To

Aai and Baba, need I say more ?

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Characterisation of the leukemic stem cell in a murine model of *CALM/AF10* positive myeloid leukemia

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Charakterisierung der leukämischen Stammzelle in einem murinen Modell der *CALM/AF10* positiven myeloischen Leukämie

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Introduction

Materials

Methods

Leukemia is the neoplastic transformation of hematopoietic cells and is amongst the most common forms of cancer in humans. Despite advances in the understanding of the mechanisms involved and therapeutic approaches, most leukemia-affected individuals succumb to the disease (Appelbaum *et al.*, 2001). A vast majority of myeloid leukemias are associated with chromosomal rearrangements. It has been demonstrated in several cases that a number of these translocations lead to the formation of fusion genes which can contribute significantly to the transformation event, underlining the need to understand the mechanisms involved in fusion-gene mediated transformation. Recent studies have offered new insights into the evolution of the disease, emphasizing the relevance of the leukemic stem cell for the pathogenesis of the disease and its implications for future therapeutic developments. The first part of the introduction deals with the complex processes of normal hematopoiesis and factors that trigger the development of leukemia with particular emphasis on the most recent literature on the cancer stem cell model. In the second part, the role of chromosomal translocations and mouse models used for the studies of leukemogenesis are described, focusing in detail on the t(10;11)(p13;q14) translocation and the resultant *CALM/AF10* fusion gene, the oncogenic potential of which we sought to characterize in this study. Also discussed in detail are the promiscuous or biphenotypic leukemias for which the current study offers valuable insights.

1.1   Hematopoiesis

1.1.1   Normal hematopoiesis

1.1.1.1   General overview: Hematopoiesis is a an orderly process of alternate expression of specific transcriptional regulators, growth factors, and growth factor receptors, the combination of which determines lineage commitment and maturation of blood cells. The blood consists of various types of cells, broadly classified into myeloid and the lymphoid cells. The myelo-erythroid lineage includes erythrocytes that transport respiratory gases, platelets that play an important role in coagulation and monocytes and granulocytes that can migrate from blood vessels into other tissues and are involved in inflammation and phagocytosis. The lymphoid cells include the B lymphocytes, which provide immunity by the production of highly specific soluble antibodies, and the T lymphocytes, which are responsible for a variety of immune functions including the elimination of cells with foreign molecules on the surface. There is a notable difference in the function as well as morphology of these cells. Despite the diversity, a lot of evidence points to the fact that all these cells derive from a common precursor, the rare hematopoietic stem cell. The other hematopoietic cell types arising from this cell but found outside the blood are the dendritic cells and mast cells.

1.1.1.2   The hematopoietic hierarchy: Hematopoietic cells of all lineages derive originally from a relatively small number of committed hematopoietic progenitors, which arise from even fewer hematopoietic stem cells (Weissman, 2000). Hematopoietic stem cells (HSCs) are self-renewing clonogenic multipotent progenitors that can produce all mature blood types. These highly self-renewing HSCs which are also termed long-term repopulating HSCs (LT-HSCs) for their ability to confer long-term engraftment on lethally irradiated mice, generate the short-term repopulating HSCs (ST-HSCs) with limited self renewal and increased proliferation. ST-HSCs subsequently give rise to multipotent progenitors (MPPs) that generate committed progenitors of different lineages, the common myeloid progenitor (CMP) for the myelo-erythroid lineage and the common lymphoid progenitor (CLP) for the lymphoid lineage. The CMP in turn gives rise to the granulocyte-macrophage progenitor (GMP) and the megakaryocyte-erythrocyte progenitors (MEP) that are restricted to churning out mature granulocytes, macrophages or mast cells and megakaryocytes or erythrocytes

respectively. The CLP generates all mature lymphoid cell types including B and T lineage cells, platelets and NK cells. Dendritic cells can be generated from both CLPs as well as CMPs. Thus the hematopoietic hierarchy is composed of the stem cells, the committed progenitors and their progeny, the mature blood cells of all lineages.
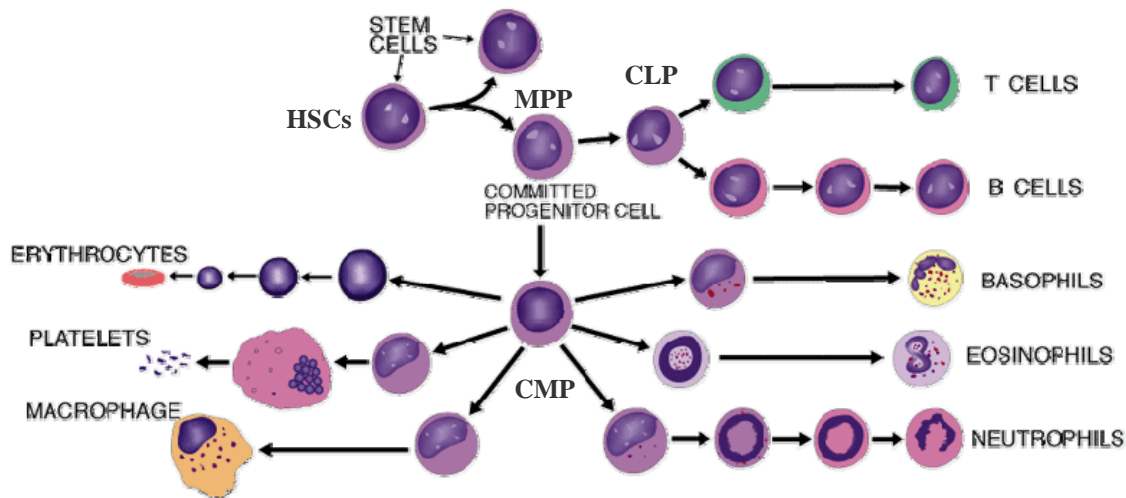
Fig. 1.1.1.2 The hematopoietic hierarchy: The hematopoietic hierarchy consists of the hematopoietic stem cells (HSC), the multipotent progenitors (MPPs) and the more downstream progenitors, the common myeloid and the common lymphoid progenitor (CMP and CLP) respectively. Collectively, these give rise to all the mature cells of the hematopoietic lineage. (With permission from Larry Rohrschneider, Fred Hutchinson Cancer Research Centre)

1.1.2   Leukemic hematopoiesis

Leukemias arise from the deregulated pathways of normal hematopoiesis. Although leukemias are heterogeneous in terms of phenotypes, there are general mechanisms underlying leukemic transformation such as block in differentiation, increased proliferation capacity, prolongation of cell survival by inhibition of apoptotic signals, increased telomere maintenance and the retention or reacquisition of enhanced self-renewal capacity (Warner *et al.*, 2004; Weissman, 2000). The development of leukemias, like cancers in general, is a stepwise process in which somatic mutations are acquired leading to an increasingly transformed clonal population of blast cells. It is therefore believed that mutations disrupting more than one of these processes are necessary for neoplastic transformation, (Hanahan and Weinberg, 2000) though it is important to note that one mutation can simultaneously disrupt more than one of these processes. In acute myeloid leukemia (AML), for example, the

PML/RAR α fusion gene, which has been shown to block differentiation, can also protect hematopoietic progenitors from apoptosis (Grignani *et al.*, 2000). The involvement and significance of each of these mechanisms in AML is explained in some detail in this chapter with examples.

1.1.2.1   Differentiation arrest: AMLs are a heterogeneous group of diseases with blasts in varying degrees of maturation arrest. In fact the most commonly followed classification of AML, the French-American-British (FAB) classification divides leukemias into eight major subtypes (M0 to M7) based on the degree of differentiation along the myeloid lineage (Bennett *et al.*, 1985). The FAB classification was recently updated in the WHO classification, taking morphology, immunophenotype and cytogenetics into account and defining acute leukemia to have more than 20% blasts in bone marrow (Brunning, 2003). As is discussed in the chapter on normal hematopoiesis, differentiation from primitive progenitors to mature cells in the hematopoietic system is governed by lineage specific transcription factors, the pathways for which have been elaborately defined (reviewed (Zhu and Emerson, 2002)). It was postulated that several of these pathways would be disrupted in leukemias and recent studies have confirmed this hypothesis (Tenen, 2003). One transcriptional factor complex that has been very frequently targeted by AML is the core binding factor (CBF) consisting of the *AML1* gene and the *CBFβ* gene. 12 different translocations have been found to target this complex accounting for 25% of all leukemias (Speck and Gilliland, 2002). It is interesting to note that in the vast majority of fusion proteins resulting from a leukemogenic event, one of the partners is a transcription factor. 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 depend on the nature of the fusion protein expressed (Huntly *et al.*, 2004). It is becoming increasingly apparent that the abnormal network of transcriptional regulation induced by leukemia associated genes leads to a block in differentiation. However, as shown in several studies, a block of differentiation is not solely sufficient to induce transformation and must be associated with an increase in proliferation.

1.1.2.2   Increased proliferation: The dominance of a malignant clone over normal cells necessitates increased proliferation for the acquisition of a significant growth advantage over normal cells. The leukemia specific fusion genes *PLM/RARα* and *AML1/ETO*

have previously been shown to disrupt normal hematopoietic differentiation without causing leukemia in mice (de Guzman *et al.*, 2002; Grisolano *et al.*, 1997; Pollock *et al.*, 1999). It has been demonstrated in experimental models that mutations in the receptor tyrosine kinase FLT3 inducing constitutive activation could provide proliferative advantage in collaboration with PML/RARα (Kelly *et al.*, 2002) and as shown recently in our laboratory, with *AML1/ETO* (Schessl C, 2005). Clinical data showing the frequent presence of activating mutations in the mitogenic *FLT3* and *KIT* receptor tyrosine kinases in AML (Reilly, 2003) supports the hypothesis that proliferative advantage could be provided to leukemic cells by activating mutations in tyrosine kinase receptors.

1.1.2.3   Inhibition of programmed cell death/apoptosis: Since the acquisition of mutations is a stepwise process, cells that have already undergone mutations, termed *preleukemic* cells, must inhibit apoptotic events and survive for a longer time period to acquire more mutations ultimately leading to an increasingly transformed cell type. It should be noted that a high level of apoptosis in myelodysplastic syndromes (MDS) distinguishes them from AML and that the progression from MDS to AML reflects an increase in the expression of anti-apoptotic versus pro-apoptotic members of the *BCL2* gene family (Davis and Greenberg, 1998). *BCL2* upregulation in leukemias has been shown to correlate with poor response to therapy (Campos *et al.*, 1993) and it has been proposed as a potential target for future therapy regimens (Konopleva *et al.*, 2000). Recent studies have demonstrated that the prevention of cell death is one of the key events in myeloid transformation that probably sets the stage for acquiring new mutations.

1.1.2.4   Telomere maintenance: The 'Hayflick limit' (Hayflick, 1997), the upper limit on the number of times a cell can divide, imposed on a cell by shortening telomeres, has to be overcome in cancer as the tumor cell divides infinitely to propagate the tumor. Enhanced telomere maintenance is therefore a prerequisite to the limitless propagation of the leukemia stem cell in AML. Indeed, increased levels of telomerase activity have been reported in 70% of all AML cases (Ohyashiki *et al.*, 2002).

1.1.2.5   Enhanced self-renewal: The incessant propagation of transformed cells requires the presence of a self-sustaining population of transformed stem cells that would continuously feed the pool of the bulk cancerous population. Neoplastic transformation

would then require the acquisition of stem cell characteristics by a targeted cell or alternatively, the direct targeting of a self-renewing stem cell to form a cancer stem cell. The aspect of stem cell properties in normal and cancerous cells is elaborated upon in the following chapter.

1.2   Stem cells and cancer

1.2.1   Stem cells and 'stemness': Stem cells can be classified into two main categories, embryonic stem cells and adult stem cells. Stem cells are clonogenic cells capable of both self-renewal and multilineage differentiation. Some adult tissues like blood, skin, gut etc. need a constant turnover of cells for tissue renewal. Tissue stem cells, which are adult stem cells, respond to this need as they consist of multipotent progenitors. Between birth and death, humans produce approximately 10^{16} blood cells of different types. These specialized cells are continuously produced from precursor cells, which in turn must be replaced by cells further up the blood hierarchy. Ultimately, the entire blood system is fed by a pool of rare hematopoietic stem cells (HSCs)(Dick, 2003). Several decades after the observation that cellular recovery can be attained following lethal irradiation by transplanting bone marrow, it was identified that the ability of such transplants to reconstitute hematopoiesis can be attributed to a few extremely rare stem cells found predominantly in the bone marrow but capable of mobilization into peripheral tissues via the blood vascular system. Since then, HSCs have been the best characterized stem cells at the phenotypic and functional level and the hematopoietic system has been the proving ground for most of the experimental procedures and conceptual paradigms for stem cell biology in general. HSCs and indeed most stem cells can be defined by certain unique properties, sometimes referred to as 'stemness', the most prominent of which are self-renewal, multipotency and quiescence. Since a discussion on stem cells in general is beyond the scope of this thesis, this part of the chapter will deal exclusively with hematopoietic stem cells.

1.2.1.1   Self-renewal: Self-renewal is the property of a stem cell to generate progeny with exact stem cell properties of the parent cell. A stem cell divides symmetrically to give rise to two daughter stem cells, each possessing stem cell properties, or alternatively, asymmetrically, wherein one daughter cell is a stem cell and the other is a rapidly cycling

downstream progenitor with a reduced capacity of self-renewal (Warner *et al.*, 2004)(Fig. 1.2.1.1). The downstream progenitor has a relatively enhanced proliferative capacity and it is necessary for clonal expansion and production of large numbers of committed progenitors and more differentiated hematopoietic cells. HSCs can be functionally separated into long-term repopulating stem cells, capable of indefinite self-renewal and short-term repopulating stem cells that self-renew for a defined interval ($\approx$ 8 weeks in mice) (Passegue *et al.*, 2003). The short-term repopulating stem cell then gives rise to the non self-renewing oligolineage progenitors which in turn give rise to progeny that are more restricted in their differentiation potential, and finally to functionally mature cells.
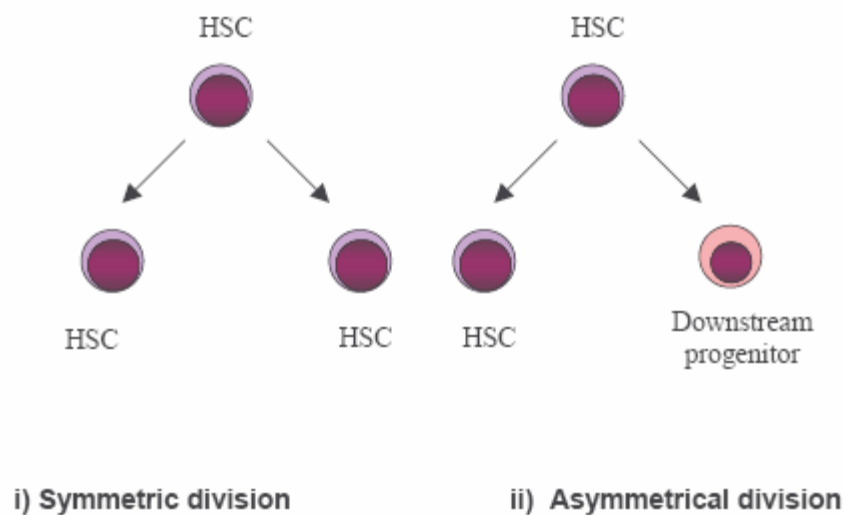
Figure 1.2.1.1 Symmetrical and asymmetrical division of hematopoietic stem cells: The symmetrical process yields two daughter HSCs from the parent HSC whereas the asymmetrical process yields one daughter HSC and one downstream progenitor.

In steady state bone marrow, hematopoietic stem cells divide rarely but mostly asymmetrically to retain their numbers as well as to produce the entire complement of cells necessary for normal hematopoiesis. Normal hematopoiesis therefore is a delicate balance between self-renewal and differentiation. This property of self-renewal, as is discussed later in this chapter, is a property shared by stem cells and cancer stem cells and acquisition of this characteristic is now believed to be a crucial turning point in the transformation process.

At the molecular level, pathways governing the process of self-renewal are poorly understood; there is a growing amount of evidence linking several gene families to the process. These include the genes belonging to the Hox gene family (Antonchuk *et al.*, 2002;

Buske *et al.*, 2002; Thorsteinsdottir *et al.*, 2002), the Wnt family (Reya *et al.*, 2003) (Willert *et al.*, 2003) and to the Polycomb group (Kajiume *et al.*, 2004; Lessard and Sauvageau, 2003a). There is also evidence suggesting a role of Notch (Karanu *et al.*, 2000) and Sonic Hedgehog (Shh) (Bhardwaj *et al.*, 2001) in HSC self-renewal though clear pathways elucidating the self-renewal process are yet to be demonstrated.

       1.2.1.2   Multipotency: The major difference between embryonic and adult stem cells is that while the former is totipotent, i.e. it can give rise to all the cells of every tissue of the organism, the latter can be described as multipotent, i.e. they can give rise to all cells of a given tissue. The hematopoietic stem cell (HSC) can be operationally defined as a long-term repopulating cell with both lymphoid (T and B) and myeloid potential (Orlic and Bodine, 1994). HSCs normally function to generate all of the lineages of mature blood cell types necessary for maintaining proper hematopoietic function (Kondo *et al.*, 2003). The ability of a single HSC to give rise to hematopoietic cells of all the different lineages (multipotency) is one of the hallmark properties of a HSC. The HSC gives rise to committed progenitors that can proliferate extensively to produce the billions of differentiated cells that enter the peripheral blood per day. Prospectively isolated hematopoietic progenitors have increasingly limited differentiation potential as they branch out from the HSC. The multipotency of HSCs is thought to be disrupted in leukemias due to blocks in differentiation and/or the dysregulation of certain lineage specific transcription factors.

       1.2.1.3   Quiescence: Quiescence, or the relatively slow cycling of HSCs, in marked contrast to the rapidly proliferating progenitors is necessary to protect the stem cell compartment from toxic and oxidative stress and to prevent consumption of the regenerative cell pool, an occurrence known as stem cell exhaustion (Cheng *et al.*, 2000). HSCs are relatively rare (1 in 10000 to 100000 cells in peripheral blood) and they reside in relatively larger numbers in the bone marrow of adult mice and humans where they are normally inactive (Bonnet, 2002). It was demonstrated by 5-bromo2'-deoxy-uridine (BrdU) incorporation studies (for the measurement of cell proliferation) in mice, that approximately 75% of long-term repopulating HSCs (LT-HSCs) were in the G_0 phase at any given time in steady state bone marrow (i.e. quiescent) (Cheshier *et al.*, 1999). Long-term repopulating stem cells are the earliest stem cells that divide very rarely to give rise to the more proliferative short-term HSCs which in turn give rise to the non self-renewing lineage

committed progenitors that produce mature hematopoietic cells in large numbers. HSCs can however, proliferate rapidly symmetrically in response to myelosuppressive chemotherapy or irradiation followed by bone marrow transplantation to give rise to committed progenitors as well as copies of more HSCs, which then return to the quiescent state (Dixon and Rosendaal, 1981). The control over quiescence is therefore especially crucial in conditions of stress, such as myelotoxic injury, to prevent hematopoietic death. There is little information about the molecular events that promote this process, though the bone marrow microenvironment or the 'stem cell niche' is believed to play an important role. Evidence that the local microenvironment is critical for controlling basic mechanisms of self-renewal and differentiation exists for normal stem cells (Lemischka, 1997; Schofield, 1983). Based on these studies, it seems likely that the tumor microenvironment is also critical for self-renewal of leukemia propagating cells with stem cells like properties or leukemic stem cells (LSCs). Thus, a major challenge for stem cell targeted therapy is to identify apoptotic stimuli that effectively target the tumor stem cell population while simultaneously sparing normal stem cells; and to do so in the context of a largely uncharacterized *in vivo* microenvironment. To meet this challenge, development and analysis of sophisticated LSC experimental systems is essential. As will be discussed below, recent findings indicate that leukemia stem cells retain several properties of stem cells, providing poor targets for therapeutic agents targeting rapidly proliferating cells (Hope *et al.*, 2004).

1.2.2   The cancer stem cell model

A marked functional heterogeneity is observed among tumor cells with regards to proliferative potential and tumorigenicity. It has been consistently demonstrated that only a small subset of cells within the bulk cancerous population in solid tumors had tumor initiating ability (as assessed by *in vitro* and *in vivo* assays) (Buick and Pollak, 1984; Mackillop *et al.*, 1983) as well as substantial proliferative potential (Mendelsohn, 1962; Wantzin and Killmann, 1977). This heterogeneity can be explained by two theories, one theory suggested that every cell within a blast cell population possesses an equal but low probability of being able to initiate the tumor by entering the cell cycle (Till *et al.*, 1964). This model, called the stochastic model, assumes that a cell capable of extensive proliferation necessary to initiate and sustain tumor growth ultimately undergoes many more divisions than a cell lacking this ability. Therefore, the majority of cells are unable to regrow the tumor because the cumulative

probability of undergoing the required number of cell divisions is very low (Reya *et al.*, 2001). The alternate hypothesis proposed by many investigators is a model in which every tumor contains a rare functionally distinct population of cells termed cancer stem cells (CSCs). The cancer stem cell is a cell that has tumor initiating function and can maintain the bulk tumor population as its clonal progeny. The cancer stem cell (CSC) hypothesis therefore suggests that neoplastic clones are maintained exclusively by this rare fraction of cells with stem cell properties.

The hematopoietic system provides for an excellent proving ground for testing these hypotheses. This is facilitated by the development of techniques over the last few decades, that allow the flow cytometric isolation of highly purified hematopoietic populations, (Akashi *et al.*, 2000; Kondo *et al.*, 1997), the development of techniques to efficiently transduce early hematopoietic progenitor cells (Dick *et al.*, 1985) and of various *in vitro* and *in vivo* assays (Morrison *et al.*, 1995)(including the use of xenograft models) (Dick *et al.*, 1991) and finally automated array systems to directly derive and compare large scale analyses of gene expression profiles of normal and leukemic purified or bulk populations (Ivanova *et al.*, 2002; Ramalho-Santos *et al.*, 2002; Venezia *et al.*, 2004). Emerging data utilizing the aforementioned techniques point to a regulated self-renewal process of both normal and leukemic stem cell hierarchies (Passegue *et al.*, 2003). The recent finding that the Polycomb group gene Bmi-1 regulates the self-renewal of both normal and leukemia stem cells (Dick, 2003; Lessard and Sauvageau, 2003b) strongly supports this theory. Recent studies have demonstrated that many pathways associated with cancer also regulate normal stem cell development, supporting the cancer stem cell theory. The HOX and WNT gene families have been demonstrated to be involved in the process of self-renewal both in normal and leukemic hematopoiesis (Buske *et al.*, 2002; Reya *et al.*, 2003) and leukemia (Buske and Humphries, 2000; Muller-Tidow *et al.*, 2004). The biology of stem cells and their intrinsic properties are now recognized as integral to tumor pathogenesis in several types of cancer. Cancer stem cells, the minor self-renewing fraction of cells within the tumor that can regrow the tumor, reflect the normal stem cells of the corresponding tissue in several aspects and among others, have been isolated for the hematopoietic system (Lapidot *et al.*, 1994) (Bonnet and Dick, 1997) breast cancer, (Al-Hajj *et al.*, 2003) and more recently, brain tumor (Singh *et al.*, 2004). Using a variety of sophisticated experimental approaches, investigators have identified, isolated and begun to characterize malignant stem cells from multiple types of cancer. Given

the newly appreciated role of stem cells in many normal organ systems, it seems likely that cancer stem cells will be described in other tumor types in the near future.

1.2.3   The need for identification of the leukemic stem cell:

As is discussed at considerable length in the previous parts of the thesis, it has been established that one of the important events necessary for leukemic transformation is the abnormal retention or reacquisition of stem cell characteristics by a transformed cell. The striking similarity of LSCs with their normal counterparts has hampered the development of therapeutic strategies selectively targeting the LSCs but sparing normal stem cells or early myeloid committed progenitors for patients with AML. The quiescent nature of leukemia stem cells in CML has been clearly demonstrated (Holyoake *et al.*, 1999). This observation is important because though the treatment of CML patients with the tyrosine kinase inhibitor imantib mesylate effectively induces remission, it cannot eradicate the disease (Bhatia *et al.*, 2003; Holtz and Bhatia, 2004). The analysis of CML stem cells treated with imatinib mesylate showed that the quiescent stem cell population is resistant to the drug *in vitro* (Graham *et al.*, 2002). Therapeutic approaches to leukemia have focussed mostly on elimination of rapidly proliferating cell, however, with the advancing knowledge about the relatively quiescent leukemia stem cell (LSC), the limitations of this approach have come to the fore. The identification of the LSCs in the bulk leukemic population that resist therapy and sustain the leukemia has therefore assumed great significance (Jamieson *et al.*, 2004). Functional studies have shown that the more primitive $CD34^+/CD38^-$ subset of multiple human AMLs are the minor fraction that can sustain the leukemia in xenograft studies in the non obese diabetic / severe combine immunodeficient (NOD/SCID) mouse model (which is employed for transplantation studies of human bone marrow cells) and that this population retains several attributes of the stem cell (Bonnet and Dick, 1997; George *et al.*, 2001), though it has also been demonstrated that the LSC compartment could also exist in the more downstream $CD34^-$ compartment (Terpstra *et al.*, 1996). This apparent paradox reflects the case that leukemia results mostly from mutations occurring in a self-renewing primitive HSC or alternatively, from the rare aberrant acquisition of stem-cell properties by downstream progenitors. In mice, the identification of the leukemia propagating cell is made easier by the use of modern purification and retroviral transduction techniques. This was elegantly demonstrated in a couple of studies which showed that a) the transduction of highly purified HSCs, CMPs as well as GMPs with the *MLL/ENL* fusion gene leads to myeloid transformation *in vitro* and *in*

vivo with maturation arrest at the same myelomonocytic stage regardless of the cell type used for transformation (Cozzio *et al.*, 2003) and b) in the case of *MLL/GAS7*, the retroviral transduction of HSCs or their immediate downstream progeny, the MPPs result in leukemias of lymphoid, biphenotypic, and myeloid characteristics (So *et al.*, 2003). It was also demonstrated that transgenic mice conditionally expressing the *BCR-ABL* and *BCL2* genes in myeloid progenitors and their myelomonocytic progeny, but not in HSCs could still propagate AML (Jaiswal *et al.*, 2003). More recently, the leukemia derived fusion gene *MOZ/TIF2* has been shown to confer self-renewal properties to normally non self-renewing GMPs in a mouse model of myeloid leukemia (Huntly *et al.*, 2004). These results indicate that leukemias can be originated in committed progenitors that acquire stem cell characteristics.

The identification and characterization of leukemia stem cells and more importantly, markers expressed differentially on leukemia and normal stem cells will therefore lead to the design of novel therapeutic approaches in leukemia. We sought to identify the leukemic stem cell in a mouse model of *CALM/AF10* positive acute myeloid leukemia. Generally, two approaches that have been used to identify cancer stem cells, one approach that has been used for human CSC identification, wherein, the different sub-populations in a cancer have each been analysed for transplantability of the tumor (Bonnet and Dick, 1997; Singh *et al.*, 2004) and the other approach, in which cells of a distinct differentiation stage are prospectively isolated, engineered to express the cancer specific mutation and tested for their ability to develop characteristics of a CSC under expression of the genetic alteration (Huntly *et al.*, 2004; So *et al.*, 2003); of these, we have used the former approach.

1.2   Translocations in leukemia

Leukemias, like all cancers are known to arise from acquired genetic changes. Generally, chromosomal aberrations contribute to a vast majority of cancers and the link between the occurrence of chromosomal aberrations and cancer has been very well established due to current advances in cytogenetics and molecular biology. Analyses of recurring chromosomal aberrations have led to the identification of numerous proto-oncogenes (Rabbitts, 1994). As regards AML, more than 80% of these leukemias are associated with at least one chromosomal rearrangement (Pandolfi, 2001) and over 100 different chromosomal translocations have been cloned (Gilliland and Tallman, 2002).

Frequently, these translocations involve genes encoding transcription factors that have been shown to play an important role in hematopoietic lineage development. It has been demonstrated that the chimeric fusion gene products or in some cases, putative proto-oncogene activation by the translocation event *per se* is responsible for the transformation (Rabbitts and Boehm, 1991). The cloning of breakpoints and the subsequent employment of techniques that allow testing them for the oncogenic potential (mouse bone marrow transplantation model) will allow the identification of new proto-oncogenes and shed light on intrinsic mechanisms of leukemic transformation.

1.3.1   The t(10;11)(p13;q14) translocation in leukemia

Chromosomal rearrangements involving the long arm of chromosome 11 and the short arm of chromosome 10 have been found in a variety of leukemias. The t(10;11)(p12-13;q23) translocation fuses the trithorax group gene *MLL* to the putative transcription factor *AF10* and it was reported that the t(10;11)(p13;q14) translocation fuses the *AF10* gene to the novel clathrin assembly lymphoid myeloid leukemia gene *CALM* (Dreyling *et al.*, 1996). Interestingly both the translocations have a poor prognosis and low survival rate (Dreyling *et al.*, 1998; So *et al.*, 2003). The latter is a rare but recurring translocation and leads to the expression of the *CALM/AF10* and in some cases the reciprocal *AF10/CALM* chimeric fusion transcripts.
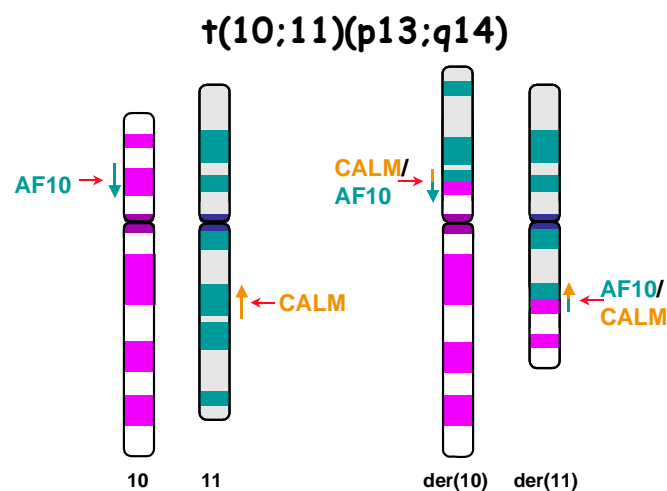
Fig. 1.3.1: A schematic representation of the t(10;11)(p13;q14) translocation: The t(10;11)(p13;q14) translocation fuses the *CALM* gene on chromosome 11 to the *AF10* gene on chromosome 10 to generate an in-

frame *CALM/AF10* fusion gene on the derivative chromosome 10 [der(10)] and a reciprocal *AF10/CALM* fusion gene on the derivative chromosome 11 [der(11)] (Figure courtesy S. Bohlander)
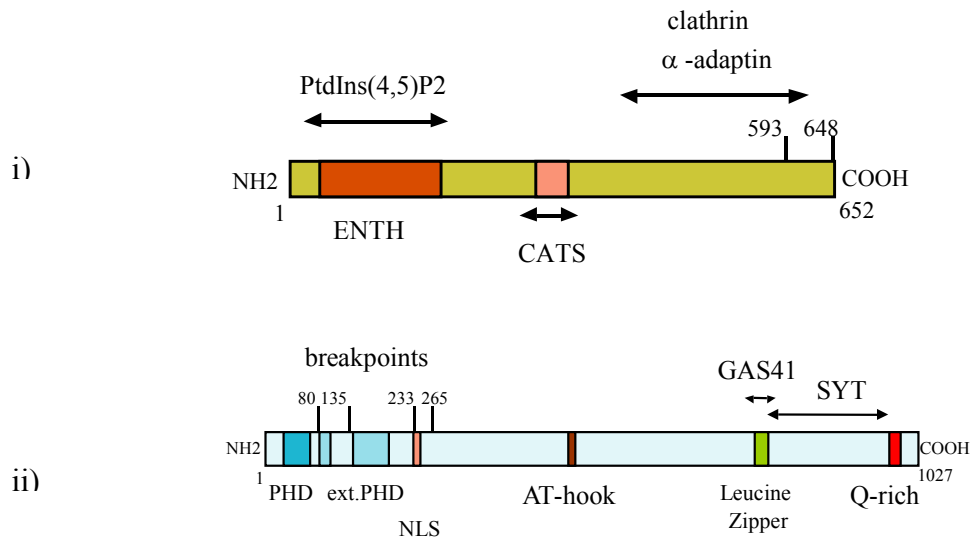
Fig. 1.3.2.a Schematic representation of *CALM* and *AF10*:

i) The CALM protein is a 652 amino acid protein with a proximal <u>E</u>psin <u>N</u> terminal <u>h</u>omology domain (ENTH domain) that bears a high homology with the N-terminal portion of the Epsin protein. Amino acids 221-294 of the CALM protein bind to CATS, a novel protein expressed strongly in the thymus and spleen.

ii) The AF10 protein is a 1027 amino acid protein with five N terminal plant homeodomain like (PHD) zinc fingers, an AT hook and a highly conserved octapeptide motif-leucine zipper domain. At the C terminal end, there is a glutamine rich domain. (Figure courtesy S. Bohlander)

1.3.2 *CALM*, *AF10*, and the *CALM/AF10* fusion:

The *CALM (PICALM)* gene was identified as a fusion partner of *AF10* in the human monocytic cell line U937 harboring the t(10;11)(p13;q14) translocation. This gene is located on chromosome 11q14 and encodes a 652 amino acid protein that is ubiquitously expressed and shares a high homology with the neuronal specific protein AP180 (Fig.1.3.2a i)(Tebar *et al.*, 1999). Studies on clathrin mediated vesicle formation, a critical step in endocytosis point to a potential role of CALM in the regulation of clathrin recruitment to the membrane and/or formation of the coated pit (Tebar *et al.*, 1999). This is thought to be mediated by the binding of CALM to the clathrin heavy chain through its C-terminal region and with phosphoinositides through its Epsin N-terminal homology (ENTH) domain. This CALM-

clathrin interaction has been shown to promote the assembly of clathrin triskelia into clathrin cages *in vitro (Ford et al., 2002; Ford et al., 2001)*. Mutations in the *AP180/CALM* gene homologs in *Drosophila (lap)* (Nonet *et al.*, 1999) and *Caenorhabditis elegans (unc11) (*Zhang *et al., 1998)* have suggested a role of these proteins in regulation of endocytic vesicle size during the clathrin assembly process. N-ethyl-N-nitrosourea (ENU) induced point mutagenesis in the murine *Picalm* gene resulted in disrupted hematopoiesis and reduced iron metabolism and retarded development in mice (Klebig *et al.*, 2003). Interestingly, in a screen for identifying CALM interacting proteins using the yeast two hybrid system, Archangelo *et al.*, identified a novel protein, termed CATS for <u>CA</u>LM interacting protein expressed in <u>t</u>hymus and <u>s</u>pleen. *CATS* expression is limited to lymphoid organs and the CATS protein interacts with CALM *in vitro* and *in vivo* (Archangelo, 2005).The fact that the *CALM* gene is involved in two distinct translocations (*CALM/AF10* and *MLL/CALM*) as well as the identification of several leukemia-associated fusion proteins in endocytosis points to a role of clathrin-mediated endocytosis (CME) in leukemia.

