligations were carried out as follow:

PCR- product	6µl
10* Ligation buffer	1µl
vector pCR2.1(3.9Kb)	2µl
T4 DNA ligase	1µl

Ligation samples were incubated at 16°C overnight.
(in a 20µl volume containing dephosphorilated vector, a two to threefold molar excess of insert, 1µl of T4 DNA Ligase (400 U/µl, New England Biolabs) and 1µl of 10 X Ligation buffer (50mM TrisHCl pH 7.6, 10mM $MgCl_2$, 1mM DTT, 1mM ATP).

3.13.3.2 Transformation of Bacteria

Preparation of competent *E.coli*

Competent bacteria were prepared using $CaCl_2$ method. Bacteria were streaked on petriplate without amphicillin and incubated overnight at 37°C. A single colony of E.coli HB101 was picked and inoculated in 5ml LB medium without ampicillin for overnight. After overnight incubation, the miniculture were transferred into 400ml fresh LB medium without amphicillin, and grown at 37°C until reaching the OD_{600} (optical denstiy with a wavelenght of 600nm) of 0.5 - 0.6. Reading the optical density at 600 nm, at which wavelenght one OD unit correspond to about 0.8x108 cells/ml, can monitor the growth of culture. When bacteria reach the desired OD, the culture was immediately chilled on ice for 15 minutes and pelleted by centrifugation at 4000 x g for 20 minutes at 4°C. The pellet was resuspended in 80ml of sterile $CaCl_2$ solution (50mM $CaCl_2$, 10mM TrisHCl pH 7.4), by circular movements on ice for 30 minutes. Cells collected by centrifugation at 4000 x g for 15 minutes at 4°C. Then, 11ml of $CaCl_2$ and 2ml of glycerol solution were added to cell pellet which is then alliqouted as 100µl in 1.5ml tubes and frozen immediately by immersing the tubes in liquid nitrogen and stored at -80°C.

Transformation of Bacteria

Aliquot of competent E.coli bacteria was taken out of the liquid nitrogen, after 10 minutes incubation on ice, 10µl ligation reaction were added and mixed by flicking the tube. Bacteria were left on ice for the next 30 minutes and then exposed to the 42°C heat shock for 2 minutes. 500µl of LB (without antibiotic) were added, and samples incubated for 1 hour in a shaking incubator.

Afterwards, bacteria were transferred onto the LB-ampicillin plates together with XGal, and following overnight incubation on 37°C, individual white colonies were picked for minipreparation.

3.13.3.3 Miniprep: A Small Scale Preparation of Plasmid DNA

The preparation of plasmid DNA was performed by alkaline lysis method (Sambrook et al., 1989).

Alkaline treatment causes proteins and linear chromosomal DNA of the host to denature irreversibly. The denatured chromosomal DNA, proteins and cellular debris are then precipitated with SDS at high concentrations of salt. For a small-scale preparation of plasmid DNA, a 2ml culture of LB ampicilin medium (0.1mg/ml) was inoculated with a single bacterial colony using a sterile toothpick. The culture was placed at 37°C in a shaking incubator for 8 to 16 hours.

Resuspension solution (Sol I)	50mM	glucose
	25mM	Tris-HCl pH 8.0
	10mM	EDTA pH 8.0
Alkaline lysis solution (Sol II)	0.2M	NaOH
	1.1% (w/v)	SDS

Neutralization solution (Sol III) 3M potassium acetate pH 5.2

1,5 ml of the overnight culture was spun at 14000 x g for 2 minutes, and the bacterial pellet resuspended in 100μl of the solution (Sol I). The cells were lysed by addition of 200μl of the alkaline lysis solution (Sol II) and mixed gently by inverting. Denatured chromosomal DNA and cell debris were precipitated by addition of 150 μl of the ice-cold neutralization solution (Sol III). The tube was inverted again, centrifuged (14000 x g, 5 minutes, 4°C), and supernatant extracted with mixture of phenol/chloroform. This mixture was used to separate and remove proteins from preparations of nucleic acids. Phenol was equilibrated with Tris, pH8.0 and chloroform used for DNA extraction was a 24:1 (v/v) mixture of chloroform and isoamylalcohol (latter referred as sevag).

The DNA supernatant was then mixed with approximately the same volume of phenol/sevag, briefly vortexed and spun (14000 x g, 2 minutes, RT), upper phase transferred in the new tubes and extraction repeated. Final supernatant was precipitated with ethanol, at low

temperature and in presence of monovalent cations. One-tenth volume of 3M sodium acetate, pH5.2, and 2,2 volumes of ice cold ethanol were added to each sample. DNA was precipitated by incubation at -20°C for 3h to overnight, and followed by centrifugation at 14000 x g for 15 minutes at 4°C. The DNA pellet was rinsed with 70% (v/v) ethanol and finally dissolved in 50µl of dd water or 10mM TE buffer.

3.13.3.4 Determining of Nucleic Acid Concentration

A quartz cuvette was filled with dd H_2O and photometer adjusted to zero. Preparation of nucleic acids was strongly diluted (in dd H_2O) and absorption spectrum of the solution measured at a wavelength of 260nm. To determine the concentration of nucleic acids, a following equation was used:

$C=OD_{260nm}$ x f x n,

Where C represents the concentration (µg/ml), OD equals optical density, f stands for dilution factor and n was set by default as: 50µg/ml for DNA, 40µg/ml for RNA and 37 µg/ml for oligonucleotids (all for 1 cm quartz cuvettes).

The ratio between the absorbance readings at 260nm and 280nm was used to estimate the purity of nucleic acid preparation. Expected ratio of a very pure DNA or RNA preparation was OD260/280 equals 1.8 or 2.0, respectively.

3.13.4 Standards for the Quantative Real Time PCR

After the measurement of the DNA concentration of the minipreprations, standards were prepared from TF-1 cells according to the calculation below.

Calculation:

$$\text{DNA(mol)} = \frac{\text{Mass (g)}}{660\text{(g/mol)x bp (vector+fragment)}}$$

Molecular weight= DNA(mol) x $6,02x10^{23}$

Dilution factor = $\frac{\text{Molecular weight(n/µl)}}{2x10^{2(n-2)}}$

Amount of stock sol= $\frac{\text{Desired volume}}{\text{Dilution factor}}$

Dilution:

10^{n-2}= amount stock sol.add 500 µl

10^{n-3}= 100 µl + 900 µl

.
.
.

10^{1}

3.13.5 cDNA Preparation

DNAase treated 10µgr total RNA that isolated from Human $CD34^{+}$ cells was combined in a 25µl final volume in the eppendorf tube together with DEPC-treated water. Then, heated at 70°C for 10 min and placed immediately on ice. Master mix were prepared and added into 25 µl RNA.

Mastermix:

Gibco 5x RT-buffer	10µl
DTT	5µl
PDN	0,25 µl
dNTPs	2µl
RNA-Guard	2µl
DEPC treated water	3,25µl
Superscript II RT	2,5µl

RT reaction were performed in this conditions:

10 min	25°C
45 min	42°C
3 min	99°C

Then cDNA samples were stored in –20°C.

3.13.6 Taqman PCR

Human HOXB4 Fw: 5′-CCT GGA TGC GCA AAG AAG TTC A -3′
Human HOXB4 Rev: 5′-AAT TCC TTC TCC AGC TCC AAG A -3′
Human HOXB4 probe: 5′-FAM TGA GCA CGG TAA ACC CCA ATT ACG CC TAMRA3′
Human GAPDH Fw: 5'-CATCTTCCAGGAGCGAGA-3'
Human GAPDH Reverse Primer - 5'-TGTTGTCATACTTCTCAT-3'
Platinium qPCR Supermix-UDG (invitrogen)
Rox (invitrogen)
For the recommended reaction volume of 25µl for the Optical 96-Well Fast Plate, each PCR reaction contains the components as listed as follows**:**

Supermix	12,5 µl
Primer 1(HoxB4, GAPDH)	0,5 µl
Primer 2 (HoxB4, GAPDH)	0,5 µl
Probe (HoxB4, GAPDH)	0,5 µl
DEPC water	8 µl
Rox	0,5 µl
cDNA/Standard	2,5 µl

The TaqMan PCR was conducted in duplicates following standard protocols using the ABI PRISM 7700 (PE Biosystems). Normalization to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was done for each sample.

3.13.7 Data Analysis

The Sequence Detector Software SDS 2.0 (Applied Biosystems) was used for data analysis. The first step was to generate an amplification plot for every sample which shown ΔRn on the *y* axis (where Rn is the fluorescence emission intensity of the reporter dye normalized to a passive reference and ΔRn is the Rn of an unreacted sample minus the Rn value of the reaction) against the cycle number, displayed on the *x* axis. From each amplification plot, a threshold cycle (Ct) value was calculated, which is defined as the cycle at which a statistically significant increase in ΔRn is first detected and is displayed in the graph as the intercept point of the amplification curve with the threshold. The threshold is automatically calculated by SDS as the 10-fold SD of Rn in the first 15 cycles. The obtained Ct values were then exported to a Microsoft Excel spreadsheet for further analysis.

The next step was to construct calibration curve plots, using Microsoft Excel as recommended in User Bulletin 2 for the ABI Prism 7700 Sequence Detection System (Applied Biosystems), showing Ct values on the *y* axis and the logarithm of the input amount of cDNA (equivalent to the amount of total RNA) on the *x* axis. All human ABC transporters were subsequently measured in the different human tissues, and the obtained Ct values were used to calculate the initial input amount. Thereafter the results were normalized to the endogenous control, *GAPDH*. In the last step, the expressions of each individual ABC transporter were compared in the complete tissue panel. Therefore, for each ABC transporter, the normalized amount of expression in the tissue that showed the lowest expression was used as a calibrator (set to 1), and the remaining tissue samples were displayed as fold changes.

4 Results

4.1 Effect of VPA as A Single Agent on HSCs

The potent histone deacetylase inhibitor (HDI) valproic acid (VPA), which regulates transcription through chromatin modeling like other HDIs, has been reported to induce differentiation of carcinoma cells, transformed hematopoietic progenitor cells and leukemic blasts from AML patients (Gottlicher*, et al* 2001). On the other hand, in a clinical study by Bug *et al*, VPA/t-RA combination therapy in advanced AML patients resulted in blast cell reduction and hypergranulocytosis. Importantly, it was possible to distinguish malignant from normal hematopoiesis in one patient, by the presence of the isochromosome (17)(q10) in the leukemic blasts. The analysis revealed that whereas the $CD34^{+}$ progenitor cells contained residual isochromosome (17)(q10), all granulocytes had a normal karyotype, suggesting dominance of normal hematopoiesis over the malignant clone most likely due to an enhancement of nonleukemic myelopoiesis and the suppression of malignant hematopoiesis rather than enforced differentiation of the leukemic blast (Bug, *et al* 2005b). Moreover, VPA has been recently demonstrated to enhance early acting cytokine effect on the maintenance and expansion of primitive human HSCs at least *in vitro* consistent with the hypothesis that VPA influences normal hematopoiesis.

Therefore, the effect of chromatin modeling through VPA on HSCs was investigated with respect to differentiation, proliferation as well as self-renewal in the present study.

4.1.1 VPA Increases the Proliferation of Human CD34$^+$ HSCs

In order to determine the effects of VPA on human HSC, the proliferation and differentiation potential of human adult or umbilical cord blood CD34$^+$ cells were analyzed in the presence both early cytokines and an external milieu that favors differentiation (supplemented by GM-CF and FBS). Firstly, the effect of increasing doses VPA on adult CD34$^+$ cells were analysed with respect to number of CD34$^+$ (stem cell marker) and CD14$^+$ (differentiation marker) cells after 7 days in liquid culture in the presence of the early acting cytokines IL-3 (10 ng/ml), TPO (20 ng/ml), SCF, and FL (50 ng/ml). According to the results, VPA prevented differentiation of bone marrow CD34$^+$ cells in a dose-dependent manner as revealed by the increase of CD34$^+$ cells and the concomitant decrease of monocytic CD14$^+$ cells upon increasing doses of valproic acid in a 7-day liquid culture (Fig. *8A*).

