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Characterization of the putative CALM/AF10 collaborator Meis1 in leukemia development

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Dedicated

To

My Family

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1 Introduction

1.1 Cancer

Cancer is a disorder characterized by uncontrolled growth and proliferation of cells. The cancerous cells, also known as malignant cells, have the ability to invade by direct growth into adjacent tissues by invasion or by implantation into distant sites through metastasis. Cancer is the leading cause of death worldwide. In 2007, cancer caused about 13% of all human deaths worldwide which accounted for almost 7.9 million deaths (Jemal *et al*., 2011). The most common cancer deaths each year are due to lung, stomach, liver, colorectal and breast cancer. However, the death rates continue to decline for lung, colorectal, breast and prostate cancer according to cancer statistics of 2012 (Siegel *et al*., 2012). Generally, people of all ages have the risk to develop cancer but the risk tends to increase with age probably due to less effective cellular repair mechanisms.

In the course of normal development and throughout adult life, there is a balance between cell growth and cell death. When this balance is perturbed due to several genetic and environmental factors, cancer develops. The transformation from a normal cell into a malignant, cancerous cell is a multistage process. Broadly, the development of cancer occurs in three stages:

Initiation – mutation of a single cell

Promotion – proliferation of the mutated cell

Progression – additional mutations in the tumor resulting in malignancy

Normal animal cells are subdivided according to their embryonic tissue of origin. Normal cells arise from one of the three embryonic cell layers: endoderm, ectoderm or mesoderm. Cancers are classified based on their cell of origin as carcinomas if they derive from endoderm or ectoderm, and as sarcomas if they derive from mesoderm. The carcinomas include the most common cancers developing in the lung, breast, prostate, pancreas and colon. The sarcomas include the cancers which initiate from the connective tissue like bone, cartilage, nerve and fat. Leukemia is a subdivision of sarcomas arising from hematopoietic cells.

The severity of symptoms depends on several factors like the affected site, character of the malignancy and occurrence of metastasis. The loss of cellular regulation in cancer is caused by mutations in tumor suppressor genes and proto-oncogenes,. The mutations in tumor suppressor genes result in inappropriate growth by inactivating the tumor suppressor function and the mutations in proto-oncogenes result in hyperactive gene products called oncogene. These mutations are caused either by carcinogens or by certain viruses that can insert their genome into the human genome.

1.2 Overview of Leukemia

Leukemia is the malignant neoplasm of blood forming tissues which is characterized by abnormal proliferation of immature white blood cells called blasts that accumulate in the bone marrow and enter the blood stream, thus interfering with the normal hematopoiesis. Leukemia is associated with relatively high incidence rate and poor survival (Kampen *et al*., 2011). Leukemia is the most common form of cancer in children aged 0-14 and accounts for about 33% of the cancer cases in children. In adults, leukemia is considered as one of the top 15 most common forms of cancer according to World Health Organization (Kampen *et al*., 2011). According to 2012 statistics, an estimated of 47,150 people will be diagnosed and around 23,540 people will die of leukemia in USA (Howlader *et al*., 2012). The incidence rates of leukemia development are higher among males than in females.

Leukemia can be broadly classified as acute or chronic based on the clinical and pathological course of the disease; and myeloid or lymphoid depending on the lineage of the malignant white blood cells involved. Acute leukemia is characterized by increased proliferation of immature cells or blasts in the bone marrow and peripheral blood and a differentiation block. If the patients suffering from acute leukemia are left untreated, death usually occurs within 6 months. Chronic leukemia results in increased numbers of mature cells. Chronic leukemia is characterized by slow progression depending on the subtype of the proliferating cell, taking months or years until the patient dies.