There is little known about the human *AF10* gene except for the fact that it is found as a fusion partner of both *CALM* as well as *MLL* and is involved in different types of leukemias and lymphomas. The human *AF10* gene is a homolog of the *Drosophila dAF10,* which is reported to play a role in the heterochromatin dependent genomic silencing of position effect variegation, "a phenomenon associated with chromosomal rearrangements that cause mosaic expression of euchromatic genes when relocated next to heterochromatin'' (Linder *et al.*, 2001). The *AF10* gene, located on chromosome 10 band 12p, encodes a 1,027 amino acid protein (Fig.1.3.2a ii). The leucine zipper-octapeptide motif domain and the plant homeodomain like zinc finger domains are both highly conserved between AF10 and its homologs AF17 and BR140. The leucine zipper of the *Drosophila* homolog of AF10 (Alhambra) has also been implicated in the inhibition of Polycomb group responsive element (PRE) mediated repression (Perrin *et al.*, 2003). Interestingly, this leucine zipper domain of *AF10,* together with the octapeptide motif, has also been shown to be the minimal portion necessary for transformation when fused to *MLL* a mouse model of the *MLL/AF10* translocation (DiMartino *et al.*, 2002). This region is conserved between human, *Drosophila* and *Cenorhabditis elegans* homologs and possesses transcriptional activation potential (DiMartino *et al.*, 2002) and also binds to a glioma amplified sequence gene GAS41 that has been shown to interact with the SWI/SNF complex (Debernardi *et al.*, 2002).
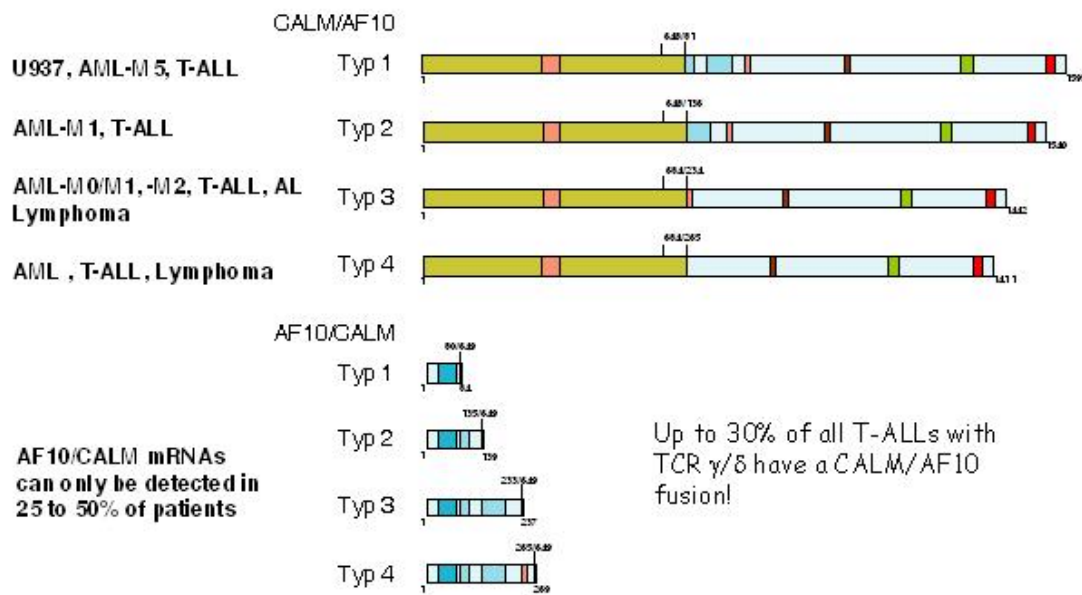
Fig. 1.3.2b The various *CALM/AF10* breakpoints: Different *CALM/AF10* breakpoints in patients with leukemia (right panel) show no correlation with the phenotype of the leukemia (left panel). All fusions generate in-frame fusion products of *CALM/AF10* and in some cases, the reciprocal *AF10/CALM*. The octapeptide motif is retained in all the *CALM/AF10* fusions, whereas none of the fusions retain the entire intact zinc finger domain.

Most t(10;11)(p13;q14) fusions generate *CALM/AF10* as well as the reciprocal *AF10/CALM* transcripts, however *AF10/CALM* transcripts could not be detected in all leukemia samples (Carlson *et al.*, 2000)indicating that it is probably the *CALM/AF10* fusion transcript that directs the transformation process. Analysis of the breakpoint region in various t(10;11)(p13;q14) leukemias has shown three different breakpoints for *CALM* and four breakpoints for *AF10* (Fig. 1.3.2b) with no noticeable correlation with the phenotype or outcome of disease (Bohlander *et al.*, 2000). While the *CALM* gene is more or less completely retained, all breakpoints in *AF10* lead to the partial or complete loss of the plant homeodomain (PHD) like zinc finger domain but retain the C-terminal octapeptide motif and the leucine zipper domains (Fig. 1.3.2b) and in all the different translocations, the open reading frames both of *CALM* and of *AF10* are maintained (Kumon *et al.*, 1999). Moreover, the CATS binding portion of CALM is retained in both *CALM/AF10* as well as *MLL/CALM* fusions (Archangelo, 2005).

The t(10;11)(p13;q14) translocation has been reported in a variety of acute myeloid (as well as megakaryocytic and eosinophilic) leukemias, (Dreyling *et al.*, 1996; Jones *et al.*, 2001; Salmon-Nguyen *et al.*, 2000) lymphoid leukemias and lymphomas (Bohlander *et al.*,

2000) Moreover, the *CALM/AF10* fusion event, like many translocations involving the *MLL* proto-oncogene, has been found to be the sole genetic abnormality in acute undifferentiated or biphenotypic leukemias (Kumon *et al.*, 1999) which will be described in some detail in the following chapter.

1.4 Acute biphenotypic leukemias

Leukemias are characterized as myeloid or lymphoid based on the expression on the surface of the blast cells, of lineage specific antigens. There is however, a small subset of rare leukemias, termed mixed lineage leukemias, acute undifferentiated leukemias or acute biphenotypic leukemias (ABLs) that present blasts with antigens specific to both lineages. (Altman, 1990; Bernier *et al.*, 1995)

1.4.1 ABLs in humans: ABLs in human patients are typically associated with a poor prognosis, reduced survival rate and a high incidence of relapse (Sulak *et al.*, 1990) These leukemias account for 3-7 % of all acute leukemias and 10-25% of pediatric acute leukemias (Altman, 1990; Sulak *et al.*, 1990) Most of the acute biphenotypic leukemia blasts coexpress surface markers of B-myeloid characteristics and less often a T-myeloid phenotype (Matutes *et al.*, 1997). It is widely suspected that the incidence of promiscuous leukemias is much higher than is currently reported due to the lack of a consistent approach of defining promiscuity, the classification of leukemias on the basis of a limited set of diagnostic criteria as well as the inherent heterogeneity amongst this unique entity of leukemias. The presence of lymphoid specific genomic rearrangements in myeloid leukemias (Schmidt *et al.*, 1995; Williams and Moscinski, 1993; Yen *et al.*, 1999) might represent previously undetected promiscuity (Grimwade *et al.*, 2002).

1.4.2 ABL models in mice: To our knowledge there are few murine models that present blasts with biphenotypic features, the prominent example being a murine model of the *MLL* proto-oncogene. Chi Wai So *et al.*, demonstrated in 2003 that the *MLL/GAS7* fusion gene transforms primitive hematopoietic cells and causes acute biphenotypic leukemias in mice with blasts that coexpress B and myeloid antigens (So *et al.*, 2003). More recently,

Gurevich *et al.* reported the appearance of the B cell specific antigen B220 on the cell surface of myeloid blasts in a subset of terminally ill mice transplanted with the NUP98/TOPOII fusion gene (Gurevich *et al.*, 2004).

1.4.3   Theories explaining biphenotypic character in ABL: Two alternative explanations have been proposed for the presence of biphenotypic cells and there has been considerable debate as to which is the valid explanation for the coexpression of dual markers in these leukemias, though most investigators agree that these two mechanisms might both be possible and furthermore, need not be mutually exclusive. The mechanisms are termed lineage infidelity and lineage promiscuity.

1.4.3.1   Lineage infidelity: This theory proposes that the coexpression of myeloid and lymphoid markers is a spurious result of malignancy as transformed cells from one lineage express cell surface markers of another lineage aberrantly due to the transformation event (McCulloch, 1987)

1.4.3.2   Lineage promiscuity: This theory states that a normal myelo-lymphoid cell or a cell with bilineage potential is the target in biphenotypic leukemias and the biphenotypic character of the blasts reflect differentiation block at this normal biphenotypic stage. (McCulloch, 1987)

The relatively low survival rate as well as the high incidence of relapse in patients with biphenotypic leukemia argues for a more primitive target cell for the disease, strengthening the lineage promiscuity theory. Indeed, Chi Wai So and colleagues demonstrated that the transformation of a normal biphenotypic multipotent progenitor but not lineage committed progenitors could give rise to an acute biphenotypic leukemia in mice (So *et al.*, 2003). As is mentioned in the discussion section however, our results indicate that there could be an alternative explanation for the appearance of cells with biphenotypic characteristics.

1.4   Mouse models of leukemia

Several mouse model systems have emerged that are likely to provide powerful means for the analysis of leukemias. These include ubiquitous or conditional knock-ins, targeted and random *in vivo* gene disruption and retrovirally transduced bone marrow transplantation into irradiated recipients. The murine bone marrow transplantation model employs *ex vivo* retroviral gene transfer of primary hematopoietic cells followed by transplantation into lethally irradiated syngenic mouse recipients. Studies including those in our laboratory have demonstrated that proto-oncogenes (Rawat *et al.*, 2004) and proto-oncogene combinations (Kelly *et al.*, 2002; Schessl C, 2005) can induce acute leukemias in mice using this model and importantly, the characteristics of disease in such models appear to recapitulate human leukemia. For example, infection of bone marrow cells with a retroviral vector expressing the *AML1/ETO* proto-oncogene or *FLT3-ITD* alone fails to induce leukemic transformation *in vivo*. When expressed together however, these two genes collaborate to cause full blown leukemia in mice, reflecting the need for a secondary mutation in leukemias involving these factors in human patients as well as their likely collaboration in human leukemia (Schessl C, 2005). Importantly, in these models disease initiates from a relatively rare subset of cells, which undergo varying degrees of differentiation and/or inappropriate growth. These models have provided valuable means for analyzing the molecular and cellular characteristics of leukemia. Their recent use, as mentioned before, in identifying the target cell of leukemias is particularly interesting as it offers new insights into the characterization of the LSC of several leukemias including biphenotypic leukemias as we have previously described. We used the murine bone marrow transplantation model for the generation of leukemia models recapitulating the *CALM/AF10* positive leukemia.

1.6   Aim of the study

Most therapies for AML target the bulk leukemic population and spare the leukemic stem cell. It is therefore critical to determine and characterize the leukemic stem cell in the various types of AML for the development of novel therapeutic targets. Since AML patients harboring the t(10;11)(p13;q14) translocation have a poor prognosis the characterization of leukemic stem cells in this subset of AML is clinically relevant and would lead to the understanding of disease progression. The purpose of the study was to employ the murine bone marrow transplantation marrow model in order to directly assess the oncogenic potential of the *CALM/AF10* fusion gene, the product of the t(10;11)(p13;q14) translocation and the characterisation of the LSCs in this disease. The mechanism of *CALM/AF10* mediated transformation is an interesting aspect to study and the bone marrow transplantation model provides an excellent tool for the structure-function analysis of various domains critical in the transformation process. We also sought to characterise the domains responsible for the hematopoietic activity of the *CALM/AF10* fusion gene, providing an insight into the mechanism of transformation.

2.1   Mice and related reagents and equipment:

Avertin solution: Stock solution was prepared by adding 15.5 ml tert-amyl alcohol to 25 grams Avertin (2-2-2 Tribromoethanol), both procured from (Sigma-Aldrich, St. Louis, MO) and dissolved overnight. For working solution, 0.5 ml stock solution was added to 39.5 ml of cell culture grade phosphate buffered saline (PBS) and dissolved with a magnetic stirrer.

5-Fluorouracil: 50 mg/ml stock solution Medac, Hamburg, Germany. Working solution was 6 ml of the above solution mixed with 4 ml of phosphate buffered saline.

Formalin: 10% solution of formaldehyde (Sigma-Aldrich, St. Louis, MO) in water.

Sterile Syringes: BD Plastipak 1 ml syringe (BD Biosciences, Palo Alto, CA) for injection of cells in mice and Kendall Monoject 3 ml syringes (Tyco Healthcare, UK) for bone marrow flushing and plating of CFCs. The stubs of 3 ml syringes were used to macerate the spleens of mice.

Sterile needles: 0.5 x 25 mm for injection of cell in mice i.v. and 0.55 x 25 mm (BD Microlance, Drogheda, Ireland) for bone marrow aspiration from living mice and flushing of bone marrow from extracted bones. 16 x 1.5 inch needles for dispensing and plating Methocult (CFC) media (Stem Cell Technologies, Vancouver, Canada)

Erythrocyte lysis buffer: 0.8% NH_4Cl with 0.1 mM EDTA (Stem Cell Technologies, Vancouver, Canada)

Heparinized capillaries: (Microvette CB 300) plastic capillaries for collection of blood, 15 I.E Lithium heparin per ml of blood (Sarstedt, Numbrecht, Germany)

Telleyesnickzky's solution: 450 ml absolute ethanol + 25 ml glacial acetic acid + 25 ml formaldehyde

2.2   Mammalian cell lines:

GP+E86:   Mouse fibroblast cell line

293T: Human embryonic kidney cell line

NIH-3T3:   Mouse fibroblast cell line

All cell lines were procured from the American Type Culture Collection (ATCC), Manassas, U.S.A

2.3   Oligonucleotides:

All nucleotides were synthesized by Metabion AG, Martinsried, Germany.

Primers for transcriptional profile analysis

Oligonucleotide	Sequence 5´ to 3´
Pax5_fw	AGGATAGTGGAACTTGCCCA
Pax5_rev	TGATGGAGTATGAGGAGCCC
MPO_fw	ACTGGCCTCAACTGCGAGAC
MPO_rev	GTGTATTGACAGCCAGCAGC
Gata2_fw	GACTATGGCAGCAGTCTCTTCC
Gata2_rev	GGTGGTTGTCGTCTGACAATT
Gata3_fw	TCGGCCATTCGTACATGGAA
Gata3_rev	GAGAGCCGTGGTGGATGGAC
EBF_fw	GCCCGTGGAGATTGAGAGGAC
EBF_rev	GTGCTTGGAGTTATTGTGGAC
c-fms_fw (MCSF-R)	GAGTCAGAAGCCCTTCGACAAA
c-fms_rev (MCSF-R)	TGCCCAGACCAAAGGCTGTAGC
Pu.1_fw	TGGAGGTGTCTGATGCAGAAG
Pu.1_rev	CCGCTGAACTGGTAGGTGA
GCSF-R_fw	TACCAGCCACAGCTCAAAGG
GCSF-R_rev	ACGTGTCCAGTCTGATGGTG
Aiolos_fw	ATCGAAGCAGTGCCGCTT
Aiolos_fw	GTGTGCGGGTTATCCTGCATTAGC
HPRT_fw	GGGGGCTATAAGTTCTTTGC
HPRT_rev	TCCAACACTTCGAGAGGTCC

CALM/AF10 sequencing primers

Oligonucleotide	Sequence 5´ to 3´
CALM/AF10_seq_1f	CTCGAGGTCGACGGTATCG
CALM/AF10_seq_2f	AACACGTTGTTTAACTTAAGCAA
CALM/AF10_seq_3f	CTTGACATCTATAAGAAGTTCC
CALM/AF10_seq_4f	CCTCATACCTCTTTAACAACTG
CALM/AF10_seq_5f	CATTTCTTCAGATGTATCTACTT
CALM/AF10_seq_6f	GAAATGGAACCACTAAGAATGATG
CALM/AF10_seq_7f	CCCCATAAGGATGGAGCTTTAA
CALM/AF10_seq_8f	CC GATAATGTCCAATACTGTG
CALM/AF10_seq_9f	TCAGCTCACAGCTCAGGTC
CALM/AF10_seq_10f	GTACCTTAATTGGCCTCCCT
CALM/AF10_seq_11f	TTTACAGAGCCTCAGTGTTG
CALM/AF10_seq_12f	CTCTCAGTCAGGCACCATC
CALM/AF10_seq_13f	AAAACCGAAGATTAGAGGAAC
CALM/AF10_seq_14f	GTCAATGGCGTGACAGTGGG
CALM/AF10_seq_15f	ACTTCAGCAGCTGCAGATCC
CALM/AF10_jn_fw	ACCCCCTGTAATGGCCTATC
CALM/AF10_jn_rev	AGTGGCTGCTTTGCTTTCTC

Cloning primers for CALM/AF10 mutants

Oligonucleotide	Sequence 5´ to 3´
Leuziptagrev_framec	GCCCTCGAGCACTGAAAGCTGTGCATTCAA
Oct+leu_neu_fw	CCGCTCGAGCCTCCAGTAGCAGCCAGC
AF10_por2_rev_tag	CGCTCGAGGATGTTTCTCAGGTGTAAACTT
CA_dellzip_neu_fw	CGGATCGATCCTTTTCCAACAATAAC
Calm_only_nonstop_rev	CCGCTCGAGCTGTGCTCCTGATACAGGGC
Calm_only_rev	CCGCTCGAGCTACTGTGCTCCTGATACAGGGC
CA_por1_rev	CGCTCGAGCCATCGATGCTGGATGCTACTGGAG

Primers for V-DJ$_H$ and DJ$_H$ recombination

Oligonucleotide	Sequence 5´ to 3´
V$_H$ 7183	CGGTACCAAGAASAMCCTGTWCCTGCAAATGASC
V$_H$ 558	CGAGCTCTCCARCACAGCCTWCATGCARCTCARC
V$_H$ Q52	CGGTACCAGACTGARCATCASCAAGGACAAYTCC
J$_H$ 3	GTCTAGATTCTCACAAGAGTCCGATAGACCCTGG
C-mu-5'	TGGCCATGGGCTGCCTAGCCCGGGACTT
C-mu-3'	GCCTGACTGAGCTCACACAAGGAGGA
B rec chk fw1	ACGTCGACTTTTGTSAAGGGATCTACTACTGT
B rec chk fw2	ACGTCGACGCGGASSACCACAGTGCAACTG
B rec chk rev	GGGTCTAGACTCTCAGCCGGCTCCCTCAGGG

2.4   Plasmids:

MSCV-IRES-GFP: A modified form of the MSCV vector, it contains a bicistronic GFP expression cassette with an internal ribosomal entry site.

Ecopac: A packaging vector coding for the gag, pol, and env viral proteins. (Clontech, Palo Alto, CA)

pCDNA6/V5-His A vector: Mammalian expression vector used for tagging proteins at the carboxyterminal end with the polyhistidine epitope tag (Invitrogen, Carlsbad, CA)

pEYFP-C1: Mammalian expression vector used for tagging genes with fluorescent reporter (Invitrogen, Carlsbad, CA)

2.5   Antibodies:

Name	Company	Label	Dilutions used
Gr-1	BD Pharmingen, Heidelberg	PE/APC	1:500
CD11b (Mac1)	BD Pharmingen, Heidelberg	PE/APC	1:800
Sca-1	BD Pharmingen, Heidelberg	PE	1:150
Ter119	BD Pharmingen, Heidelberg	PE	1:150
B220	BD Pharmingen, Heidelberg	PE/APC	1:200
CD4	BD Pharmingen, Heidelberg	PE	1:150
CD19	BD Pharmingen, Heidelberg	PE	1:200
CD23	BD Pharmingen, Heidelberg	PE	1:200
CD24	BD Pharmingen, Heidelberg	PE	1:200
CD43	BD Pharmingen, Heidelberg	PE	1:200
sIgM	BD Pharmingen, Heidelberg	PE	1:200
F4/80	Caltag Laboratories, CA	PE	1:200

CD117 (c-kit)	BD Pharmingen, Heidelberg	APC	1:500
CD8	BD Pharmingen, Heidelberg	APC	1:150
CALM/AF10S19	Santa Cruz Biotech. Inc., CA	-	1:1000
CALM/AF10G17	Santa Cruz Biotech. Inc., CA	-	1:1000
CALM/AF10C18	Santa Cruz Biotech. Inc., CA	-	1:1000
GFP	Molecular Probes Inc., OR	-	1:5000
Anti-His	Invitrogen, Carlsbad, CA	HRP	1:3000
Goat Anti-Mouse	Invitrogen, Carlsbad, CA	HRP	1:2000

2.6   Reagents, media and apparatus:

2.6.1: Molecular biology:

Agarose: Molecular biology tested (Sigma-Aldrich, St. Louis, MO)

Protamine sulfate: (Salamine) from Salmon, cell culture tested (Sigma-Aldrich, St. Louis, MO) 5 mg/ml stock solution

DNeasy mini kit: Genomic DNA extraction kit for small cell numbers (Qiagen GmbH, Hilden, Germany)

Small-scale plasmid preparation: GFX miniprep kit for isolation of plasmid DNA from bacteria (Amersham Biosciences GmbH, Freiburg, Germany)

Gel Elution of DNA and PCR or DNA cleanup: GFX gel elution and PCR purification kit for DNA elution from gels and clean up of PCRs (Amersham Biosciences GmbH, Freiburg, Germany)

Southern blot: Microspin S-300 HR columns and Megaprime DNA labeling system (Amersham Biosciences GmbH, Freiburg, Germany)

Pre-hybridisation solution: 0.2 g skimmed milk and 2.0 g dextran sulphate were dissolved in 17 ml water and 6ml 20 X SSC, 2 ml formamide, 1 ml 20% SDS and 80 µl 500 mM EDTA were added to the mixture. (All chemicals were individually obtained from Sigma-Aldrich, St. Louis, MO)

Denaturation solution: A solution of 1.5 M NaCl, 0.5 N NaOH in water.

20 X SSC: 175.3 g sodium chloride and 88.2 g sodium citrate were dissolved in 800 ml deionised water and pH adjusted to 7.0 and the final volume to one litre.

DNA Crosslinking: GS Gene linker UV chamber (BIO-RAD Laboratories, Hercules, CA)

Western blot: ECL Western blotting analysis system (Amersham Biosciences GmbH, Freiburg, Germany)

Total RNA and genomic DNA isolation: Total RNA isolation reagent (TRIZOL) and Genomic DNA isolation reagent DNAZOL (Invitrogen, Carlsbad, CA)

Molecular weight markers: Nucleic acid size standards, 1 kb ladder, 1 kb plus ladder and 100 bp ladder (Invitrogen, Carlsbad, CA)

Enzymes: Ligase, Calf intestine phosphatase, *Xho I, Cla I, Eco RV, Eco RI, Hpa I,* and *Pme I* all from New England Biolabs (NEB, Beverly, MA)

RT and PCR: Platinum Taq DNA polymerase kit, ThermoScript kit, RT-PCR kit and DNaseI DNA inactivating enzyme kit (all from Invitrogen, Carlsbad, CA) PCR soft tubes (0.2 ml) (Biozym Scientific GmBH, Hess.Oldendorf, Germany)

Real time PCR kit: LightCycler FastStart DNA Master SYBR green I kit (Roche Diagnostics, Mannheim, Germany) LightCycler Carousel and carousel centrifuge (Roche Diagnostics, Mannheim, Germany)

dNTP mix: 10 mM each of dATP, dTTP, dCTP and dGTP (Invitrogen, Carlsbad, CA)

2.6.2: Cell and tissue culture:

Methylcellulose media: Methocult 3434 for the culture of myeloid CFC assays and Methocult 3630 for the pre-B CFC assays (Stem Cell Technologies, Vancouver, Canada).

Filtration units: Millex syringe driven filter units 0.22 μm and 0.45 μm filters (Millipore, Billerica, MA)

Cell strainer: BD Falcon 40 μm Nylon strainer for macerating the spleen and filtering the tissue (BD Biosciences, Palo Alto, CA)

Cell Scrapers: 25 cm sterile cell scrapers (Sarstedt, Newton, NC)

Cell culture pipettes (2, 5, 10 and 25 ml): Sterile disposable pipettes (Corning Inc., Corning, NY)

Cell culture plates and dishes: Sterile 96 well, 24 well, 6 well plates (Sarstedt, Numbrecht, Germany) 100 mm x 20 mm dishes for adherent cells (Corning Inc., Corning, NY), and Petri dishes for suspension cells (Becton Dickinson Labware, Franklin Lakes, NJ) 150 mm x 20 mm dishes for adherent cells (Greiner Bione, Frickenhausen, Germany)

Calcium Chloride solution for transfection: 2.5 M $CaCl_2$ (Sigma-Aldrich, St. Louis, MO) solution in water.

Hepes Buffered Saline: (HBS) (Invitrogen, Carlsbad, CA)

Media: Dulbecco's Modified Eagle's Medium (DMEM) 4,5 g/l glucose, l-glutamine, sodium pyruvate and 3,7 g/l $NaHCO_3$ (PAN biotech GmbH, Aidenbach, Germany)

Fetal Bovine Serum (FBS): 0,2 μm-filtered mycoplasma screened (PAN biotech GmbH, Aidenbach, Germany)

Dulbecco's phosphate buffered saline (DPBS): without magnesium and calcium, sterile filtered (PAN biotech GmbH, Aidenbach, Germany)

Trypsin – EDTA: 1 X in HBS without calcium and magnesium with EDTA (Invitrogen, Carlsbad, CA)

Penicillin/Streptomycin: Antibiotic solution with 10,000 u/ml Pen G sodium and 10,000 µg/ml Streptomycin sulfate in 0,85% saline. Used 5 ml per 500 ml medium bottle (Invitrogen, Carlsbad, CA)

Murine cytokines: mIL3, mIL6, mSCF, mG-CSF, mM-CS and mGM-CSF (lyophilized) (Tebu-bio, Offenbach, Germany)

Ciprofloxacin: Ciprofloxacin 400 solution, (Bayer AG, Leverkusen, Germany)

2.6.4: Miscellaneous:

Giemsa: Giemsa's Azure Eosin Methyleneblue solution modified. (Merck KGaA, Darmstadt, Germany)

May-Gruenwald: May-Gruenwald's Eosin Methyleneblue solution for microscopy (Merck KGaA, Darmstadt, Germany)

Cytospin apparatus: Cytospin 2 Shandon Apparatus (Thermo Electron corporation, U.S.A)

Cytospin slides: Marienfield pre-cleaned twin frosted slides for fixing single cell suspensions and blood smears (Marienfield, Lauda-Königshofen, Germany)

Cytospin filter cards: ThermoShandon thick white 5991022 filter cards for cytospins (Histocom AG, Zug, Switzerland)

Flow cytometry: BD FACS Calibur System (BD Biosciences, Palo Alto, CA)

Fluorescence Activated Cell Sorting: BD FACSVantage SE System (BD Biosciences, Palo Alto, CA)

Sequencing mix and apparatus: BigDye Terminator v1.1 Cycle Sequencing Kit and the ABI Prism 310 Genetic Analyzer (Applied Biosystems, Foster City, CA)

Microscope: Leitz Diavert Inverted Microscope (Ernst Leitz Wetzlar GmBH, Wetzlar, Germany)

2.6.5   Software:

The software used for Flow cytometry and FACS sorting was the CellQuest Version 3.1(f) (BD Biosciences, Palo Alto, CA). Calculations of survival curve were performed using Sigma Plot 2001 Version 7 (SPSS Inc. Chicago, IL) and those for the frequency of the leukemia propagating cell using the L-Calc Limiting dilution analysis software Version 1.1 (StemSoft Inc., Vancouver, Canada). The Roche LightCycler software Version3 (Roche Diagnostics, Mannheim, Germany) was used for real time PCR runs and data analysis. The ABI Prism 310 sequencing software (Applied Biosystems, Foster City, CA) was used for sequencing and analysis of sequences. The Openlab software 3.0.8 (Improvision Deutschland, Tuebingen, Germany) was used for visualizing and photographic documentation of cell morphology. Primers were designed using the Primer3 program, Whitehead Institute, Massachusetts Institute for Technology, MA (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi).

3.1   Cloning of constructs:

The 5.2 kb full length *CALM/AF10* fusion gene initially cloned from the U937 monocytic cell line was sub-cloned by blunt end ligation into the *Hpa I* site in the multiple cloning site (MCS) of the modified murine stem cell virus (MSCV) 2.1 vector upstream of the internal ribosomal entry site (IRES) and the enhanced GFP fluorescent protein gene. The *CALM*Δ3' mutant was cloned by ligating a PCR amplified (primers: Calm_only_fw and Calm_only_nonstop_rev) 2 kb fragment of the truncated *CALM* gene in frame to the polyhistidine epitope of the pCDNA6/V5-His A plasmid. For the *CALM*/OM-LZ mutant, a PCR amplified 200 bp fragment (primers: Oct+leu_neu_fw and Leuziptagrev_framec) encoding the *AF10* octapeptide motif (OM) and the leucine zipper (LZ) was cloned in frame into the *Xho I* site at the 3' end of the *CALM* Δ3' construct, also in frame to the polyhistidine tag. The *CALM/AF10* ΔOM-LZ mutant was constructed in two steps; first by ligating a portion of PCR amplified DNA (primers: Calm_only_fw and CA_por1_rev) from the start of *CALM/AF10* till the octapeptide motif of the *AF10* gene by an *Eco RV-Xho I* digestion into the pCDNA6/V5-His A vector. A *Cla I* site was introduced in frame with the cDNA just before the 3' end of the reverse primer before the XhoI site. In the second step a PCR amplified *Cla I-Xho I* fragment of the remaining portion of *AF10* after the leucine zipper (primers: CA_dellzip_neu_fw and AF10_por2_rev_tag) was inserted into the *Cla I-Xho I* digested first clone. *Cla I* and *Xho I* were inserted into the 5' and 3' regions of the primers respectively for this second fragment; the primers were designed to remove the stop codon and maintain the frame with the polyhistidine tag. All these mutants were then sub-cloned into the modified MSCV vector for retroviral transduction experiments. All clones were sequenced with the *CALM/AF10* sequencing primers.

3.2   Preparation of high titre stable virus producing cell lines:

1.5x 10^6 293T cells were plated in a 15 cm dish and on the following day used for transient transfection. Medium was changed 4 hours prior to the transfection and 30 μg plasmid DNA each of the gene of interest and of the retroviral packaging construct Ecopac were added to sterile water and a sterile solution of 100 μl 2.5M $CaCl_2$ was added drop wise to the water-DNA mixture. The volume of water added initially was calculated so as to make the total volume 1 ml. This was added slowly to a tube containing 1 ml sterile Hepes buffered saline solution (pH 7.2). After gentle mixing and incubating at room temperature for 3-4 minutes, this mixture was added drop wise to the medium covering the whole plate and without agitating the cells. The medium was changed the next day and supernatant was collected from the cells every 12 hours (thrice totally) and fresh medium added. This supernatant was filtered with a 0.45 mm Millipore filter and stored as VCM at −80° for later use or used directly to transduce GP+E86 fibroblasts or murine bone marrow.

5x 10^4 GP+E86 fibroblasts were plated into 6 well plates one day prior to transduction. The next day, medium was withdrawn from these cells and 500 μl or 1 ml of fresh or frozen VCM was layered on top of the cells with the addition of a final concentration of 10 μg/ml protamine sulfate. Fresh medium was added after 4 hours and the transduction procedure was repeated every 12 hours for three-four times. The cells were expanded and two days were allowed for GFP expression. Green fluorescent cells were sorted using the fluorescence activated cell sorter (FACS) sorter, propagated and used as stable virus producing cell lines to transduce murine bone marrow. Using these protocols, *CALM/AF10/GFP*, only *GFP*, Δ*CALM/GFP*, *CALM+LZ/GFP*, or Δ*LZ/GFP* GP+E86 cell lines were constructed and used for experiments.

In some cases where viral titres of bulk cell lines were low, single cells were sorted into 96 well plates and after expansion their viral titres determined on NIH-3T3 cells. Clones producing highest titres were expanded, frozen and used for experiments.

Titration was performed by plating 2 x 10^5 NIH3T3 cells per well in 6 well plates and layering them with 500 μl VCM the next day with the addition of a final concentration of 10 μg/ml protamine sulfate. Fresh medium was added after 3-4 hours to stop transduction. This was performed every 12 hours (thrice totally). Two days following transduction with VCM, cells were analysed for GFP expression at the FACS calibur.