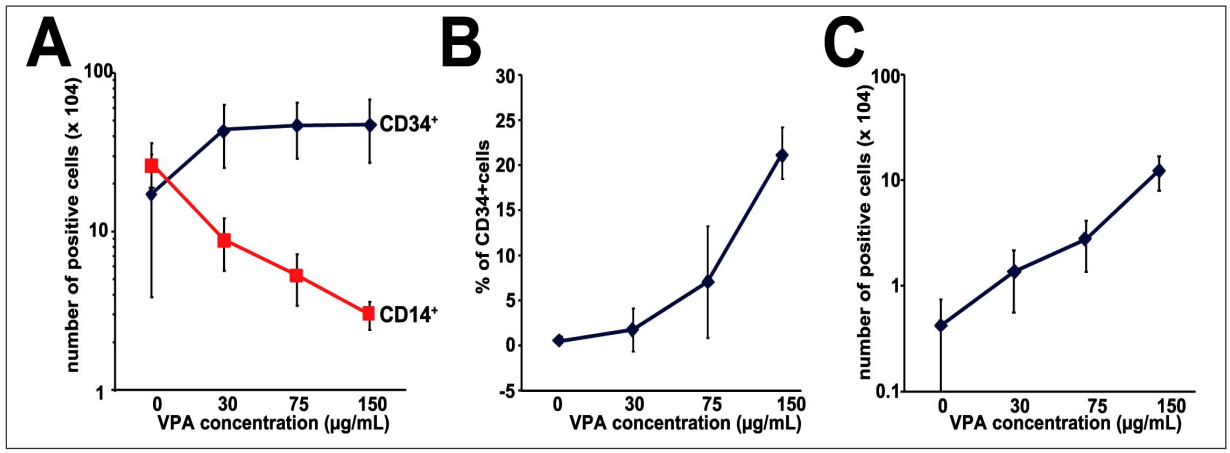

Figure 8. Proliferation and Differentiation of Human HSC on exposed to VPA. ***A***, expression of CD34 and CD14 in bone marrow CD34$^+$ on increasing concentrations of VPA (0, 30, 75, and 150 µg/mL). ***B***, percentage of CD34$^+$ cells in colonies of bone marrow CD34$^+$ plated in semisolid medium and cultured in the presence of FCS and G-CSF for 10 days on increasing concentrations of VPA (0, 30, 75, and 150 µg/mL). ***C,*** total number of the umbilical cord blood CD34$^+$ cells plated in semisolid medium and cultured in the presence of FCS and G-CSF for 10 days on increasing concentrations of VPA (0, 30, 75, and 150 µg/mL).

Secondly, the functional properties, proliferation and differentiation potential of HSC even in the presence of high differentiation pressure supplemented by FBS and GM-CSF in the semisolid medium were assayed by clonogenic CFU assay. The effect of increasing doses of VPA on adult $CD34^+$ was analysed with respect to percentage of $CD34^+$ cells after 10 days in methylcellulose. In spite of the higher differentiation pressure in a semisolid medium supplemented with FBS and Granulocyte /macrophage colony-stimulating factor (GM-CSF), 150 µg/mL of VPA increased the percentage of $CD34^+$ cells from 0.5% to more than 21% (Fig. *8B*).

Lastly, the effect of increasing doses of VPA on umbilical cord blood $CD34^+$ cells, which were more enriched in the early stem cell fraction ($CD34^+/CD38^-$) than BM cells (De Felice*, et al* 2005, Mayani and Lansdorp 1998) was analysed through CFU assay. Umbilical cord blood $CD34^+$ cells harvested and analysed after 10 days of culture in semisolid medium with respect to number of $CD34^+$ cells. VPA augmented the number of $CD34^+$ cells in a dose-dependent manner more than 1 log with respect to untreated cells (Fig. *8C*).

These data clearly indicate that VPA does not induce differentiation, but proliferation in HSC.

4.1.2 Treatment with VPA Increases CFU and the Replating Efficiency of Murine HSCs

To compare the effect of VPA on HSC with the known enhancing effect of all-trans retinoic acid (t-RA) on colony formation potential of murine HSCs (Collins 2002, Purton*, et al* 1999), the investigation was extended to murine $Sca1^+/lin^-$ HSCs. Thus, the effect of VPA (150 µg/mL) on the replating efficiency of $Sca1^+/lin^-$ HSC was compared with that of t-RA (1 µmol/L) in the semisolid medium.

VPA did not only enhance the number of colony-forming units (CFU;Fig.*9A*) but also allowed a second plating with a constant number of CFU, in contrast to that of t- RA which enhances only replating efficiency (Fig.9*A*). VPA-treated cells did not differentiate as shown by the high levels of the stem cell markers Sca1 and c-Kit (Fig.*9B).*

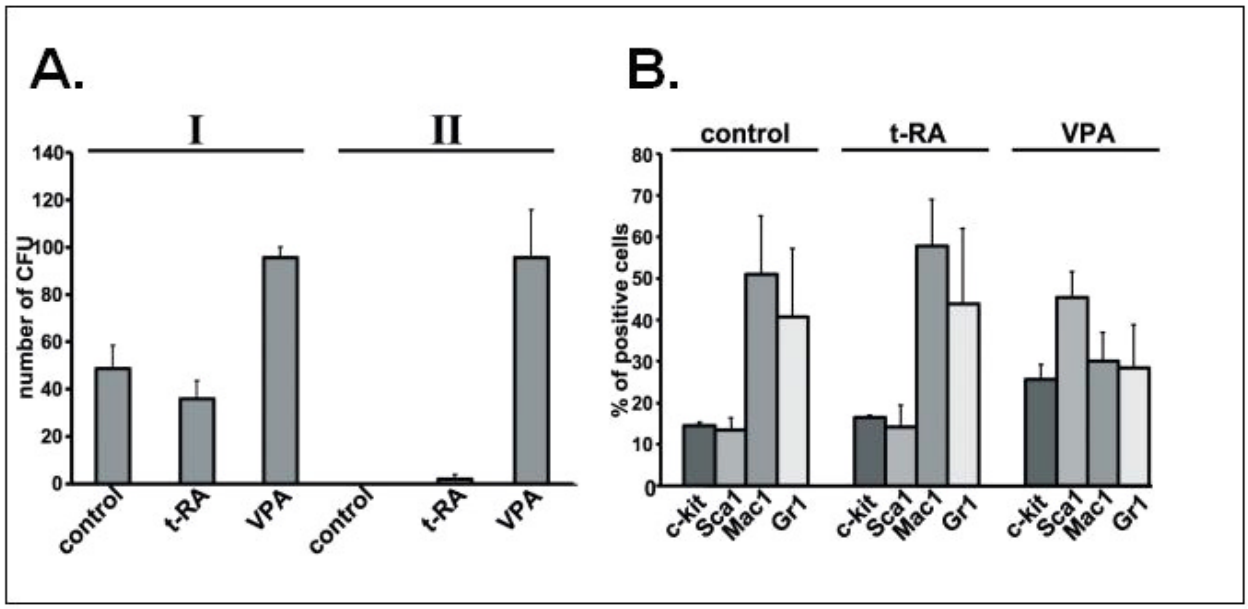

Fig.9. Colony Formation of Sca^+/lin^- HSC exposed to VPA or t-RA. ***A***, replating efficiency of murine Sca^+/lin^- HSC exposed to VPA (150 µg/mL). Reported are numbers of platings (*I*, *II*) and CFU. ***B***, differentiation of Sca^+/lin^- HSC on exposure to VPA (150 µg/mL) cultured in semisolid medium for 10 days; c-Kit as well as Sca1 were used as stem cell markers and Gr1 and Mac1 as myeloid differentiation markers.

In brief, VPA prevents murine HSCs from differentiation, increases proliferation as well as replating potential of murine HSCs.

4.1.3 VPA Increases Self-renewal of the Murine HSCs

In order to figure out whether the effect of VPA on HSC *in vitro* is related to an increase of their potential for self-renewal, the effect on VPA on the generation of CFU-S on day 12 from cultured Sca^+/lin^- HSCs was investigated. Murine Sca^+/lin^- HSCs were treated for 2 days with t-RA or with VPA and inoculated into lethally irradiated recipient mice. At day 12 the spleen colony-forming units (CFU-S12) were analyzed. VPA -treated HSC gave origin to a higher number of CFU-S D12 as compared with that of control and t-RA- treated HSC as revealed by differences in the spleen size (Fig.*10A*). Exposure to VPA led to a higher percentage of c-Kit and Sca1-expressing cells in the CFU-S D12 as compared with that of control (Fig.*10B*).

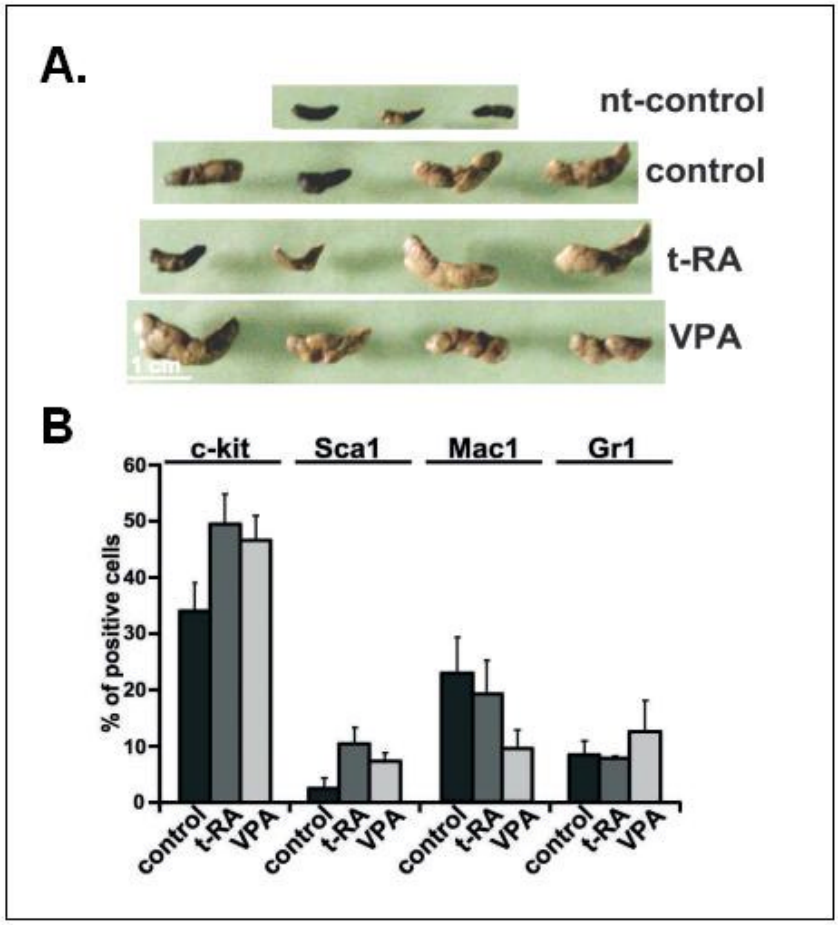

Figure 10. ***A,*** CFU-S D12 Assay of Sca^+/lin^- HSC exposed to VPA or t-RA for 2 days *in vitro*. *nt-controls,* not transplanted recipients; *control,* untreated Sca^+/lin^- HSC; *t-RA,* t-RA treated Sca^+/lin^- HSC; *valproic acid,* valproic acid treated Sca^+/lin^- HSC. Shown are the spleens from one of two experiments which yielded similar results. ***B,*** analysis of surface marker expression in the CFU-S Sca^+/lin^-HSC exposed to VPA or t-RA. Sca1 and c-Kit: stem cell markers; Mac1 and Gr1: myeloid differentiation markers.

CFU-S cells are far more differentiated than primitive HSCs and transient repopulation CFU-S assay, which is limited to myeloid cell types, often fail to predict primitive function HSCs that repopulate both the lymphoid and myeloid systems. Hence, the effect of VPA on the more primitive ST-HSCs than CFU-S cells was examined through competitive repopulation assay. Murine Ly5.1 Sca^+/lin^- HSCs were exposed to VPA or t-RA for 2 days *in vitro*. Then, Untreated or VPA-, t-RA-treated Ly5.1 Sca^+/lin^- HSCs together with fresh normal Ly5.2 bone marrow cells were inoculated into lethally irradiated Ly5.2 recipients and analyzed 12 weeks after the transplantation. FACS analysis, using antibodies specific for the Ly5.2 (host) and

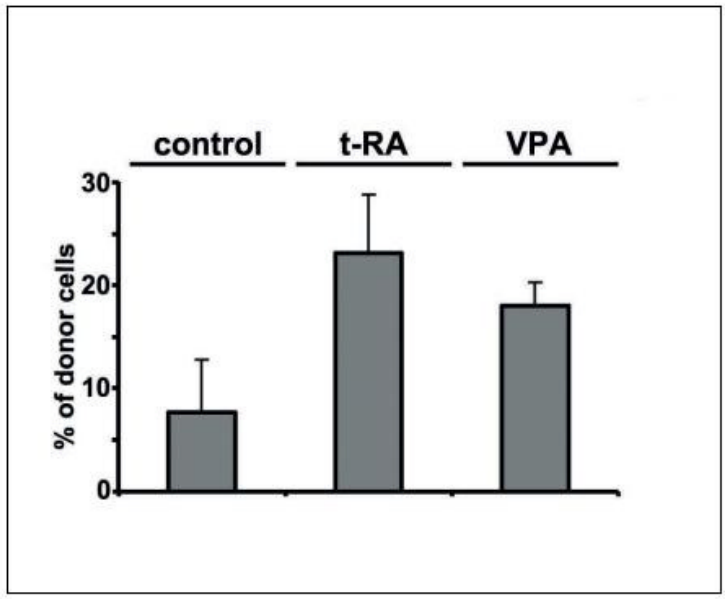

Figure 11. The effect of VPA and t-RA on Primitive Short-term Repopulating Hematopoietic Stem Cells (ST-HSCs) of Sca^+/lin^- HSC exposed to valproic acid or t-RA for 2 days *in vitro*; Competitive Repopulation Assay: donor cells are Ly5.1-positive cells in comparison with Ly5.2 recipient hematopoietic cells. *Columns,* mean (6 mice/group); *bars,* SD. Given is one experiment of two that yielded similar results.