Based on the above classification the following four types of leukemia can be distinguished:

Acute myeloid leukemia

Acute lymphoblastic leukemia

Chronic myeloid leukemia

Chronic lymphocytic leukemia

1.2.1 Acute myeloid leukemia (AML)

AML is a disease that progresses rapidly and is characterized by the accumulation of blasts or immature cells of granulocyte or monocyte precursors in the bone marrow and blood (Tenen, 2003). According to 2012 statistical estimate, a total of 13,780 people will be diagnosed with and 10,200 people will die of AML in USA (Howlader *et al*., 2012). The incidence rate of AML is 3.6 per 100,000 men and women per year. It is more common in adults with the median age of 66 years (Howlader *et al*., 2012). The most common classification schemes for AML are the French-American-British (FAB) system and the newer World Health Organization (WHO) system. In addition to these two schemes, a different classification scheme for AML is the Medical Research Council (MRC) classification. In this classification, AML patients are divided into favorable, intermediate and unfavorable subtypes based on their survival. (Tenen, 2003).

1.2.1.1 FAB classification of AML

This is the traditional classification system in which the cell morphology has been used to describe the different subtypes of AML on the basis of differentiation status (Table 1.2.1.1). This was first proposed in 1976. Using this classification system, AML is divided into eight subtypes, M0 through M7, based on the cell type from which leukemia has developed and the degree of maturation (Bennett *et al*., 1976).

FAB subtype	**Description**
M0	Minimally differentiated
M1	Myeloblastic leukemia without maturation
M2	Myeloblastic leukemia with maturation
M3	Promyelocytic leukemia

M4	Myelomonocytic leukemia
$M4_{Eo}$	Myelomonocytic with bone-marrow eosinophilia
M5	Monocytic leukemia
M6	Erythroleukemia
M7	Megakaryoblastic leukeima

Table 1.2.1.1 FAB classification of AML

1.2.1.2 WHO classification of AML

It is sometimes difficult to identify the heterogeneity of AML based on morphology alone, but it can be better appreciated by taking the underlying genetic aberrations into account (Caceres-Cortes, 2012). Therefore, the aim of the WHO classification of AML is to incorporate and correlate morphology, cytogenetics, molecular genetics and immunologic markers that are universally applicable as well as prognostically relevant (Jaffe *et al*., 2001). This classification scheme uses different prognostic parameters to separate between more homogeneous classes and also identify groups of patients responding to specific drugs or treatment (Table 1.2.1.2a). Thus, the WHO classification is more advanced compared to the FAB classification. However, some changes were made to the 2001 edition of WHO classification and were introduced into the 2008 WHO classification of AML.

WHO classification of AML (2001)
AML with recurrent genetic abnormalities
AML with t(8;21)(q22;q22); RUNX1-RUNX1T1
Acute promyelocytic leukemia with t(15;17)(q22;q12); PML-RARA
AML with inv(16)(p13.1q22) or t(16;16)(p13.1;q22); CBFB-MYH11
AML with 11q23 / MLL abnormalities

AML with multilineage dysplasia
With prior MDS
Without prior MDS
AML with myelodysplastic syndrome, therapy-related
AML not otherwise categorized

Table 1.2.1.2a AML subtypes defined by WHO classification (2001)

As compared to the 2001 edition, changes were introduced into the 2008 WHO classification of AML (Table 1.2.1.2b). The category with recurrent genetic abnormalities was expanded, AML with multilineage dysplasia was renamed and the features with myeloid proliferations were described. These changes have benefited the diagnostic and prognostic approaches for AML patients (Falini *et al*., 2010; Vardiman *et al*., 2009).

The new classification of AML and precursor-related neoplasms (WHO classification, 2008) is as follows:

WHO classification of AML 2008
AML with recurrent genetic abnormalities
AML with t(8;21)(q22;q22); RUNX1-RUNX1T1
AML with inv(16)(p13.1q22) or t(16;16)(p13.1;q22); CBFB-MYH11
Acute promyelocytic leukemia with t(15;17)(q22;q12); PML-RARA
AML with t(9;11)(p22;q23); MLLT3-MLL
AML with t(6;9)(p23;q34); DEK-NUP214
AML with inv(3)(q21q26.2) or t(3;3)(q21;q26.2); RPN1-EVI1
AML (megakaryoblastic) with t(1;22)(p13;q13); RBM15-MKL1
AML with mutated NPM1*