3.3   Retroviral transduction of primary bone marrow:

Bone Marrow Transplantation Model
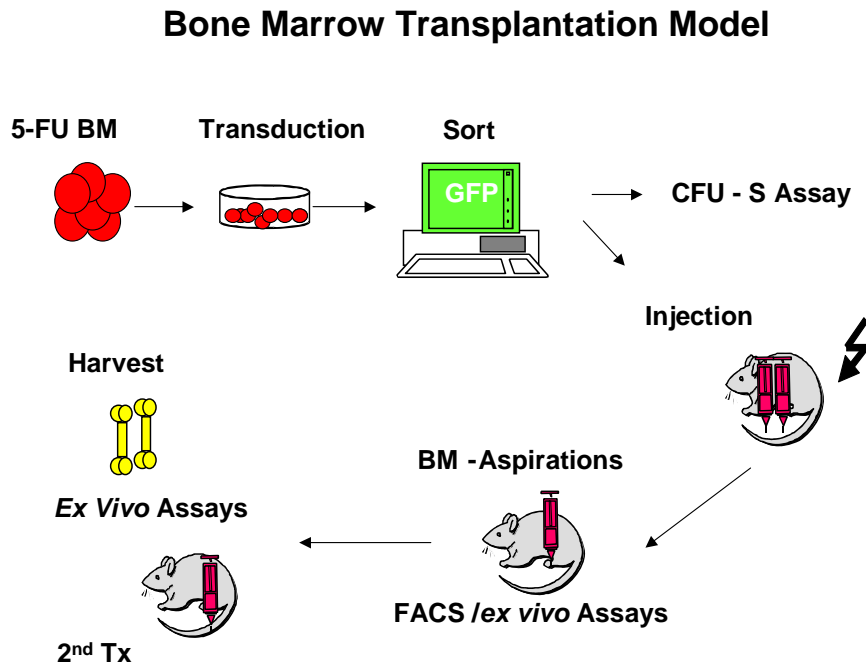
Fig. 3.3a Experimental design of bone marrow transplantation of *CALM/AF10* and control mice

Parental strain mice were bred and maintained at the GSF animal facility. The mice were fed with autoclaved chow and supplied with drinking water containing ciprofloxacin and acetic acid and housed in individually vented cage systems. Donors of primary BM cells were > 8-wk-old (C57BL/6Ly-Pep3b x C3H/HeJ) F_1 (PepC3) mice. 150 milligrams of 5-Fluorouracil (5-FU) was injected per kg of mouse body weight to eliminate cycling cells and to enrich for hematopoietic progenitor cells. On the fifth day following 5-FU injection, these mice were sacrificed and their femurs and tibia flushed with serum-supplemented medium to obtain bone marrow cells. This bone marrow was prestimulated by culturing for 2 days in a cytokine cocktail (10 ng/ml mIL-6, 6 ng/ml mIL-3 and 100 ng/ml murine stem cell factor) in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 15% FBS. On day 3, transduction was performed by layering the bone marrow cells on top of adhered GP$^+$E86 cell lines (coculture). Cell lines were irradiated for 40cGy plated on adherent 150 mm x 20 mm dishes one day prior to the transduction. 10 μg/ml protamine sulfate was always added to the medium during viral transduction. On day 5, following

transduction for two days, bone marrow was removed gently but completely without disturbing the adhered monolayer of the GP+E86 cell line. Bone marrow was cultured in DMEM 15% FBS and 2 more days were allowed for GFP expression. On day 7, GFP positive cells were sorted by FACSVantage and used for bone marrow transplantation or for *in vitro* culture. Bone marrow was always cultured in DMEM 15% FBS medium supplemented with 10 ng/ml mIL-6, 6 ng/ml mIL-3 and 100 ng/ml murine stem cell factor.

3.4   Bone marrow transplantation and assessment of mice:
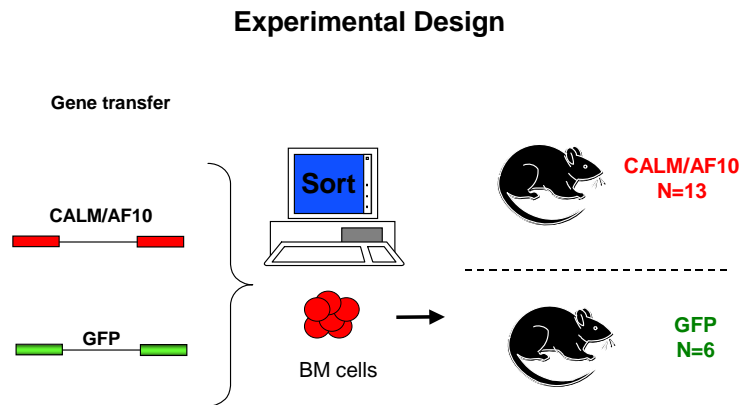
Fig. 3.4 a Experimental design of bone marrow transplantation of *CALM/AF10* and control mice

This is a representation of the experimental set of mice from the *CALM/AF10* and the MSCV-IRES-GFP arms. A total of 13 mice were injected with bone marrow cells transduced with *CALM/AF10* and six mice with the bone marrow cells transduced with the empty vector.

Recipients were > 8- to 12-wk-old (C57BL/6J x C3H/HeJ) F_1 (B6C3) mice. These mice were lethally irradiated (0.80 cGy) a few hours prior to receiving bone marrow transplants. Transduced bone marrow or bone marrow from leukemic mice was injected with or without addition of mock transduced or non-transduced bone marrow cells intravenously into the tail vein of mice using a sterile 0.5 x 25 mm needle. Mice were assessed periodically

for signs of leukemic symptoms by blood withdrawal from the tail vein using sterile scalpels and bone marrow aspiration from the tibia of anaesthetised animals or by the observance of symptoms that included crouching, frizzled body hair, paleness in the feet, heavy breathing and disturbed gait. Mice were considered moribund when one of these symptoms was starkly visible.

Expt. no	Gene	Transduced cells	Mock cells
3493b	*CALM/AF10*	200000	200000
3493b	*CALM/AF10*	200000	200000
3493b	*CALM/AF10*	200000	200000
3493b	*CALM/AF10*	400000	0
3493b	*CALM/AF10*	400000	0
3515	*CALM/AF10*	200000	200000
3515	*CALM/AF10*	200000	200000
3515	*CALM/AF10*	200000	200000
3521	*CALM/AF10*	100000	300000
3724	*CALM/AF10*	100000	250000
3724	*CALM/AF10*	100000	250000
3724	*CALM/AF10*	100000	250000
3746	*CALM/AF10*	50000	100000
3493a	*GFP*	400000	0
3493a	*GFP*	400000	0
3493a	*GFP*	400000	0

Fig. 3.4 b Injection of transduced and mock transduced bone marrow in *CALM/AF10* and MSCV-IRES-GFP mice

This is a schematic representation of the number of cells injected and the respective amount of mock transduced added for transplantation into each mouse. Mock transduced cells were cells sorted from the same sample but negative for GFP expression. For secondary and tertiary mice, $1x\ 10^6$ bone marrow cells from a leukemic primary or secondary mouse respectively were injected with the

addition, to each mouse, of $1x \, 10^6$ bone marrow cells from a syngenic wild type mouse (non-transduced mock cells).

Moribund mice were sacrificed by CO_2 asphyxiation and bone marrow was aspirated as described before. Spleens were dissected and macerated to produce single cell suspensions and peripheral blood was drawn with a sterile 0.5 x 25 mm needle by puncturing the heart immediately after sacrificing the mice. Red blood cell (RBC) lysis for peripheral blood, bone marrow and spleen cells was performed by incubating the cells in ammonium chloride buffer for 10 minutes at room temperature.

3.5   FACS analysis of murine cells:

Single cell suspensions of cells were immunostained with various fluorescence-conjugated antibodies. Staining was performed in PBS with the fluorescence-conjugated antibodies using a 1: 200 dilution for each antibody. Samples were incubated at 4°C for 20 minutes and subsequently washed with PBS to remove excess antibody. Cells were centrifuges and after decanting the supernatant, resuspended in FACS buffer (2% fetal bovine serum and 2 μg/ml propidium iodide in phosphate-buffered-saline). Antibodies used for FACS were labelled with phycoerythrin for Gr-1, CD11b (Mac1), Sca-1, Ter119, CD4, CD19, CD23, CD24, CD43, sIgM, F4/80 and allophycocyanin conjugated CD11b (Mac-1), CD117 (c-kit), B220, and CD8. Fluorescence was detected using a FACSCalibur flow cytometer and analyzed using the CellQuest software. Dead cells were gated out by high PI staining and forward light scatter.

3.6   *Ex Vivo* proliferation and CFC Assays:

Cell proliferation was assessed in DMEM supplemented with 15% FBS 10 ng/ml mIL-6, 6 ng/ml mIL-3 and 100 ng/ml murine stem cell factor (standard medium). Differentiation of clonogenic progenitors was analysed by plating cells in methylcellulose

supplemented with cytokines (Methocult M3434) or pre-B cell assays (Methocult M3630). Replating was performed every week in appropriate dilutions.

IL-3-dependent cell populations expressing *CALM/AF10* were established *in vitro* directly after sorting in DMEM 15% FBS with IL-3 alone (6 ng/ml). Single cell lines were generated by sorting these cells into 96 well plates using the BD FACS Vantage in 200 μl DMEM 50% FBS and 6 ng/ml IL3. After 2 weeks individual wells were assessed for growth and growing cells expanded as single cell clones.

The differentiation capacity of cultured cells was tested in DMEM 15% FBS supplemented with granulocyte colony-stimulating (G-CSF) factor 100 ng/ml or macrophage colony-stimulating factor (M-CSF) 10 ng/ml. After 5 days, the morphology was determined by Wright–Giemsa stained cytospin preparations.

3.7 Cytospin preparations and Wright Giemsa staining:

Cytospins of single cell suspensions were performed by resuspending cells in PBS at a concentration of 2-6x 10^5 cells per 200 μl and this volume was introduced into the cytospin apparatus. The cells were permanently fixed on glass slides by centrifugation at 500 rpm for 10 minutes and subsequently air-dried. Modified Wright Giemsa staining was performed by immersing the slides in an undiluted solution of May-Grunwald stain for 5 minutes. This was followed by immersing the slides in 1:50 diluted Giemsa stain for 1 hour. Slides were dipped in water to remove excess stain between the two staining steps and after the staining procedure and air-dried for observance under the inverted light microscope.

For histological analysis, the peritoneum of sacrificed leukemic mice was dissected so as to expose all organs and most of the blood drained by cutting the peritoneal artery and absorbing the blood with a tissue paper. The mice were fixed in an aqueous solution of formaldehyde (10% v/v) and sections of selected organs were prepared and hematoxylin-eosin stained using standard protocols.
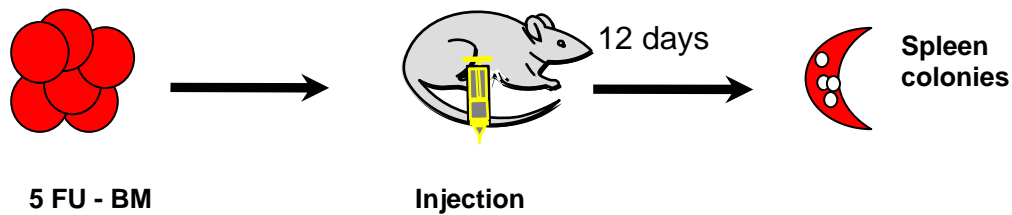
3.8 Colony-Forming Unit–Spleen (CFU-S) Assay:

Fig. 3.8a   Schematic representation of the Colony Forming Units in Spleen Assay (CFU-S)

Primary BM cells from F_1 (PepC3) donor mice that had been primed 5 days previously with an i.v. injection of 150 mg/kg 5-fluorouracil were retrovirally transduced (as described in 3.3 and 3.4) with the different viruses and cells were highly purified based on expression of GFP by using a FACSVantage. Transduced cells were injected directly after sorting into lethally irradiated F_1 (B6C3) recipient mice. The recovery of CFU-S cells was quantified by determining the number of macroscopic colonies on the spleen at day 12 post-injection after immersion in Telleyesnickzky's solution.

3.9 Quantification of the Leukemia propagating cell frequency:

B220APC+/Mac1PE- *(B population)*, B220APC+/Mac1PE+ *(BM population)* and B220APC-/Mac1PE+ *(M population)*, cells were sorted from the flushed bone marrow of a sacrificed primary *CALM/AF10* mouse. The sort purity of these cells was checked with the FACSCalibur and determined to be over 95%. Tenfold serial dilutions of these cells were injected intravenously (max. 5x 10^5, min. 50 cells) into lethally irradiated secondary recipient mice as previously described (Section 3.4). 1x 10^6 cells from a syngenic disease free mouse bone marrow were added to each sample for radioprotection. Mice were assessed for signs of leukemia as described previously and sacrificed when moribund. The day of sacrifice/death was noted and immunostaining of cells from various organs was done as described (Section

3.3). The frequency of leukemia propagating cells was calculated using the L-Calc limiting dilution analysis software.

3.10   RNA and genomic DNA isolation and cDNA preparation:

The Trizol method for RNA isolation described by the manufacturer was used to extract RNA with the addition of 1 ml of Trizol solution per million cells. Equal amounts of RNA as quantified by a spectrophotometer were added to each reaction (in a set) used for cDNA preparation for the semi-quantitative PCRs. Each sample was treated with DNaseI for prevention of genomic DNA contamination in cDNA samples. This was performed for each sample prior to cDNA preparation according to the manufacturer's instructions.

Genomic DNA was isolated from a minimum of $1x\ 10^7$ cells for Southern blotting from various murine organ cells using the DNAZOL reagent and the protocol for the same according to the manufacturer. Genomic DNA for the V-DJ and D-J PCRs was isolated using the DNeasy mini kit using supplied methods. Genomic DNA was resuspended in sterile water and quantified using a spectrophotometer after proper dissolution.

cDNA was prepared from DNaseI treated RNA. First-strand cDNA synthesis was done with ThermoScript kit. In a 20 μl reaction volume, 1 μg RNA and 1 μg of oligo (dT) were mixed to a final volume of 11 μl and incubated 10 minutes at 70°C. Then, 4 μl of 5 X first-strand buffer, 2 μl of DTT 0.1 mol/L, 1 μl of 10 mmol/L deoxynucleoside triphosphate mix, and 2 μl of ThermoScript reverse transcriptase were added. The sample was incubated 1 hour at 42°C and used for PCRs.

3.11   Southern and Western (immuno) blotting:

3.11.1   Southern blot analysis:

Southern blot analysis to assess proviral integration was performed by isolating DNA from bone marrow, spleen and peripheral blood of leukemic mice using DNAZOL reagent as recommended by manufacturer. Southern blot was performed using standard protocols. DNA was digested with *Eco RI,* which cuts the proviral DNA once, to release a

fragment specific to the proviral integration site. To check the full-length integration, DNA was digested with *Nhe I*, which cuts in the long terminal repeats (LTRs) to release the proviral genome. After digestion DNA was loaded on a 0.7% agarose gel with 0.5 ug/ml ethidium bromide. After electrophresis, the DNA was depurinated by soaking the gel in 0.2 N HCL for 8 minutes, and subsequently for 45 minutes in denaturation buffer. After denaturation, the DNA was transferred on zeta-Probe GT membrane by capillary action in a 10X transfer buffer. Cross-linking of the DNA with membrane was done by incubating the membrane at 150 mjoule in a UV gene linker. The probe used was a 700 bp GFP fragment, which was digested out from the pEGFP-C1 plasmid and labelled with α-^{32}P dCTP using Megaprime DNA labelling system. Probe was purified using Microspin S-300 HR columns. Hybridization was done with α-^{32}P GFP labelled overnight at 62^{0}C. After two rounds of washing the membrane was dried, covered with a plastic film and put in a cassette for exposure of the film. The film was put on the membrane in a dark room and the exposure was done at variable exposing times between 48 hours and one week, depending on the visualization signal observed.

3.11.2   Western Blotting (Immunoblotting):

Sample preparation and cell lysis (total cell extract):

Proof of protein expression was performed using the 4D6 E86 *CALM/AF10* cell line. The cells were lysed using 150 µl RIPA buffer with fresh added protease inhibitors and detached using a cell culture scraper. The cells with RIPA buffer were transferred to an Eppendorf microcentrifuge tube and mixed by inversion for 30 minutes at 4°C. After the homogenization, the sample was centrifuged at 14000 rpm for 30 minutes at initialized. After centrifugation, the supernatant was transferred to a new Eppendorf tube and either frozen at -80°C, or kept on ice for determination of protein concentration. As a control, 293T cells from an 80% confluent 15 mm cell culture dish (between 5 and 10x 10^7 cells) were transiently transfected with 10 µg of pEYFP-*CALM/AF10* DNA. Lysates were prepared using the method described above.

Determination of Protein Concentration:

The method used for measuring the protein concentration was the Bradford method. The assay is based on the observation that the absorbance maximum for an acidic solution of Coomassie Brilliant Blue G-250 shifts from 465 nm to 595 nm when binding to protein occurs. Both hydrophobic and ionic interactions stabilize the anionic form of the dye, causing a visible color change. The assay is useful since the extinction coefficient of a dye-albumin complex solution is constant over a 10-fold concentration range. Within the linear range of the assay (5-25 µg/ml), the more protein present, the more Coomassie binds. The protein concentration of the sample was determined by comparison to values obtained for the measure of the known range of protein standards. The protein standard used was Bovine Serum Albumin (BSA). Six different albumin concentrations (2.5 µg, 5 µg, 10 µg, 15µg, 20µg and 25µg) were diluted in distilled water to a final volume of 800 µl. One microliter of cell lysate was diluted in distilled water for the measure. 200 µl of Protein Assay solution was added to the tubes. The tubes were incubated at RT for 15 minutes and the content was further transferred to polystyrol cuvettes. A determination of the standard curve of the spectrophotometer with distilled water and the protein standards was done using the specific program for protein in the spectrophotometer. The sample was measured following the standard curve determination.

SDS PAGE of Cell Extracts:

Total cell extract (TCE) proteins were separated on a denaturing gel consisting of 8% Tris-glycine gel was used and a 5% stacking gel. The concentration of the separation gel was chosen considering the sizes of wt CALM protein (70kDa) and *CALM/AF10* (about 145 kDa) as indicated in molecular protocols (Sambrook *et al.*, 1989). The sample was homogenized and diluted 1:1 with 2x loading buffer and heated in a boiling water bath for 10 minutes. 80 µg protein was loaded on each gel lane. The electrophoresis was performed under 100 v for 1hour and 30 minutes in the cold room at 4^oC.

Protein Blotting:

After the electrophoresis, the gel was taken from the cassette and washed once with TBS. For the blotting, the wet system was used. To permit a better transfer of large

molecular weight proteins as CALM/AF10, which has a predicted size of about 170 kDa, a PVDF membrane was chosen. The membrane was wetted in methanol for 30 seconds, rinsed in distilled water and equilibrated in transfer buffer for 10 minutes. The system was assembled putting a sponge on the bottom of the sandwich (in contact to the negative pole), a 0.8 mm filter paper in contact to the sponge, and the gel over the paper. A 10 ml pipette was used to eliminate the eventually formed air bubbles. On the membrane, another filter paper was put, a second sponge and the chamber was closed. The PVDF membrane was oriented to the positive pole to permit the protein (negatively charged) to migrate from the gel to the membrane (on the positive pole). The transfer system was submitted to constant amperage of 250 milliamp for 4 hours at 4°C with agitation. The observation of the high molecular weight proteins of the pre-stained protein standard on the membrane was an indicator of successful transfer.

Protein detection on the blotting membrane with HRP-marked antibodies:

The antibody-detection of protein was performed following the instructions of the antibody's supplier. After the transfer, the membrane was blocked to prevent non-specific binding of antibodies to the membrane by incubating with BlottoA buffer for one hour at room temperature or overnight at 4°C in constant shaking. The membrane was further washed once with TBS for five minutes and incubated with the primary antibody at 1:1000 dilution in BlottoA overnight. The concentration used for the antibodies was adjusted according to the intensity and background. After incubation with the primary antibody, the membrane was washed three times with TBS with 0.05% Tween-20 (TBST). The secondary antibody conjugated with Horse Radish Peroxidase (HRP) was diluted 1:2000 in BlottoA and put on the membrane for 45 to 90 minutes incubation at room temperature. The membrane was rinsed with distilled water, washed again tree times with TBST and once with TBS for 5 minutes under agitation. To detect the antibodies on the membrane a commercial chemiluminescence kit was used according to the manufacturer's instructions. After washing, the ECL detection solution was put on the membrane for 90 seconds. The membrane was dried, covered with a plastic film and put in a cassette for exposure of the film. The film was put on the membrane in a dark room and the exposure was done at variable exposing times between 15 seconds and 10 minutes, depending on the visualization signal observed.

3.12   PCRs:

3.12.1 PCR for transcriptional profile analysis:

PCRs were performed to check the expression of various lineage specific transcripts in highly purified B220+, B220+/Mac+ and Mac+ cells. These cells were sorted from a cell line obtained after the propagation of a single B220+/Mac1- cell. Sort purity of cells was analysed and determined to be over 95% in each case. PCR was performed for *Aiolos*, *MCSF-R*, *GSCF-R*, *EBF*, *MPO*, *Pax5*, *Pu.1*, *Gata2*, and *Gata3* using primers described in the methods section. The housekeeping gene *HPRT* was used to normalize input cDNA. Initially a test PCR with all cDNAs employing 20, 25 and 30 cycles for *HPRT* was performed to determine and avoid saturation related pseudo-normalization. If the intensity of one sample in the lowest cycle PCR was different from the other, cDNAs were recalibrated till a PCR at that cycle number with bands of equal intensity for each cycle was observed. Then, the PCRs were performed for each gene with 30 cycles at different conditions.

3.12.2 PCR for V-D-J recombination status:

D-JH rearrangements in the Ig locus were detected by a PCR strategy employing two upstream degenerate primers binding 50 of the DFL/DSP element or the DQ52 element. The reverse primer was complementary to a binding site downstream of the JH4 segment. All three primers were used in a single PCR reaction in a multiplex PCR and the following reaction used in germline configuration, the DQ52 and JH4A primers will amplify the 2.15-kb germline fragment. D-J_H1, D-J_H2, D-J_H3, and D-J_H4 rearrangements involving either D_{FL}, D_{SP}, or D_{Q52} elements will be detected by the emergence of bands of ~1.46, ~1.15, ~0.73, and ~0.20 kb, respectively. The amplification protocol was an initial denaturation at 94°C for 1 minute followed by 35 cycles of 1 minute at 94°C, 1 minute at 60°C, and 1 minute 45 seconds at 72°C. Final extension was carried out at 72°C for 10 minutes. The concentration of genomic DNA taken was always between 20-300 ng/μl.

The PCR assay for V to D-J rearrangement employs three degenerate primers oligonucleotides (each in separate reactions) homologous to the conserved framework region 3 (FR3) sequences of the three V_H gene families (V_H 7183, V_H558 and V_H Q52) and the J_H reverse primer. This results in amplified VDJ rearrangements of ~1,058, ~741, or ~333

nucleotides. Wild-type murine spleen genomic DNA was used as a positive control and genomic DNA from the myeloid cell line 32D as a rearrangement negative control. PCR was carried out after an initial denaturation step of 94°C for 4 min for 35 cycles with 94°C for 1 minute, 60°C for 1 minute and 72°C for 1 minute 45 seconds. Final extension was carried out for 7 minutes at 72°C. All PCR products were evaluated on a 1.5% agarose gel by gel electrophoresis.

3.13   Statistical Analysis:

Data were evaluated by using the t test for dependent or independent samples (Microsoft EXCEL). Differences with P values < 0.05 were considered statistically significant. For calculations of frequency of leukemia propagating cells, the L-Calc software was used. Cell numbers were entered as doses, the number of mice per cohort as test and the number of mice dead as the response for the frequency calculation.

4.1   Cloning and expression of *CALM/AF10*: We sought to determine the effect of constitutive expression of *CALM/AF10* in hematopoietic stem and progenitor cells upon hematopoietic reconstitution of lethally irradiated mice. For this purpose, a full length *CALM/AF10* cDNA from the monocytic cell line U937 was sub-cloned in a MSCV vector with an IRES driven GFP co-expressing cassette to track transduced cells. This vector has been shown to efficiently transduce hematopoietic stem cells and the GFP co-expression enables tracking and purification of transduced cells (Rawat *et al.*, 2004). In each experiment, GFP expressing empty vector control transduced cells were used as controls. A *PmeI-PmeI CALM/AF10* fragment from a pcDNA6 V5 His-A *CALM/AF10* clone was sub-cloned in MSCV-IRES-GFP vector linearized with *HpaI* with a blunt end ligation. This construct was used to produce a stable virus producing GP+E86 cell line. Western blots were performed with this GP+E86 cell line for proof of protein expression.

a) | LTR |————————| IRES | GFP |—| LTR |

b) | LTR |—| CALM/AF10 | IRES | GFP |—| LTR |
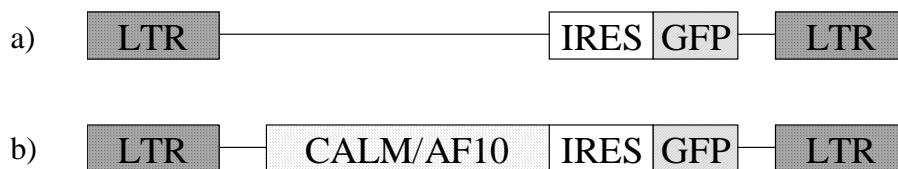
Fig. 4.1.a A cartoon representation of the vectors used for bone marrow transplantation experiments: a) empty vector control and b) full length *CALM/AF10* cDNA flanked by long terminal repeat (LTR) sequences. The internal ribosomal entry site (IRES) facilitates co-expression of the GFP.
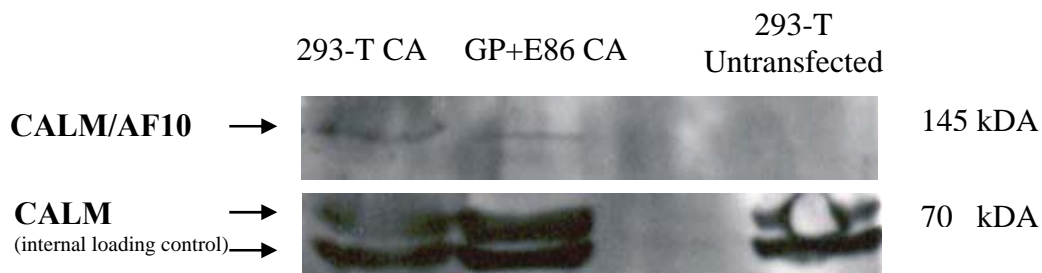
Fig. 4.1.b Protein expression of CALM/AF10: Protein expression of CALM/AF10 was observed by immunoblotting of the whole protein lysate from the *CALM/AF10* GP+E86 (GP+E86 CA) cell line used for transduction of murine bone marrow. As a positive control, a cell line transduced with the *CALM/AF10* fusion gene (293-T CA) was used; the untransfected 293T cells were used as the negative control. The two alternatively spliced forms of CALM were visible in all samples and acted as internal loading controls.

In order to directly assess the *in vivo* effects of *CALM/AF10* transduced bone marrow cells in syngenic mice we performed bone marrow transplantation experiments on lethally irradiated murine recipients. Bone marrow cells enriched for stem and progenitor cells by 5-fluorouracil treatment of donor mice were retrovirally transduced with *CALM/AF10* or the empty vector and injected into syngenic recipient mice. Lethally irradiated recipients normally die of bone marrow failure if injected cells fail to engraft the marrow. Following injection of bone marrow cells; it is therefore possible to assess the effects on stem cell activity as well as leukemogenic ability of genes retrovirally targeted into these cells.

4.2 *CALM/AF10* **expression enhances the short-term engraftment potential of bone marrow progenitors:** Stem and progenitor cells injected into lethally irradiated mice provide short and long-term engraftment, which can be measured at 4 and 8 weeks respectively. Analysis of peripheral blood samples of syngenic mice that had received *CALM/AF10* transduced bone marrow revealed high levels of short-term engraftment as assessed by the percentage of transduced versus non-transduced cells at 4 weeks post injection. GFP was used as a marker of transduction.
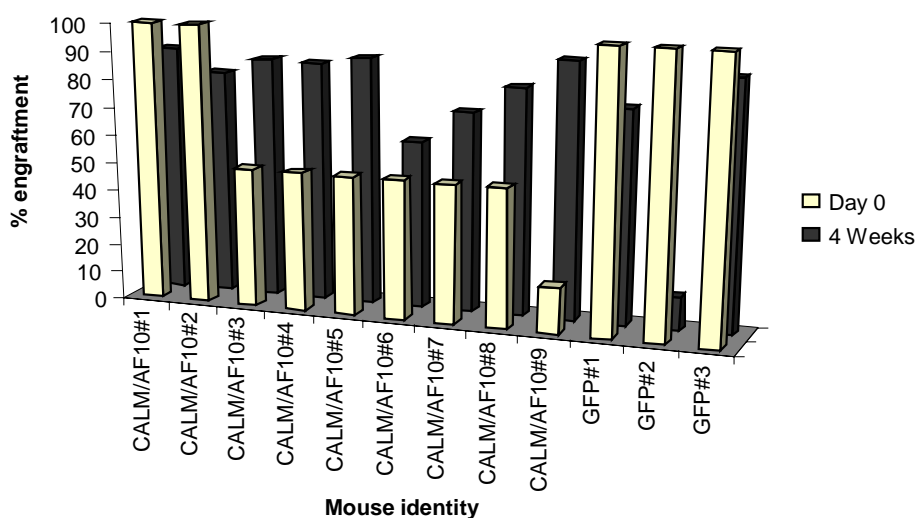

Fig. 4.2. Percentage engraftment of *CALM/AF10* mice at 4 weeks post transplantation: The percentage of transduced cells in the *CALM/AF10* mice were detected by flow cytometric analysis. The percentages of transduced cells injected at day 0 are shown in light coloured bars and the percentage engraftment at 4 weeks in dark coloured bars. *CALM/AF10* mice (labelled CALM/AF10#1 through 9) showed an average of 82 (±10) % engraftment in the peripheral blood with transduced bone marrow cells. In comparison, cells transduced with the GFP vector control, (labelled GFP#1, 2 and 3) injected with 100 % transduced bone marrow cells each, showed an average of 58 (±49) % of engraftment after 4 weeks.

The high engraftment of mice with CALM/AF10 transduced cells was despite the injection of an average of 57 (±26) % transduced cells at the day of injection (day 0) and the addition of a remainder of mock-transduced cells, indicating that *CALM/AF10* cells had a competitive growth advantage *in vivo* over non-transduced cells (For the exact numbers of transduced versus non-transduced cells injected in each mouse, see Fig. 3.4b (page 34).

4.3 **CALM/AF10 causes an aggressive acute leukemia in mice:** Mice injected with *CALM/AF10* and *GFP* transduced bone marrow were monitored for symptoms of leukemia that included frizzled body hair, paleness in the feet and lethargy. Moribund mice were sacrificed and analysed for leukemia. The examination of leukemic symptoms includes the measurement of leukocyte and erythrocyte numbers in the peripheral blood from sacrificed leukemic mice (Fig. 4.4) and the measurement of spleen weight (Fig. 4.5). Various organs of leukemic mice were fixed in formalin for histopathological examination (Fig. 4.6).
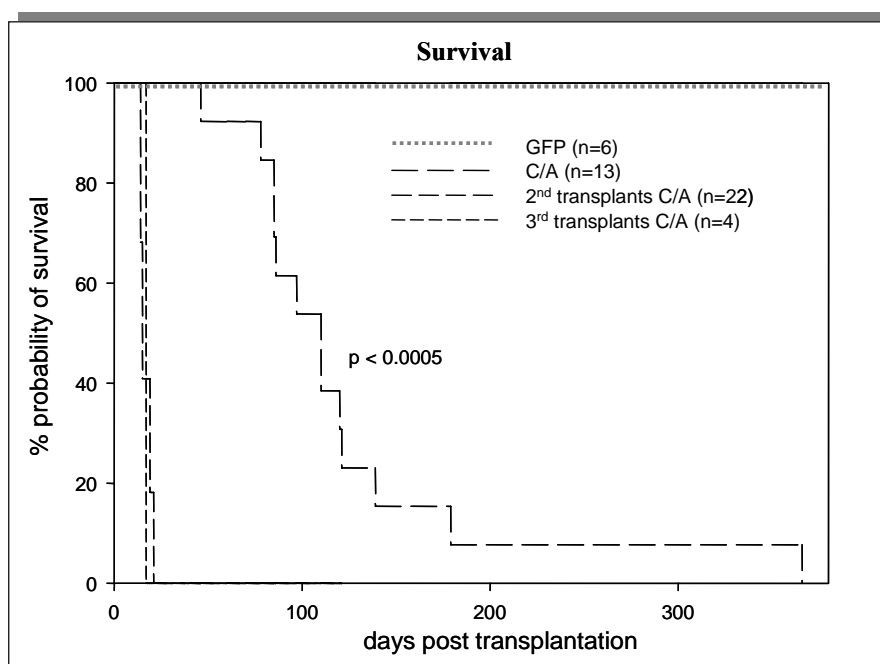
Fig. 4.3. Kaplan-Meyer plot of lethally irradiated mice injected with *CALM/AF10* transduced bone marrow: Mice injected with *CALM/AF10* transduced bone marrow cells (n=13) rapidly succumbed to an acute leukemia with a median survival time of 125 days (range 46 to 366 days) (*P* < 0.0005 compared to control mice). Secondary mice (mice injected with leukemic cells from the bone marrow of a primary leukemic mouse) and tertiary transplanted mice (injected from the bone marrow of sacrificed leukemic secondary recipients) (n=22 and 4, respectively) also succumbed to an acute leukemia with a median latency of 17 days post transplantation (*P* < 0.0005 compared to control mice).

This data shows that in the bone marrow transplantation model CALM/AF10 induces a transplantable acute leukemia with a short latency time.