Ly5.1 (donor) epitopes enabled the quantification of the percentage of residual host and donor Ly5.2 (untreated) versus Ly5.1 donor (treated) cells in the mononuclear bone marrow cells.

As compared with the untreated controls (8%), exposure to valproic acid increased the short-term competitive repopulation potential (≤ 4 months) of HSCs (18%) but to a lower extent than t-RA (23%; Fig.*11*).

To sum up, VPA increase the number of CFU-S D12 and self-renewal or generation of murine primitive ST-HSCs.

4.1.4 VPA Accelerates Cell Cycle Progression of hematopoietic stem cells and down-regulates $p21^{cip-1/waf-1}$.

Following the proliferation and self-renewal analysis of murine HSC, a cell cycle analysis was performed in order to investigate the effect of VPA on cell cycle progression of the HSCs. The cell cycle analysis was assessed using $Sca1^+/lin^-$ HSC cultured 7 days in semisolid medium in the presence or absence of 150 µg/mL valproic acid. In contrast to t-RA, VPA increased the percentage of the cells in S phase (23% and 38%, respectively) as compared with that of untreated control cells (20%) with a concomitant reduction of cells in G_1 phase (Fig.*12A*).

To investigate the effect of VPA on cell cycle regulators in the hematopoietic cascade, the expression of the cyclin-dependent kinase inhibitors (CDKIs) $p21^{cip-1/waf-1}$ (p21), which governs the entry of HSC into cell cycle and possesses a differentiation stage –specific function in HSCs was examined. Murine $Sca1^+/lin^-$ HSC and human $CD34^+$ cells were cultured in semisolid medium in the presence of VPA (150 µg/mL) for 7 days and for 2 days, respectively and analysed for p21 expression by western blotting.

In contrast to t-RA, VPA reduced the expression level of p21 in both human $CD34^+$ HSC (at day 2) and murine $Sca1^+/lin^-$ HSC even at day 7 (Fig. *12B,C*).

In conclusion, these data indicate that VPA accelerates cell cycle progression of HSC accompanied by a down-regulation of p21.

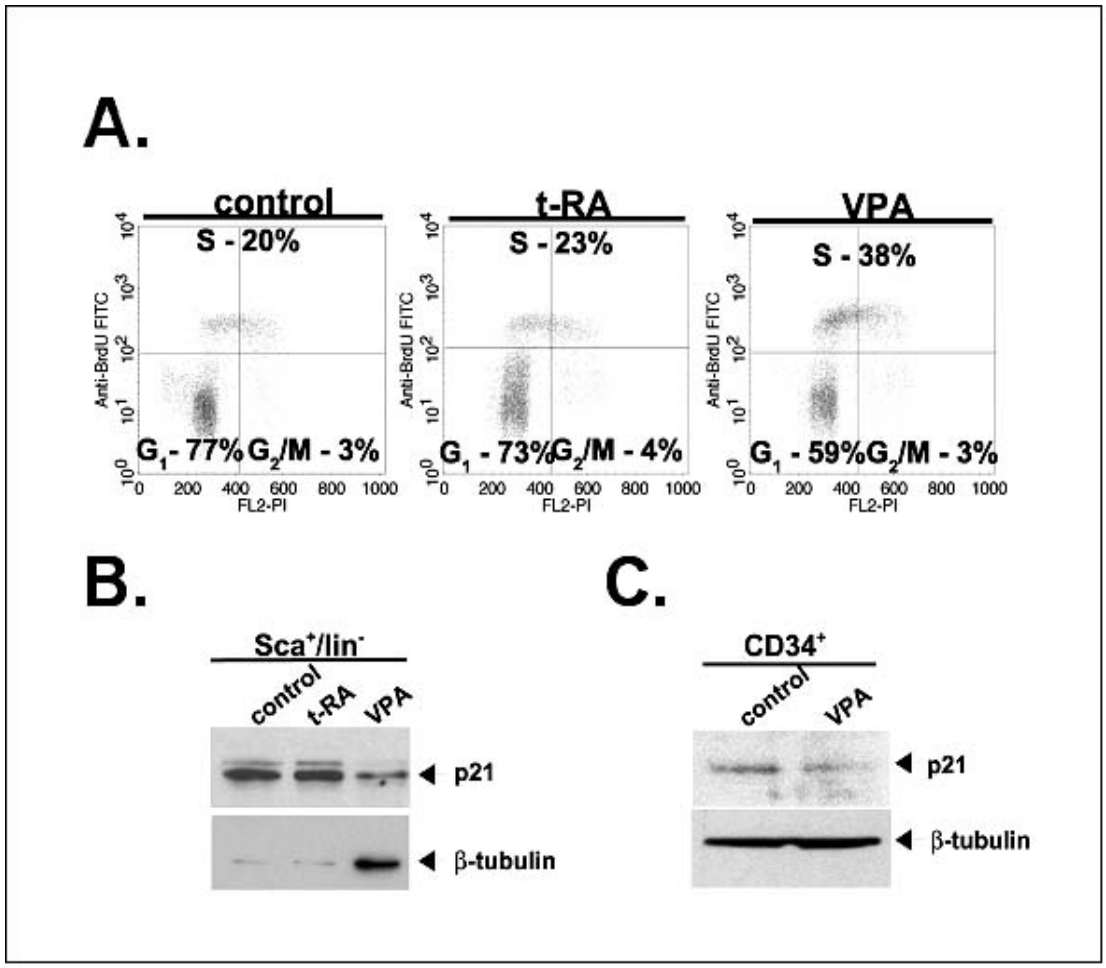

Figure 12. Cell Cycle Progression of Sca^+/lin^- HSC exposed to VPA (150 µg/mL) and t-RA (1 µmol/L). ***A,*** propidium iodide staining and BrdUrd incorporation of Sca^+/lin^- HSC on exposure to valproic acid (150 µg/mL) and t-RA (1 µmol/L). The percentages of cells in G_1, S, and G_2-M are given in the respective gates. One representative experiment of three is given. ***B,*** regulation of $p21^{cip\text{-}1/waf\text{-}1}$ in Sca^+/lin^- HSC on exposure to VPA (150 µg/mL) and t-RA (1 µmol/L). Assessment of ß-tubulin was used as loading control. ***C,*** regulation of $p21^{cip\text{-}1/waf\text{-}1}$ in $CD34^+$ HSC on exposure to valproic acid (150 µg/mL). ß-tubulin staining serves as loading control.

4.1.5 VPA Inhibits GSK3ß and Up-regulates HoxB4 in HSCs.

One of the key components in Wnt signalling pathway is GSK3_ inhibition of which leads to stabilization of *ß*-catenin and finally self-renewal of HSC. Recently, VPA has been demonstrated to exert an inhibitory activity on GSK3_ by phoshorylation on Ser9 and to stimulate AKT in human neuoroblastoma cells (Su*, et al* 2004). To clarify the mechanisms by which valproic acid induces proliferation and self-renewal of HSC, the effect of VPA on GSK3ß expression and phoshorylation were investigated in both human and murine HSC as well as human acute myeloid leukemia KG-1 cells through western blotting. KG-1 cells, express the CD34 antigen characteristic of the hematopoietic stem cells, were cultured under conditions (Xvivo10 medium) that about 80% of cell population were CD34+/CD38- (data not shown).

Exposure to VPA induced a strong modification of GSK3ß in bone marrow $CD34^+$ cells at 48 hours (Fig. *13A)* as well as in the murine $Sca1^+/lin^-$ at day 7 (Fig. *13B*). In fact, GSK3ß was Ser9-phosphorylated in HSC on exposure to VPA but not to t-RA (Fig.*13B*) indicating an inhibition of GSK3ß. This effect was also observed in KG-1 cells (Fig. *13C*).

Owing to the fact that GSK3ß is known to be Ser9-phosphorylated by activated Akt (Jope and Johnson 2004, Doble, *et al* 2003), the activation of Akt was also investigated in both HSC and KG-1 cells. In $CD34^+$, Akt was activated after 48 hours (Fig. *13D*), whereas in $Sca1^+/lin^-$ Akt was not activated by VPA exposure at day 7 (Fig. *13E*). This is most likely due to the late time point of analysis as shown in KG-1 cells in which activated Akt returned to control levels after 96 hours on exposure to VPA (Fig.*13F*)

Furthermore, Ser9-phosphorylated GSK3ß is known to stabilize ß-catenin and then lead to self-renewal of HSC. After it has been shown that GSK3ß is inhibited by Ser-9 phosphorylation by exposure to VPA, the expression of ß-catenin was also examined by western blotting. Given the fact that in both $CD34^+$ and $Sca1^+/lin^-$ endogenous ß-catenin was hardly detectable using a variety of antibodies (Fig. *13E.* and data not shown), up-regulation

of ß-catenin on exposure to VPA was confirmed in KG-1 cells, which are >80% $CD34^+/CD38^-$ (Fig. *13F*).

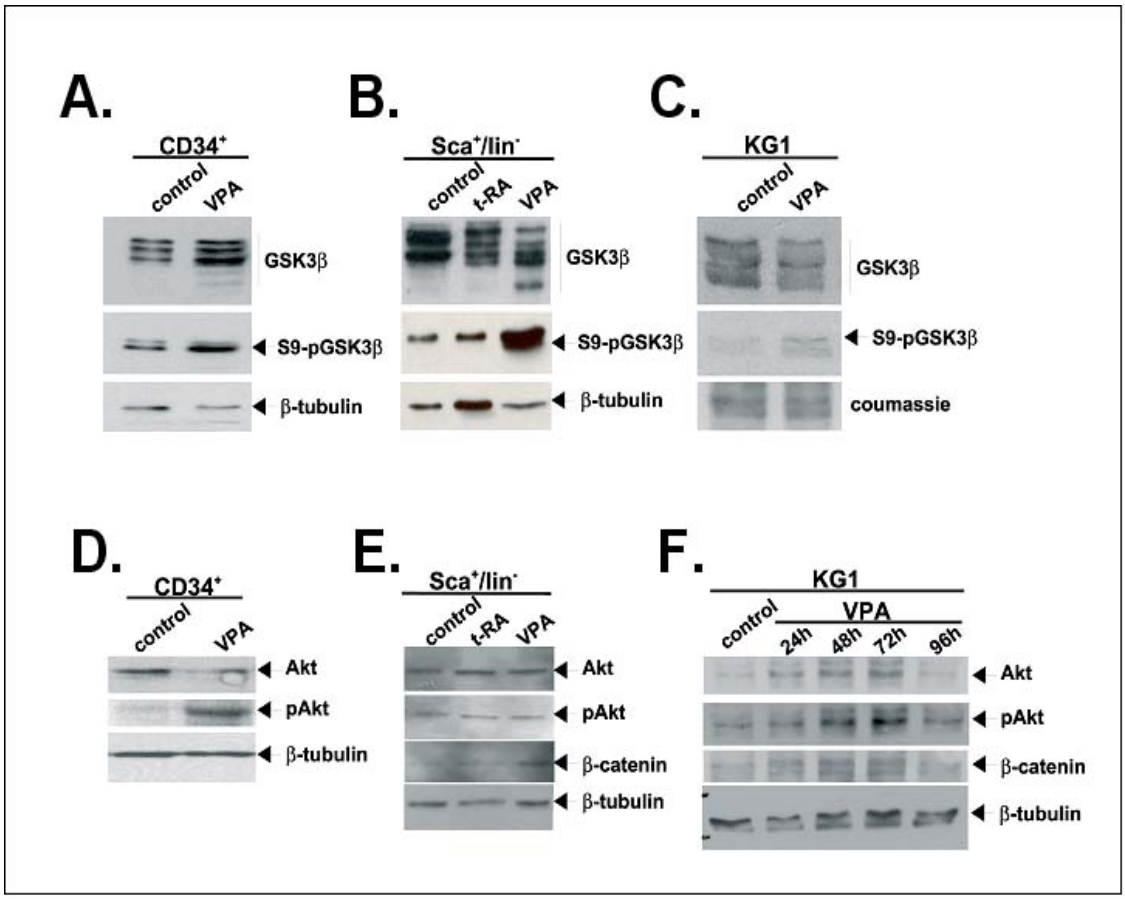

Figure 13. Regulation of Wnt-pathway components ***A***, influence of VPA (150 µg/mL) on the expression as well as the Ser9 phosphorylation of GSK3ß in $CD34^+$at 48 h; ß-tubulin: loading control. ***B***, influence of VPA (150 µg/mL) on the expression as well as the Ser9 phosphorylation of GSK3ß in Sca^+/lin^- HSC at day 7 of culture in semisolid medium. ß-tubulin: loading control. ***C***, influence of VPA (150 µg/mL) on the expression as well as the Ser9 phosphorylation of GSK3ß in $CD34^+/CD38^-$ KG-1 cells on exposure to VPA (150 µg/mL) at 48 h; Coomassie: loading control. ***D***, influence of VPA (150 µg/mL) on the activation of Akt (direct proportion between p-Akt and Akt) in $CD34^+$at 48 hours; ß-tubulin: loading control. ***E***, influence of valproic acid (150 µg/mL) on the activation of Akt and on the expression level of ß-catenin in Sca^+/lin^- HSC at day 7 of culture in semisolid medium; ß-tubulin: loading control. ***F***, influence of VPA (150 µg/mL) on the activation of Akt and on the expression level of ß-catenin in $CD34^+/CD38^-$ KG-1 cells on exposure to VPA (150 µg/mL) at 48 hours; ß-tubulin: loading control.