AML with mutated CEBPA*
AML with myelodysplasia-related changes
Therapy-related myeloid neoplasia
Acute Myeloid Leukemia, Not Otherwise Specified (NOS)
AML with minimal differentiation
AML without maturation
AML with maturation
Acute myelomonocytic leukemia
Acute monoblastic/monocytic leukemia
Acute erythroid leukemia
- Pure erythroid leukemia
- Erythroleukemia, erythroid/myeloid
Acute megakaryoblastic leukemia
Acute basophilic leukemia
Acute panmyelosis with myelofibrosis
Myeloid sarcoma
Myeloid proliferations related to Down's syndrome
Transient abnormal myelopoiesis
Myeloid leukemia associated with Down's syndrome
Blastic plasmacytoid dendritic cell neoplasm

* Provisional entities

Table 1.2.1.2b AML and related precursor neoplasia defined by WHO classification (2008)

1.2.2 Acute lymphoblastic leukemia (ALL)

ALL is characterized by the accumulation of malignant, immature lymphoblasts in the bone marrow and peripheral blood. ALL is more common in children than in adults with a peak incidence at 2-5 years of age. The incidence rate of ALL is 3 per 100,000 children per year (Heim and Mitelman, 1995). It is estimated that 6,050 people will be diagnosed and 1,440 people will die of ALL in 2012 in USA (Howlader *et al*., 2012).

1.2.2.1 FAB classification of ALL

The FAB classification scheme is widely used for subtyping various forms of ALL (Bennett *et al*., 1976) (Table 1.2.2.1).

Cytological features	**L_1**	**L_2**	**L_3**
Cell size	Mainly small	Large, heterogeneous	Large, homogeneous
Nuclear chromatin	Fairly homogeneous	Heterogeneous	Finely stippled, homogeneous
Nuclear shape	Mainly regular	Irregular; clefting and indentation common	Regular; oval or round
Nucleolus	Not visible or small	Usually visible, often large	Usually prominent
Amount of cytoplasm	Scanty	Variable, often abundant	Moderately abundant
Basophilia of cytoplasm	Slight to moderate	Variable	Strong
Cytoplasmic vacuolation	Variable	Variable	Often prominent

Table 1.2.2.1 FAB classification of ALL

1.2.2.2 WHO classification of ALL

The WHO international panel advocates the use of immunophenotypic classification as follows:

1- Acute lymphoblastic leukemia/ lymphoma (Former FABL1/L2)

(i) Precursor B acute lymphoblastic leukemia. Cytogenetic subtypes as follows:

- t(12;21)(p12,q22) TEL/AML-1
- t(1;19)(q23;p13) PBX/E2A
- t(9;22)(q34;q11) ABL/BCR
- T(V,11)(V;q23) V/MLL

(ii) Precursor T acute lymphoblastic leukemia/ lymphoma

2- Burkitt's leukemia (Former FAB L3)

3- Biphenotypic acute leukemia (Brunning, 2003)

1.2.3 Chronic myeloid leukemia (CML)

CML (also known as chronic granulocytic leukemia) is characterized by increased and unregulated growth of granulocytes in the bone marrow and accumulation of these cells in the blood. CML occurs mostly in the middle-aged and elderly group of people. The annual incidence of CML is 1-2 per 100,000 people. In CML, blood cell differentiation occurs in an orderly manner without any differentiation block. Most CML cases have a t(9;22)(q34;q11), which is a balanced chromosomal translocation between chromosomes 9 and 22. The derivative chromosome 22 is known as Philadelphia chromosome. This translocation leads to the fusion of a portion of the *ABL* gene from chromosome 9 with the *BCR* gene on chromosome 22. The resulting *BCR/ABL* fusion gene is known to play the crucial role in the pathogenesis of CML (Daley *et al*., 1990; Pear *et al*., 1998). CML usually has a triphasic course, starting from an initial chronic phase but always progresses over time into an intermediate accelerated phase and a terminal blast crisis (Bhatia *et al*., 2003).

Chronic phase – This phase lasts for about three years, is usually asymptomatic and progresses to accelerated phase.