4.4 **Leukemic *CALM/AF10* mice exhibit hyperleukocytosis and anemia:** One of the hallmarks of the leukemia was a manifold expansion of white blood cells (hyperleukocytosis) in the hematopoietic organs and the relative and absolute reduction in red blood cell numbers (anemia) in *CALM/AF10* mice as compared to the control mice.
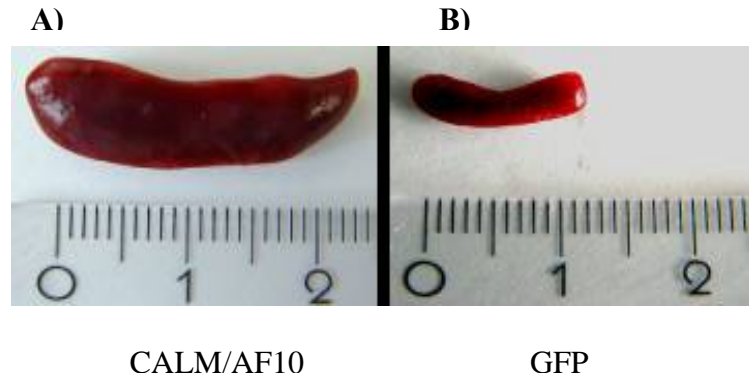
Mouse no.	Retroviral construct	Peripheral blood	Peripheral blood
		RBC per ml X $10E^9$	WBC per ml X $10E^6$
3493b # 2	CALM/AF10	0.75	50
3493b # 4	CALM/AF10	0.75	50
3493b # 5	CALM/AF10	0.6	85
3515 # 3	CALM/AF10	0.82	52
3724 # 1	CALM/AF10	0.6	48
3724 # 2	CALM/AF10	1.25	32
	GFP 1	6	4.5
3493a # 1	GFP 2	4.8	3.2
3493a # 3	GFP 3	5	3.6

Fig. 4.4. RBC and WBC counts in the peripheral blood, bone marrow and spleen of *CALM/AF10* and control mice: Paleness in the feet was one of the first visible signs of leukemia, which resulted from a severe anemia in mice. Leukemic *CALM/AF10* mice showed a marked decrease in RBC counts in peripheral blood (4.2 fold, $P < 0.005$) and an elevated circulating WBC count (13.3 fold $P = 0.044$) as compared to vector control mice with up to 50 x 10^6 circulating WBCs per ml of peripheral blood.

4.5   **Spleens of leukemic *CALM/AF10* mice are typically enlarged:** Enlargement of the spleen of *CALM/AF10* mice was a typical feature with the presence of visible white spots on the surface indicating infiltration with blast colony forming cells.

Mouse no.	Retroviral construct	Spleen weight (mg)
3493b # 2	CALM/AF10	700
3493b # 4	CALM/AF10	400
3493b # 5	CALM/AF10	250
3515b # 3	CALM/AF10	300
3724 # 1	CALM/AF10	200
3725 # 2	CALM/AF10	400
3515a# 1	GFP control 1	150
3493a # 1	GFP control 2	200
3493a # 3	GFP control 3	200

Fig. 4.5. Splenomegaly in *CALM/AF10* mice: The spleens of diseased *CALM/AF10* mice (A) were typically larger compared to GFP control mice (B). Diseased *CALM/AF10* mice showed an average spleen weight of 370 mg as compared to an average of 183 mg in GFP mice.

4.6   Leukemic blasts infiltrate multiple organs of *CALM/AF10* mice: Leukemia progression is marked by the infiltration of blasts in various organs and therefore we assessed sacrificed moribund mice for these signs by making histopathological sections of fixed organs and immunostaining followed by microscopy.
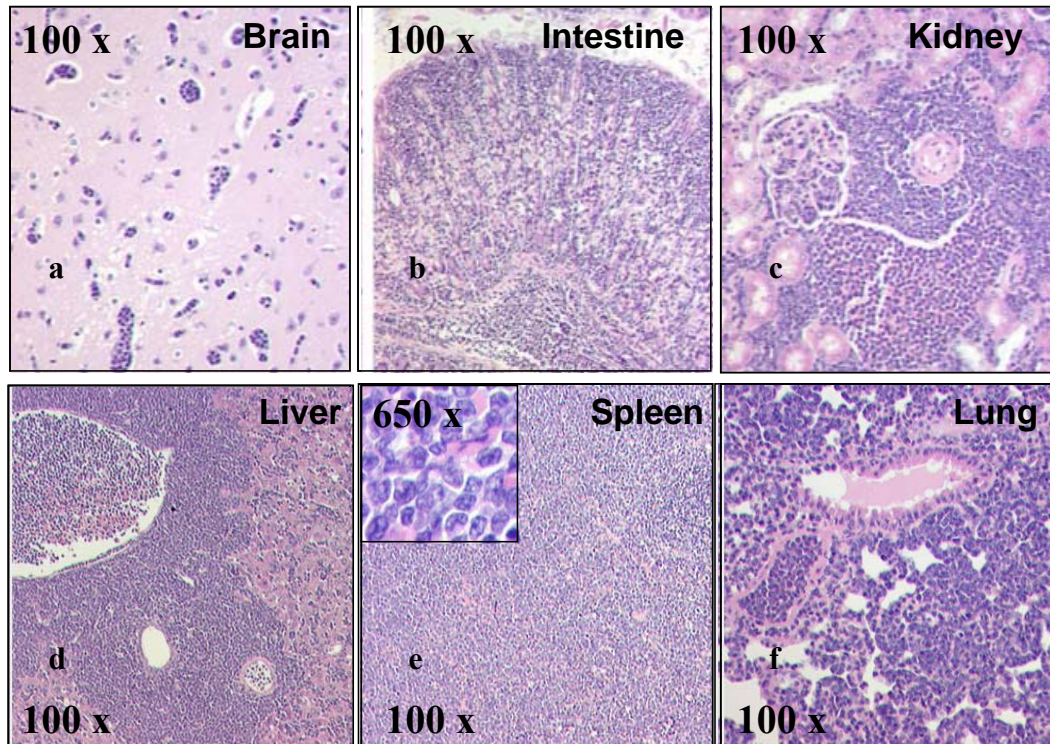
Fig. 4.6: Immunohistopathology of diseased *CALM/AF10* mice: A study of the   histological sections demonstrated infiltration of myeloid blasts in multiple organs, including non-hematopoietic tissues. Of note, the blast population crossed the blood-brain barrier resulting in perivascular infiltration in the brain of leukemic mice. The cerebellum of the leukemic mouse brain showed extensive perivascular infiltration with hemorrhage and necrosis (a). The intestine also showed infiltration of blast cells and the kidneys showed glomerular and tubular infiltration (b&c). There was extensive sinusoidal and portal infiltration in the liver (d). The spleen was diffusely infiltrated with no residual lymphoid cells (e). The lungs were also diffusely infiltrated (f).

The infiltration of leukemic blasts to the non-hematopoietic organs in general and to the brain in particular was striking, highlighting the aggressive nature of the disease.

4.7 **Leukemic blasts in *CALM/AF10* mice stain for myeloid markers:** The type and maturity of the cells involved in leukemia can be identified by analysing cells under a microscope after staining with histochemical and immunohistochemical stains distinguishing between various cell types. The myeloperoxidase stain distinguishes between immature cells in acute myeloid leukemia (cells stain positive) and acute lymphoid leukemia (cells stain negative) (Fischbach, 1996). Lymphoid blasts are characteristically negative for myeloperoxidase and chloracetate esterase. Immunochemical stains against B220 and CD3 aid in the detection of B and T lymphoid blasts respectively. In order to determine the nature of the blasts and to confirm the myeloid nature of the disease, we performed various stainings of the organs from primary leukemic mice.
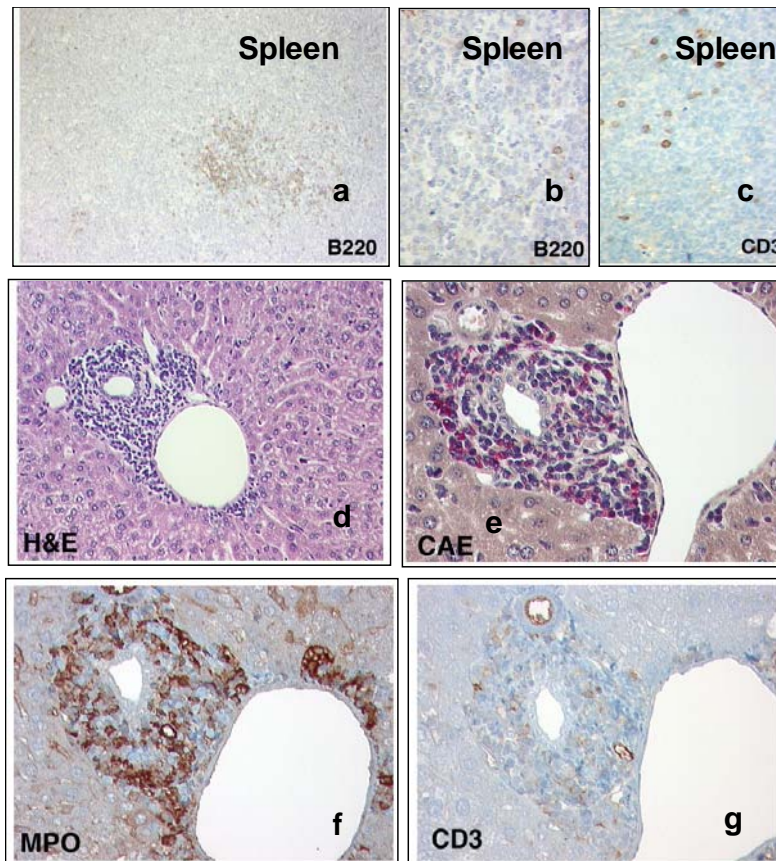
Fig. 4.7: Histochemical and immunohistochemical staining of leukemic blasts: Immunohistochemistry on histological slide preparations of leukemic mice with different stainings showed the blast like nature of the cells by hematoxylin-eosin staining **(d)**, negativity of the blasts for B220 **(a and b)** and CD3 **(c and g)** and positivity for chloracetate esterase **(e)** and myeloperoxidase **(f).**

These stainings revealed the myeloid nature of the bulk population confirming the diagnosis of acute myeloid leukemia.

4.8 **Cells from hematopoietic organs of leukemic *CALM/AF10* mice are predominantly myeloid in appearance with a high number of infiltrating blast like cells:**

Cytological slide preparations were made and assessed by the observation of morphology of the leukemic samples fixed on slides and stained with the Wright-Giemsa protocol.

Fig. 4.8.a: Blast like cells from *CALM/AF10* Mouse organs: Wright-Giemsa stained cytospins of single cell suspensions from organs of *CALM/AF10* mice or blood smears revealed differentiated myeloid cells (dashed arrows) and a number of large cells (dark arrows) with a high nucleus to cytoplasm ratio and two to three nucleoli confirming their blast like nature.
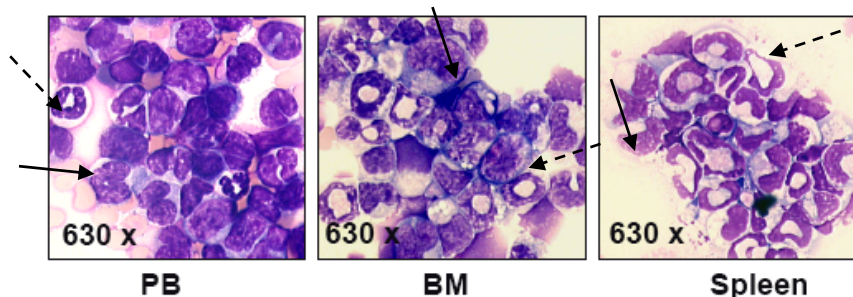
Mouse no.	% Blast like cells BM	% Blast like cells PB	% Blast like cells Spleen	% myeloid PB	% lymphoid PB	Lymphoid/ Myeloid ratio (PB)
CALM/AF10 # 1	30	27	32	89	3	0.03: 1
CALM/AF10 # 2	40	33	30	91	9	0.09:1
CALM/AF10 # 3	65	61	60	94	6	0.06:1
CALM/AF10 # 4	38	31	38	96	4	0.04:1
CALM/AF10 # 5	78	40	n.d	n.d	n.d	n.d
CALM/AF10 # 6	n.d	42	32	96	4	0.04:1
GFP # 1	0	0	0	20	80	4:1
GFP # 2	0	0	0	32	68	2:1
GFP # 3	0	0	0	35	65	2:1

Fig. 4.8.b: Percentage of blast like cells from *CALM/AF10* Mouse organs

100 cell differential counts from organs sacrificed leukemic mice showed a very high percentage of monocytoid blast like cells. Panoptic staining of PB smears and cytospin preparations of BM and spleen cells revealed an accumulation of myeloid blasts with a median of 32, 40 and 39 %, respectively in all organs.

The reversal in the lymphoid to myeloid ratio in leukemic mouse peripheral blood indicated massive myeloid proliferation and/or inhibited lymphoid growth in this compartment.

4.9   A subset of leukemic blasts from *CALM/AF10* mouse bone marrow co-express lymphoid and myeloid markers: We analysed various organs of leukemic mice by flow cytometric analysis of cell suspensions of the organs after staining with various lineage specific antibodies. We could consistently detect the expression on a subset of leukemic bone marrow cells of the B cell surface marker B220. Since most cells expressed the myeloid markers Mac-1 and/or Gr-1, it could be inferred that some cells would co-express the lymphoid B220 and the myeloid Mac-1 and/or Gr-1 markers. To confirm this, we performed a co-staining of bone marrow cells from a leukemic mouse for B220 and Mac-1 or Gr-1.
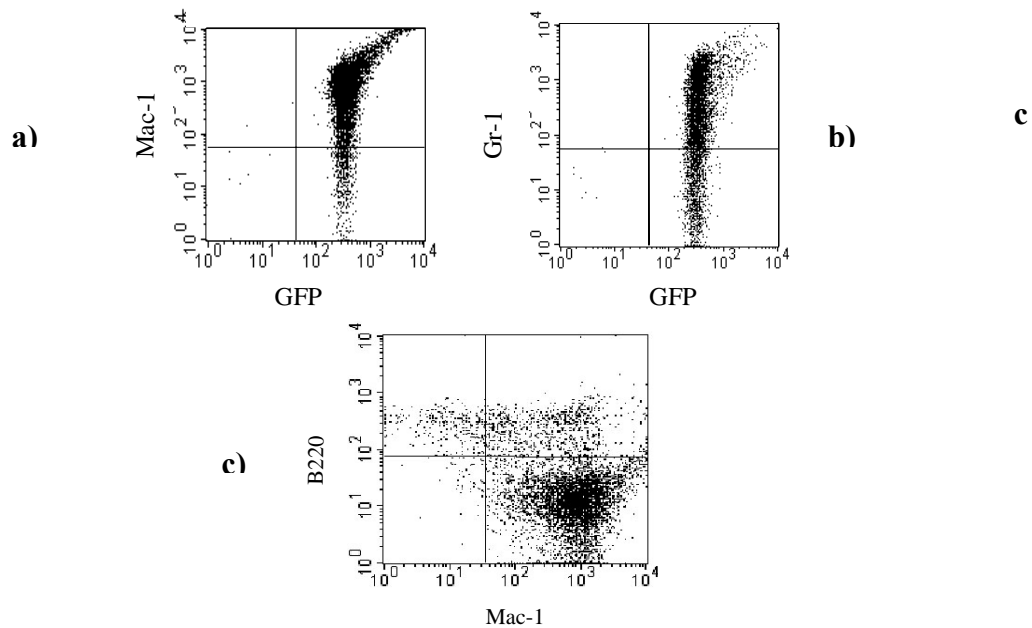
Fig. 4.9: Immunostaining analysis of bone marrow cells from CALM/AF10 mice: A majority of bone marrow cells from leukemic mice stain for the myeloid markers Mac-1 (a) and Gr-1 (b). CALM/AF10 positive cells are tracked by the analysis of GFP (X axis). Typical co-staining of bone marrow cells from a representative *CALM/AF10* leukemic mouse with the B220 and Mac-1 markers shows a minor population of cells that are co-express Mac-1 and B220 *(B/M population)* and a smaller population of Mac-1 negative B220 marker positive cells (*B population)* (c).

In the bone marrow of diseased mice on an average 6.74 % of the cells were positive for B220 and negative for myeloid markers compared to 9.39 % in the GFP control mice. Furthermore, an average of 26.04 % ($\pm$ 17.18) co-expressed B220 and Mac1 in the leukemic mice versus 2.06 % in control mice, while 32.53 % ($\pm$ 26.32) of the cells co-expressed B220 and Gr1 in leukemic mice compared to 1.28 % in the controls. An average of 57.08 % cells in the bone marrow of diseased mice were B220$^-$/Mac1$^+$ and 60.79 % were B220$^-$/Gr-1$^+$ compared to 85.81 % and 84.79 % respectively in the controls.

4.10   The CALM/AF10 induced leukemia is derived from several transformed clones: In order to check the clonality of the disease, we performed Southern blots with the genomic DNA extracted from the cells of leukemic mice. For this purpose, we digested purified genomic DNA from leukemic mice with *EcoR1*, which cuts once in the vector. The other *EcoR1* site would be at a random distance in the genome, distinct in every clone in which the retroviral construct is integrated. Each clone therefore would generate a fragment of a distinct size, which can be probed by Southern blotting using a PCR amplified probe in the GFP region. Bands of different sizes and in addition varying intensities would therefore indicate the presence of different clones in the leukemic samples.
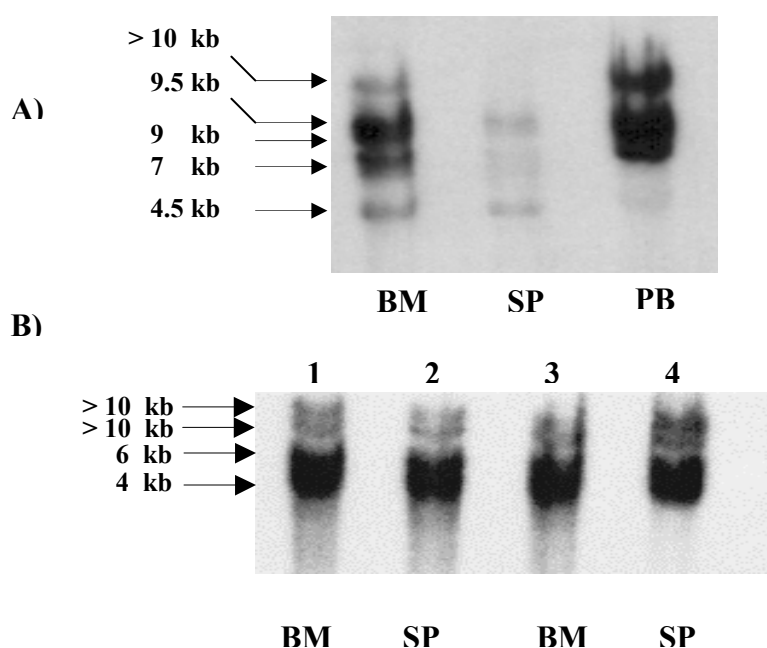
Fig. 4.10: Evidence of oligoclonality of leukemia in diseased *CALM/AF10* mice

A) Southern blot analysis of *EcoRI* digested genomic DNA from the bone marrow (BM), peripheral blood (PB), and spleen (SP) of a representative primary leukemic *CALM/AF10* mouse (mouse no.3983 # 3). Signals with different intensity, marked by arrows, indicate the presence of different leukemic clones proving the oligoclonal nature of the disease.

B) Analyses of samples from a primary leukemic mouse (mouse no.3746) bone marrow (BM) and spleen (SP) (lane 1 and 2) and that of a secondary mouse (mouse no.3833 # 1) (lane 3 and 4) derived from primary mouse (mouse no.3746) shows the presence of various bands of different intensities (marked by arrows) indicating different clones and the clonal propagation of these individual clones in the secondary disease.

The presence of bands of varying sizes and intensity demonstrated the oligoclonal nature of the *CALM/AF10* induced leukemia indicating that probably few additional mutations are required for *CALM/AF10* mediated transformation.

4.11   Identification of the leukemia propagating sub-fraction in the leukemic bone marrow population: As shown in Fig. 4.9, the leukemic bulk population consistently showed three sub-populations, the B220$^+$/myeloid marker$^-$ population or the *B population*, the B220$^+$/myeloid marker $^+$ or the *B/M population* and the B220$^-$/myeloid marker $^+$ *M population*.

For the sake of consistency and to avoid confusion, only the italicised terms *B*, *B/M* and *M populations* will be used throughout the thesis to denote the B220$^+$/myeloid marker$^-$, B220$^+$/myeloid marker$^+$ and the B220$^-$/myeloid marker$^+$ fractions respectively.

The consistent detection of cells with lymphoid markers in an acute myeloid leukemia was interesting to study. For this purpose, we examined the three populations for their morphology (Fig. 4.11), differentiation capability (Fig. 4.12) and the frequency of leukemia propagating cells (Fig. 4.13).

B population	*B/M population*	*M population*

B220$^+$/Mac1$^-$ sorted cells   B220$^+$/Mac1$^+$ sorted cells   B220$^-$/Mac1$^+$ sorted cells
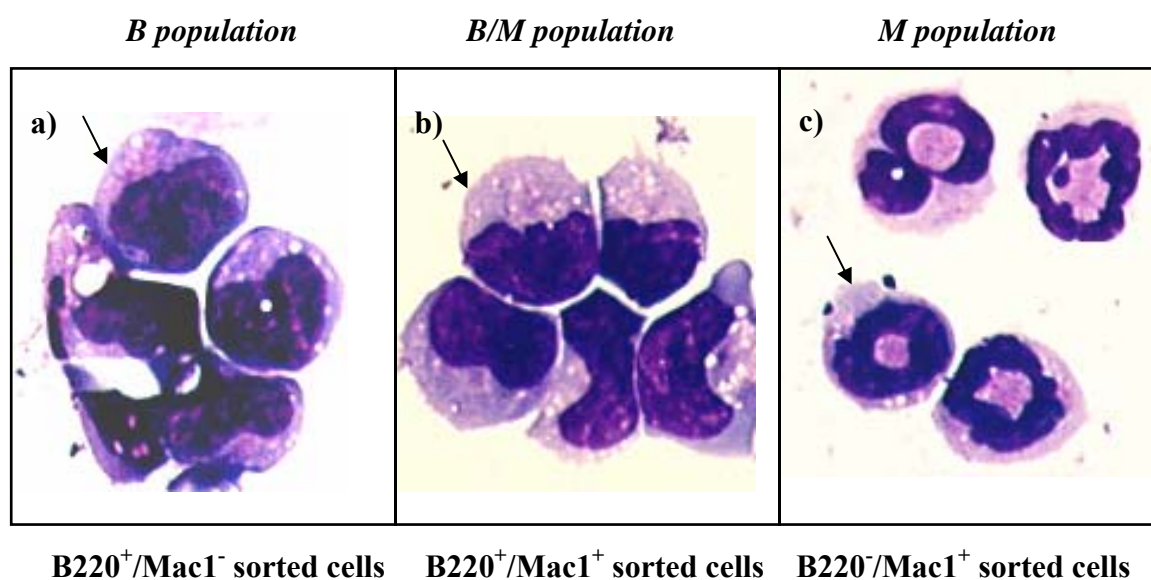
Fig. 4.11: Cytospin preparations of the three different cell populations from a leukemic *CALM/AF10* mouse (no. 3746) : *B population* cells **(a)** appear as monocytoid blasts with a high nucleus to cytoplasm ratio with multiple nucleoli and moderate vacuolation, *B/M population* cells **(b)** are also blast like, with an indented nucleus with relatively more cytoplasm and vacuolation. *M population* (**c**) cells have a distinctly differentiated appearance with a segmented or circular nucleus and a comparatively smaller size.

Since the morphology of the cells positive for the B220 marker was more undifferentiated as compared to the B220 negative population, it indicated that the leukemic blasts resided in the B220 marker positive compartment and not in the bulk B220 negative compartment.

4.12 ***B population* cells have a higher proliferative potential at the single cell level compared to the *B/M* and the *M population*:** We plated the cells from the three different sub-populations as single cells in 96 well plates to assess the proliferative capacity of each sub-population as well as the clonal differentiation ability at the single cell level.
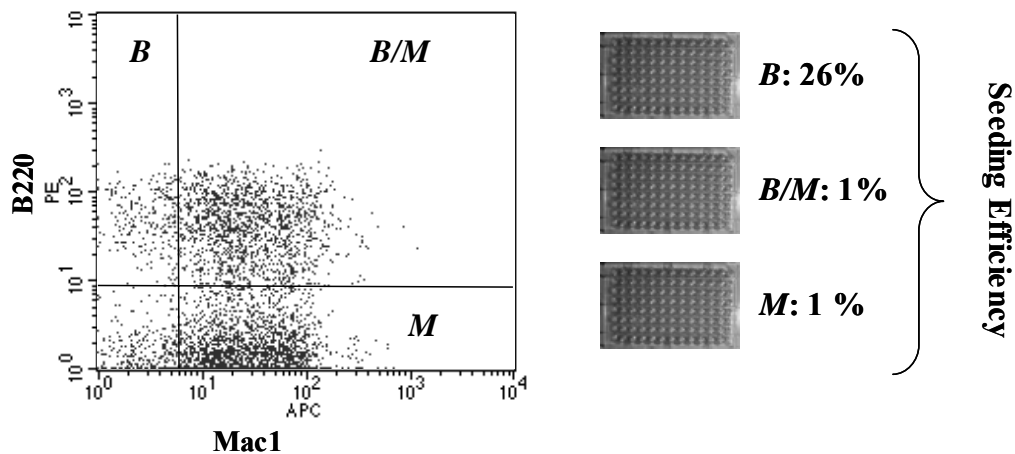
Fig. 4.12: Single cell seeding efficiency of the different populations: Of the three populations in the leukemic bulk cell population propagated in IL3, only the *B population* displayed efficient clonal proliferative potential at the single cell level with a seeding efficiency of 26 % after single cell sorting compared to 1 % in the other two populations.

This data proved that the *B population* cells in the rapidly proliferating IL3 dependent leukemic cell population had the highest seeding efficiency in comparison to the other two populations.

4.13 **The frequency of leukemia propagating cell is the highest in the *B population* as compared to the *B/M population* and the *M population*:** Since the *B*, *B/M* and *M* sub-populations were present in each leukemic mouse and since the *B population* (or cells with the B220 marker) were the cells with the blast like morphology and not the bulk myeloid population, it was critical to determine the population where the frequency of the leukemia propagating cell (LPC) was the highest. In order to determine the frequency of the leukemia propagating cell in each sub-population and to determine the compartment in which the leukemic stem cells reside, limiting–dilution secondary transplantation assays were performed by highly purifying the three different populations from leukemic primary recipients and injecting them at different cell dosages into cohorts of animals and observing for signs of leukemic engraftment.
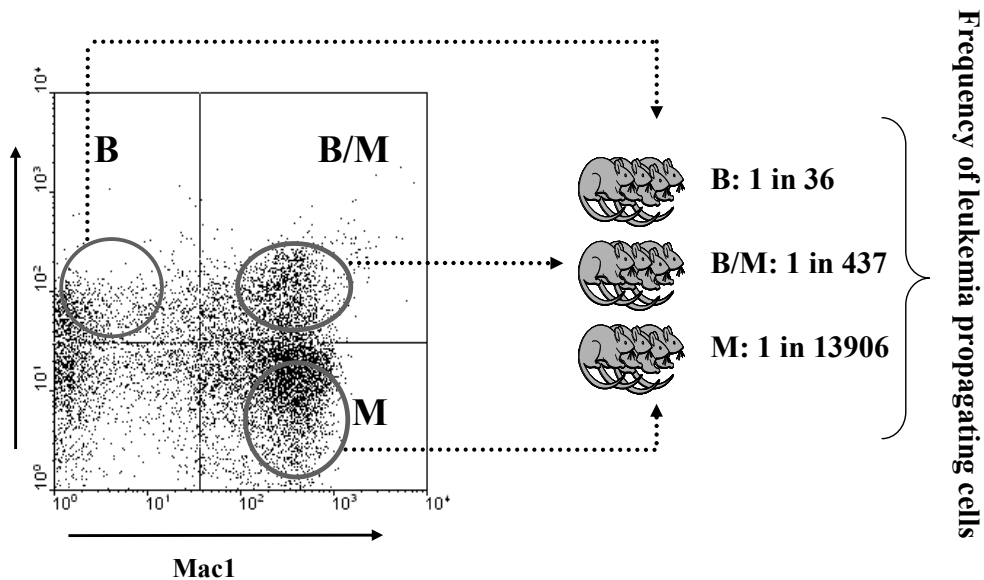
Fig. 4.13: Limiting dilution analysis for determining LPC frequency: The frequency of the LPC was more than 380 fold higher in the *B population* (1 in 36 cells) than in the *M population* (1 in 13906 cells) and more than 12 fold increased compared to the *B/M population* (1 in 437 cells).

This demonstrates that the B220$^+$/myeloid marker negative population in the leukemic mice contains the highest frequency of cells that are able to propagate leukemia in transplanted mice supporting the argument that these are the stem cell candidates in this model of myeloid leukemia.

4.14   Transformed *CALM/AF10 B population* blasts can differentiate into *B/M* and functionally myeloid *M population* cells *in vitro*: Single sorted *B population* cells seeded with a high efficiency and could be propagated indefinitely in culture medium supplemented with IL3. We analysed the differentiation capability of the cells at the single cell level. For this purpose, we did the flow cytometric analysis of cells derived from single *B population* cells cultured in IL3 supplemented medium for 2 weeks. Cells were co-stained with the B220 and Mac-1 or B220 and Gr-1 markers. To determine if the *M population* cells were indeed functionally myeloid, they were incubated with heat inactivated *S. cerevisiae* and stained with Wright Giemsa stain after fixing on slides.
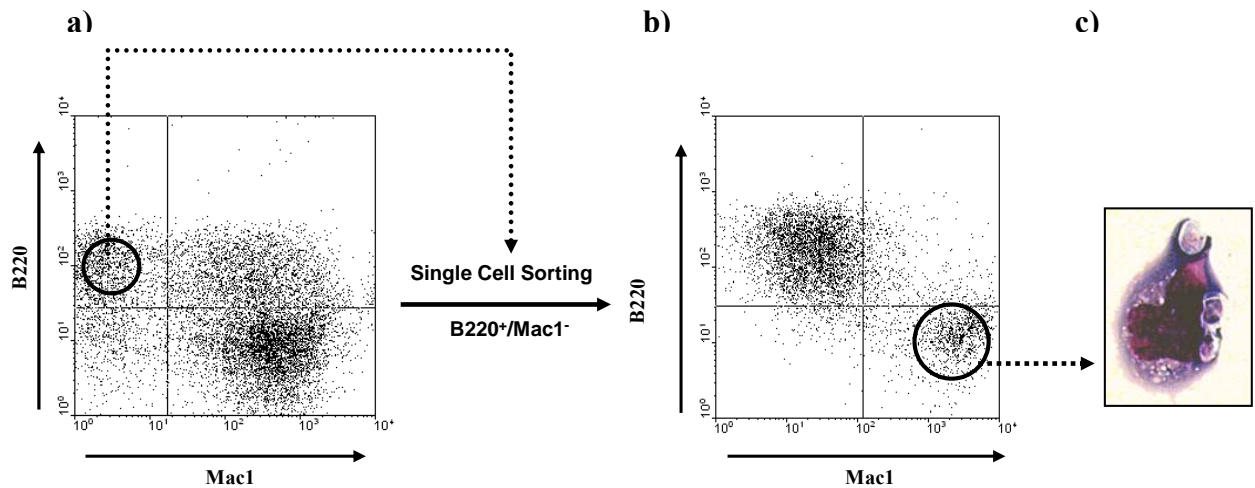
Fig. 4.14: *In vitro* myeloid differentiation of single sorted *B population* cells: Representative example of a highly purified cell of the *B population* from bulk leukemic cells cultured in IL3 **(a)** gave rise to the *B/M* and the *M populations in vitro* **(b)**. The *M population* cells, apart from expressing the myeloid markers Mac-1 and/or Gr-1 (data not shown), were determined to be functionally myeloid as is seen by the active phagocytosis of *S.cerevisiae* by these cells *in vitro* **(c)**.

4.15   Clonal D-J$_H$ rearrangements can be detected in all sub-populations derived from *B population* cells: Since functionally myeloid B220 marker negative cells could be derived from highly purified single cell sorted B220 positive myeloid marker negative cells, this proved that the *B population* cells in the *in vitro* cultured leukemic blast population could differentiate spontaneously under the given culture conditions into the *B/M population* and the *M population.* We sought to determine if the B220 positive cells showed any other lymphoid cell characteristics and to analyse whether these characteristics were also present in the cells derived from the *B population* cells. Genomic rearrangements that join the diversity (D) and junction (J) regions of the immunoglobulin locus are signatures of lymphoid cells. We assessed D-J rearrangements of the heavy chain of the immunoglobulin locus in all the three populations propagated from a single *B population* cell using a multiplex PCR strategy. This strategy detects the most common D-J$_H$ rearrangements. Cells of the *B population* showed D-J$_H$ rearrangements. To confirm that the *B/M* and *M populations* indeed arose from a pure single-cell sorted B population cell, we checked for D-J$_H$ rearrangements in both the derived populations.
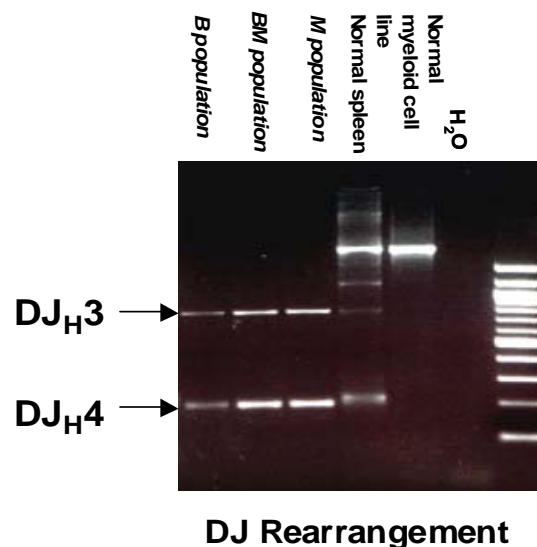
DJ Rearrangement

Fig. 4.15: ***B/M* as well as *M populations* derived from a single B *population* cell show clonal D-J rearrangements:**   Single cell sorted *B population* cells were analysed for D-J rearrangements. In this representative case, *B/M* (lane2) and *M* (lane3) *population* cells generated from a single *B population* cell exhibited the same D-J rearrangements (D-J$_H$3/D-J$_H$4) as the parent *B population* clone (lane1). Lane4 and lane5 are the positive (wild type mouse splenic cells) and negative controls (32D murine myeloid cell line) for D-J$_H$ recombination respectively.

This confirmed the data from 4.14 that the B/M and M populations generated in vitro from cultured purified B population cells were indeed derived from the B population cell riling out the possibility of contamination from other sources or improper sorting procedures.