HoxB4, a key factor in the regulation of the self-renewal and the proliferation of HSC, is one of the target genes of ß-catenin (Reya, *et al* 2003). The expression level of HoxB4 in human bone marrow $CD34^+$ cells was assessed by quantitative real-time PCR after 48 hours exposure to VPA. VPA increased the number of HoxB4 transcripts about 3.8 times in these cells (Fig. 14*A*). In $Sca1^+/lin^-$ murine HSC the expression level of HoxB4 was measured by Western blotting at day 7 of treatment. In contrast to t-RA, which seemed to reduce the amount of HoxB4, valproic acid increased the expression of HoxB4 (Fig.14*B*).

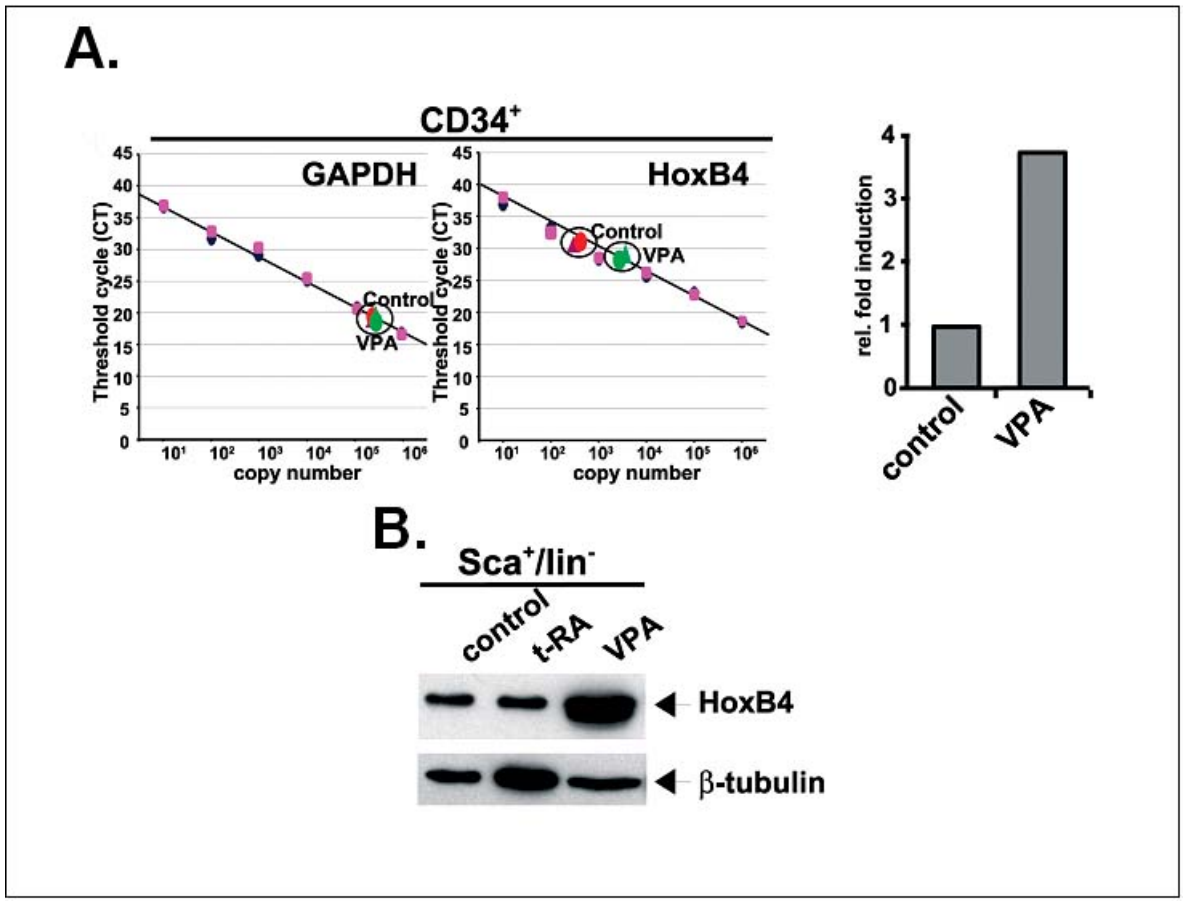

Fig 14..HoxB4 Expression. ***A,*** induction of HoxB4 by VPA (150 µg/mL) in $CD34^+$ HSC in comparison with untreated cells (*Control*) monitored by real-time PCR; number of copies defined by the comparison to a plasmid-based standard curve. GAPDH expression was done as reference. The fold change between untreated controls and VPA-treated cells is represented graphically. Representative experiment of three yielding similar results. ***B***, induction of HoxB4 by VPA (150 µg/mL) in Sca^+/lin^- HSC by Western blotting; ß-tubulin: loading control

These data indicate that VPA influences signaling pathways relevant for both self-renewal and proliferation of HSC by the inhibition of GSK3ß and upregulation of HoxB4.

4.2 Effect of t-RA/VPA Combination Treatment on HSCs

All-trans retinoic acid (t-RA) is predominantly known for its differentiating effects, being a potent inducer of terminal differentiation of malignant promyelocytes (Huang*, et al* 1988). However, it has been also shown that t-RA has different effects on cultured hematopoietic stem cells depending on their maturational state (Collins 2002, Purton, *et al* 1999). It was indicated that t-RA enhanced the maintenance and/or generation of short-and long-term repopulating cells from lin^- $ckit^+$ $sca1^+$ hematopoietic precursors (Purton*, et al* 2000).

It was observed that VPA as a single agent stimulates proliferation and self-renewal of HSC (Bug, *et al* 2005a). In order to figure out whether the effect of VPA on murine HSC could be further enhanced by the combination with t-RA, the effect of combination of t-RA/VPA (1 µmol/L, 150 µg/mL, respectively) on HSCs were compared with that of valproic acid (150 µg/mL) through colony forming unit potential with serial replating, colony forming unit spleen assay (CFU-S) and competitive repopulating assay.

4.2.1 Treatment of VPA in Combination with t-RA further Increase Maintenance of HSC up to the 6^{th} replating.

Murine $sca1^+/lin^-$ HSCs were plated in methyl cellulose. Colony forming units were counted and colony pictures were taken at day 10 after plating, cells washed out from the methylcellulose for subsequent analysis of surface marker expression, and replated until the end of the colony formation under the same conditions.

Combination of VPA with t-RA did not enhance the colony forming units as indicated by colony counts in contrast to VPA, on the other hand, increased replating efficiency up to the 6^{th} cycle as opposed to other treatment groups (Fig.15 *A).* In other words, t-RA maintained the incidence of HSCs until the 6^{th} replating in comparison to other treatment groups. t-RA treatment enabled cells to give colonies up to 4th replating, whereas, VPA treatment

maintained the colony formation for only up to the 3rd replating in comparison to untreated cells which did not give rise to any colonies after the 1st replating.

Combination of VPA with t-RA resulted in small homogeneous blast colonies of the progenitor cells similar to that of t-RA treated cells.

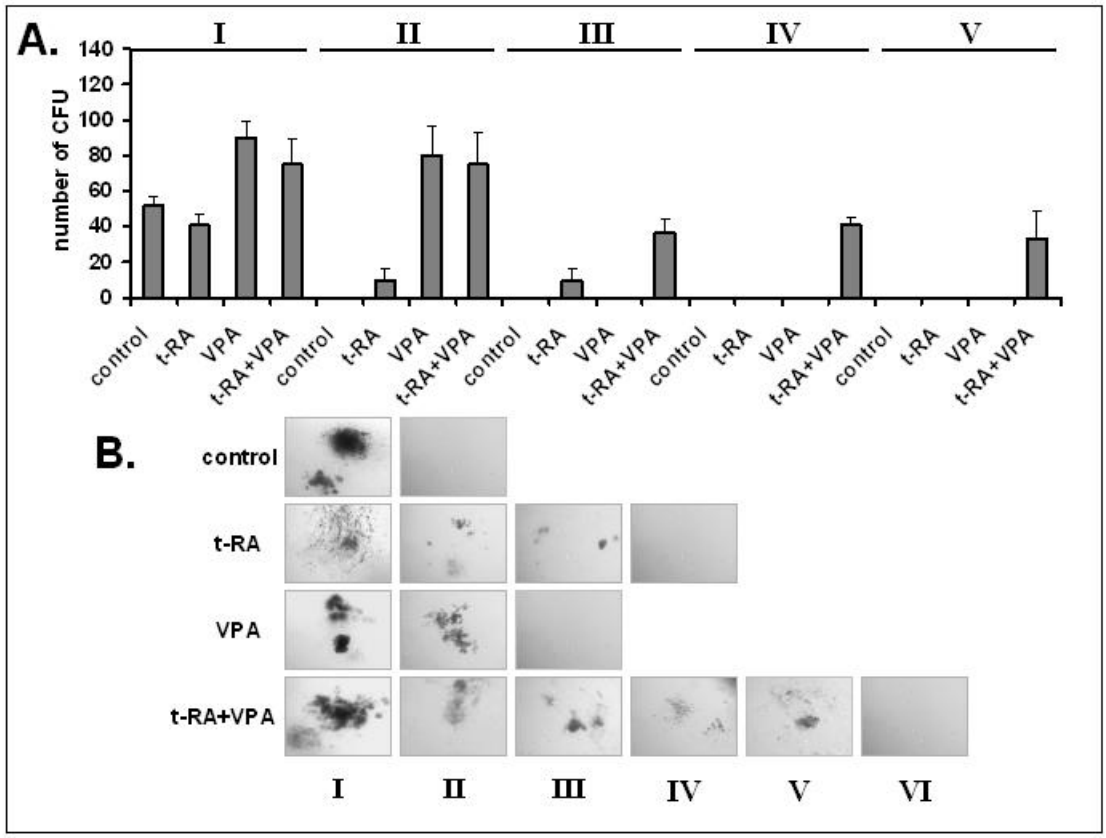

Fig.15 Colony Formation of Sca^+/lin^- HSC exposed to combination of t-RA/VPA, valproic acid or t-RA. ***A***, Replating efficiency of murine Sca^+/lin^- HSC on exposure to t-RA/VPA (1 µmol/L /150 µg/mL, respectively). Reported are numbers of platings (*I*, *II, III, IV,V*). ***B***,colonie pictures of HSCs exposed to combination or as a sinle agent t-RA, VPA from each passage.