4.16   IgH D-J rearrangements can be detected in myeloid populations of cells from leukemic *CALM/AF10* mice: The presence of genomic D-J$_H$ rearrangements in the myeloid cells derived *in vitro* from the *B* population cells implied that the *M* population cells in the leukemia would also show genomic D-J$_H$ rearrangements if they were derived similarly form *B* population cells *in vivo*. In order to determine whether the bulk myeloid leukemic population was derived from transformed D-J$_H$ rearranged progenitors, we analysed the various sorted sub-populations of bulk leukemic samples for D-J$_H$ rearrangements in the spleen of diseased mice.
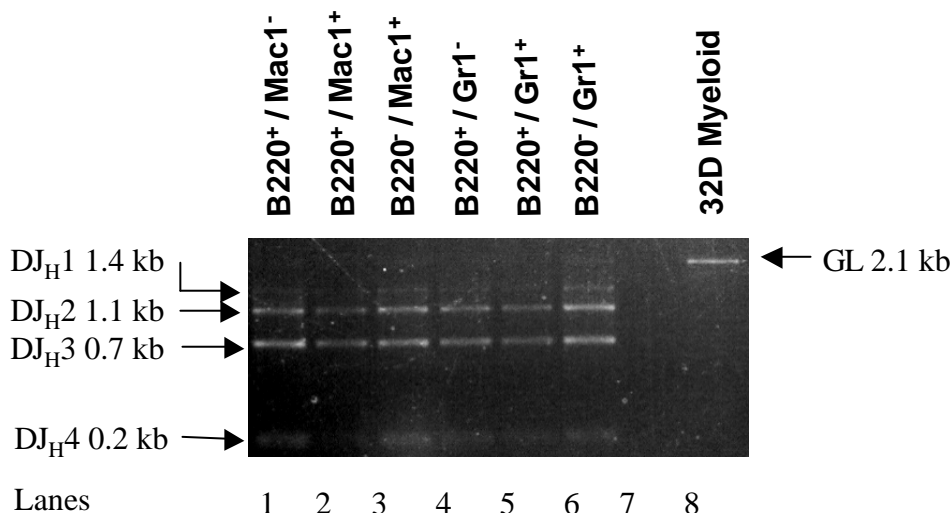
Fig. 4.16: PCR analysis of IgH D-J rearrangements: This is a representative example of the analysis of genomic rearrangements at the IgH loci from a leukemic mouse. All the populations isolated from leukemic mice displayed DNA bands corresponding to all recombination positive D-J$_H$ rearrangements (D-J$_H$1, D-J$_H$2, D-J$_H$3 and D-J$_H$4), which are detectable by the used multiplex PCR. Lane 1-6: B220$^+$/Mac1$^-$, B220$^+$/Mac1$^+$, B220$^-$/Mac1$^+$, B220$^+$/Gr-1$^-$, B220$^+$/Gr-1$^+$ and B220$^-$/Gr-1$^+$ cells purified from a leukemic mouse spleen. Lane 8: 32D monocytic cell line, myeloid germline control.

The presence of D-J$_H$ rearrangements in all the populations suggested that these populations, including those expressing only myeloid markers, arose from D-J$_H$ recombined progenitors. This data strongly supported the argument that the B220 positive D-J$_H$ rearranged progenitor propagated the myeloid leukemia in mice.

Results from the above experiments clearly demonstrated that the leukemic stem cell candidate in the myeloid leukemia seen in mice transplanted with the *CALM/AF10* transduced bone marrow in this murine model was a D-J_H rearranged B220 positive myeloid marker negative cell. Under normal circumstances, murine B220 positive D-JH rearranged cells do not possess myeloid differentiation capacity. Studies from Busslinger *et al.* (Heavey *et al.*, 2003) (Rolink *et al.*, 2000) however have demonstrated that B220$^+$ D-J_H rearranged B lymphoid progenitors from Pax5 -/- mice can differentiate into the myeloid lineage. These cells also show promiscuous expression of certain myeloid transcription factors such as myeloperoxidase (MPO). We therefore analysed the three populations generated from the single sorted *B population* cell for the expression of various transcription factors by semiquantitative PCR.

4.17   B population cells are Pax5 negative but express the early B cell factor gene
EBF: The genes analysed for transcriptional profiling were lineage specifying genes or those expressed by hematopoietic stem cells and various T and B cell progenitors.
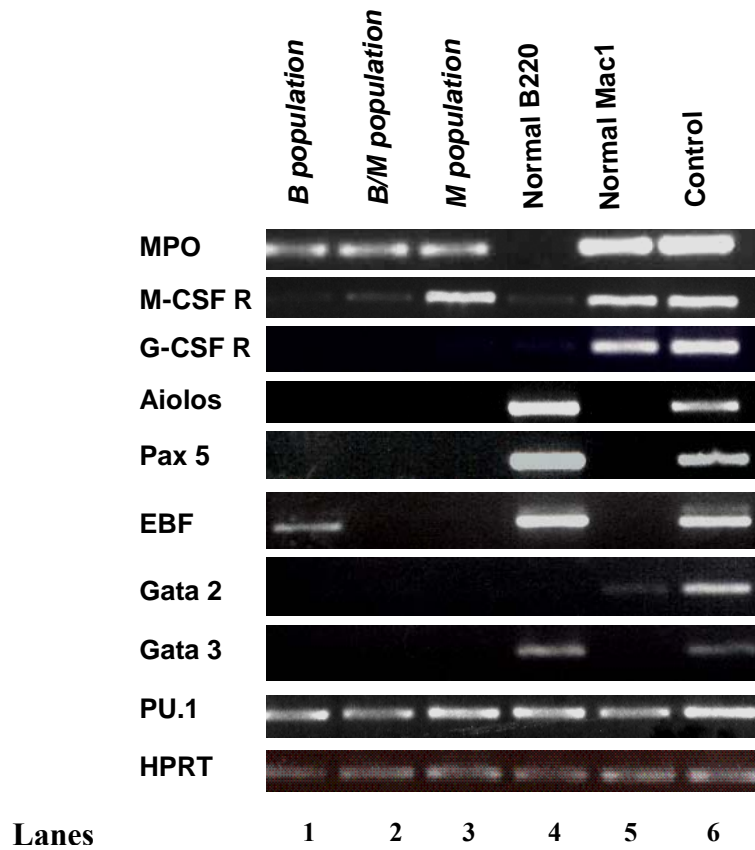
Fig. 4.17: An analysis of various transcripts specific to the hematopoietic lineage in the three sub-populations: We employed a semiquantitative PCR to analyse the transcriptome pattern of the three subpopulations in a cell line derived from a single *B population* cell. The B *population* cells (lane 1) expressed the B cell specific transcription factor *EBF* but lacked *Pax5* expression. They also expressed the myeloid marker (myeloperoxidase) but failed to express other transcription factors expressed in stem and other progenitor cells indicating a unique transcriptional profile. The expression of the myeloid colony stimulating factor (M-CSF) receptor was progressively higher with differentiation from the *B population* (in which it was undetectable) to the *M population* cell (lane 3) through the *B/M population* intermediate (lane 2). The controls used were B220 positive cells sorted from a normal wild type mouse spleen (lane 4), Mac-1 sorted cells from a wild type mouse bone marrow (lane 5) and whole bone marrow (lane 6) as a positive control for all polymerase chain reactions.

The expression of the early B cell factor EBF in the *B population* cells, taken together with the expression of B220 and D-J$_H$ rearrangements indicate that these cells resemble early B lineage cells. In addition, the absence of Pax5 expression could explain the block in B cell differentiation and promiscuous myeloid differentiation capability *B population* progenitors.

4.18   The *B population* cells express various early B lineage markers: Since the *B population* cells resembled early B lineage cells, we tested them for the presence of various early surface B lineage markers to determine the stage of B lymphoid differentiation and to ascertain their identity as lymphoid progenitors.
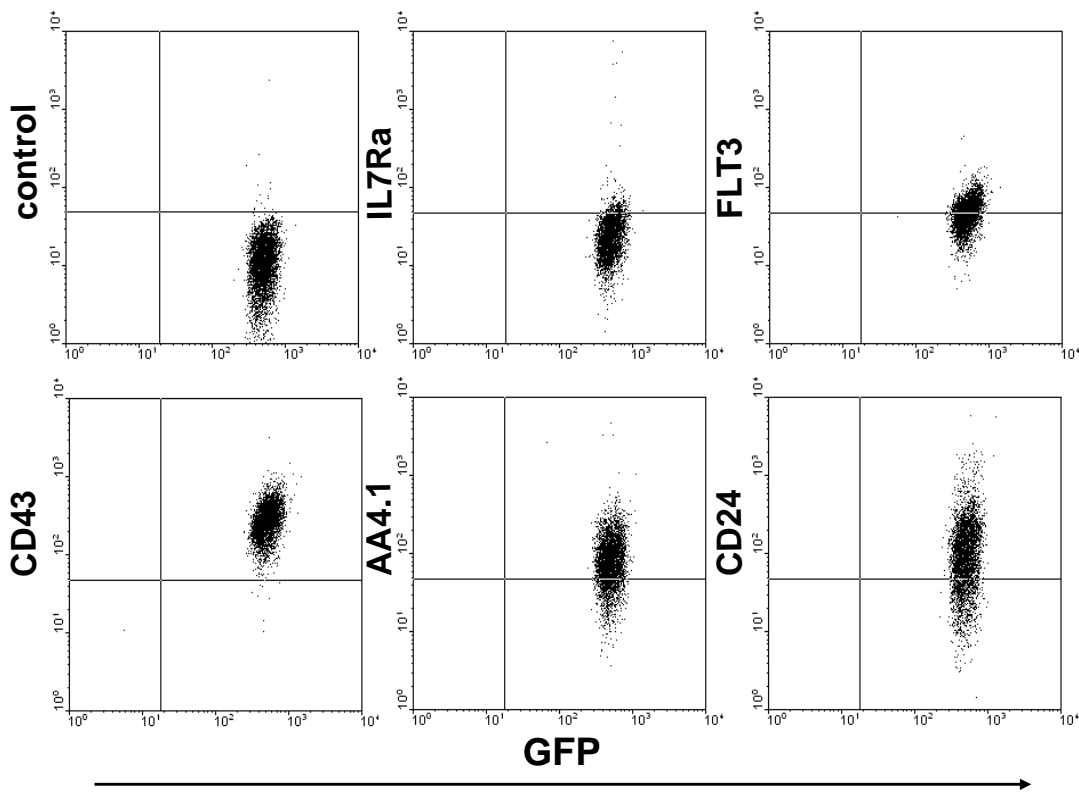
Fig. 4.18: Immunophenotypic characterization of the *B population* cells: Flow cytometric analysis of bone marrow cells from the leukemic bulk population propagated in IL3 supplemented medium and stained with various markers characterized the *B population* cells as being CD43$^+$/AA4.1$^+$/HSA$^{low-pos}$/FLT3R$^+$/IL-7R$^{low-neg}$ and negative for CD19, c-kit, Sca1 and CD4. (data not shown).

This immunophenotypic analysis, taken together with the transcription profile analysis indicates that the B *population* cell is a Pax-5 negative pro B or pre BI like cell according to the Basel nomenclature of B cell development (Li *et al.*, 1996)

4.19   The expression of *CALM/AF10* in bone marrow progenitors enhances the recovery of day 12 CFU-S: The number of short-term hematopoietic stem cells in a cell population can be assayed by the <u>C</u>olony <u>F</u>orming <u>U</u>nits in <u>s</u>pleen (CFU-S) assay that quantifies the frequency of these cells by the formation of visible spleen colonies 12 days after injection. To investigate the effect of CALM/AF10 on primitive hematopoietic cells, using this assay, cells transduced with the different viruses were injected into lethally irradiated mice, and spleen colony formation was quantified 12 days later. (Fig. 4.19A) Colonies can be visualized after immersion of the spleen of sacrificed mice in Telleyesnickzky's solution (representative example Fig. 4.19B).
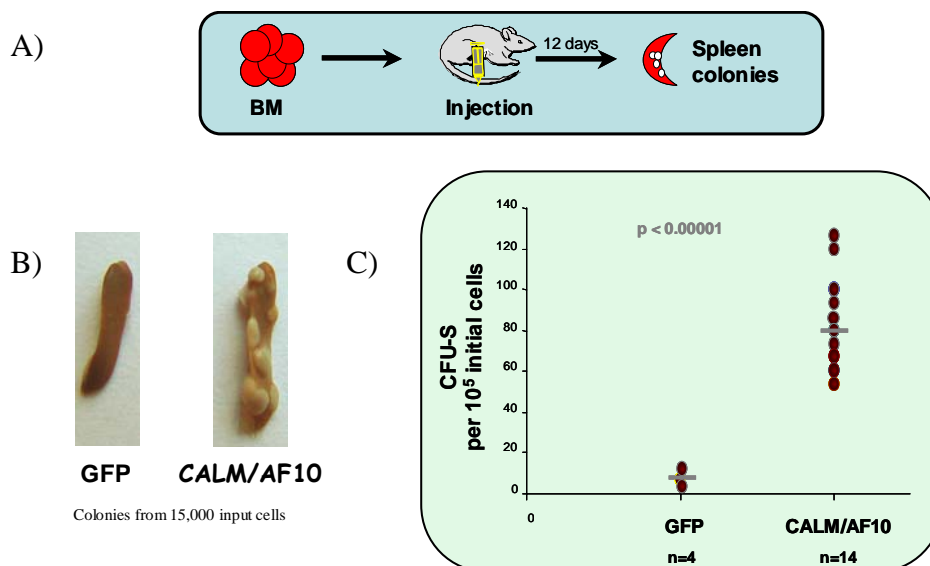
Fig. 4.19: Effect of *CALM/AF10* expression on short-term hematopoietic stem cells: An average of 75($\pm$23) day12 CFU-S colonies per input 10^5 cells could be recovered from bone marrow cells expressing the *CALM/AF10* fusion gene as compared to 2 ($\pm$3) colonies with cells expressing the empty GFP vector. (Fig. 4.19C)

Therefore, the expression of *CALM/AF10* in hematopoietic progenitors and their subsequent injection into mice leads to a more than 45 fold increase (*P < 0.0005*) in the number of short-term repopulating progenitors as assessed by the day-12 CFU-S assay content (number of colonies on the spleen 12 days post injection).

4.20   The leucine zipper-octapeptide motif domain is critical for the hematopoietic activity of CALM/AF10: We used the CFU-S assay to perform structure function analysis of the hematopoietic activity of CALM/AF10. Retroviral transduction of bone marrow progenitors with various mutants of *CALM/AF10* was performed to test their effect in the CFU-S assay in which the expression of the wild type *CALM/AF10* fusion gene shows an enhancement in day 12 CFU-S numbers as compared to the empty vector.
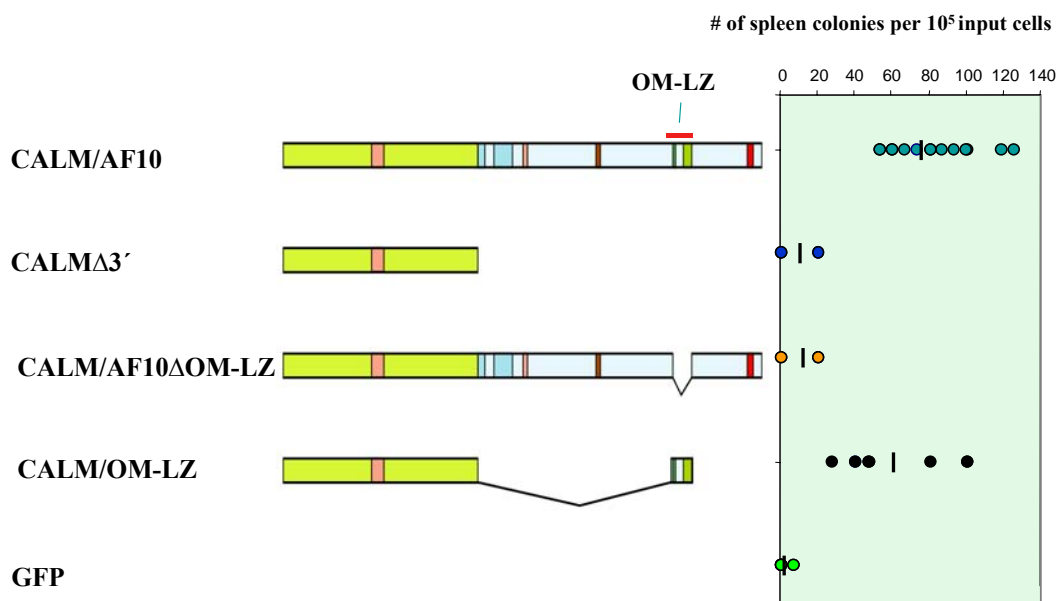
Fig. 4.20: Structure function analysis of the hematopoietic activity of CALM/AF10: The average number of CFU-S recovered 12 days after injection with cells expressing the CALM gene truncated to the breakpoint of *CALM/AF10* (*CALM*Δ3') or the *CALM/AF10* fusion gene with a deleted octapeptide motif - leucine zipper domain (*CALM*/OM-LZ) were 10 (±11) and 12 (±11) respectively per input 10^5 bone marrow cells. In contrast, the number of day 12 CFU-S recovered from cells expressing the truncated *CALM* fused to the octapeptide motif–leucine zipper domain was 68 per input 10^5 cells. This shows that the deletion of the AF10 portion or the octapeptide-motif-leucine zipper domain from the *CALM/AF10* fusion gene causes a loss in enhancement of ST-HSC numbers seen with the *CALM/AF10* fusion gene. In contrast, the expression of a construct fusing the octapeptide motif-leucine zipper domain of *AF10* 3' of the truncated *CALM* construct (*CALM*/OM-LZ) caused a 40 fold expansion in the number of day 12 CFU-S (*P=0.0006*) compared to the vector control (GFP), demonstrating that the octapeptide motif leucine-zipper domain of AF10 is highly relevant for the hematopoietic activity of the *CALM/AF10* fusion gene.

This data implicated the octapeptide-leucine zipper domain of AF10 in the enhancement of CFU-S recovery in hematopoietic progenitors by CALM/AF10 and indicated that it might be playing a role in CALM /AF10 mediated transformation.

The t(10;11)(p13;q14) translocation is a rare but recurring translocation in several different leukemias and lymphomas (Bohlander *et al.*, 2000). Using the murine bone marrow transplantation model, we assessed the oncogenic potential of the human leukemia specific *CALM/AF10* fusion gene cloned from the U937 monocytic cell line. The failure of CALM/AF10 to induce transformation of hematopoietic progenitors *in vitro* (data not shown) possibly reflects a requirement of specific growth factor/s for the proliferation of the leukemia initiating cell or alternatively, the specific targeting, by CALM/AF10, of a unique progenitor cell incapable of *in vitro* proliferation in the conditions provided. We report that the ectopic expression of the *CALM/AF10* fusion transcript in 5-FU mobilized bone marrow causes an aggressive acute leukemia *in vivo*. The injection of *CALM/AF10* transduced bone marrow cells into lethally irradiated mice led to a rapid engraftment with transduced progenitors indicating that CALM/AF10 confers engraftment capability on bone marrow progenitors. All the mice transplanted with *CALM/AF10* transduced bone marrow succumbed to an aggressive acute leukemia with relatively short latency indicating that probably few additional mutations are required for CALM/AF10 mediated leukemogenesis. Massive infiltration in non-hematopoietic organs of leukemic *CALM/AF10* mice highlights the aggressive character of the leukemia. Infiltration of the central nervous system, which we observed in *CALM/AF10* mice, is a feature more common to acute lymphoblastic leukemias probably supporting the lymphoid origin of this predominantly myeloid leukemia; though the alternative explanation that this only reflects the more aggressive nature of the disease cannot be ruled out.

The myeloid nature of the leukemia was confirmed by immunohistology and staining for specific myeloid and lymphoid markers. The bulk leukemic population in all the leukemic mice was predominantly myeloid as analysed by morphology and immunophenotype ($B220^-$/myeloid marker$^+$). There was however, a biphenotypic population of cells ($B220^+$ and $Mac1^+$ with or without Gr1) and an even smaller cell population that displayed only lymphoid markers ($B220^+$/$Mac1^-$/$Gr1^-$).

Leukemias that present blasts with characteristics of two different lineages (acute biphenotypic leukemias) are a unique identifiable subset of leukemias and have been linked to poor prognosis (Sulak *et al.*, 1990). The target cell of these leukemias has been widely postulated to be a primitive hematopoietic progenitor. As is discussed in the introduction, the presence of biphenotypic cells in leukemias has been attributed either to the transformation of a normally occurring biphenotypic cell (lineage promiscuity) or to the transformation of a lineage committed cell acquiring markers of the other lineage as a result of malignancy

(lineage infidelity) (McCulloch, 1987). Earlier observations that the biphenotypic cells in a mouse model of MLL-GAS7 leukemia appear due to the malignant transformation of normal IgH locus naïve biphenotypic multipotent progenitors lends credence to the promiscuity theory (So *et al.*, 2003). However, we observed genomic DJ rearrangements at the IgH loci in all three cell fractions and also demonstrated that the frequency of in-vivo leukemia propagating cells as assessed by the transplantibility of leukemia (leukemia stem cells) is significantly higher in the B220$^+$/Mac1$^-$/Gr1$^-$ fraction as compared to the other two fractions (B220$^+$/myeloid marker$^+$ and the B220$^-$/ myeloid marker$^+$). Furthermore, we demonstrate that the two fractions B220$^+$/myeloid marker$^+$ and the B220$^-$/ myeloid marker$^+$ can be clonally generated in vitro from single cell sorted DJ rearranged B220$^+$/Mac1$^-$/Gr1$^-$ leukemic cells. Furthermore, the B220$^+$/Mac1$^-$/Gr1$^-$ cells are also the only cells in the three fractions that can seed efficiently when plated at the single cell level. Moreover, immunophenotypic characterization of the B220$^+$/Mac1$^-$/Gr1$^-$ fraction revealed the expression, on these cells, of markers specific to early B cell progenitors specifically AA4.1, CD43 and Heat Stable Antigen (HSA). B220$^+$/CD43$^+$/AA4.1$^+$/HSA positive cells have been characterized as B lineage precursors. Refined analysis of the cell surface phenotype of the B220$^+$ cells characterized them as CD43$^+$/AA4.1$^+$/HSA^{lo-pos}/CD19$^-$/FLT3R$^+$/IL-7R$^{low-neg}$ c-kit$^{low-neg}$, and negative for Sca1 and CD4. Transcriptional profiling demonstrated positivity for the B-lymphoid transcription factor EBF, but negativity for Pax5. Importantly, the cells expressed myeloperoxidase (MPO) but not the myeloid factors G-CSF R, M-CSF R, or Gata-2. With regard to T-cell associated factors there was no detectable transcription of Gata-3, or Aiolos. Taken together, these data indicate that in this mouse model of myeloid leukemia, the leukemic stem cell candidate propagating the leukemia resides in the minor lymphoid like B220$^+$/CD43$^+$/AA4.1$^+$/HSA$^+$/CD19$^-$ progenitor cell compartment. Even though this progenitor resembled cells of the early B lineage, the classification of this CALM/AF10 leukemia propagating cell according to the different B cell classification systems remains difficult because of its leukemogenic characteristics and the potential impact of the fusion gene on its cell surface phenotype and transcriptional network. However, the B220$^+$/CD19$^-$ cell surface phenotype resembles the phenotype described for the pro-B/pre-B-I differentiation stage of the Basel nomenclature, the pre-pro B of the Philadelphia nomenclature and the fraction A$_1$/A$_2$ of B-cell precursors according to Hardy *et al.* (Busslinger *et al.*, 2000; Martin *et al.*, 2003; Osmond *et al.*, 1998). This cell surface phenotype discriminates them on the one hand from the B220$^+$/CD19$^+$ cell stage, which is associated with entire commitment to the B cell

lineage (Hardy, 2003)and on the other hand from the earliest lymphoid progenitors, the common lymphoid progenitors (CLPs), which lack expression of lineage markers such as B220 (Kondo *et al.*, 1997).

This is a significant observation as we demonstrate, for the first time that leukemia with predominant characteristics of one lineage could arise from progenitor cells bearing characteristics of another lineage. It is difficult to determine whether this reflects the transformation of a normal lymphoid like progenitor with transdifferentiation capabilities or the active reprogramming of normal lymphoid progenitors by the expression of *CALM/AF10*. This however, raises the interesting possibility that the leukemic stem cells or leukemia propagating cells of some biphenotypic leukemias or even leukemias diagnosed as myeloid could be transformed lymphoid precursors.

Earlier, Robert Slany and co-workers have demonstrated that hematopoietic progenitors transformed with the MLL-ENL fusion gene and cultivated in lymphoid conditions could generate an indefinitely propagating B220$^+$/CD19$^-$ cell population that was blocked in differentiation, could cause leukemia in mice and could give rise to a B220$^+$/Mac1$^+$ biphenotypic population (Zeisig *et al.*, 2003). The biphenotypic cells might recapitulate a situation where B lymphoid differentiation in the B220$^+$/Mac1$^-$ target cell is blocked and the cells are driven to myeloid differentiation possibly directed by the fusion gene. We propose that biphenotypic cells would then be preserved as relics of this transdifferentiation. The differentiation of transformed human lymphoid cell lines to myeloid or lympho-myeloid cells *in vitro* is well documented (Matsuo and Drexler, 1998) as is the observation of lymphoid cell specific genomic rearrangements or configurations in human myeloid leukemias (Schmidt *et al.*, 1995; Yen *et al.*, 1999). It is interesting to note that patients with *CALM/AF10* positive AML have been shown to display rearrangements at the TCR locus (Asnafi *et al.*, 2003), a phenomenon that has been observed in patient samples from other non-lymphoid leukemias (Schmidt *et al.*, 1995; Yen *et al.*, 1999). The role of hematopoietic transcription factors in the control of hematopoietic cell fate decisions is well documented. Ectopic expression studies of transcription factors (CEBPα) (Xie *et al.*, 2004), cytokine receptors (IL2R and GM-CSFR) (Kondo *et al.*, 2000) or oncogenes (c-myc, v-raf) (Klinken *et al.*, 1988) in murine lymphoid progenitors or cell lines have been shown to drive the differentiation of these cells into the myeloid lineage while blocking their lymphoid development. This is thought to be either by their active reprogramming, or by the induction of alternative choices that are still available to the progenitors. Moreover, transcription factor dysregulation has been shown to be a key

leukemogenic event. Taken together, reprogramming of progenitors by the dysregulation of transcription factors, cytokine receptors or the activation of oncogenes could give rise to the promiscuity observed in some leukemias. Studies from Busslinger et.al. have demonstrated that B220$^+$/CD19$^-$ pro B cells, deficient in the B cell specific transcription factor Pax5 are blocked in B cell development but gain unprecedented plasticity and can differentiate into myeloid cells (Rolink *et al.*, 2002a; Rolink *et al.*, 2002b). This scenario is recapitulated by the CALM/AF10 transformed progenitors bearing lymphoid characteristics. This indicates that reprogramming of early B progenitors or more likely, the release of suppression of available alternative lineage choices could be a potential mechanism of CALM/AF10 induced transformation in this model. It is relevant to note here that we failed to detect Pax5 expression in the B220$^+$/myeloid marker$^-$ cells.

Patients harbouring the t(10;11) translocation have been reported to have a poor survival rate and prognosis with higher relapse rates (Dreyling *et al.*, 1998). These leukemias include those involving the MLL fusion gene and leukemias involving CALM, both fused to AF10 on chromosome 10. *CALM/AF10* leukemias share some similarities with MLL leukemias such as promiscuity (Kumon *et al.*, 1999; Mitterbauer-Hohendanner and Mannhalter, 2004) and the aberrant dysregulation of HOX genes (Armstrong *et al.*, 2002) and (Krause A et.al and Delabesse E, et.al., unpublished data). Interestingly, cell lines from a human MLL lymphoma have been shown to bear B lymphoid markers and monocytoid appearance as well as macrophage differentiation capabilities (Bertrand *et al.*, 2003).

The dysregulation of Hox genes was also found in patients with AML and was recently reported in patients harbouring the t(10;11)(p12;q13) translocation. The leucine zipper of AF10 could play a role in this dysregulation as the octapeptide motif-leucine zipper domain of Alhambra; the Drosophila homolog of AF10 has been shown to positively regulate Hox genes. In support of these findings, our structure function analyses pinpoint the octapeptide motif-leucine zipper domain to be the minimal portion of AF10, which is necessary for the increased hematopoietic activity of to the fusion gene. This domain has been shown previously to be essential and sufficient to transform hematopoietic cells when fused to the MLL oncogene (DiMartino *et al.*, 2002) indicating a wider role for this motif in leukemogenesis especially in leukemias in which the expression of Hox genes is deregulated.

Finally, the observations in this model that myeloid leukaemia can arise from a B lymphoid progenitor might have clinical implications. In this leukemia model the expression of the B220 antigen would discriminate this cell from the normal hematopoietic stem cell phenotype in the murine *CALM/AF10* leukaemia model. In particular differences in the expression of cell surface antigens between the leukemic stem cell and the normal hematopoietic stem cell would facilitate the development of treatment strategies that eradicate the leukemic but spare the normal stem cell (Buske *et al.*, 2002). The expression of B cell antigen on the leukemic stem cell candidate in AML would open the intriguing possibility to target the leukemic stem cell in myeloid leukaemia by B-cell specific antibodies, which is a well established elements of multimodal treatment strategies for patients with aggressive and indolent lymphoma (Buske *et al.*, 2004; Coiffier, 2004).

In conclusion, we have demonstrated, for the first time, that an acute leukemia with predominantly myeloid characteristics can be propagated by a lymphoid progenitor in a mouse model of the t(10;11) (p13;q14) translocation. Mice transplanted with bone marrow retrovirally engineered to express the leukemia specific *CALM/AF10* fusion gene consistently developed an acute leukemia with a short latency. The leukemia showed characteristic myeloid features such as the presence of myeloid marker positive cells infiltrating multiple hematopoietic and non-hematopoietic organs, the positivity of blasts for myeloid specific histochemical stainings and the depletion of the lymphoid compartment in lymphoid organs. Apart from the major population of cells expressing myeloid but not lymphoid markers (*M population*), a smaller population of cells expressing myeloid markers as well as the lymphoid marker B220 (*B/M population*) and a smaller population expressing only the B220 marker (*B population*) could be detected in all mice.

We determined that the frequency of leukemia propagating cells was the highest in the *B population* and that this population could give rise to the other two populations of cells, namely the *B/M* and the *M populations*. This indicated that the leukemic stem cell candidate for the myeloid leukemia in this model of *CALM/AF10* induced transformation is a $B220^+$ cell. Further characterization of these candidate LSCs revealed the presence of $D-J_H$ rearrangements and the absence of *Pax5* transcription. These cells were characterised as being $CD43^+/AA4.1^+/HSA^{low-pos}/CD19^-/FLT3R^+/IL-7R^{low-neg}$ $c-kit^{low-neg}$ and expressing the early B cell factor (*EBF*) transcripts as well as transcripts for the myeloperoxidase (*MPO*) gene, bearing a resemblance to *Pax5* knockout preBI cells.

These findings indicate that the leukemia-propagating cell in a subset of acute myeloid leukemias could be a cell with lymphoid characteristics. The fact that this progenitor cell expressed markers different from those expressed by the bulk leukemic population but could still propagate the leukemia raises the interesting possibility of selectively targeting these cells using novel therapeutic strategies that aim to eliminate these LSCs.

Erstmalig wurde in dieser Arbeit gezeigt, dass eine Akute Leukämie mit vorherrschend myeloischen Merkmalen durch einen lymphoiden Vorläufer in einem Mausmodell der Translokation t(10;11) (p13;q14) verursacht werden kann. Mäuse, deren transplantiertes Knochenmark durch retrovirale Transduktion das leukämiespezifische Fusionsgen *CALM/AF10* exprimiert, entwickeln innerhalb kurzer Zeit eine Akute Leukämie. Diese Leukämie zeigt charakteristische myeloische Merkmale, wie hämatopoetische und nichthämatopoetische Organe infiltrierende Zellen, welche für myeloische Marker positiv sind, Blasten, die in für das myeloische System spezifischen histopathologischen Färbungen positiv sind und eine Verarmung der lymphoiden Kompartimente in den lymphoiden Organen. Neben der nur myeloische Marker exprimierenden Hauptpopulation (*M population*) und einer kleineren, die die myeloischen und den lymphoiden Marker B220 zusammen exprimiert (*B/M population*), konnte in allen Mäusen eine noch kleinere Population detektiert werden, die nur den lymphoiden Marker B220 exprimiert (*B population*).

Wir konnten feststellen, dass die Häufigkeit von Leukämie hervorrufenden Zellen in der *B Population* am höchsten war und dass aus dieser Population die beiden anderen, die *B/M-* und die *M Population*, entstehen können. Dies ließ folgern, dass der Kandidat der leukämischen Stammzelle (LSC) in diesem Modell einer *CALM/AF10* induzierten Transformation eine B220 exprimierende Zelle ist. Die weitere Charakterisierung offenbarte das Vorhandensein des D-J_H Rearrangements und den Verlust der *Pax5* Transkription. Diese Zellen sind des weiteren CD43$^+$/AA4.1$^+$/HSA$^{low-pos}$/CD19$^-$/FLT3R$^+$/IL-7R$^{low-neg}$ c-kit$^{low-neg}$. exprimieren den Frühen B-Zell Faktor (*EBF*), sowie die Transkripte der Myeloperoxidase (*MPO*) und haben somit Ähnlichkeit zu *Pax5 knockout* preBI Zellen.

Diese Ergebnisse weisen darauf hin, dass die die Leukämie verursachende Zelle in einer Untergruppe Akuter Myeloischer Leukämien lymphoide Charakteristika zeigen kann. Die Tatsache, dass diese Vorläuferzelle andere Marker als die eigentliche leukämische Hauptpopulation exprimiert und trotzdem die Leukämie verursachen kann, weißt auf die interessante Möglichkeit hin, mit neuen therapeutischen Strategien diese leukämische Stammzellen gezielt zu eliminieren.

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Generally, an extensive acknowledgement follows every good thesis but the lack of a vocabulary profound enough to express my feeling of gratitude towards the people who have assisted me severely limits this endeavour. Also, the oddity of having an acknowledgement section that outnumbers the thesis in terms of the number of pages would be striking. So with the limitations of language and space, I shall complete the ritual.