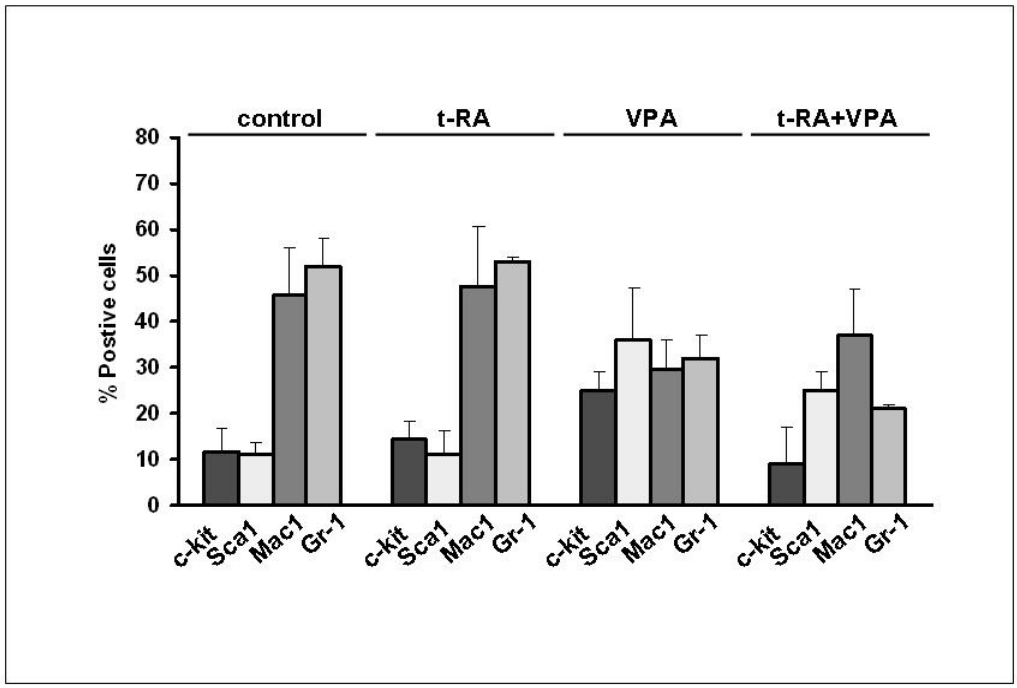

Fig 16. Differentiation of Sca^{+}/lin^{-} HSC upon exposure to t-RA/VPA (1 µmol/L //150 µg/mL) cultured in semisolid medium for 10 days; c-Kit as well as Sca1 were used as stem cell markers and Gr1 and Mac1 as myeloid differentiation markers.

To delineate the enhancement effect of replating efficiency up to the 6th serial replating *in vitro,* murine $Sca1^{+}/lin^{-}$ HSC treated for 2 days with t-RA, VPA, or t-RA/VPA were inoculated into lethally irradiated recipient mice.

At day 12 or week 12, the spleen colony-forming units (CFU-S) or competitive repopulation efficiencies between VPA alone and combination of t-RA/VPA were compared. According to the both transient and short-term competitive repopulation analyses, combination of t-RA/VPA did not further enhance self-renewal of HSCs with respect to VPA as a single agent. (data not shown).

To sum up, combination of t-RA with VPA increase the replating efficiency up to the 6th replating meaning that maintains the HSCs at least in *vitro* up to 6th replating, but does not further enhance self-renewal potential of HSCs as indicated by repopulation analyses.

4.3 Comparison of LAQ824 and VPA effects on HSCs

LAQ-824, a hydroxamate-based HDAC inhibitor like TSA, has been shown to inhibit tumor cell growth at submicromolar concentrations and induces apoptosis in several types of leukemia including acute myeloid leukemia, acute lymphoblastic leukemia and chronic myeloid leukemia cells (Romanski*, et al* 2004, Weisberg*, et al* 2004).

In order to confirm the data of VPA on HSC and to investigate whether other classes of HDIs are also able to influence the fate of early HSCs, the effect of LAQ824 on HSCs was also investigated and compared with VPA either alone or in combination with t-RA with respect to proliferation and cell cycle progression.

4.3.1 LAQ824 Enhances CFU and Replating Efficiency of HSCs

To assess colony formation potential, 5000 murine Sca1^{+}/lin^{-} HSC were plated in methyl cellulose. Colony forming units were counted and colony pictures were taken at day 10 after plating, cells washed out from the methylcellulose for subsequent analysis of surface marker expression, and replated until the end of the colony formation under the same conditions.

According to the CFU assay, LAQ824 did not only enhance the number of CFU but also enables HSC to form colonies for the second passage similar to VPA. After the 2nd passage, both LAQ824- and VPA-treated HSCs failed to form colonies (Figure.17*A*) as shown by colony count and pictures. Besides, addition of t-RA into LAQ824- treated HSCs enabled cells to form colonies for one more cycle in comparison to LAQ824 alone. On the other hand, the t-RA/LAQ824 combination did not maintain hematopoietic precursors *in vitro* after the 3rd replating in contrast to t-RA/VPA combination treatment, which further enhanced the maintenance up to the 6th replating.

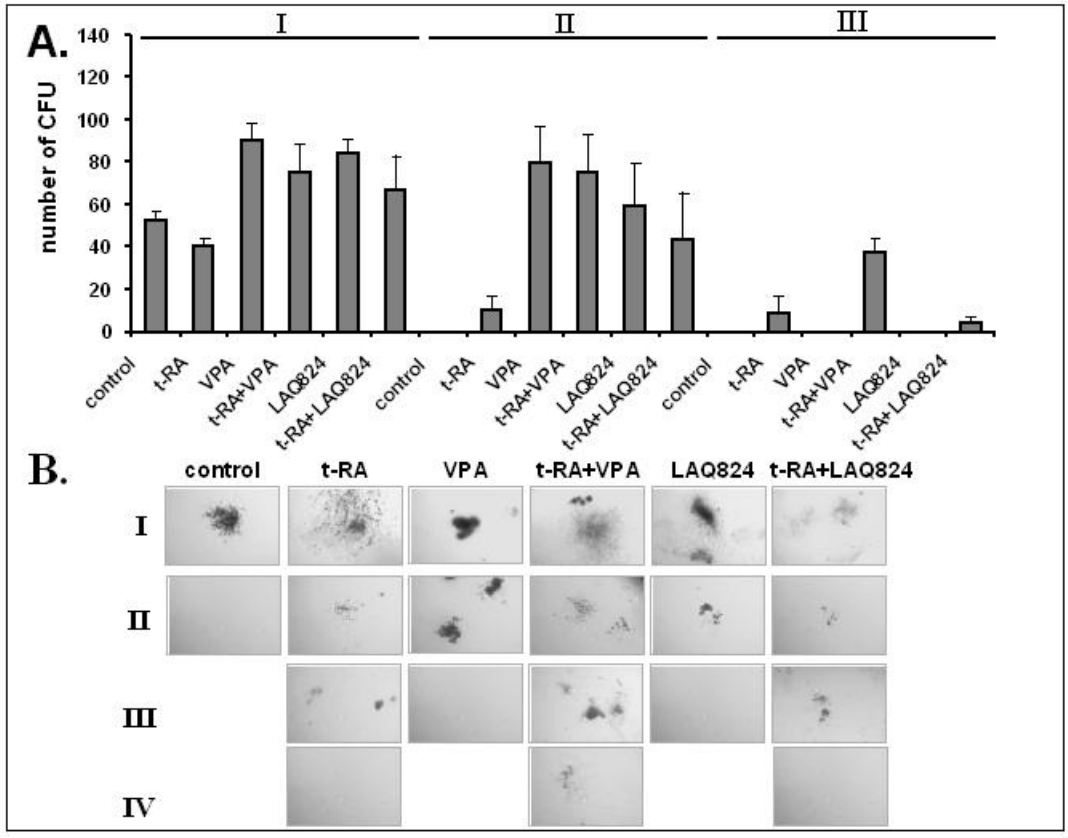

Fig. 17. Colony Formation of Sca^{+}/lin^{-} HSC exposed to LAQ824 alone or a combination of t-RA/LAQ824. ***A,*** replating efficiency of murine Sca^{+}/lin^{-} HSC on exposure to t-RA/LAQ824 (1 µmol/L /10 nmol/L)Reported are numbers of platings (*I*, *II, III, IV*) from the CFU assay. ***B***,colony pictures of HSCs exposed to LAQ824, t-RA and VPA as a single agents or combinations.

In spite of the fact that LAQ824 and VPA showed a similar pattern for CFU number and formation, the treated cells differed in the cell surface marker. LAQ824 treatment slightly increased the percentage of c-kit and Mac1-expressing cells, whereas, VPA treatment highly increased the percentage of Sca1 and c-kit –expressing cells with respect to untreated control cells. Combination of LAQ824/t-RA treatment slightly decreased the percentage of c-kit expressing cells, whereas increased Gr1-expressing cells with respect to LAQ824 treatment.

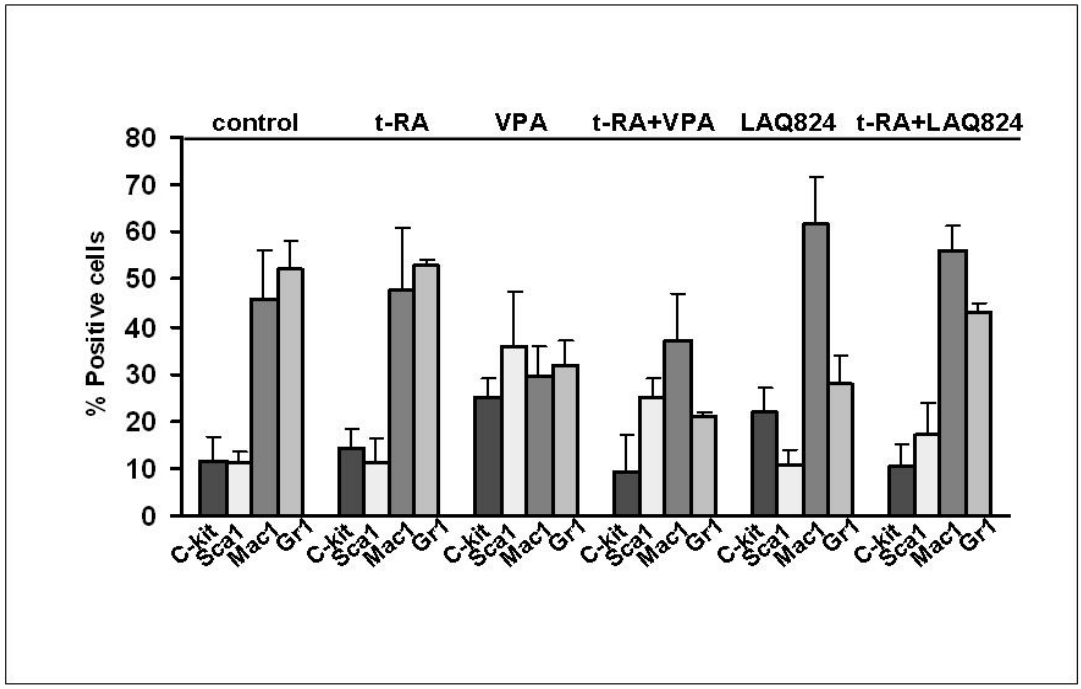

Fig 18. Differentiation of Sca^{+}/lin^{-} HSC upon exposure to LAQ824 alone or t-RA/LAQ824 (1 µMol/L /10 nMol/L) cultured in semisolid medium for 10 days; c-Kit as well as Sca1 were used as stem cell markers and Gr1 and Mac1 as myeloid differentiation markers.

These data suggest that LAQ824 enhances the proliferation and replating efficiency of HSC similar to VPA. On the other hand, this effect might result from the effect of LAQ824 on more mature HSCs with regard to HSCs targeted by VPA as shown by the different cell surface marker expressing cells.

4.3.2 Effect of LAQ824 on Cell Cycle Progression of HSCs.

To further evaluate and compare the effect of LAQ824 on HSCs with that of VPA, cell cycle progression of Sca1+lin- cells exposed to both LAQ824 and VPA was analysed after 2 days in liquid culture in the presence of SCF, IL3 and IL6.

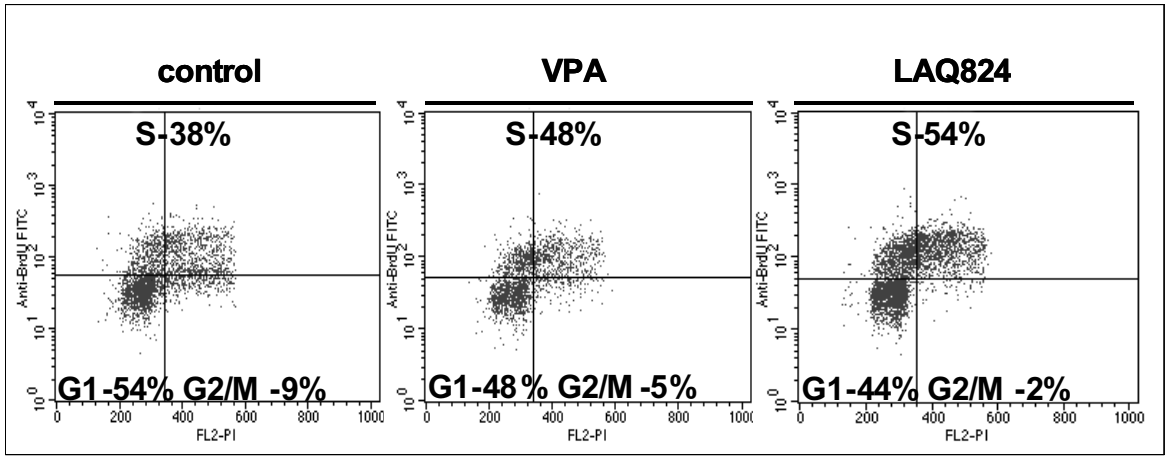

Fig.19. Cell Cycle Progression of Sca^{+}/lin^{-} HSC upon exposure to LAQ824 (10 nmol/L) or VPA(150 µg/mL) exposure. Propidium iodide staining and BrdUrd incorporation of Sca^{+}/lin^{-} HSC on exposure to VPA (150 µg/mL) LAQ824 (10 nmol/L) .The percentages of cells in G_1, S, and G_2-M are given in the respective gates.