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RESEARCH EXPERIENCE

National Centre for Cell Sciences, Pune, India **Junior research student** – "A transgenic mouse model of Smar1, a T cell specific MAR binding protein" Initiated a SMAR1 murine transgenic project, mapped genomic SMAR1 and cloned the SMAR1 promoter from a prescreened human genomic library	**1999-2001**
Max Planck Institute for Biochemistry, Martinsried, Germany **Research student** – "Isolation and characterization of genes involved in replicative senescence" Worked on a secreted frizzled related protein (SFRP) and its role in replicative senescence	**2001**
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PUBLICATIONS

- Ectopic expression of the homeobox gene Cdx2 is the transforming event in a mouse model of t(12;13)(p13;q12) acute myeloid leukemia **Proc Natl Acad Sci U S A**. **2004 Jan 20**;**101(3):817-22**

Vijay P. S. Rawat, Monica Cusan, Aniruddha. J. Deshpande, Wolfgang Hiddemann, Leticia Quintanilla-Martinez, R. Keith Humphries, Stefan K. Bohlander, Michaela Feuring-Buske, and Christian Buske

- The AML1-ETO fusion gene and the FLT3 length mutation collaborate in inducing acute leukemia in mice **J Clin Invest**. **2005 Aug 1**;**115(8):2159-2168**

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- Acute myeloid leukemia is propagated by a leukemic stem cell with lymphoid characteristics in a mouse model of calm/af10 positive leukemia **(manuscript submitted)**

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Ectopic expression of the homeobox gene *Cdx2* is the transforming event in a mouse model of t(12;13)(p13;q12) acute myeloid leukemia

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Creation of fusion genes by balanced chromosomal translocations is one of the hallmarks of acute myeloid leukemia (AML) and is considered one of the key leukemogenic events in this disease. In t(12;13)(p13;q12) AML, ectopic expression of the homeobox gene *CDX2* was detected in addition to expression of the *ETV6-CDX2* fusion gene, generated by the chromosomal translocation. Here we show in a murine model of t(12;13)(p13;q12) AML that myeloid leukemogenesis is induced by the ectopic expression of *CDX2* and not by the *ETV6-CDX2* chimeric gene. Mice transplanted with bone marrow cells retrovirally engineered to express *Cdx2* rapidly succumbed to fatal and transplantable AML. The transforming capacity of *Cdx2* depended on an intact homeodomain and the N-terminal transactivation domain. Transplantation of bone marrow cells expressing *ETV6-CDX2* failed to induce leukemia. Furthermore, coexpression of *ETV6-CDX2* and *Cdx2* in bone marrow cells did not accelerate the course of disease in transplanted mice compared to *Cdx2* alone. These data demonstrate that activation of a protooncogene by a balanced chromosomal translocation can be the pivotal leukemogenic event in AML, characterized by the expression of a leukemia-specific fusion gene. Furthermore, these findings link protooncogene activation to myeloid leukemogenesis, an oncogenic mechanism so far associated mainly with lymphoid leukemias and lymphomas.

T he molecular dissection of balanced chromosomal translocations in patients with acute leukemia has greatly advanced our knowledge of the pathogenesis of this disease, demonstrating that chromosomal translocations often affect genes that regulate hematopoiesis. Chromosomal translocations involve mainly two mechanisms that lead to malignant transformation: deregulation of the expression of a protooncogene by juxtaposition of a potent enhancer or promoter elements or creation of a fusion gene (1–3). Although both mechanisms are found in lymphoid leukemia or lymphoma, formation of a fusion gene predominates in acute myeloid leukemia (AML). In fact, to date, there are no experimentally confirmed instances in which the transcriptional deregulation of a protooncogene is the key leukemogenic event in a fusion gene-positive AML.

The oncogenic potential of fusion genes has been well documented experimentally. However, emerging data, mostly from murine *in vivo* models, have demonstrated that many of these fusion genes are not able to induce leukemia on their own. This observation suggests an important role for other genetic alterations that cooperate with fusion genes in patients with AML (4–6). The intriguing differences in the oncogenic potential of fusion genes are well documented for the large family of chimeric genes involving the *ets* transcription factor *ETV6*, located at 12p13. *ETV6* is one of the genes most frequently involved in chromosomal translocations. Chromosomal translocations affecting the *ETV6* locus have been reported with >40 different partners (7). Fusion partners of *ETV6* can be phosphotyrosine kinases (PTK) or transcription factors and genes of unknown function, dividing *ETV6* fusion genes into two distinct groups. Fusions of *ETV6* with PTKs such as *PDGFRB*,

JAK2, *ABL1*, *ABL2*, or *NTRK3* create highly leukemogenic proteins in murine experimental models (8–12). In the group of *ETV6*-transcription factor fusions, the N-terminal portion of *ETV6* is fused to the partner gene in most cases, retaining (e.g., *ETV6-AML1*) or losing the *pointed* domain (e.g., *ETV6-CDX2*, *ETV6-MDS1/EVI1*) (13–15). Although data about the leukemogenic potential of this group of fusion genes are still limited, extensive analyses of the most frequent *ETV6* chimeric transcription factor, *ETV6-AML1*, failed to show any major transforming activity in a transgenic or bone marrow (BM) transplantation mouse model (16, 17). Based on these data, expression of an *ETV6*-transcription factor fusion might not be sufficient to induce disease. Indeed, recent evidence corroborates that *ETV6* acts as a tumor suppressor gene and that, in almost all cases of *ETV6/AML1*-positive acute lymphoblastic leukemias, there is a deletion or loss of expression of the nonrearranged *ETV6* allele (18, 19). Furthermore, several chromosomal translocations involving the *ETV6* locus associated with myeloid malignancies such as t(4;12), t(5;12), or t(12;17) do not form any functional fusion gene at all, pointing to a key variant oncogenic mechanism in these cases (20, 21). In this regard, the t(12;13)(p13;q12) associated with the *ETV6-CDX2* fusion gene in human AML is of notable interest. The translocation breakpoint leaves the *CDX2* gene intact, and expression of both the fusion gene and full-length *CDX2*, normally restricted to intestinal epithelial cells, was observed in leukemic cells, thus raising the possibility that ectopic expression of *CDX2* is the key pathogenic event (14).

To clarify this particular issue and to gain insight into alternative mechanisms of transformation in patients with AML and *ETV6* rearrangements, we established a mouse model for t(12;13)(p13;q12) human AML. We demonstrate that ectopic expression of *Cdx2* is the key transforming event that induces fatal AML in transplanted mice. In contrast, expression of the ETV6-CDX2 fusion protein is unable to induce leukemia. Furthermore, we show that the transforming potential of *Cdx2* depends on the integrity of its DNA-binding domain and the N-terminal domain of Cdx2. Our data point to a previously uncharacterized mechanism of leukemogenesis in patients with AML, in which a balanced chromosomal translocation contributes to malignant transformation by activating the expression of a protooncogene, a mechanism so far associated mainly with lymphoid leukemias or lymphomas (3).

Materials and Methods

cDNA Constructs and Retroviral Vectors. cDNAs of *ETV6-CDX2* and *Cdx2* (93% overall and 98% identity in the homeodomain between
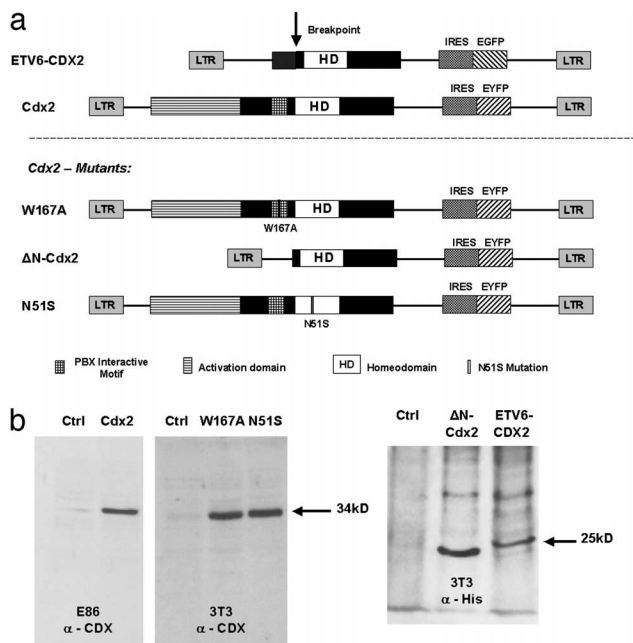

MEDICAL SCIENCES

Fig. 1. (a) Retroviral vectors used to express *ETV6-CDX2*, *Cdx2*, and the different *Cdx2* mutants in murine BM. IRES, internal ribosomal entry site. (b) Western blot analysis of cellular extracts from NIH 3T3 or E86 cells transfected with the different constructs. The molecular mass is indicated.

the human and murine proteins) were kindly provided by D. G. Gilliland (Division of Hematology/Oncology, Harvard Medical School, Boston) and N. Cross (Department of Haematology, Hammersmith Hospital, London). A histidine-tagged version of *ETV6-CDX2* was constructed by ligating a PCR product of the fusion gene in frame to the 3′ end of the histidine epitope of the pCDNA6/V5-His A plasmid (Invitrogen), *Cdx2* mutants were created that were previously shown to inactivate a putative PBX1 interacting motif (W167A-Cdx2) (22) or to inactivate the DNA-binding homeodomain (N51S-Cdx2) (23) by using the QuikChange XL Site-Directed Mutagenesis Kit (Stratagene). The *Cdx2* mutant lacking the first 179 N-terminal amino acids, which are deleted in the *ETV6-CDX2* fusion, was generated and histidine tagged by PCR following standard procedures (ΔN-Cdx2) (4). For retroviral gene transfer into primary BM cells, the different constructs were subcloned into the multiple cloning site of the modified murine stem cell virus (MSCV) 2.1 vector (4) upstream of the internal ribosomal entry site (IRES) and the enhanced GFP or yellow fluorescent protein (YFP) gene. As a control, the MSCV vector carrying only the IRES-enhanced GFP cassette was used.

Production of high-titer helper-free retrovirus was carried out following standard procedures by using the ecotropic packaging cell line GP+E86 (4). The number of provirus integrants was determined by *Eco*RI digestion and full length integration by *Nhe*I digestion, followed by Southern blot analysis using standard techniques (24). Protein expression of the *ETV6-CDX2*, *Cdx2*, and *Cdx2* mutant plasmids was documented by Western blotting using standard procedures. Membranes were probed with an antihistidine monoclonal antibody (Sigma) for ETV6-CDX2 and the ΔN-Cdx2 mutant or with an anti-CDX2 monoclonal antibody (kindly provided by DCS Innovative, Hamburg, Germany) for expression of the Cdx2, W167A-Cdx2, and N51S-Cdx2 mutants (25) (Fig. 1).

***In Vitro* Assays.** Cell proliferation was assessed in DMEM supplemented with 15% FBS/10 ng/ml mIL-6/6 ng/ml mIL-3/100 ng/ml murine stem cell factor (standard medium) (Tebu-bio, Offenbach, Germany). Differentiation of clonogenic progenitors was analyzed

by plating cells in methylcellulose supplemented with cytokines (Methocult M3434, StemCell Technologies, Vancouver). IL-3-dependent cell populations expressing Cdx2 or coexpressing ETV6-CDX2 and Cdx2 were established *in vitro* directly after sorting in DMEM/15% FBS with IL-3 alone (6 ng/ml). The differentiation capacity of cultured cells was tested in DMEM/15% FBS supplemented with granulocyte colony-stimulating factor 100 ng/ml or macrophage colony-stimulating factor 10 ng/ml (R & D Systems) and *all-trans* retinoic acid at 1 μM final concentration. After 5 days, the morphology was determined by Wright–Giemsa-stained cytospin preparations (4, 25).

Mice and Retroviral Infection of Primary BMC. Parental strain mice were bred and maintained at the GSF animal facility. Donors of primary BM cells were >12-wk-old (C57BL/6Ly-Pep3b × C3H/HeJ) F₁ (PepC3) mice, and recipients were >8- to 12-wk-old (C57BL/6J × C3H/HeJ) F₁(B6C3) mice. Primary mouse BM cells were transduced as described (4). For transduction, cells were cocultured with irradiated (40 Gy) ETV6-CDX2/GFP or Cdx2/YFP GP+E86 producers or with a mixture of 40–50% Cdx2/YFP and 50–60% ETV6-CDX2/GFP producers in cotransduction experiments.

Colony-Forming Unit–Spleen (CFU-S) Assay. Primary BM cells from F₁(PepC3) donor mice treated 4 days previously with 5-fluorouracil were transfected with the different viruses, and retrovirally transduced cells were highly purified based on expression of GFP or YFP by using a FACSVantage (Becton Dickinson). Transduced cells were cultured 7 days in standard medium. The day 0 equivalent of 2.5–3 × 10⁴ cells was injected into lethally irradiated F₁(B6C3) recipient mice. The recovery of CFU-S cells was quantified by determining the number of macroscopic colonies on the spleen at day 12 postinjection after fixation in Telleyesnickzky's solution.

BM Transplantation and Assessment of Mice. Recipient F₁(B6C3) mice (8–10 wk old) were irradiated with 850 cGy from a ¹³⁷Cs γ-radiation source. FACS-purified transduced BM cells, or a defined ratio of transduced and untransduced cells was injected into the tail vein of irradiated recipient mice. Peripheral blood (PB) or BM cell progeny of transduced cells were tracked by using the GFP or YFP fluorescence (26). The lineage distribution was determined by FACS analysis as described (4): phycoerythrin-labeled Gr-1, ScaI, Ter-119, CD4, and allophycocyanin-labeled Mac1, cKit, B220, or CD8 antibodies were used for analysis (all PharMingen). For histological analyses, sections of selected organs were prepared and hematoxylin/eosin-stained by using standard protocols.

RT-PCR. Expression of *Hoxa9* and *Meis1* was assayed by RT-PCR in Sca-1⁻Lin⁺ cells sorted from a mouse repopulated with Cdx2 expressing BM cells or a control animal. Total RNA was isolated by using Trizol reagent (GIBCO/BRL) and treated with DNase I (amp grade) to remove contaminating genomic DNA. First-strand cDNA was synthesized from 1 μg of total RNA by using the thermoScript RT-PCR system (all reagents from Invitrogen). Equal amounts of cDNA originating from 50 ng of starting RNA were loaded to assess transcription levels. Intron-spanning primer pairs were selected to avoid amplification of contaminating genomic DNA. The annealing temperatures were 58°C and 60°C for *Meis1* and *Hoxa9*, respectively. The number of PCR cycles for each gene was chosen to stop the reaction in the linear phase of amplification (25 cycles for mβ-2 microglobulin, 35 cycles for *Meis1* and *Hoxa9*).

Statistical Analysis. Data were evaluated by using the *t* test for dependent or independent samples (Microsoft EXCEL). Differences with *P* values < 0.05 were considered statistically significant.
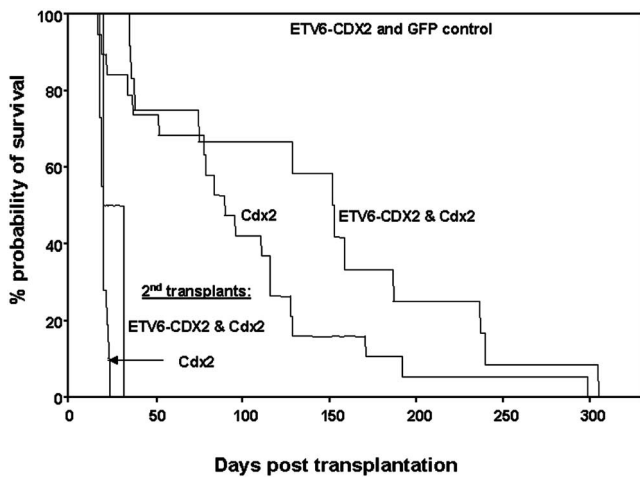
Fig. 2. Survival curve of mice transplanted with BM cells expressing *Cdx2* (n = 18), *ETV6-CDX2* (n = 9), or coexpressing *Cdx2* and the fusion gene (n = 13). The control group was injected with BM infected with the GFP empty retrovirus (n = 7). The survival time of secondary recipient mice, transplanted with BM from diseased primary *Cdx2* or *ETV6-CDX2* and *Cdx2* recipients, is indicated.

Results

Ectopic Expression of *Cdx2* Causes AML in Transplanted Mice. To analyze whether expression of the t(12;13)-associated *ETV6-CDX2* fusion gene and/or the ectopic expression of the homeobox gene *Cdx2* is able to transform early murine hematopoietic progenitors *in vivo*, we generated MSCV-based retroviral constructs and documented full-length protein expression by Western blotting (Fig. 1). Murine hematopoietic progenitors constitutively expressing *ETV6-CDX2* or *Cdx2* were highly purified by FACS based on GFP$^+$ or YFP$^+$ expression, respectively, and injected into lethally irradiated recipient mice directly after sorting (3–3.5 × 10^5 and 2–3.6 × 10^5 cells per mouse for *Cdx2* and *ETV6-CDX2*, respectively).

Mice transplanted with BM cells expressing *Cdx2* became moribund after a median of 90 days posttransplantation (n = 18) (Fig. 2). Diseased mice were characterized by cachexia, shortness of breath, and lethargy when they were killed for further analysis. In
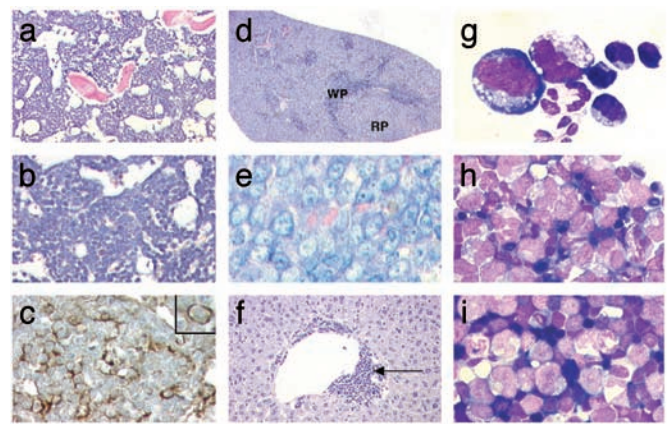

Fig. 3. Histological analysis of diseased *Cdx2* mice. (*a*) BM [hematoxylin/eosin (H&E)]. Immunohistochemistry of the BM (×200) for *N*-acetyl-chloroacetate esterase (×400) (*b*) and CD34 expression (×640) (*c*). Histology of the spleen H&E (×25) (*d*) and Giemsa staining (×640) (*e*) and liver with perivascular infiltration (×200) (*f*). Cytospin preparations from PB (*g*), BM (*h*), and spleen (*i*) (all ×1,000).

striking contrast, mice transplanted with *ETV6-CDX2*-expressing cells did not succumb to terminal disease (n = 9) (Fig. 2). Diseased *Cdx2* mice were characterized by elevated peripheral white blood count (WBC) (3.8-fold) with up to 48 × 10^6 circulating WBC per milliliter. Furthermore, moribund mice were anemic, with a 5-fold decrease in peripheral erythrocyte count (P < 0.001) (Table 1). All *Cdx2* mice analyzed (n = 7) suffered from splenomegaly, with an average spleen weight of 0.6 g (range 0.4–0.9; P < 0.01 compared to control animals) (Table 1). More detailed hematological analyses demonstrated that animals suffered from AML with a high percentage of blasts in the BM (42% ± 6), PB (14% ± 3), and spleen (35% ± 5) (n = 8; P < 0.01 compared to the control animal) (Table 1). Furthermore, leukemic mice showed multiple organ infiltration with blast cells. Thirty percent of the blasts expressed CD34 but were negative for *N*-acetyl-chloroacetate esterase, periodic acid/ Schiff reagent, and terminal deoxynucleotidyltransferase, as shown by immunohistochemistry, consistent with an undifferentiated myeloblastic phenotype of the disease (Fig. 3). Immunophenotypic

Table 1. Hematological parameters of experimental mice

Mouse no.	Retroviral construct	Day of death	RBC per ml ×10^9	WBC per ml ×10^6	Spleen weight, mg	BM % blasts	Spleen % blasts	PB % blasts	Lymphoid/myeloid ratio in PB
1	GFP	90	6	4.5	150	0	0	0	5:1
2	GFP	90	4.8	3.2	200	0	0	0	2:1
3	GFP	90	5.0	3.6	200	0	0	0	2:1
1*	Cdx2	128	1.0	3.2	400	28	21	8	0.5:1
2*	Cdx2	79	2.0	37	650	40	35	12	0.4:1
3*	Cdx2	52	0.7	9	600	38	30	15	0.2:1
4*	Cdx2	116	0.4	48	nd	ND	60	14	0.4:1
5*	Cdx2	37	0.6	5	400	25	22	5	0.3:1
6*	Cdx2	171	0.8	24	900	71	48	18	0.3:1
7*	Cdx2	192	1.1	10	800	60	41	30	0.5:1
8*	Cdx2	84	0.4	28	400	32	24	8	0.8:1
1*	++	168	1.0	3.2	400	25	18	3	0.6:1
2*	++	230	1.1	8	500	45	30	10	0.1:1
3*	++	151	0.2	8	600	58	37	16	0.4:1
4*	++	237	1.5	24	300	25	18	5	0.6:1
5*	++	187	0.5	25	900	50	43	8	0.3:1
1	ETV6-CDX2	375	6.5	2.4	160	10	8	0	0.3:1
2	ETV6-CDX2	375	5	3.2	200	25	15	0	0.4:1
3	ETV6-CDX2	375	5.2	6	180	15	9	0	2:1

*, diseased; ++, ETV6-CDX2 and Cdx2; RBC, red blood cell count; WBC, white blood cell count; ND, not determined.
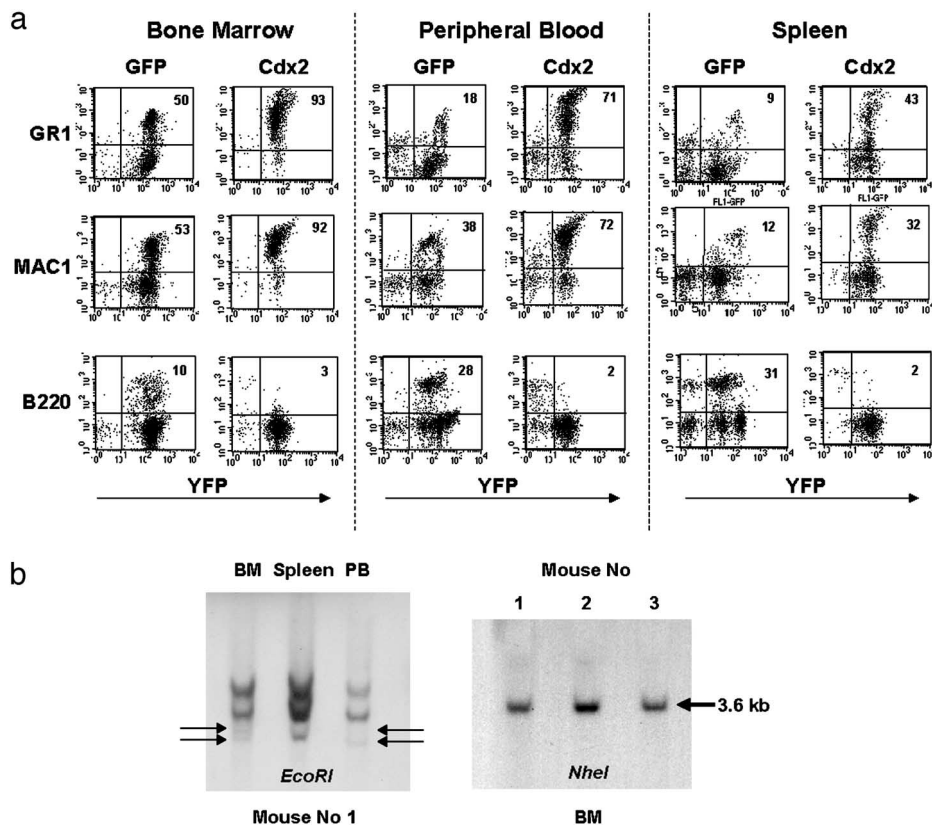
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Fig. 4. (*a*) Flow cytometry from a representative leukemic *Cdx2* mouse from PB, BM, and spleen in comparison to a GFP control animal. Cells were stained for the myeloid markers Gr1 and Mac1 and the lymphoid marker B220. The proportion of positive cells within the GFP$^+$ compartment is indicated. (*b*) Southern blot analyses of genomic DNA from BM, PB, and spleens of representative leukemic *Cdx2* mice. Genomic DNA was digested with *Eco*RI, which cuts once in the provirus, to determine the number of provirus integrants. Signals with different intensity, indicating the presence of different leukemic clones, are indicated. Full-length provirus integration was documented by digestion with *Nhe*I, which cuts only in the LTRs of the provirus.

characterization of PB, BM, and spleen in diseased mice confirmed the predominance of myeloid Mac1$^+$ and Gr-1$^+$ cells (84% ± 10 and 73% ± 15 in the PB, 65% ± 14 and 53% ± 14 in the spleen, respectively; $n = 4$) compared to the GFP control mice (Mac1$^+$ and Gr-1$^+$ cells 47% ± 5 and 25% ± 3 in the PB, 14% ± 9 and 10% ± 1 in the spleen, respectively; $n = 4$). Furthermore, diseased mice were characterized by a greatly reduced normal B220$^+$ lymphoid population in the spleen and PB compared to controls (1.8% ± 1 vs. 35% ± 8 and 1.3% ± 0.5 vs. 46% ± 21 in the PB and in the spleen, respectively; $n = 4$) (Fig. 4*a*). Mice transplanted with *Cdx2*-expressing BM cells were characterized by a 19-fold increased frequency of clonogenic cells in the PB and a >100-fold increase in the spleen compared to the control as quantified by *ex vivo* CFC assays (248 vs. 13 clonogenic cells per 1×10^6 cells/ml in the PB and 1,400 clonogenic cells vs. 13 per 1×10^6 cells/ml in the spleen, respectively) ($n = 3$). Twenty-eight percent (±3) of these clonogenic progenitors were not able to terminally differentiate and formed blast colonies in methylcellulose with high serial replating capacity (data not shown).

The *Cdx2*-induced AML was transplantable and all lethally irradiated mice ($n = 11$) injected with BM cells of diseased *Cdx2* animals died within 24 days posttransplantation (Fig. 2). Analysis of the clonality of the disease by Southern blot analysis demonstrated different intensities and patterns of proviral signals in the different hematopoietic organs consistent with an oligoclonal nature of the disease (Fig. 4*b*).

To analyze whether the *ETV6-CDX2* fusion caused subtle perturbations in hematopoietic development, healthy animals ($n = 3$) were killed 44 wk after transplantation with *ETV6-CDX2*-expressing BM cells. Interestingly, two of three animals showed an expansion of the mature neutrophil compartment in the PB with an inversion of the lymphoid/myeloid ratio (Table 1) and 87% and 68% Mac1$^+$/Gr1$^+$ cells in the GFP-positive compartment. Furthermore, spleens from all mice were infiltrated with terminally differentiated myeloid cells (86% ± 0.9 Gr1$^+$/Mac1$^+$ cells). How-

ever, none of the animals suffered from anemia, splenomegaly, or the emergence of a blast population in the PB (Table 1). Thus, *ETV6-CDX2* was able to induce a myeloproliferation without causing disease but failed to induce leukemic transformation.

In addition, 13 mice were transplanted with a mixture of *ETV6-CDX2*, *Cdx2*, and *Cdx2* and *ETV6-CDX2* coexpressing cells, containing between $1.9-4.5 \times 10^4$ *Cdx2* and *ETV6-CDX2* cells and <4,000 *Cdx2* cells per mouse. The addition of *Cdx2* and *ETV6-CDX2* coexpressing cells did not accelerate the course or change the phenotype of the disease compared to only *Cdx2*-expressing cells. All animals succumbed to AML, and the leukemic population consisted of *Cdx2*- and *ETV6-CDX2*-coexpressing or *Cdx2*-expressing cells in all mice analyzed ($n = 4$) (Fig. 2). These data indicate that aberrant expression of the wild-type *Cdx2* gene is crucial for malignant transformation in this model.

The Transforming Potential of *Cdx2* Depends on the N-Terminal Transactivation Domain and the Intact Homeodomain. In an effort to characterize the contribution of different motifs of *Cdx2* to the transforming capacity of the gene, three different mutants were designed: a mutant inactivating the homeodomain (N51S-Cdx2), a Cdx2 mutant with an inactivating mutation in the putative PBX1-interacting motif (W167A-Cdx2), and a mutant lacking the N-terminal portion of Cdx2, which is not present in the *ETV6-CDX2* fusion (ΔN-Cdx2). Protein expression of the mutants was confirmed by Western blot analysis (Fig. 1*b*). Expression of wild-type Cdx2 and W167A-Cdx2 in primary bone marrow cells rapidly induced the outgrowth of IL-3-dependent cell populations in liquid cultures. The cells showed blast morphology, were Gr$^+$/Mac1$^+$-positive, and had lost their differentiation capacity when incubated with macrophage colony-stimulating factor, granulocyte colony-stimulating factor, or *all-trans* retinoic acid (data not shown). Furthermore, mice transplanted with 1×10^6 of these cells developed leukemia 8 wk posttransplant in contrast to mice injected with nontransduced or GFP-expressing control cells. Cells expressing
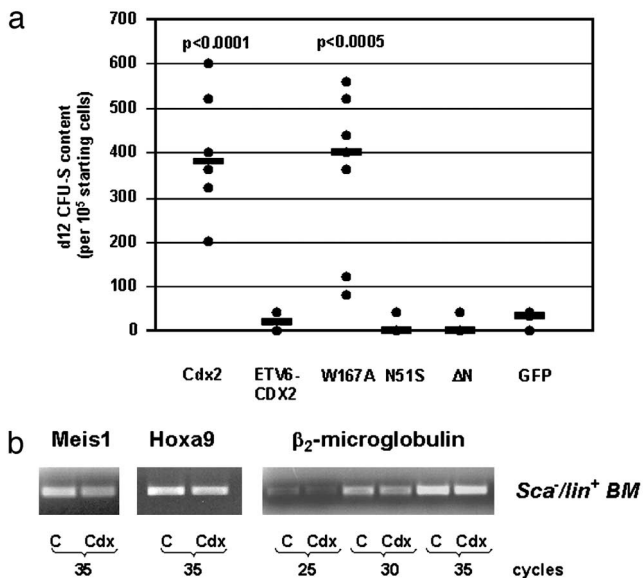
a

b

Fig. 5. (*a*) Total number of d12 CFU-S colonies derived per culture initiated with 1×10^5 cells transduced with the different viruses after 1 wk in liquid culture. The median is indicated. (*b*) Expression of *Meis1* and *Hoxa9* analyzed by RT-PCR in Sca⁻lin⁺ BM cells isolated from a *Cdx2* mouse or a control mouse. The number of PCR cycles for each gene was chosen to stop the reaction in the linear phase of the amplification (25 cycles for mβ-2 microglobulin, 35 cycles for *Meis1* and *Hoxa9*). C, control; Cdx, Cdx2.

ETV6-CDX2, the ΔN-Cdx2, or the N51S mutant as well as the control cells were not able to form blast cell populations *in vitro*. When colony formation was tested, Cdx2-positive cells generated a higher number of primary CFC in methylcellulose compared to GFP (76 ± 22 vs. 41 ± 20 per 500 initially plated cells, respectively; $n = 5$; $P < 0.02$). Furthermore, Cdx2-positive colonies contained $\approx$10 times more cells per colony than the controls (33×10^3 vs. 3.9×10^3 per colony, respectively; $n = 5$; $P < 0.004$). The expression of the other constructs did not change the size or number of colonies compared to the control. To investigate the effect of the different mutants on primitive hematopoietic cells, cells infected with the different viruses were injected into lethally irradiated mice after 7 days of *in vitro* culture, and spleen colony formation was quantified 12 days after injection in killed mice (CFU-S assay). Cdx2 expression as well as expression of the W167A-Cdx2 mutant induced a significant >10-fold increase in the yield of day 12 CFU-S compared to the GFP control ($n = 8$; $P < 0.0001$). In contrast, deletion of the N-terminal portion of Cdx2 ($n = 5$) or inactivation of the homeodomain ($n = 5$) resulted in complete loss of the Cdx2 activity in these assays. ETV6-CDX2 ($n = 6$) did not show any increase in CFU-S compared to the GFP control (Fig. 5*a*).

The Expression of *Hoxa9* and *Meis1* Is Not Increased by Ectopic Expression of *Cdx2*. Given the role of *Cdx2* as an upstream regulator of *Hox* gene expression, we asked whether *Cdx2* would perturb expression of leukemogenic homeobox genes such as *Hoxa9* or *Meis1*. First, expression of *Hoxa9* and *Meis1* was determined by RT–PCR in the 32D cell line transduced with the Cdx2, the ETV6-CDX2, or the GFP virus. Compared to the control, *Cdx2* did not increase expression of Hoxa9 or Meis1 (data not shown). In addition, Sca⁻lin⁺-differentiated cells were recovered and highly purified from a mouse transplanted with *Cdx2*-expressing BM cells and a control animal, a cell population with normally no detectable expression of *Hoxa9* and *Meis1* (27): specific amplification products were not detectable by RT-PCR after 25 cycles in both experimental arms. Amplification products could be detected after 35 cycle but without considerable differences in the intensity between

Cdx2-transduced and control cells (Fig. 5*b*). Thus, ectopic expression of *Cdx2* was not associated with up-regulation of *Meis1* or *Hoxa9* in this model system.

Discussion

The formation of fusion genes with oncogenic properties by balanced chromosomal rearrangements is considered one of the crucial steps for leukemic transformation in patients with AML. By using the murine BM transplantation model, we now provide direct evidence that the ectopic expression of the protooncogene *Cdx2* and not the expression of the fusion gene *ETV6-CDX2* is the key transforming event in t(12;13)(p13;q12)-positive AML. Activation of protooncogenes by balanced chromosomal translocations is a well-known oncogenic mechanism in lymphoid leukemias or lymphomas but has, to our knowledge, not been functionally demonstrated for AML and translocations involving *ETV6* (3). In addition, these data present evidence that the homeobox gene and *Hox* gene upstream regulator *Cdx2*, which so far has been linked to intestinal metaplasia and colon cancer (28), is highly leukemogenic when aberrantly expressed in hematopoietic progenitor cells.