As shown in the Figure.19*,* lAQ824 like VPA increased the percentage of the cells in the S phase of the cell cycle in comparison to untreated cells. There were no differences by the addition of t-RA into both LAQ824 and VPA with respect to single VPA or LAQ824 treatment on cell cycle progression (data not shown). According to this data, LAQ824 seems to trigger cell cycle progression of HSCs similar to VPA.

5 Discussion

A significant role of histone deacetylases (HDAC) in hematopoiesis is highlighted by findings that aberrant chromatin modeling and interaction of acute myeloid leukaemia (AML)- fusions with co-repressor/HDAC complexes plays a key role in the pathogenesis of AMLs (Grignani, *et al* 1998, Minucci and Pelicci 1999). Therefore, HDAC inhibitors (HDIs) received considerable interest due to ability to promote differentiation and/or apoptosis in leukemic blasts regardless of their primary genetic lesion, an effect achieved either alone or in combination with differentiating agents, such as all-trans retinoic acid (t-RA) (Ferrara, *et al* 2001, Gottlicher, *et al* 2001).

VPA is one of the most promising HDI, raising the hope that a successful differentiation therapy of AML might be possible according to recent studies. Contrary to recent opinion, novel effects of VPA on hematopoietic stem cells (HSCs) were reported in the present study, requesting reconsideration of the future role of VPA as well as other HDIs in AML therapy.

As opposed to recent report (Gottlicher, *et al* 2001) which shows that valproic acid induces differentiation and/or apoptosis of carcinoma, PML/RARα transformed hematopoietic progenitor cells and leukemic blasts from AML patients , VPA blocks differentiation of normal HSCs as revealed by the fact that it increases the fraction of HSCs rather than differentiated cells. In other words, VPA does not induce differentiation, but proliferation in HSCs in comparison to its effect on leukemic blasts. Furthermore, this novel effect is also contrary to the effects of other HDIs which induce differentiation in leukemic blasts in *vitro* and in *vivo* (Ferrara, *et al* 2001, Warell, *et al* 1998, Grignani, *et al* 1998, Minucci and Pelicci 1999)). In the study by Gottlicher et al, it has been reported that VPA has no toxic effect on normal hematopoietic progenitor cells (lin- cells) as revealed by colony formation and viability of the progenitor cells. On the other hand, the effect of VPA on primitive HSCs was not further investigated.

The data observed in the present study compatible with the hypothesis that favorable effects of the combination treatment with t-RA/VPA in patients with advanced acute myeloid leukemia could result from an enhancement of nonleukemic myelopoiesis and the suppression of malignant hematopoiesis rather than enforced differentiation of the leukemic blast.

Present result also consistent with the data indicates that VPA significantly enhances the effect of early acting cytokines on the amplification of human HSC and selectively increases the early human HSC compartment (De Felice*, et al* 2005). In the present study; however, VPA increases the percentage and number of human HSCs not only in the presence early acting cytokines, but also in the presence of the external milieu that favors differentiation. These effects were confirmed in HSCs from BM and CB, which contains relatively higher percentage of the early fraction ($CD34^{+}/CD38^{-}$) of HSCs with respect to BM. In another study, the combination of HDI TSA with DNA hypomethylating agent 5AzaD in the presence of cytokine cocktail favoring differentiation has been also reported to result in significant retention of human stem cell phenotype and number. However, HSCs exposed to cytokines and to either TSA alone or 5AzaD alone had a more limited expansion of primitive compartment of HSCs with regard to combination of TSA/5AzaD (Milhem*, et al* 2004). TSA is a hydroxamic based HDI, which is functionally and structurally different from VPA.

Furthermore, VPA enhances CFU, percentage of stem cell markers-expressing cell and replating efficiency with a constant number of CFU of murine HSCs with respect to t-RA which only enhances replating efficiency, suggesting proliferation and self-renewal potential of murine HSCs exposed to VPA. Moreover, addition of t-RA into VPA treatment shows a synergistic effect on replating efficiency of HSCs, meaning the maintenance of HSC pool at least in long-term culture conditions (Guel*, et al* 2003). Based on the recent reports, It is known that t-RA slows proliferation of primitive HSCs, whereas enhances ex vivo maintenance/self-renewal of primitive hematopoietic stem cells (Collins 2002, Purton*, et al* 1999)

Importantly, LAQ824, hydroxamic acid HDI, has been also demonstrated to have similar effect of VPA on murine HSCs as demonstrated by an increase in both CFU and replating efficiency. On the other hand, there are differences of the phenotype of HSCs between LAQ824- and VPA- treated cells as well as decrease in the maintenance of HSCs exposed to LAQ824/t-RA (unpublished data). These data suggest that these differences between VPA- and LAQ824-treated HSCs might result from targeting different types of HSCs by these two distinct types of HDIs. HSCs are heterogeneous and include long-term HSC, short-term HSC and multipotent progenitors (MPP) which can be identified with respect to surface marker expression and functional readout assays (Christensen and Weissman 2001, Morrison and Weissman 1994). Thus, the difference observed between the distinct two distinct types of HDIs might be further delineated by surface marker expression and functional stem cell assays *in vivo*.

According though the present data, VPA is also confirmed to increase self-renewal of Murine HSCs as shown by CFU-S D12 and competitive repopulation assay similar to t-RA (Collins 2002, Purton*, et al* 2000). Enhanced production of CFU-S D12 in the VPA-treated cultures suggests that VPA enhances transient repopulating ability due to the direct effect on hematopoietic progenitor cells or more primitive short-term repopulating cells (ST-HSC) which could also result in enhanced CFU-S production but can not be detected by CFU-S (Harrison 1980, Harrison*, et al* 1978, Purton*, et al* 2000, van der Loo*, et al* 1994). According to the short-term competitive repopulation analysis ($\leq$ 4 months), functional assay of more primitive cells (ST-HSCs) than CFU-S, VPA increases the short-term competitive repopulation of HSCs similar to t-RA, suggesting the self-renewal of primitive HSCs. However, this effect of VPA on the ST-HSCs could even result from self-renewal of the most primitive long-term HSCs (LT-HSCs). These possibilities could be distinguished by long-term competitive reconstitution analysis ($\geq$ 6 months) which would be the topic of the further research.

Conversely, combination of t-RA/VPA did not give any additive or synergistic effect on the self-renewal of HSCs. But, combination of t-RA/VPA most probably maintains primitive HSC pool like t-RA (Collins 2002) rather than expands in comparison to VPA according to *in vitro* results obtained by CFU serial replating and phenotype analysis in this study. This synergistic effect of t-RA/VPA on replating efficiency meaning maintenance of HSC *in vitro* might be further delineated by serial CFU-S or competitive repopulating assay as short- and long term maintenance *in vivo*, which may be also the one topic of further research.

An asymmetrical induction of apoptosis in the HSC population by valproic acid was excluded by the evidence that valproic acid accelerates the cycle progression of HSC accompanied by a down-regulation of $p21^{cip\text{-}1/waf\text{-}1}$. However, this effect is in contrast to the effect of valproic acid as well as other HDIs which increase $p21^{cip\text{-}1/waf\text{-}1}$ expression in cell line models of acute leukemia followed by differentiation or apoptosis (Gurvich*, et al* 2004, Romanski*, et al* 2004). Therefore, Valproic acid affects normal hematopoietic stem cells (HSC) in a manner that is distinctly different from that exerted on leukemic blast cells. There is a direct relationship between the differentiation status and the response to HDIs, meaning that very immature cells respond to HDI with a down-regulation of $p21^{cip\text{-}1/waf\text{-}1}$ and cell cycle progression, whereas at a more advanced differentiation stage, cells respond to valproic acid with a up-regulation of $p21^{cip\text{-}1/waf\text{-}1}$ and differentiation or apoptosis. $p21^{cip\text{-}1/waf\text{-}1}$ is one of the cyclin-dependent kinase inibitor (CDKI) which plays critical roles in the regulation of cell cycle kinetics in the hematopoietic cascade and governs the entry of HSC into cell cycle and possesses differentiation stage –specific function in HSCs (Cheng*, et al* 1999, Cheng*, et al* 2000, LaBaer*, et al* 1997, Stier*, et al* 2003). Furthermore, another potent HDI LAQ824 seems to accelerate cell cycle progression similar to VPA (unpublished data). Yet, further studies should be conducted before the last statement about the effect of LAQ824 on HSCs.

It could be hypothesized that VPA increases self-renewal of HSCs by the inhibition of GSK3 β or "transcriptional reprogramming " of these cells through chromatin modeling. But, it is most likely that both of these mechanisms are involved in VPA-induced self-renewal of HSCs.

One of the mechanism by which valporic acid increases self-renewal of HSC might be explained by the inhibition of GSK3β, which is the negative regulator of the Wnt-signalling pathway.

In the present study, VPA has been demonstrated to inhibit GSKβ by phosphorylation on Ser9 as claimed previously (Su*, et al* 2004) Furthermore, GSK3β is phosphorylated by activated Akt as shown both in $CD34^+$ and acute myeloid leukemia (AML) cell line KG1 cells that are >80% $CD34^+/CD38^-$. On the other hand, Akt was not activated by VPA exposure in $Sca1^+/lin^-$ most likely due to the later time point of analysis in this cells with respect to KG-1 as well as $CD34^+$ cells. Akt has been demonstrated to return baseline levels after 96 hrs in VPA-treated KG1 cells. According to the recent studies, GSK3_ is known to be inhibited through phosphorylation on Ser9 by activated Akt (Doble and Woodgett 2003, Jope and Johnson 2004). Moreover, up-regulation of β-catenin on exposure to VPA was confirmed in KG-1 cells. It is known mechanism that The Ser9 phosphorylation of GSK3β reduces its kinase activity on β-catenin enabling it to transcriptionally activate Wnt target genes.

The activation of the Wnt-signaling pathway by valporic acid is also confirmed as demonstrated by the up-regulation of Wnt-target gene as well as transcription factor, *HoxB4.* Evidences suggest that Wnt-signalling pathway is a critical pathway utilized by LT-HSC in self-renewing divisions. (Austin*, et al* 1997, Reya*, et al* 2003, Shizuru*, et al* 2005, Willert*, et al* 2003) Activation of Wnt signalling results in signaling events such that HSC enter the cell cycle with little differentiation out of the LT-HSC pool (Reya *et al* 2003). Furthermore, activation of Wnt-signalling in HSCs induces upregulation of genes such as *hoxb4* (Reya 2003), genes independently implicated in proliferation as well as self- renewal of HSC (Antonchuk *et al*., 2001&2002, Amsellem*, et al* 2003, Krosl, *et al* 2003)

In fact, in the present study, VPA increases self-renewal of murine HSCs as shown in both CFU-S as and competitive repopulation assays, suggesting its self-renewal effect on primitive HSCs similar to t-RA (Purton et al., 2000). However, one should be careful in comparing the result of the studies on humans and on mice. Therefore, investigation of the effect of VPA on

self-renewal and/or engraftment potential of human primitive HSCs using NOD-SCID mice might be useful.

Another mechanism involved in VPA induced self-renewal of HSCs might be a „transcriptional reprogramming" of these cells through chromatin modelling. VPA is known to induce histone acetylation as well as DNA demethylation in a dose-dependent manner (Gottlicher, *et al* 2001, Detich *et al*., 2000). In addition to its potent and specific inhibition of HDAC, VPA is the first therapeutic agent shown to erase DNA methylation patterns in a replication-independent manner by stimulating the accessibility for a demethylase enzyme. (Detich, *et al* 2003).

Modification of chromatin structure is an important factor in determining whether a particular gene is expressed or not. Histone acetylation by HAT is associated with the activation of genomic regions, whereas deacetylation of histone leads to a chromatin conformation resulting in gene silencing. In addition, DNA methylation is associated with the silencing of gene expression through DNA methyl transferases.

Therefore, actual HSC fate decisions may be governed by the expression patterns of transcription factors such as HoxB4 and may be under the control of methylation and deacetylation of chromatin, while commitment or differentation might be triggered and regulated through external regulatory pathways, activated by interactions of HSCs with cytokines or the marrow microenvironment.

The data presented here suggest that VPA can act as transcriptional regulator through chromatin modeling due to its capacity to induce histone acetylation and active DNA demethylation in the self-renewal of primitive HSCs and commitment of more mature progenitor cells into different lineages. Furthermore, it seems that VPA treatment resulted in interruption of the ongoing differentiation process driven by external factors such as cytokines and reprogramming of HSCs fate decision.

During the course of this study another group has reported that human bone marrow $CD34^+$ cells exposed in combination with DNA demethylating agent (5aza 2'deoxycytidine, 5azaD) and HDAC inhibitor trichostatin A (TSA) resulted in a significant expansion of primitive HSCs capable of repopulating immunodeficient mice even in the presence of cytokines that promote differentiation (Milhem, *et al* 2004). In addition, this effect has been reported to be maintained by transcriptional reprogramming of HSCs through both acetylation of histone and DNA demethylation. It is important to point out that the addition of TSA alone to the cultured cells led to more limited expansion of primitive HSCs cell number but not function, whereas the addition of 5AzaD alone preserved and function of primitive human HSCs, an effect significantly increased by the combination of TSA/5AzaD. VPA is a different chromatin modeling agent from TSA as well as 5AzaD with respect to structure and function. However, it has capacity to induce both histone acetylation and DNA hypomethylation and increase repopulation efficiencies of murine HSCs, suggesting its effect on early HSCs due to both acetylation of histone and demetylation of DNA. However, one should be careful in comparing the results of the studies on humans and mice. Therefore, it might be useful to investigate the effect of VPA on primitive HSCs by NOD-SCID assay for human marrow repopulating cells.

Furthermore, another study (De Croce, *et al* 2005) assesing the effect of VPA on human HSC in the presence of early cytokines indicated that VPA enhanced significantly the cytokine-induced ex vivo expansion of early compartment of human HSCs and increased histone H4 acetylation leves at specific regulatory sites on *hoxb4* in human HSC, indicating the potentiality of novel epigenetic approaches to modify HSC fate *in vitro*. However, self-renewal and/or engraftment of long-term repopulating stem cells in animal models were not analyzed.

Moreover, it is important to point out that present study as well as other recent studies discussed in this report support the recent hypothesis (Akashi et al) that maintenance of self-renewal and multipotential capability requires that HSCs express a broad set of transcription factors and wide-open chromatin structure. In other words, the mechanism regulating HSC

fate decision are likely to be alterable and under the contol of chromatin modeling program, which guides transcriptinonal accesssibilty for each hematopoietic stage.

In the present study; thus, a potent HDI VPA, known to induce differentiation or apoptosis in leukemic blasts, has been demonstrated to stimulate the proliferation and self-renewal of primitive HSCs through most likely both inhibition of GSK3β and transcriptional reprogramming of HSCs

This novel effect of VPA on HSCs may have important therapeutic consequences. Among the various forms of leukemia, the myeloid diseases have been best characterized with regard to a stem cell origin. Interestingly, acute myelogenous leukemia (AML) is known to display marked clinical heterogeneity (types M0-M7) according to the French-American-British (FAB) classification system. However, at the stem cell level, there appears to be substantial conservation. A clone of leukemia cells seems to be organized as a hierarchy that originates from a stem cell pool like normal hematopoietic system (Bonnet and Dick, 1997). Conversely, LSCs could also be more restricted progenitor which would have first to reacquire self-renewal capability. But, it is clear that leukemic cells require self-renewal capability to propagate the disease (Passegue, *et al* 2003). This idea suggests similarities in the molecular regulation of both normal and AML stem cells.

Anti-leukemia treatments have historically sought to eliminate proliferating cells. But like normal HSCs, leukemic stem cells are dormant and rarely proliferate, therefore, resistant to such therapies. Valproic acid induced entry of quiescent hematopoietic and leukemic stem cells into the cell cycle could render them more susceptible to conventional chemotherapy, resulting either in a prolonged aplasia (bone marrow failure to produce blood cells after chemotherapy) due to a higher efficiency of the therapy or in a shortened aplasia owing to the enforced proliferation of the normal hematopoiesis. The recent clinical data indicate that exposure to valproic acid increases the response to chemotherapeutic agents (Bug, *et al.* 2005b).

Therefore, the data reported in this study suggest reconsideration of the role of HDIs from desired differentiation inducers to a coadjuvant factors for increasing the response to conventional therapy in AML. However, more extensive knowledge of the effect of chromatin modeling events or players on HSCs is required. As there are many connections between normal and malignant stem cells, these studies will provide a much clearer picture of the leukemic stem cells and thus help to increase the efficiency of not only existing therapeutic approaches but also to initiate novel, biology-based, rational therapeutic approaches for the treatment of leukemia.

6 Summary

Acute myeloid leukemia (AML) is characterized by the accumulation of a large number of abnormal, immature blast cells. Recently, histone deacetylase inhibitors (HDIs), which regulate transcription through modification of chromatin structure, received considerable interest on the ground of their ability to overcome the differentiation block in these leukemic blasts regardless of the primary genetic alteration, an effect achieved either alone or in combination with differentiating agents, such as all-*trans* retinoic acid (t-RA).

Valproic acid (VPA), a potent HDI, is now under clinical evaluation owing to its potent differentiation effect on transformed hematopoietic progenitor cells and leukemic blasts from AML patients. Conversely, in a clinical study by Bug et al., the favorable effects of the combination treatment with t-RA/VPA in advanced acute myeloid leukemia patients were reported to be most likely due to an enhancement of nonleukemic myelopoiesis and the suppression of malignant hematopoiesis rather than enforced differentiation of the leukemic cells. Moreover, there is evidence that the wide-open chromatin structure required for HSCs multipotentiality and the lineage potential is hierarchically controlled during early hematopoiesis most likely through the control of chromatin modeling program. Consistent with this hypothesis, VPA has been reported to enhance early acting cytokine effect on the maintenance and expansion of primitive normal human HSCs at least *in vitro*. Hence, the effect of chromatin modeling by VPA on HSCs was investigated with regard to proliferation, differentiation as well as self-renewal in order to evaluate potential for clinical usage of HDIs in acute myeloid leukemia (AML) therapy.

According to the data observed in the present study, VPA increases both proliferation and self-renewal of HSCs as shown by immunophenotypical features, both *in vivo* and *in vitro* functional HSC characteristics as well as cell cycle and gene expression analyses.

It is clearly indicated that VPA enhanced the stem cell phenotype and/or increased the number of human HSCs isolated from both adult bone marrow (BM) and umbilical cord blood (CB) rather than differentiated cells either in the presence of early acting cytokines or an external milienu favoring differentation.

Furthermore, VPA enhances proliferation and self-renewal of murine HSCs as demonstrated by the increase in CFU, the percentage of stem cell marker-expressing cells as well as the replating efficiency of cultured murine HSCs with constant CFU *in vitro.* The enhancing effect of VPA on the replating efficiency of HSCs due to their self-renewal was also confirmed *in vivo* by an increase in the colony forming unit spleen on day 12 (CFU-S D12) and competitive repopulation efficiency of VPA-treated HSCs.

Based on the cell cycle analysis, valproic acid accelerates murine HSC cell cycle progression from G1 phase to S phase as indicated by the increase in the percentage of cells in S phase and the concomitant decrease in those in G1 phase. In addition, this process is accompanied by a down-regulation of $p21^{cip\text{-}1/waf\text{-}1}$ as demonstrated in both human and murine HSCs.

On the molecular level, VPA inhibits GSK3ß by phosphorylation on Ser9 accompanied by an activation of the Wnt signaling pathway in HSCs. Moreover, HoxB4, a transcription factor as well as a target gene of Wnt signaling, is up-regulated upon exposure to VPA in human and murine HScs. Both, the Wnt signaling pathway and HoxB4 are known to directly stimulate the proliferation of HSC and to expand the HSC pool.

To sum up, valproic acid, a potent histone deacetylase inhibitor known to induce differentiation and/or apoptosis in leukemic blasts, stimulates the proliferation and self-renewal of hematopoietic stem cells. Therefore, the data reported in this study suggest to reconsider the role of histone deacetylase inhibitors from a desired differentiation inducer to a coadjuvant factor for increasing the response to conventional therapy in acute myeloid leukemia.

7 Zusammenfassung

Akute myeloische Leukämie (AML) ist gekennzeichnet durch die Akkumulation einer großen Zahl abnormaler Zellen, die nicht in funktionelle Granulozyten oder Monozyten differenzieren können. Die überwiegende Mehrheit der AML-Patienten ist über 60 Jahre alt. Standardtherapie ist eine dosisintensive Chemotherapie mit dem Ziel einer kompletten Remission, gefolgt von unterschiedlichen Formen einer Postremissionstherapie. Ein kleiner Teil der Patienten kann mit diesem Therapieansatz geheilt werden. Die meisten Patienten sterben jedoch innerhalb der ersten 2 Jahre aufgrund einer refraktären oder rezidivierten Erkrankung oder an Therapiefolgen.

Verschiedene genetische Änderungen resultieren in einem gemeinsamen Muster deregulierter Genexpression, welches zu einem Differenzierungsblock führt, der die myeloide Leukämogenese fördert. Zusätzlich zur Induktion des leukämischen Phänotyps zeigen sich bei AML aberrante Chromatinremodellierung und Interaktionen von AML-Fusionsproteinen mit Corepressor/Histon-Deacetylase-Komplexen. Man nimmt an, dass Histon-Deacetylasen (HDACs) durch Erniedrigung des Acetylierungslevels der core-Histone unter anderem auch Gene reprimieren, die eine Rolle in Differenzierung und Zellzykluskontrolle spielen. Die starke Toxizität und die limitierte Effizienz heutiger chemotherapeutischer Strategien, sowie die der AML zugrunde liegenden biologischen Mechanismen, haben zur Entwicklung alternativer Therapieansätze geführt, wie z.B. der Differenzierungs-induzierenden Therapie mit Histon-Deacetylase-Inhibitoren (HDIs). HDIs sind Substanzen, die die Hyperacetylierung von core-Histonen induzieren können und somit zur Verringerung des Chromatin-Kondensationsgrades führen. Dies wiederum erhöht die Zugänglichkeit der DNA für Transkriptionsfaktoren und aktiviert somit Genexpression. Es ist gezeigt worden, dass HDIs wie z.B. Trichostatin A (TSA) oder Valproinsäure (VPA) entweder alleine oder in Kombination mit Differenzierungs-induzierenden Agenzien wie all-*trans*-Retinsäure (t-RA), den Differenzierungsblock in leukämischen Blasten unabhängig von der primären genetischen Veränderung überwinden können.

VPA (2-Propyl-Pentansäure) ist eine kurzkettige, verzweigte Fettsäure mit günstigen pharmakokinetischen Eigenschaften, welche schon seit Jahrzehnten in der Behandlung von Epilepsien eingesetzt wird. Neuere Untersuchungen zeigten, dass Valproinsäure neben ihrer seit langem genutzten Wirkung auch einen Einfluss auf das Epigenom haben kann, indem sie Histonacetylierung und DNA-Methylierung induziert. Darüber hinaus befindet sich VPA aufgrund seiner potenten Differenzierungseffekte auf Karzinomzellen, transformierte hämatopoetische Vorläuferzellen und leukämische Blasten von AML-Patienten in der klinischen Erprobung.

Auf der anderen Seite wurde in einer klinischen Studie allerdings gezeigt, dass eine VPA/t-RA-Kombinationstherapie bei AML-Patienten in fortgeschrittenen Stadien zu einer erheblichen Reduktion der Zahl an Blasten und einer peripheren Hypergranulozytose führt. Überdies war es in einem Patienten möglich normale und maligne Hämatopoese anhand des Vorkommens des Isochromosoms (17)(q10) in den leukämischen Blasten zu unterscheiden. Während CD34-positive Progenitorzellen zu einem geringen Teil noch dieses Isochromosom enthielten, hatten reife Granulozyten einen normalen Karyotyp, was nahe legt, dass es nicht zur forcierten Differenzierung kommt, sondern dass die normale Hämatopoese dominant gegenüber dem malignen Zellklon ist.

Außerdem wird zur Zeit gemutmaßt, dass eine offene Chromatinstruktur essentiell für die Multipotenz hämatopoetischer Stammzellen (HSCs) ist und das Differenzierungspotential während der frühen Hämatopoese hierarchisch kontrolliert wird, sehr wahrscheinlich über Chromatinmodellierung. In Einklang mit dieser Hypothese führte eine Kombination des Hydroxamsäure-basierten Histon-Deacetylase-Inhibitors Trichostatin A mit DNA-demethylierenden Agenzien zu einer signifikanten Expansion hämatopoetischer Stammzellen, welche zur Repopulation immundefizienter Mäuse in der Lage waren. Zudem konnte gezeigt werden, dass Valproinsäure frühe Zytokin-Effekte auf den Erhalt und die Expansion primitiver menschlicher HSCs zumindest *in vitro* verstärken kann. Daher wurde in der vorliegenden Arbeit der Effekt von Valproinsäure-induzierter Chromatinmodellierung auf das Schicksal hämatopoetischer Stammzellen untersucht, um damit die Möglichkeiten des Einsatzes von Histon-Deacetylase-Inhibitoren in der AML-Therapie zu evaluieren.