Cdx2 belongs to the large group of homeobox genes, which were originally described as master regulators of embryonic body development. The *Cdx* genes and their homologues *caudal* in *Drosophila* and *Xcad* in *Xenopus* belong to the ParaHox cluster, which is considered an ancient paralog of the *Hox* gene cluster (29). Although the *Cdx* genes show similarities to the 5′-located Abdominal-B like genes of the *Hox* gene cluster, they possess a Pbx recognition motif, a characteristic of 3′-located *Hox* genes (30). *Cdx* genes play a key role in the homeobox regulatory network, acting as upstream regulators of several *Hox* genes (30, 31). Thus, perturbation of *Cdx2* might be linked to critical alterations in downstream *Hox* genes that are central regulators of normal early hematopoietic development in the adult with a distinct expression profile in human and murine early progenitor cells (27, 32, 33). Gene expression profiling of acute leukemias using DNA microarray technology linked aberrant expression of *Hox* genes such as *HOXA9*, *HOXA10*, and of the nonclustered homeobox gene *MEIS1* to leukemogenesis (34–37). Retrovirally enforced expression of these genes induced severe perturbations of normal hematopoietic development in human and murine experimental models (24, 38). Altered expression of several *Hox* genes might be one of the reasons for the strong oncogenic potential of *Cdx2* (30, 39–42). However, RT–PCR analyses in the 32D cell line model and in Sca⁻/lin⁺ BM population of a *Cdx2* repopulated mouse did not indicate gross up-regulation of *Meis1* and *Hoxa9* by *Cdx2*. However, this does not exclude that perturbation of other *Hox* genes might play a role in the transformation process initiated by ectopic *Cdx2* expression.

Of note, perturbed expression of *Hox* genes such as *HOXA9* or *HOXA10* in hematopoietic progenitor cells is not able to induce frank AML in transplanted mice after a short latency time but requires collaboration with the *Hox* co-factor *MEIS1*. In striking contrast, constitutive expression of *Cdx2* rapidly caused leukemia in recipient mice. The underlying cause for the difference in the leukemogenic activity between *Cdx2* and *HOXA9* or *HOXA10* is not known. But, in contrast to *HOXA9* and *HOXA10*, which are normally expressed at high levels in progenitor cells, *CDX2* is not expressed in hematopoietic cells (14). Thus, ectopic expression of *CDX2* in leukemia patients might result in the activation of *de novo* downstream pathways, which are normally silent in early blood development.

Despite the differences in the oncogenic potential, many of the *in vitro* and *in vivo* hematopoietic effects induced by *Cdx2* are highly reminiscent of the effects of retrovirally overexpressed hematopoietic *HOX* genes as well as leukemia-specific fusion genes such as *NUP98-HOXD13* with regard to the impact on short-term repopulating CFU-S or clonogenic progenitors (4, 38, 43). The striking similarities of the phenotypes induced by the over-expression of

homeobox genes of the *Hox* cluster and of *Cdx2* as a member of the ParaHox complex (29) point to a high level of functional redundancy among homeobox proteins in hematopoiesis.

The hematopoietic activity of *Cdx2* strictly depended on its intact homeodomain, implicating that DNA binding of *Cdx2* is essential for its transforming activity. Furthermore, deletion of the Cdx2 N-terminal portion resulted in a complete loss of activity in our assays. Of note, it was demonstrated that the N-terminal part of Cdx2 is necessary for transcriptional activation of *Hox* genes, supporting the concept that activation of downstream *Hox* genes is a potential key mechanism of *Cdx2*-induced transformation (44). Furthermore, it was demonstrated that the transcriptional activity of CDX proteins depends on the interaction of the p38 mitogen-activated protein kinase and the N-terminal transactivation domain of Cdx2 (45). As a consequence, N-terminal deletion would diminish the transactivation capacity of CDX2. Importantly, the *ETV6-CDX2* fusion gene lacks the N-terminal portion of *CDX2*, presumably hampering its capability to transactivate target genes. This would explain the obvious discrepancy in the oncogenic potential between *Cdx2* and the *ETV6-CDX2* fusion gene; this is supported by our data, which demonstrate a complete loss of activity when this N-terminal portion of *Cdx2*, which is not present in the *ETV6-CDX2* fusion gene, is deleted in the ΔN-Cdx2 mutant. Notably, mice transplanted with BM cells expressing the chimeric gene developed myeloproliferation after a long latency time but without any clinical symptoms. These data indicate that, despite the loss of the N-terminal portion, the fusion gene is able to perturb hematopoietic development, although to a significantly lesser extent than full-length *Cdx2*. However, it cannot be excluded from our experiments that the first 54 amino acids of ETV6, which are fused to CDX2, are responsible for or at least contribute to the observed disturbances of hematopoiesis. Taken together, our data propose a model in which the chromosomal translocation t(12;13)(p13;q12) causes AML by inducing the ectopic expression of CDX2. The mechanism of transcriptional induction is not precisely known, but it was demonstrated that the chromosome 13 breakpoint lies upstream of the *CDX2* gene. Therefore, one possible explanation for the ectopic expression of CDX2 could be that the translocated protooncogene might now be under the control of one of the two alternative ETV6 enhancer/promoters, located between exons 2 and 3 of ETV6 (14). Intriguingly, it was recently shown that the homeobox gene GSH2 and IL-3 are ectopically expressed in patients with AML and the translocations t(4;12)(q11–12;p13) and t(5;12)(q31;p13), respectively. Both translocations involve *ETV6* but do not create any functional fusion genes (20). This observation suggests that activation of protooncogenes is a more common phenomenon in ETV6-associated leukemias than previously thought. Taking into consideration that several AML-associated fusion genes are not leukemogenic on their own, it is tempting to speculate that activation of protooncogenes by chromosomal rearrangements might be quite a widespread mechanism in myeloid leukemogenesis. This hypothesis is supported by observations in AML cases not affecting ETV6, in which expression of the putative protooncogene EVI1 is activated by juxtaposition to the enhancer sequences of the ribophorin-I gene in patients with AML and 3q21 alterations (46). Our data provide compelling evidence that myeloid leukemogenesis can be initiated by this mechanism and emphasize the relevance of protooncogene activation for the development of AML.

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The *AML1-ETO* fusion gene and the *FLT3* length mutation collaborate in inducing acute leukemia in mice

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The molecular characterization of leukemia has demonstrated that genetic alterations in the leukemic clone frequently fall into 2 classes, those affecting transcription factors (e.g., *AML1-ETO*) and mutations affecting genes involved in signal transduction (e.g., activating mutations of *FLT3* and *KIT*). This finding has favored a model of leukemogenesis in which the collaboration of these 2 classes of genetic alterations is necessary for the malignant transformation of hematopoietic progenitor cells. The model is supported by experimental data indicating that *AML1-ETO* and *FLT3* length mutation (*FLT3-LM*), 2 of the most frequent genetic alterations in AML, are both insufficient on their own to cause leukemia in animal models. Here we report that *AML1-ETO* collaborates with *FLT3-LM* in inducing acute leukemia in a murine BM transplantation model. Moreover, in a series of 135 patients with AML1-ETO–positive AML, the most frequently identified class of additional mutations affected genes involved in signal transduction pathways including *FLT3-LM* or mutations of *KIT* and *NRAS*. These data support the concept of oncogenic cooperation between *AML1-ETO* and a class of activating mutations, recurrently found in patients with t(8;21), and provide a rationale for therapies targeting signal transduction pathways in AML1-ETO–positive leukemias.

Introduction

The cloning of recurring chromosomal translocations and, increasingly, the molecular characterization of point mutations in patients with acute leukemia have substantially contributed to the understanding of the pathogenesis of this disease. In acute myeloid leukemia (AML), chromosomal translocations most frequently target transcription factors involved in the regulation of normal hematopoietic differentiation, whereas point mutations often affect genes involved in signal transduction pathways associated with cell proliferation (1–3). The systematic analyses of genetic alterations in patients with AML have demonstrated that genetic lesions of more than 1 transcriptional regulator, such as *AML1-ETO* (*RUNX1-MTG8*), *HOX* fusion genes, or *PML-RARA*, rarely occur in the leukemic clone. Similarly, patients with concurrent mutations of *FLT3*, *KIT*, or *NRAS* are rare. However, there are numerous examples in which fusion genes are identified together with activating mutations of receptor tyrosine kinases, exemplified by *PML-RARA* and the *FLT3* length mutation (*FLT3-LM*), which occur together in up to 35% of all patients with t(15;17)–positive AML (4).

These observations have favored a model of pathogenesis of acute leukemia in which the 2 groups of genetic alterations, 1 affecting transcriptional regulation and hematopoietic differentiation, the other altering signal transduction cascades associated with cell proliferation, collaborate in inducing acute leukemia (5). This concept is supported by experimental data demonstrating that *AML1-ETO*, one of the most frequent fusion genes in AML, is not able, on its own, to induce leukemia in experimental in vivo models but requires additional mutations in yet unknown genes for induction of hematological disease. In a conditional AML1-ETO murine model, for example, only mice treated additionally with *N*-ethylnitrosourea (ENU) developed AML as well as T cell lymphoblastic lymphoma, whereas untreated AML1-ETO mice showed only minimal hematopoietic abnormalities (6). Similar observations were reported from an hMRP8–AML1-ETO transgenic mouse model, which developed AML as well as T–acute lymphoblastic leukemia/lymphoma (T-ALL/lymphoma) only after ENU mutagenesis (7), and from a murine BM transplantation model inducing constitutive expression of AML1-ETO in hematopoietic progenitor cells by retroviral gene transfer (8). In a recent report, mice targeted to express AML1-ETO in the HSC compartment developed a nonlethal long-latency myeloproliferative syndrome but failed to develop acute leukemia (9).

To test the hypothesis of oncogenic cooperation between different classes of mutations, we analyzed a series of 135 patients with AML1-ETO–positive AML for the occurrence of activating mutations involving signal transduction pathways (*FLT3-LM*, *FLT3D835*, *KITD816*, *NRAS* codon 12/13/61). Because almost one-third of all AML1-ETO–positive patients had such activating mutations, we asked whether AML1-ETO would be able to collaborate with 1 of

Table 1
Genetic alterations in patients with *AML1-ETO* rearrangement

	No. of patients analyzed	No. of patients with mutation detected
FLT3-LM	135	11 (8.1%)
FLT3D835	135	3 (2.2%)
KITD816	135	11 (8.1%)
NRAS codon 12/13/61	135	13 (9.6%)
FLT3, *KIT*, or *NRAS* mutation	135	38 (28.1%)
MLL-PTD	87	0

these alterations to induce leukemia. Here we demonstrated that retrovirally engineered coexpression of *AML1-ETO* and *FLT3-LM* potently synergizes to trigger the development of aggressive leukemia in a murine transplantation model.

This model will allow valuable insights into the pathogenesis of core-binding factor (CBF) leukemias and demonstrates, for the first time to our knowledge, the functional collaboration of AML1-ETO with a class of activating mutations frequently found in patients with t(8;21)–positive leukemia.

Results

AML1-ETO occurs frequently together with activating mutations involving signal transduction pathways in patients with AML. In order to characterize genetic alterations that occur together with the *AML1-ETO* fusion gene in AML, 135 patients with *AML1-ETO* (93 male, 42 female; median age 50.9, range 15.8–89.1) were screened for activating mutations in the receptor tyrosine kinases *FLT3* and *KIT* as well as in *NRAS* (*KITD816*, *NRAS* codon 12/13/61). Patients included 118 with newly diagnosed AML, 4 in first relapse, and 13 classified as having therapy-related AML. Activating mutations were detected in 38 patients (28.1%) and included mutations in the receptor tyrosine kinase *FLT3* or *KIT* (25 patients in total) or in *NRAS* (13 patients). In contrast, no *MLL-PTD* (partial tandem duplication) mutations were detected in 87 samples subjected to this analysis (Table 1). These data demonstrate that genetic alterations occurring with the *AML1-ETO* fusion gene frequently affect signal transduction pathways.

AML1-ETO cooperates with FLT3-LM in inducing acute leukemia in transplanted mice. To test the functional significance of the association of AML1-ETO with mutations involving critical signal transduction cascades, we used the murine BM transplantation model. Murine stem cell virus–based (MSCV-based) retroviral constructs carrying the AML1-ETO cDNA upstream of an internal ribosomal entry site–green fluorescent protein (IRES-GFP) cassette or the FLT3-LM cDNA upstream of an IRES–yellow fluorescent protein (IRES-YFP) cassette were generated to transduce and track hematopoietic cells expressing AML1-ETO (GFP[+]), FLT3-LM (YFP[+]), or both AML1-ETO and FLT3-LM (GFP[+]/YFP[+]) in vitro and in vivo (Figure 1). In order to investigate the impact of expression of AML1-ETO or FLT3-LM individually on primary primitive hematopoietic progenitor cells, we performed the colony-forming spleen assay (CFU-S). BM cells transduced with the *AML1-ETO*/GFP or *FLT3-LM*/YFP vector or both vectors were highly purified 96 hours after the start of infection by FACS. Their ability to form spleen colonies (day 0 equivalent) was measured by transplantation of transduced cells after purification into lethally irradiated recipient mice and quantification of spleen colony formation 12 days after injection. Constitutive expression of FLT3-LM did not

increase the CFU-S content compared with the GFP control. In contrast, AML1-ETO increased the CFU-S content 3.1-fold compared with the control ($P < 0.002$). Strikingly, coexpression of FLT3-LM together with AML1-ETO increased CFU-S numbers a further 2.1-fold for a net increase of 6.5-fold CFU-S compared with the control ($P < 0.013$), thus demonstrating functional collaboration of these 2 genetic alterations in enhancing the CFU-S frequency (Figure 2A). In an effort to characterize the domains responsible for the collaboration of the 2 aberrations, an FLT3-LM and an AML1-ETO mutant were generated: the FLT3-LM mutant with loss of its kinase activity (kinase dead [KD]) (FLT3-LM-KD) and the AML1-ETO mutant with an L148D point mutation in the Runx1 domain of AML1-ETO (AML1-ETO-L148D), previously reported to lack DNA-binding activity. Expression of the constructs was tested by Western blot and FACS analysis, and FLT3-LM-KD was also tested for autophosphorylation as a surrogate marker for kinase activity and for its capacity to induce IL-3–independent growth in Ba/F3 cells (Figure 1, B, D, F, and G). Of note, AML1-ETO-L148D was not able to collaborate with FLT3-LM. Furthermore, the collaboration between AML1-ETO and FLT3-LM was dependent on the kinase activity of FLT3, as FLT3-LM-KD did not collaborate with the fusion gene (Figure 2A). Inhibition of the kinase activity of FLT3-LM by the protein tyrosine kinase (PTK) inhibitor PKC412 was tested in a ΔCFU-S assay after 48 hours of incubation with the inhibitor. The compound induced a 62% reduction of the day 0 equivalent of the CFU-S frequency (42 versus 16 per 1×10^5 initiating BM cells) of cells cotransfected with *FLT3-LM* and *AML1-ETO* compared with the untreated control, whereas the CFU-S frequency of cells infected with the GFP control vector was unchanged by the inhibitor (Figure 2B).

To further assess the potential collaboration of AML1-ETO with FLT3-LM, we carried out long-term BM transplantation studies using BM transduced with *AML1-ETO* or *FLT3*-LM alone or with both together. Over an observation period extending to 20.6 months, no disease developed in recipients of BM singly transduced with *AML1-ETO* (3×10^5 to 4×10^5 highly purified GFP^{+} cells; $n = 9$) or *FLT3-LM* (7×10^4 to 2×10^5 highly purified YFP^{+} cells together with 3×10^5 to 1×10^6 nontransduced helper cells; $n = 9$). To obtain mice engrafted with AML1-ETO/FLT3-LM–coexpressing BM cells, mice were injected with a mixture of GFP[+]/YFP^{+} cells (range 1×10^3 to 5.5×10^4 cells) and nontransduced normal BM cells (range 2.3×10^5 to 1.9×10^6). All recipients of doubly transduced BM ($n = 11$ from 5 independent experiments) succumbed to an aggressive acute leukemia after a median latency time of 233 days post-transplantation (Figure 3). These mice were engrafted with GFP/YFP–coexpressing cells that were positive for AML1-ETO and FLT3-LM transcripts in the RT-PCR analysis (Figure 1C). At diagnosis the mice were moribund, cachectic, and short of breath and suffered from splenomegaly (median spleen weight 441 mg) (Table 2). Peripheral blood and BM contained a high proportion of blasts, and peripheral blood wbc counts were highly elevated in 5 of 11 animals (range 2×10^6 to 430×10^6 cells/ml) compared with the GFP control (range 3.5×10^6 to 9×10^6) (Table 2), consistent with a diagnosis of acute leukemia. Additionally, mice were anemic, with a 45% reduction in erythrocyte counts compared with the mean count in the control.

Coexpression of AML1-ETO and FLT3-LM causes both acute myeloblastic and lymphoblastic leukemia. In 7 animals the morphology of the blasts was myeloblastic (Figure 4A), whereas 4 animals were characterized by a lymphoblastic cell population (Figure 4, B and C, and Table 2). In 2 of the 7 animals with AML (mice nos. 16 and 24) the blast population was accompanied by a dominant mast cell
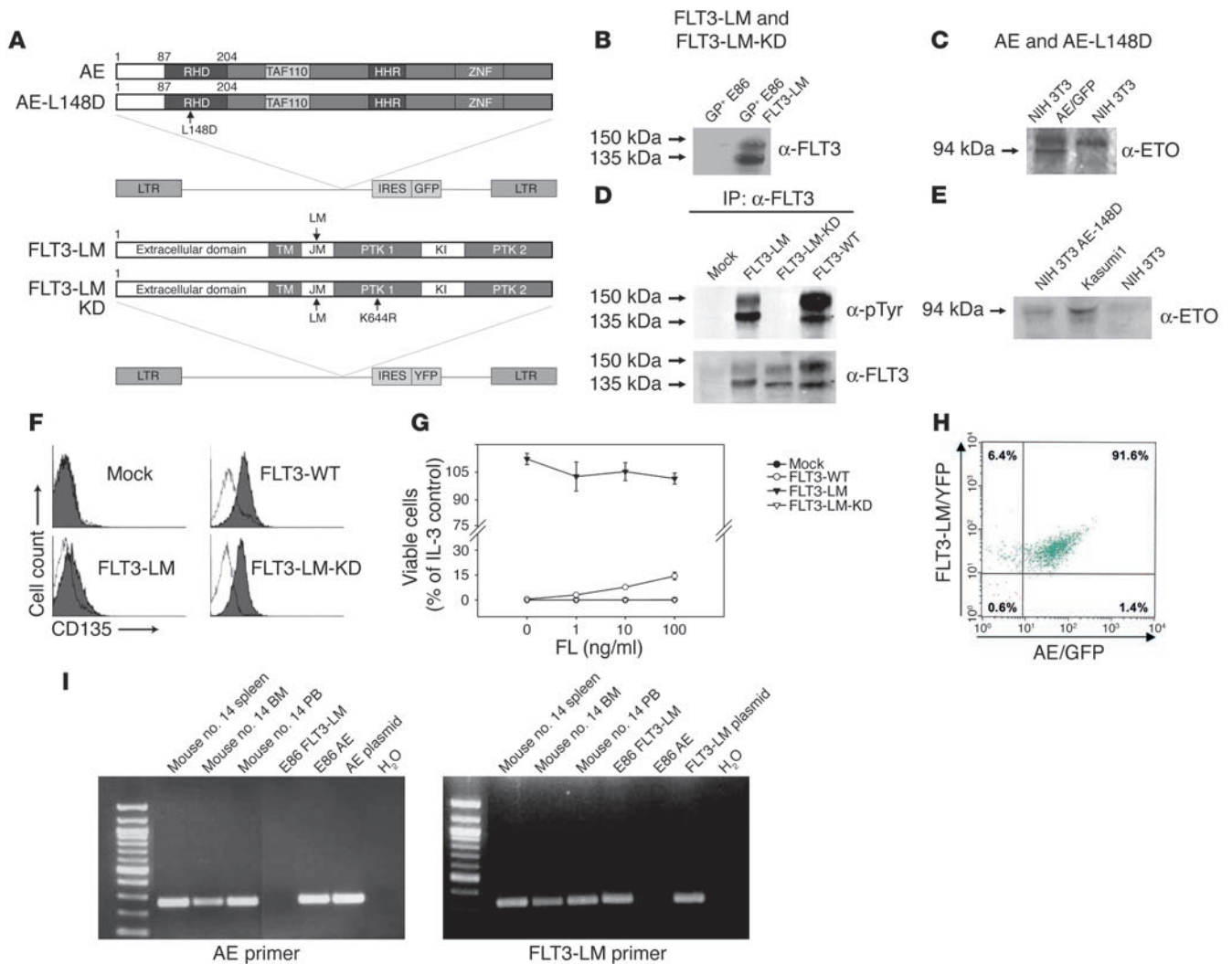
Figure 1
Schematic diagram and analysis of expression of different constructs. (**A**) Retroviral constructs for expression of AML1-ETO and of the AML1-ETO-L148D (31, 47), FLT3-LM, and FLT3-LM-KD mutant proteins. The GFP vector served as a control. AE, AML1-ETO; LTR, long-terminal repeat; RHD, runt homology domain; TAF110, TATA-binding protein–associated factor 110; HHR, hydrophobic heptad repeat; ZNF, zinkfinger; TM, transmembrane; JM, juxtamembrane; PTK, protein tyrosine kinase; KI, kinase insert. (**B**, **C**, and **E**) Western blot analysis of cellular extracts from GP+ E86 and NIH 3T3 cells transfected with the different constructs (the molecular mass is indicated). Kasumi cells served as a positive control. (**D**) α-pTyr plot demonstrating phosphorylation of FLT3-LM and FLT3-WT but not of FLT3-LM-KD. (**F**) FACS analysis of Ba/F3 cells transduced with the FLT3 constructs. (**G**) Growth of IL-3–dependent Ba/F3 cells infected with the different constructs. (**H** and **I**) Flow cytometry and RT-PCR analysis of cells coexpressing FLT3-LM/YFP and AML1-ETO/GFP, isolated from a representative leukemic mouse. FL, FLT3 ligand; PB, peripheral blood.

population with fine metachromatic granulation in the panoptic staining (Figure 4A). The BM and spleen were infiltrated with up to 80% and 80% blasts, respectively, in the mice with AML and up to 85% and 95%, respectively, in the mice suffering from ALL (Table 2 and Figures 4 and 5).

In order to determine more precisely the immunophenotype of the leukemic population, flow cytometric analyses from BM cells were performed. Seven animals suffered from AML with a Gr-1/Mac-1–positive cell population in the transduced compartment, which coexpressed Sca-1 (45.2%, range 19–73%). Of note, in 4 of the 7 animals with AML, coexpression of CD4 was detected in 21%, 27%, 31%, and 32% of BM cells (animals nos. 15, 16, 22, and 23, respectively; Table 2). Three mice suffered from B-lymphoblastic leukemia, with

90.4% of the transduced cells expressing B220 (range 85–97%) and lacking expression of myeloid antigens (Gr-1–positive 1.5%, range 0.4–2.3%; Mac-1–positive 1.7%, range 1.4–1.9%). One animal developed T cell leukemia, with coexpression of CD4 and CD8 (99% CD8+, 86% CD4+ in the transduced compartment) and expression of Sca-1 in 76% of all cells (Figure 4C). Histological tissue sections and immunohistochemistry were performed in 2 diseased mice with AML, including 1 of the animals with an increase in mast cells in the peripheral blood (mouse no. 16). Both animals showed multiple-organ infiltration into hematopoietic and nonhematopoietic organs with effacement of the normal follicular architecture of the spleen (Figure 5, D and E [right side]) and an infiltration with leukemic blasts in the liver and spleen (Figure 5, A–D and F). Immunohistochemistry
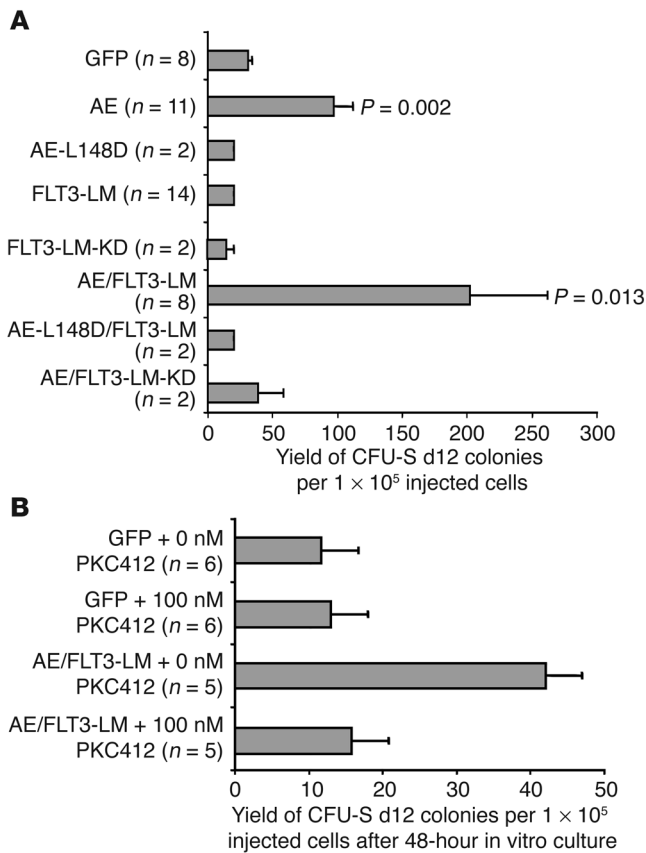
A

B

Figure 2
Analyses of CFU-S frequencies. (**A**) Primary BM cells retrovirally transduced with GFP, AML1-ETO, AML1-ETO-L148D, FLT3-LM, or FLT3-LM-KD vectors or with combinations of the different vectors were isolated by FACS 48 hours after infection and injected into lethally irradiated mice to assess initial (day 0) CFU-S numbers. CFU-S frequency per 1×10^5 initiating BM cells was determined in 3 independent experiments. The number of analyzed mice and the P value compared with the GFP control are indicated. (**B**) CFU-S frequency of primary BM cells infected with GFP or with both AML1-ETO and FLT3-LM and treated with the inhibitor PKC412 for 48 hours compared with untreated controls.

respectively. Furthermore, 31 of 52 patients (59.6%) expressed the B cell antigen CD19, 27 of 52 patients cyCD22 (51.9%), and 38 of 39 patients cyCD79a (97.4%) (Figure 7). There was no difference in the extent of coexpression of lymphoid antigens in AML1-ETO–positive AML with additional activating mutations of *FLT3*, *KIT*, and *NRAS* ($n = 14$) versus cases without this class of mutations ($n = 38$) (data not shown). This indicates that coexpression of lymphoid and myeloid antigens in myeloblastic leukemia, which is detected in the murine model, is a common characteristic in patients with AML1-ETO–positive AML.

Discussion
The translocation t(8;21)(q22;q22), which generates the AML1-ETO fusion gene, is one of the most frequent chromosomal translocations, detected in 12% of all AML patients and in up to 40% of FAB-M2 AML patients (10, 11). The translocation targets *AML1* (*RUNX1*), a member of the RUNX family characterized by a DNA-binding *Runt* domain at the amino terminus that is retained in the fusion gene (12). This domain is necessary for DNA binding and heterodimerization of AML1 with CBFB, the non–DNA-binding subunit of the complex. As predicted by the discovery that the *AML1* gene is rearranged in human hematopoietic disease, the AML1/CBFB complex was shown to be a key regulator of definitive hematopoiesis, and loss of either of these genes resulted in embryonic lethality with complete lack of definitive HSCs (13). In addition, it was recently reported that AML1$^{+/-}$ adult mice suffer from a 50% reduction of long-term repopulating stem cells (14). Although it is yet not fully understood how the

confirmed the diagnosis of AML: blasts were positive for myeloperoxidase but showed differentiation into more mature myeloid cells with positivity for chloracetatesterase (mouse no. 14; Table 2 and Figure 5, B, C, and F). In the second mouse with AML, infiltration of organs with cells expressing mast cell–specific tryptase and CD117 could be confirmed in the primarily and secondarily transplanted mouse, indicating the presence of a malignant infiltrating mast cell population in this animal (mouse no. 16).

The leukemias were readily transplantable and had the same histomorphology within 106 days after transplantation (median survival 68 days, range 57–106 days; $n = 5$) (Figure 3 and Figure 5, G–O). Southern blot analyses of BM from leukemic mice revealed modest numbers of proviral integrations, consistent with double infection and monoclonal or, at most, oligoclonal disease (Figure 6A). Monoclonal or oligoclonal disease is consistent with the relatively small transplant doses used but could also reflect a possible contribution of retroviral insertional mutagenesis to the transformation process. To further explore this latter possibility, 10 retroviral integration sites were subcloned and sequenced from 4 leukemic mice; all 10 sites were unique, and thus there was no indication of a common integration site associated with the leukemic transformation. Moreover, 5 sites were intergenic or not linked to known genes. The remaining sites were in introns in a 5′ to 3′ orientation most likely to lead to gene knock down rather than activation (Figure 6B and Table 3).

Since we observed coexpression of CD4 in leukemic cells of the majority of mice who developed AML in our model, we analyzed expression of CD4 and cytoplasmic (cy) CD3 in patients with AML1-ETO–positive AML; 17 of 52 patients analyzed (32.7%) and 39 of 50 patients analyzed (78%) were positive for CD4 or cyCD3,
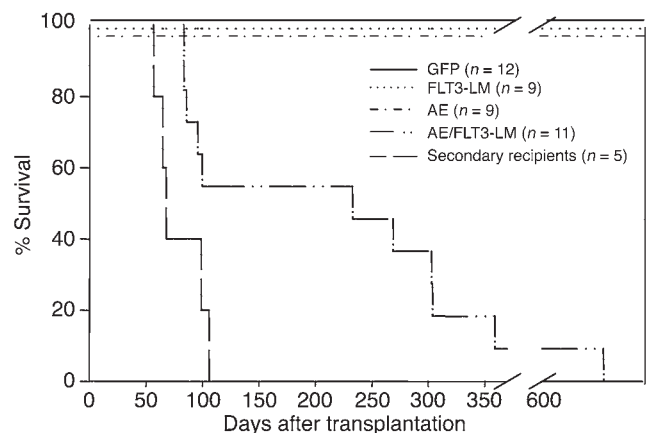

Figure 3
Survival of transplanted mice. Survival curve of mice transplanted with BM cells expressing AML1-ETO ($n = 9$), FLT3-LM ($n = 9$), or GFP ($n = 12$), of mice transplanted with marrow cells coexpressing AML1-ETO and FLT3-LM ($n = 11$), and of secondarily transplanted mice ($n = 5$).

Table 2
Hematological parameters of analyzed experimental mice

Mouse no.	Retroviral construct	Day of sacrifice	rbc/ml $\times 10^9$	wbc/ml $\times 10^6$	Spleen size (mm)	Spleen weight (mg)	BM % blasts	Spleen % blasts	PB % blasts
1	GFP	ND	4.8	7.6	ND	ND	1	0	0
2	GFP	ND	6.4	8.1	ND	ND	0	0	0
3	GFP	90	7	5	14 × 4	51	2	0	0
4	GFP	ND	5.4	9	ND	ND	0	0	0
5	GFP	689	5.6	4.5	15 × 4	78	0	0	0
6	GFP	721	7.25	3.5	ND	ND	3	0	0
7	AE	444	6	13	13 × 3.5	63	8	0	0
8	AE	479	3.1	15	14 × 4	60	11	0	0
9	AE	493	5.7	6.9	14 × 4	82	2	0	0
10	AE	615	5	7.6	16 × 5	117	14	0	0
11	FLT3-LM	88	4.5	10	15 × 4	95	4	ND	0
12	FLT3-LM	ND	4.5	13	ND	ND	2	ND	0
13	FLT3-LM	ND	5.6	8.3	ND	ND	1	ND	0
14[A]	AE/FLT3-LM	233	ND	23	27 × 7	600	80	52	20
15[A]	AE/FLT3-LM	100	0.85	26.5	19 × 6	166	48	80	75
16[A]	AE/FLT3-LM	612	1.7	12.5	14 × 4	118	20	50	25
17[B]	AE/FLT3-LM	84	ND	430	24 × 9	572	80	22	60
18[B]	AE/FLT3-LM	84	4.4	3.3	21 × 7	270	85	43	62
19[B]	AE/FLT3-LM	94	7.7	7.2	12 × 4	ND	85	20	95
20[B]	AE/FLT3-LM	96	3.8	60	21 × 6	310	40	95	60
21[A]	AE/FLT3-LM	269	4	2	28 × 9	650	38	62	30
22[A]	AE/FLT3-LM	303	2.6	10	15 × 3	200	24	76	38
23[A]	AE/FLT3-LM	304	2.6	2.5	29 × 10	1,400	27	55	78
24[A]	AE/FLT3-LM	359	2.5	35	28 × 9	760	39	77	64

[A]AML; [B]ALL. PB, peripheral blood; ND, not determined; AE, AML1-ETO.

AML1-ETO fusion gene contributes to leukemogenesis, it is thought that 1 key mechanism is the suppression of AML1- and C/EBPα-dependent activation of genes responsible for myeloid development (15, 16). Perturbation of hematopoiesis by expression of *AML1-ETO* results in an increase in the replating capacity of murine clonogenic progenitors and in the growth of primitive human progenitor cells in vitro (6, 17). Furthermore, in vivo and ex vivo analyses demonstrated alterations in the differentiation pattern and proliferative capacity of murine hematopoietic cells expressing the fusion gene (8, 9, 18, 19). However, numerous murine in vivo models documented that *AML1-ETO* on its own is not able to induce leukemia (6, 7, 9, 18, 19). The observation that *AML1-ETO* as a single factor is nonleukemogenic is further supported by findings that nonleukemic *AML1-ETO*–expressing progenitor cells can be isolated from healthy individuals as well as AML patients in remission, which suggests that additional mutations in these AML1-ETO–positive progenitors are necessary for the transformation into leukemia-initiating cells (20–22). The importance of collaborating genetic events in the pathogenesis of AML1-ETO–positive leukemias has indeed been shown in different murine models, such as a conditional *AML1-ETO* murine model as well as an hMRP8–*AML1-ETO* transgenic mouse model. Only mice treated additionally with ENU developed AML or T cell lymphoma (6, 7). Furthermore, retrovirally expressed *AML1-ETO* induced myeloblastic transformation in vivo only in a background deficient in the IFN-regulatory factor IFN consensus sequence-binding protein (18). These data strongly suggest that genetic alterations cooperating with *AML1-ETO* play a role in inducing leukemia.

In order to characterize genetic alterations that potentially collaborate with *AML1-ETO*, we screened 135 patients with AML for activating mutations of signal transduction pathways or mutations affecting the *MLL* gene. Whereas *MLL-PTD* mutations, which exemplify genetic alterations involved in transcriptional regulation, were not found at all, 28% of the patients were positive for activating mutations such as *FLT3-LM*, *FLT3D835*, *KITD816*, or *NRAS*. The frequent coexistence of such mutations with *AML1-ETO* fits well in the model of leukemogenesis in which the collaboration of 2 classes of genetic alterations, 1 affecting transcription factors associated with hematopoietic differentiation, the other affecting signal transduction pathways associated with cell proliferation, is necessary for the malignant transformation of hematopoietic progenitor cells (3). Using the murine BM transplantation model, we obtained direct evidence for a functional collaboration of *AML1-ETO* with *FLT3-LM* in inducing leukemia, supporting the aforementioned model of leukemogenesis. Furthermore, these data demonstrate the collaboration of the 2 most frequent genetic alterations in AML, providing an important model for the understanding of both the AML1-ETO–positive and the FLT3-LM–positive leukemias.

Of note, 4 of 7 AML mice reported here expressed the T cell antigen CD4. Although the mechanisms underlying the coexpression of myeloid and lymphoid antigens in AML1-ETO–positive myeloid leukemia are not clear, one possibility is that in this AML subtype an early progenitor cell with a lineage-overlapping mixed phenotype is the target of leukemogenic transformation, as recently proposed for hematological malignancies (23). Of note, it was recently demonstrated that, in AML1-ETO–positive leukemia, evidence of lineage overlap is not restricted to the expression of cytoplasmic or surface antigens but extends to the transcriptional apparatus, since *PAX5* is selectively expressed in one-third of patients with t(8;21) AML in
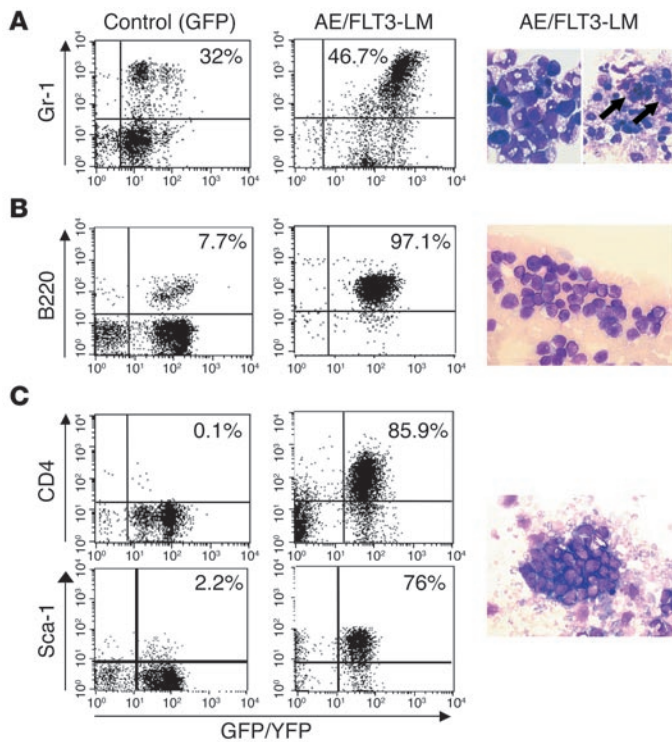
Figure 4
Immunophenotype and morphology of hematopoietic cells recovered from leukemic mice. The plots show representative FACS profiles from BM cells in comparison with cells from GFP control animals, with indication of the proportion of positive cells within the GFP+/YFP+ compartment. The photographs show cytospin preparations (H&E; magnification, ×630) from peripheral blood (**A**, right image; **B** and **C**) and from BM (**A**, left image). (**A**) AML with a dominant mast cell population (marked by arrows) (mouse no. 16). (**B**) B-ALL (mouse no. 17). (**C**) T-ALL (mouse no. 20).

tive B cell or a mixed lymphoid-myeloid phenotype (28–30). The observations that ETV6-PDGFBR, which already by itself causes a lethal myeloproliferative syndrome in transplanted mice, induces exclusively a myeloblastic leukemia when coexpressed with *AML1-ETO* might point to the importance of the collaborating partner for the phenotype of the induced leukemia (31). Another possible explanation for the development of lymphoid malignancies in our model is that, in individual mice, lymphoid-committed stages of differentiation were hit by the retrovirus, resulting in lymphoblastic leukemia in these animals. This would potentially be a key difference from the human situation, in which both *AML1-ETO* and *FLT3-LM* are already present in the HSC pool (21, 32). An important question is whether the results were influenced by retroviral insertional mutagenesis. Most of the leukemic animals were transplanted with a low transplant dose and then suffered from monoclonal or oligoclonal disease. Although the number of retroviral integration events was low in the mice, insertional mutagenesis might have contributed to the leukemogenesis. However, analyses of the retroviral integration sites in the diseased animals showed integration into intergenic regions or introns of genes, more likely resulting in their knock down than in their activation. These data suggest that retroviral insertional mutagenesis might not play the key part in disease development, an issue that might be more accurately addressed in mouse models expressing *AML1-ETO* from an endogenous promoter. The long latency of the leukemias, even of secondary disease, however, strongly argues that additional secondary in vivo genetic events in the animals contributed to disease development.

To our knowledge, this is the first functional evidence of a leukemogenic collaboration of *AML1-ETO* with a complementary class of mutation, recurrently found in patients with t(8;21). It facilitates our understanding of acute leukemias associated with 2 of the most frequent genetic alterations in this disease. Furthermore, our experimental data support recent reports that show the functional relevance of activating mutations in patients with CBF leukemias [*AML1-ETO* or *CBFB-MYH11*] by demonstrating a significantly shortened overall and event-free survival for AML1-ETO–positive leukemias harboring activating mutations of *FLT3* or *KIT* compared with those without these mutations. In contrast, *RAS* mutations did not affect the treatment outcome (S. Schnittger, unpublished observations) (33, 34). In line with these findings, it was recently shown that in patients with AML1-ETO–positive leukemia, most leukemic cells at diagnosis additionally harbored mutations in *KIT*, whereas in 3 patients analyzed in complete remission, only the fusion gene, but not the *KIT* mutation, could be detected by PCR; this strongly supports the concept of a stepwise development of disease involving 2 collaborating genetic aberrations (35). These observations encourage the systematic screening of activating mutations in patients with CBF leukemias in prospective clinical trials to evaluate more precisely their prognostic impact, and they form a rationale to consider treatment strategies targeting the signal transduction apparatus in this AML subtype.

Methods

Patient samples. BM samples from 135 adult patients with newly diagnosed AML — de novo AML (*n* = 118), secondary AML after treatment of a previous malignancy (*n* = 13), and AML at relapse (*n* = 4) — were analyzed. The diagnosis of AML was performed according to the French-American-British criteria and the WHO classification (36, 37). Cytomorphology, cytochemistry, cytogenetics, and molecular genetics were applied in all cases

contrast to all other cytogenetically defined AML subtypes (24). Furthermore, both myeloblastic and lymphoblastic leukemias of B and T cell type developed in this model of *AML1-ETO* and *FLT3-LM* cooperation. This observation was also reported in other murine models: in a conditional *AML1-ETO* murine model, mice treated additionally with ENU developed AML as well as T cell lymphoblastic lymphoma, although most of the T cell neoplasms did not express the fusion gene (6). Similar observations were reported from an hMRP8–*AML1-ETO* transgenic mouse model, which developed AML as well as T-ALL/lymphoma after ENU treatment (7). In addition, expression of *AML1-ETO* might contribute to the lymphoid phenotype of the leukemias in our model, as it was reported that *FLT3-LM* is able to induce a long-latency T cell lymphoma–like disease in the C57BL/C3H background (25). However, the association of *FLT3-LM* with a lymphoid disease seems to depend on the genetic background of the mouse strain, as *FLT3-LM* induced a myeloproliferative syndrome in BALB/c mice (26). In our model, also using the C57BL/C3H background, constitutive expression of *FLT3-LM* alone did not induce any perturbation of the hematopoietic development in vivo; this result was also recently reported in a mouse model of collaboration of *FLT3-LM* with *MLL-SEPT6* using the C57BL/6 strain (27).
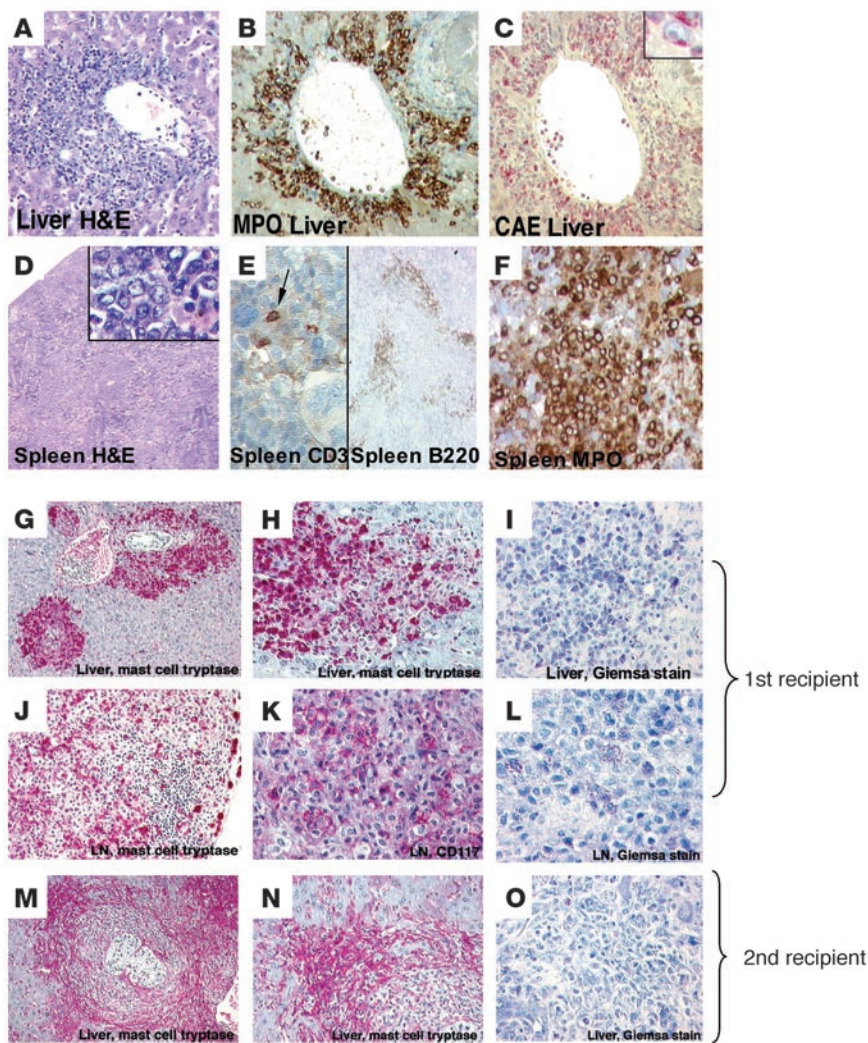
Of note, overexpression of FLT3 and activating FLT3 mutations are associated with ALL in humans, in particular in cases of ALL with hyperdiploidy or *MLL* rearrangement, characterized by a primi-

Figure 5
Histological analyses of leukemic mice. (**A**–**F**) Histological analyses of AML (mouse no. 14). Original magnifications: **A**–**C**, ×200; inset in **C**, ×1,000; **D** and right side of **E**, ×250; inset in **D**, ×650; left side of **E**, ×400; **F**, ×400. (**G**–**O**) Histological analyses of AML with a dominant mast cell population (mouse no. 16). (**G**–**L**) Primary recipient. (**M**–**O**) Secondary recipient. Original magnifications: **G** and **M**, ×100; **H**, **J**, and **N**, ×200; **I**, **K**, and **O**, ×400; **L**, ×600. Mast cells with metachromatic granulation in the Giemsa stain are indicated by an arrow. MPO, myeloperoxidase; CAE, *N*-acetyl-chloroacetate esterase.

5′-TGAACTGGTTCTTGGAGCCTCCT-3′ (corresponding to positions nucleotide 395 and nucleotide 633 of GenBank accession number D13979, respectively) were used; for *FLT3-LM* the primer forward 5′-GCAATTTAGGTATGAAAGCCAGC-3′ and the primer reverse 5′-CTTTCAGCATTTT-GACGGCAACC-3′ (corresponding to positions nucleotide 1,704 and nucleotide 1,920 of GenBank accession number NM_004119, respectively) were used. The annealing temperature was 57°C. The number of PCR cycles for each gene was chosen to stop the reaction in the linear phase of amplification (35 cycles for AML-ETO and FLT3-LM). The integrity of the RNA in all samples was confirmed by mβ-2 microglobulin RT-PCR.

For the linker-mediated PCR (LM-PCR), integrated long-terminal repeats (LTRs) and flanking genomic sequences were amplified and then isolated using a modification of the bubble LM-PCR strategy (42, 43). Aliquots of the cell lysates from leukemic mice were digested with *Pst*I or *Ase* (New England Biolabs Inc.), and the fragments were ligated overnight at room temperature to a double-stranded bubble linker (5′-CTCTCCCTTCTCGAATCGTAACCGTTCG-TACGAGAATCGCTGTCCTCTCCTTG-3′ and 5′-ANTCAAGGAGAGGACGCTGTCTGTCGAAGG-TAAGGAACGGACGAGAGAAGGGAGAG-3′). Next, a first PCR (PCR-A) was performed on 10 μl (one-tenth) of the ligation product using a linker-specific Vectorette primer (5′-CGAATCGTAACC-GTTCGTACGAGAATCGCT-3′) (Invitrogen Corp.) and an LTR-specific primer (LTR-A: 5′-CAACACA-CACATTGAAGCACTCAAGGCAAG-3′) under the following conditions: 1 cycle of 94°C for 2 minutes, 20 cycles of 94°C for 30 seconds and 65°C for 1 minute, and 1 cycle of 72°C for 2 minutes. The bubble linker contains a 30-nucleotide nonhomologous sequence in the middle region that prevents binding of the linker primer in the absence of the minus strand generated by the LTR-specific primer. A 1-μl aliquot of the PCR-A reaction (one-fifteenth) was then used as a template for a second nested PCR (PCR-B) using an internal LTR-specific primer (LTR-B: 5′-GAGAGCTCCCAGGCTCA-GATCTGGTCTAAC-3′) and the same linker-specific Vectorette primer as was used in PCR-A, with the following conditions: 1 cycle of 94°C for 2 minutes, 30 cycles of 94°C for 60 seconds and 72°C for 1 minute, and 1 cycle of 72°C for 2 minutes. Ten microliters (one-half) of the final PCR-B product was electrophoresed using 2% agarose tris-acetate-EDTA gel. Individual bands were excised and purified using the QIAEX II Gel Extraction Kit (QIAGEN) and then cloned into PCR2.1 (Invitrogen Corp.) before sequencing of the integration site of the retrovirus.

Multiparameter flow cytometry. Immunophenotypic analyses were performed as previously described (44). The following combinations of antibodies were used: CD34/CD2/CD33, CD7/CD33/CD34, CD34/CD56/CD33, CD11b/CD117/CD34, CD64/CD4/CD45, CD34/CD13/CD19, CD65/CD87/CD34, CD15/CD34/CD33, HLA-DR/CD33/CD34, CD4/CD13/CD14, CD34/CD135/CD117, CD34/CD116/CD33, CD90/CD117/CD34, CD34/NG2(7.1)/CD33, CD38/CD133/CD34, CD61/CD14/CD45,

as described below. Both animal and human studies were approved by the Ethics Committee of Ludwig Maximilians University and abided by the tenets of the revised World Medical Association Declaration of Helsinki (http://www.wma.net/e/policy/b3.htm).

Cytogenetic and FISH analysis. Cytogenetic analyses were performed using standard techniques. For FISH, a commercially available AML1-ETO probe was used according to the manufacturer's instructions (Vysis Inc.) (38).

PCR. Molecular genetic analysis for *AML1-ETO* (38), *MLL-PTD* (39), *FLT3-LM* (4), *NRAS* mutations, *FLT3D835*, and *KITD816* (40) in patient samples was performed as has been described previously (41). In leukemic mice, expression of AML1-ETO and FLT3-LM was assessed by RT-PCR in animals transplanted with BM cells coexpressing AML1-ETO/GFP and FLT3-LM/YFP. Preparation of cDNA was performed as previously described (41). For AML1-ETO the primer forward 5′-ATGACCTCAGGTTTGTCGGTCG-3′ and the primer reverse
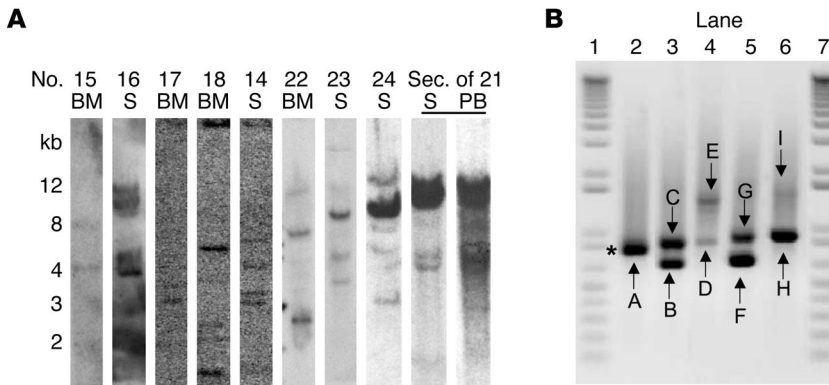
A

B

Figure 6
Analysis of proviral integrations. (**A**) Southern blot analysis of genomic DNA from different primary mice and a secondary recipient to detect clonal proviral integrations. DNA was digested with *Eco*RI, which cuts once in the proviral sequence, and blots were hybridized to a GFP/YFP probe. The mouse numbers are indicated (corresponding to those in Table 2). S, spleen; PB, peripheral blood; sec. of 21, second mouse of 1° mouse no. 21. (**B**) Bubble PCR analyses of retroviral integration sites in diseased mice. The bands (A–I) were isolated, subcloned, and sequenced. A description of the PCR products is given in Table 3. Asterisk indicates resolution as 2 unique bands after subcloning and sequencing of integration sites.

CD36/CD235a/CD45, CD15/CD13/CD33, CD9/CD34/CD33, CD38/CD34/CD90, CD34/CD79a/CD19, TdT/cyCD33/cyCD45, myeloperoxidase/lactoferrin/cyCD15, TdT/cyCD79a/cyCD3, and TdT/cyCD22/cyCD3. All antibodies were purchased from Beckman Coulter Inc., except for CD64 and CD15 (Medarex Inc.), CD133 (Miltenyi Biotec), and myeloperoxidase and lactoferrin (CALTAG Laboratories). For the analysis of cytoplasmic antigens, cells were fixed and permeabilized before staining with FIX & PERM (CALTAG Laboratories). Multiparameter flow cytometry analysis was performed with a FACSCalibur flow cytometer (BD).

The purity of all samples was 80–100%. Furthermore, immunophenotyping was performed with triple staining in all cases as indicated above, testing simultaneous expression of myeloid and lymphoid antigens to exclude contaminating normal lymphoid cells.

In mice, immunophenotypic analysis of single-cell suspensions from BM, spleen, and peripheral blood was performed by flow cytometry (FACS-Calibur cytometer; BD) using PE-labeled Sca-1, Gr-1, Ter-119, and CD4 antibodies and allophycocyanin-labeled Mac-1, Kit, B220, and CD8 antibodies (all from BD Biosciences — Pharmingen), as previously described (45). The surface expression of the FLT3-LM construct and the FLT3-LM-KD mutant of Ba/F3 cells was confirmed by FACS analysis (Figure 2B) using anti–human CD135–PE mAb (BD) and an isotype-matched IgG1-PE control (Beckman Coulter Inc.).

cDNA constructs and retroviral vectors. For retroviral gene transfer into primary BM cells, AML1-ETO cDNA was subcloned into the multiple cloning site of the modified murine stem cell virus (MSCV) 2.1 vector (41) upstream of the enhanced GFP (EGFP) gene and the internal ribosomal entry site (IRES). The FLT3-LM cDNA was subcloned into the identical MSCV vector construct carrying the enhanced YFP (Figure 1A). The MSCV vector carrying only the IRES-EGFP cassette was used as a control. The cDNA of FLT3-LM was kindly provided by D.G. Gilliland (Harvard Medical School, Boston, Massachusetts, USA) and contained a 28–amino acid duplicated sequence (CSSDNEYFYVDFREYEY-DLKWEFPRENL) inserted between amino acids 610 and 611. The AML1-ETO cDNA was provided by S.W. Hiebert (Vanderbilt University School of Medicine, Nashville, Tennessee, USA).

The FLT3-LM-KD mutation K672R (a point mutation of Lys644 to Arg that disrupts an ion pair with Glu661 that is critical for nucleotide binding in FLT3-WT; ref. 46), and the L148D AML1-ETO point mutation (31, 47) to prevent AML1-ETO DNA binding, were generated from the full-length human FLT3-LM cDNA and the AML1-ETO cDNA, respectively, using the QuikChange Site-Directed Mutagenesis Kit (Stratagene) according to the manufacturer's instructions. The correct sequences of the constructs were confirmed by complete nucleotide sequencing, and expression was proved by Western blot and FACS analysis (Figure 1).

Cell culture. Gag-pol and envelope (GP+ E86) packaging cells, NIH 3T3 cells, and 293T cells were grown in DMEM with 10% FBS and 1% penicillin/streptomycin in a humidified incubator at 37°C and 5% CO_2. Primary murine BM cells were plated in transplant medium consisting of DMEM supplemented with 15% FBS, 1% penicillin/streptomycin, 6 ng/ml IL-3, 10 ng/ml IL-6, and 100 ng/ml SCF (tebu-bio GmbH). IL-3–dependent Ba/F3 cells stably expressing the empty vector alone, FLT3-WT, FLT3-LM, and FLT3-LM-KD were seeded at a concentration of 0.05×10^6 per milliliter in the presence or absence of IL-3 and FLT3 ligand, as described previously (48). At 72 hours, viable cells were counted in a standard hemacytometer after staining with trypan blue.

Retrovirus production. High-titer helper-free retrovirus was produced with the constructs above by individual cotransfection of each construct with Ecopac (Cell Genesys Inc.) into 293T cells using calcium chloride precipitation. The retrovirus was subsequently collected in the conditioned medium.

Table 3
Identity of retroviral integration sites in diseased mice

Lane[A]	PCR product[A]	Gene	Protein family	Chromosome	Mouse no.
2	A 1	Intergenic		17qE3	15
	A 2	Intron 1 of Rnf8 alias AIP37 and 5 kbp 3′ of Pim1	Ring finger protein 8	17qA3	
3	B	Intergenic		15qE2	16
	C	Intron 1 of Ptp4a3	Protein tyrosine phosphatase 4a3	15qD3	
4	D	Hypothetical gene		10qC2	12
	E	Intron 3 of GATA	Transcription factor	6qD1	
5	F	Intergenic		18qD3	
	G	Intron 4 of SF3b	Splicing factor subunit b3	8qE1	
6	H	Intergenic in intron of hypothetical gene		3qF1	18
	I	Intergenic in intron of hypothetical gene		16qA1	

[A]Lanes and PCR products according to Figure 6B.
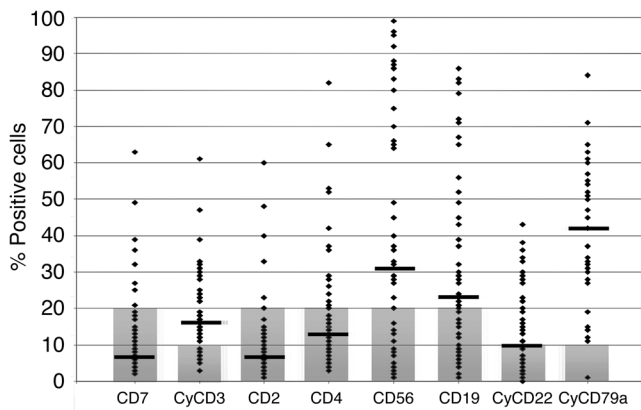
Figure 7
Expression of lymphoid antigens on 52 samples of patients with AML1-ETO–positive AML, determined by immunophenotyping. Samples were defined as negative for expression of cytoplasmic antigens when less than 10% of the cells stained with the antibody, and as negative for expression of surface antigens when less than 20% of the cells stained with the antibody (shaded areas).

To optimize transduction efficiency, the virus-containing medium (VCM) of different constructs was used to transfect GP$^+$ E86 cells to establish stable packaging cell lines, or to directly infect 5-FU–mobilized BM cells in the case of FLT3-LM. Ba/F3 cells were transfected with the different constructs as previously described (48, 49).

Mice and retroviral infection of primary BM cells. Parental-strain mice were bred and maintained at the GSF animal facility. Donors of primary BM cells [(C57BL/6Ly-Pep3b × C3H/HeJ) F$_1$ (PepC3) mice] and recipient mice [(C57BL/6J × C3H/HeJ) F$_1$ (B6C3)] were more than 8 weeks old. Primary mouse BM cells were transduced as previously described (41). For transduction of AML1-ETO, cells were cocultured in transplant medium with AML1-ETO/GFP producer cells irradiated with 40 Gy of ^{137}Cs γ-radiation. For infection with the FLT3-LM virus, BM cells were cultured in FLT3-LM/VCM, supplemented with cytokines (IL-3, IL-6, and SCF), to achieve optimal transduction efficacy. For coinfection with the FLT3-LM and AML1-ETO retroviruses, BM cells were cultured on a mixture of 30–50% AML1-ETO/GFP and 50–70% FLT3-LM/YFP producer cells in transplant medium or FLT3-LM/VCM supplemented with IL-3, IL-6, and SCF. Retroviral transfection of primary BM cells with the AML1-ETO mutant L148D and the FLT3-LM-KD mutant was performed as described for FLT3-LM, by cultivation of the BM in VCM supplemented with IL-3, IL-6, and SCF. All transductions were performed with the addition of 5 μg/ml protamine sulfate. Infected cells were highly purified (FACSVantage; BD) based on expression of GFP (for AML1-ETO alone), expression of YFP (for FLT3-LM alone), or coexpression of GFP and YFP (for AML1-ETO/FLT3-LM cotransduction) before transplantation.

BM transplantation and assessment of mice. FACS-purified transduced BM cells or ratios of transduced and nontransduced cells (if less than 3 × 10^5 transduced cells per recipient were available) were injected into the tail vein of 8- to 10-week-old irradiated recipient F$_1$ (B6C3) mice (800 cGy from a ^{137}Cs γ-radiation source). Peripheral blood or BM cell progeny of transduced cells were tracked using the GFP and/or YFP fluorescence in vivo (41). For transplantation of secondary mice, 1 × 10^6 to 2 × 10^6 cells of diseased primary animals were injected into the recipients after 800 cGy irradiation.

CFU-S and ΔCFU-S assay. 5-FU–mobilized primary BM cells from F$_1$ (PepC3) donor mice were retrovirally transduced with AML1-ETO; AML1-ETO-L148D; FLT3-LM; FLT3-LM-KD; both AML1-ETO and FLT3-LM;

both AML1-ETO and FLT3-LM-KD; or both AML1-ETO-L148D and FLT3-LM. Cells transfected with the empty GFP vector served as control. Successfully transduced cells were isolated 48 hours after termination of infection by FACS (FACSVantage; BD). To assess initial (day 0) CFU-S numbers, purified cell populations were injected into lethally irradiated F$_1$ (B6C3) recipient mice 96 hours after the start of infection (45). To study the effect of the selective protein tyrosine kinase inhibitor PKC412 on double-positive cells, freshly sorted cells were cultured in transplant medium with 0 and 100 nM PKC412. After 48 hours, the cells were injected into lethally irradiated mice as described above, and the day 0 equivalent of the CFU-S frequency was calculated for both experimental groups. In this ΔCFU-S assay the base-line frequency of CFU-S is lower than in the CFU-S assay. The recovery of CFU-S cells was quantified by determination of the number of macroscopic colonies on the spleen at day 12 postinjection after fixation in Telleyesniczky's solution.

Southern blot. Genomic DNA was isolated from BM, spleen, and peripheral blood of diseased mice with DNAzol as recommended by the manufacturer (Invitrogen Corp.). Southern blot analysis was performed as previously described (45). DNA was digested with *Eco*RI and probed with a ^{32}P-labeled GFP/YFP DNA. Hybridizing bands were visualized by autoradiography.

Western blot. Protein expression of AML1-ETO, AML1-ETO-L148D, FLT3-LM, and FLT3-LM-KD was demonstrated by Western blotting using standard procedures (41). Membranes were probed with an anti-ETO polyclonal goat antibody and an anti-FLT3 polyclonal rabbit antibody (Santa Cruz Biotechnology Inc.). Protein expression of FLT3-LM showed 2 bands, as previously reported: in detail, the FLT3 receptor occurs in 2 different forms due to glycosylation that can be resolved in SDS-PAGE gradient gels — a 158- to 160-kDa membrane-bound protein that is glycosylated at N-linked glycosylation sites in the extracellular domain and an unglycosylated 130- to 143-kDa protein that is not membrane bound (50–52). Phosphorylation of FLT3 was tested by Western blot using 293T cells that were starved for 12 hours at 37°C, 5% CO$_2$. After cell harvesting and lysis, 300 μg of the lysates was immunoprecipitated with polyclonal rabbit anti-FLT3 antibody (s-18; Santa Cruz Biotechnology Inc.). Immunoprecipitates were analyzed by SDS-PAGE with mouse monoclonal anti-phosphotyrosine antibody (PY-99; Santa Cruz Biotechnology Inc.) and reprobed with anti-FLT3 antibody.

Histology. For histological analyses, sections of selected organs were prepared and stained at the Academic Pathology Laboratory, GSF (Munich, Germany), using standard protocols, as previously described (41). The mast cell–specific tryptase and the CD117 antibody were purchased from Dako-Cytomation. All the tumors were histopathologically classified according to the Bethesda proposals for classification on nonlymphoid and lymphoid hematopoietic neoplasms in mice (53, 54).

Statistical analysis. Data were evaluated using the 2-tailed Student's *t* test for dependent or independent samples (Microsoft Excel 2002, Microsoft Corp.). Differences with *P* values less than 0.05 were considered statistically significant.

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Christian Buske and Michaela Feuring-Buske contributed equally to this work.

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A Lymphoid Progenitor Propagates AML in a Mouse Model of CALM/AF10 Positive Leukemia.

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Abstract

The identification of cancer stem cells is a major step towards the understanding of the pathogenesis of solid and hematological neoplasias and might have direct implications for the development of innovative therapeutic strategies aiming at the eradication of the tumor propagating cell. Here we describe that acute myeloid leukemia (AML), induced by the CALM/AF10 fusion gene, is propagated by a transformed lymphoid progenitor in a murine bone marrow (BM) transplantation model of t(10;11)(p13;q14) positive AML. When mice were transplanted with BM cells retrovirally engineered to express the C/A fusion, all animals (n=13) died from AML showing DJ rearrangement of the heavy chain of the IgH locus after a median of 110 days post transplantation. Diseased mice showed an accumulation of myeloid $Gr1^+/Mac1^+$ cells in the peripheral blood and spleen and a multi-organ infiltration by myeloperoxidase and chloracetate esterase positive cells in immunohistochemical sections. In the leukemic mice only a minor population counting for 6.7 % (± 2.1) cells in the BM displayed the B220 lymphoid antigen and lacked myeloid markers (on average 9.4 % ± 3). The majority of cells expressed myeloid markers (on average 82.9 % (± 8.6) $Mac1^+$ cells, 86.4 % (± 3.7) $Gr-1^+$ cells). Additionally, in the leukemic mice an average of 26.0 % (± 8.6) and 32.5 % (± 13.2) of these cells co-expressed B220 and Mac1 or B220 and Gr1, respectively, compared to 2.1 % (± 0.7) and 1.3 % (± 0.3), respectively, in GFP controls. Importantly, *in vitro* only the $B220^+/Mac^-$ cell population had growth potential at the single cell level (seeding

efficiency 29 %) compared to the B220$^+$/Mac$^+$ (1%) and B220$^-$/Mac$^+$ cells (1%). When the frequency of leukemia propagating cells (LPC) of the three different populations isolated from primary leukemic mice was determined by limiting dilution transplantation and Poisson statistics the frequency of the LPC was more than 380 fold higher in the 'B220$^+$/Mac1$^-$' population (1 in 36 cells) than in the 'Mac1$^+$/B220$^-$' bulk population (1 in 13906 cells) and more than 12fold increased compared to the B220$^+$/Mac$^+$ cells (1 in 437 cells). *In vitro* a single B220$^+$/Mac1$^-$ cell isolated from a leukemic mouse was able to give rise to the B220$^+$/Mac1$^+$ as well as the Mac1$^+$/ B220$^-$ population, both populations showing the identical genomic DJ rearrangement at the IgH locus as the initial B220$^+$/Mac1$^-$ cell, demonstrating its capacity to differentiate into the myeloid lineage at the single cell level. The B220$^+$/Mac1$^-$ population displayed a CD43$^+$/AA4.1$^+$/HSA$^+$/CD19$^-$/IL-7R$^-$ phenotype, was promiscuous in its transcription profile with positivity for *EBF*, but also *MPO* and lacked *Pax5*. Taken together, this murine leukemia model indicates that AML can be propagated from an early transformed lymphoid progenitor cell. The transformation of an early lymphoid cell, which is re-directed into the myeloid lineage by appropriate oncogenes, could explain recurrent observations of immunoglobulin rearrangements in patients with AML and provide a rationale for therapies, aiming at the elimination of the leukemia propagating cell with lymphoid characteristics, but sparing normal HSCs.