Zur Bestimmung der Effekte von Valproinsäure auf menschliche hämatopoetische Stammzellen in der Gegenwart sehr früh agierender Zytokine sowie in einem externen, Differenzierung favorisierenden Milieu, wurden die Proliferation und das Differenzierungspotential menschlicher HSCs aus adultem Knochenmark und von Nabelschnurblutzellen, welche einen höheren Prozentsatz an frühen HSCs enthalten, analysiert. VPA verstärkte den Stammzell-Phänotyp und erhöhte nicht die Zahl an differenzierten Zellen, sondern die der Stammzellen, sowohl in den Knochenmarks- als auch in den Nabelschnurblutzellen.

Für einen Vergleich der Effekte von Valproinsäure mit dem bekannten Verstärkereffekt von all-*trans*-Retinsäure (t-RA) auf das Potential von Maus-HSCs zur Koloniebildung wurden die Untersuchungen auf hämatopoetische Stammzellen der Maus ausgedehnt. Dazu wurde der Effekt von VPA auf die Replatierungs-Effizienz muriner HSCs in einem Koloniebildungs-Versuch dem von t-RA gegenübergestellt. VPA verstärkte nicht nur die Koloniebildung, gemessen als Kolonie-bildende Einheiten (colony forming units, CFU), sondern auch die Replatierungs-Effizienz bei konstanter CFU, währenddessen t-RA nur letztere beeinflusst. Außerdem kam es bei VPA-behandelten Zellen nicht zur Differenzierung, was sich an dem hohen Prozentsatz an Zellen zeigte, welche Stammzell-Marker exprimierten. Dies deutet auf eine Erhöhung sowohl der Proliferationsrate, als auch der Selbsterneuerung der VPA-behandelten hämatopoetischen Stammzellen hin.

Um herauszufinden, ob der *in vitro* Effekt von Valproinsäure auf Maus-HSCs auch auf einem erhöhten Selbsterneuerungspotentials beruht, wurde der Einfluss einer VPA-Behandlung auf die Bildung von Milzkolonien am Tag 12 (colony forming units-spleen, CFU-S D12) und auf die kompetitive Repopulation als Indikator für primitive hamätopoetische Stammzellen, die zu einer zumindest vorübergehenden Repopulation eines Wirtstieres in der Lage sind (short term HSCs, ST-HSCs), untersucht. Ähnlich wie t-RA verstärkt VPA die Selbsterneuerung von HSCs, messbar anhand der hohen Zahl von CFU-S D12 und einer Splenomegalie. Zudem erhöhte sich auch die Effizienz in den kompetitiven Repopulationsversuchen, ein Hinweis darauf, dass VPA einen Einfluss auf primitive hamätopoetische Stammzellen hat.

Ergänzend zu den Analysen zur Proliferation und Selbsterneuerung der Maus-HSCs wurde auf zellbiologischer Ebene der Zellzyklus-Status und die Expression des Cyclin-abhängigen Kinase-Inhibitors (cyclin dependent kinase inhibitor, CDKI) $p21^{cip\text{-}1/waf\text{-}1}$ in VPA-behandelten HSCs analysiert, um den Einfluss auf die Zellzyklusregulation in der hämatopoetischen Kaskade beurteilen zu können. Von Histon-Deacetylase-Inhibitoren ist bekannt, dass sie einen Zellzyklusarrest durch die Aktivierung von $p21^{cip\text{-}1/waf\text{-}1}$ induzieren können, worauf es zur Differenzierung und/oder Apoptose sowohl von leukämischen Blasten, als auch von Tumorzellen kommt. Überdies gibt es Hinweise darauf, dass $p21^{cip\text{-}1/waf\text{-}1}$ möglicherweise Stadien-spezifische Funktionen in der frühen Hämatopoese hat. In der vorliegenden Arbeit wurde gezeigt, dass Valproinsäure, im Gegensatz zu t-RA, die Zellzyklusprogression muriner HSCs von der G1- in die S-Phase beschleunigt, was sich in einer Erhöhung des Anteils von S-Phasen-Zellen bei gleichzeitiger Verringerung des Prozentsatzes von Zellen in der G1-Phase äußerte.

Außerdem führte VPA zu einer Herunterregulation von $p21^{cip\text{-}1/waf\text{-}1}$ in menschlichen und murinen hämatopoetischen Stammzellen. Diese Daten deuten auf einen direkten Zusammenhang zwischen dem Differenzierungslevel und der Antwort auf Histon-Deacetylase-Inhibitoren hin. So scheinen sehr unreife Zellen mit einer Herunterregulation von $p21^{cip\text{-}1/waf\text{-}1}$ und Zellzyklusprogression auf VPA zu reagieren, während es in fortgeschrittenen Differenzierungsstadien zu einer Hochregulation von $p21^{cip\text{-}1/waf\text{-}1}$ und Differenzierung oder Apoptose kommt.

Es ist bekannt, dass Wnt-Signale und der Transkriptionsfaktor HOXB4 direkt die Proliferation von hämatopoetischen Stammzellen stimulieren und damit zu einer Expansion des Pools an HSCs führen. Glykogen-Synthase-Kinase 3β (GSK3β) ist ein negativer Regulator des Wnt-Signatransduktionswegs, welcher zu Stabilisierung von β-Catenin und letztendlich zur Selbsterneuerung von HSCs führt. Vor kurzem wurde gezeigt, dass es nach VPA-Behandlung zur Inhibition von GSK3β durch Phosphorylierung an Serin 9 des Proteins kommt. Um die molekularen Mechanismus aufzuklären, durch welche Valproinsäure die Proliferation und Selbsterneuerung von HSCs induzieren, wurde der Effekt von VPA auf die Expression und Modifikation von GSK3β in menschlichen und murinen HSCs, sowie in KG-1-Zellen, welche eine frühe Population menschlicher HSCs repräsentieren (~80%

$CD34^+/CD38^-$), untersucht. VPA induzierte die Inhibiton von GSK3β durch Phosphorylierung an Serin 9 bei gleichzeitiger Aktivierung des Wnt-Signalweges. Zusätzlich wurde eine quantitative Analyse der Expression des *HoxB4*-Gens in HSCs aus Mensch und Maus durchgeführt. Zum einen ist *HoxB4*ein Zielgen des Wnt-Signalweges, zum anderen stellt der von ihm codierte Transkriptionsfaktor ein Schlüsselmolekül in der Regulation von Selbsterneuerung und Proliferation hämatopoetischer Stammzellen dar. Die Genexpressionsanalysen zeigten deutlich, dass die Behandlung mit Valproinsäure einer Erhöhung der *HoxB4*-RNA Level im Vergleich mit t-RA behandelten und unbehandelten Zellen führt.

Zusammenfassen lässt sich konstatieren, dass der Histon-Deacetylase-Inhibitor Valproinsäure, der für die Induktion von Differenzierung und/oder Apoptose in leukämischen Blasten bekannt war, die Proliferation und Selbsterneuerung hämatopoetischer Stammzellen stimuliert. Diese Effekte lassen sich wahrscheinlich sowohl auf eine Inhibition von GSK3β in HSCs, als auch auf eine transkriptionelle Reprogrammierung dieser Zellen durch Chromatinmodelling zurückführen.

Diese neu beschriebenen Effekte von Valproinsäure auf hämatopoetische Stammzellen könnten wichtige Konsequenzen für die AML-Therapie haben. Der VPA-induzierte Eintritt von ruhenden hämatopoetischen und leukämischen Stammzellen in den Zellzyklus könnte diese empfindlicher gegenüber konventioneller Chemotherapie machen. In der Tat konnte vor kurzem in einer klinischen Studie gezeigt werden, dass Valproinsäure das Ansprechverhalten gegenüber einer Chemotherapie verbessert.

Die in der vorliegenden Arbeit erhobenen Daten weisen darauf hin, dass die Rolle von Histon-Deacetylase-Inhibitoren in der AML-Therapie überdacht werden sollte und sie weniger als Differenzierungs-Induktoren, sondern eher als Coadjuvantien mit dem Potential zur Verbesserung des Ansprechverhaltens auf konventionelle Chemotherapien gesehen werden können.

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Abbreviations

A	ALL	Acute lymphoblastic leukaemia
	AML	Acute myeloblastic leukaemia
	APL	Acute promyelocytic leukaemia
	ATP	Adenosintriphosphat
B	BM	Bone marrow
	BCR	Breakpoint cluster region
	Bp	Base pair
	BrdU	Bromodeoxyuridine-Triphosphat (Br-dUTP)
	BSA	Bovine Serum Albumin
C	CB	Umbilical Cord Blood
	CDKI	Cyclin-dependent kinase inhibitor
	cDNA	Copy DNA
	CFU	Colony forming unit
	CFU-S	Colony forming unit spleen
	CLL	Chronic lymphoblastic leukaemia
	Cm	Centimeter
	CML	Chronic myeloblastic leukaemia
	CSF	Colony stimulating factor
D	DEPC	Diethylpyrocarbonat
	DMEM	Dulbecco's Modified Eagle Medium
	DMSO	Dimethylsulfoxid
	DNA	Deoxyribonucleic acid
E	E. coli	Escherichia coli
	ECL	Enhanced chemiluminescence
	EDTA	Ethylendiamin-N,N,N',N'-Tetra-Acetat
F	FAB-classification	French-American-British-classification
	FACS	Fluorescence activated cell sorting
	FCS	Fetal calf serum
	FITC	Fluorescein-Isothiocyanat

G	GM-CSF	Granulocyte-macrophage colony-stimulating factor
	G-CSF	Granulocyte colony-stimulating factor
	Gy	Gray
H	HDAC	Histon deacetylase
	HDI	Histone deacetylase inhibitor
	HSC	Hematopoietic stem cell
I	IgG	Immunoglobulin G
	IL-3	Interleukin-3
	IL-6	Interleukin-6
	IMDM	Iscove's Modified Dulbecco's Medium
K	kDa	Kilodalton
L	LB-Medium	Luria-Bertani-Medium
M	M	Molarity
	µg	Microgramm
	Min	Minute
	Ml	Microliter
	µM	Micromolar
	MNC	Mononuclear cell
	mRNA	Messenger ribonucleic acid
N	Ng	Nanogramm
P	PB	Peripheral blood
	PBS	Phosphate-buffered saline
	PCR	Polymerase-chain reaction
	PI	Propidiumjodid
	PML	Promyelocytic leukemia gene
R	RARα	Retinoic acid receptor alpha
	RARE	RAR specific regulatory element
	RNA	Ribonucleic acid
	Rpm	Rounds per minute
	RPMI-Medium	Roswell Park Memorial Institute-Medium
	RT	Reverse Transkriptase

	RXR	Retinoic X receptor
S	Sec	Second
	Sca-1	Stem cell antigene 1
	SCF	Stem cell factor
	SDS	sodium dodecyl sulphate
	SDS-PAGE	SDS-polyacrylamid gel electrophoresis
T	TBS	Tris-buffered saline
	TBST	Tris-buffered saline + Tween 20
	TEMED	N,N,N',N'-Tetramethylethylendiamin
	t-RA	All-trans retinoic acid
	Tris	Tris-(hidroxymethyl)-Aminoethan
	t(15;17)	Translocation of chromosome 15 and 17
	t(8;21)	Translocation of chromosome 8 and 21
	t(9;22)	Translocation of chromosome 9 and 22
	t(12;21)	Translocation of chromosome 12 and 21
U	U	Unit
V	V	Volt
	v/v	Volume per volume
	VPA	Valproic acid
W	w/v	Weight per volume

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Papers

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Ehrenwörtliche Erklärung

Ich erkläre hiermit ehrenwörtlich, dass ich die dem Fachbereich Biochemie, Chemie und Pharmazie zur Promotionsprüfung eingereichte Arbeit mit dem Titel

„Effect of Chromatin Modeling by Histone Deacetylase Inhibitors (HDIs) on Hematopoietic Stem Cell (HSC) Fate“

im Zentrum der Inneren Medizin, Medizinische Klinik II, Abteilung Hämatologie des Universitätsklinikums Frankfurt bei Prof. Dr. Dieter Hoelzer und unter Leitung von Prof. Dr. Rolf Marschalek mit Unterstützung von PD Dr. habil. Martin Ruthardt ohne sonstige Hilfe selbst durchgeführt und bei der Abfassung der Arbeit keine anderen als die in der Dissertation angeführten Hilfsmittel benutzt habe.

Ich habe bisher an keiner in- oder ausländischen biologischen Fakultät ein Gesuch um Zulassung zur Promotion eingereicht noch die vorliegende Arbeit als Dissertation vorgelegt.
Die vorliegende Arbeit wurde im Publikationsorgan veröffentlicht: Cancer Res 65(7):2537-41 (2005).

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D.Hilal Gül, M.Sc.