Accelerated phase – This is a malignant phase, disease progresses by acquiring additional chromosomal abnormalities in addition to the Philadelphia chromosome, and transforms to blast crisis.

Blast crisis – This represents the final phase and behaves like an acute leukemia with rapid progression and short survival (Tefferi, 2006). The morphologic features of the leukemic cells might be myeloblastic or lymphoblastic.

1.2.4 Chronic lymphocytic leukemia (CLL)

CLL is the most common type of leukemia and is characterized by increased proliferation and accumulation of small B cells in the bone marrow and peripheral blood. CLL is more common in adults. More than 75% people diagnosed with CLL are over the age of 50 and majority of them are men. CLL is presumed to be a neoplasm of a normal subset of physiologic B cells which are CD5 positive (Bagg, 2007). The clinical course of the disease is benign.

The four main genetic aberrations found in CLL are:

- Deletion of 17p (found in 5-10% of patients with CLL) which targets the *TP53* gene.
- Deletion of 11q (found in 5-10% of patients with CLL) which targets the *ATM* gene.
- Deletion of 13q (found in 50% of patients with CLL) is the most common abnormality.
- Trisomy 12 (found in 20-25% of patients with CLL) imparts an intermediate prognosis.

1.2.5 Complex acute leukemias

Morphologic studies, cytochemistry and immunophenotypic analysis allows to classify a vast majority (>95%) of acute leukemias into AML or ALL (Thalhammer-Scherrer *et al*., 2002).

However, at least 20% of AML and ALL cases have aberrant or cross lineage expression, i.e. AML coexpressing lymphoid antigens and ALL coexpressing myeloid antigens (Thalhammer-Scherrer *et al*., 2002). In addition to this, there are fewer than 5% cases of acute leukemias with extremely complex immunophenotypes, which includes acute leukemia of ambiguous lineage, acute mixed-lineage leukemia, hybrid acute leukemia, biphenotypic acute leukemia and acute bilineal leukemia (Bagg, 2007).

1.2.5.1 Acute leukemia of ambiguous lineage

This category of acute leukemia includes three major types: undifferentiated acute leukemia, bilineal acute leukemia and biphenotypic acute leukemia (Brunning *et al*., 2003).

Undifferentiated acute leukemia: In undifferentiated acute leukemia the leukemic blasts lack lineage specific antigenic and morphologic markers but may express non-specific antigens like HLA-DR, CD34, CD38, CD7 and TdT.

Bilineal or Biclonal acute leukemia: Bilineal acute leukemia is characterized by co-existence of two distinct immunophenotypic blast populations, for example, myeloid and lymphoid or B and T.

Acute biphenotypic leukemia: In this type of leukemia, the myeloid and lymphoid, or both B and T lineage markers are co-expressed on individual leukemic blasts (Altman, 1990; Legrand *et al*., 1998). But it has been observed that cases with both lymphoid lineages (B and T), or involving three lineages (triphenotypic) are very rare. B-lymphoid and myeloid surface marker coexpression is more common than T-lymphoid and myeloid in acute biphenotypic leukemia blasts (Matutes *et al*., 1997). Acute biphenotypic leukemia associate with cytogenetic abnormalities and have bad prognosis (Carbonell *et al*., 1996; Legrand *et al*., 1998). This type of leukemia is more common in infants and children than adults (Altman, 1990). Several findings have shown *IgH* and *TCRß* gene rearrangements in myeloid leukemias (Caudell *et al*., 2007; Deshpande *et al*., 2006; Yen *et al*., 1999).

Two hypotheses have been proposed for the biphenotypic nature in the leukemic blasts of acute biphenotypic leukemias: lineage infidelity and lineage promiscuity.

Lineage infedility: The transformed or leukemic cells from one lineage start expressing surface markers from another lineage aberrantly (genetic reprogramming) due to the transformation event (Altman, 1990; Bagg, 2007; McCulloh, 1987).

Lineage promiscuity: Neoplastic transformation of a bipotential or multipotential progenitor cell and the differentiation block at this stage results in the biphenotypic character of the leukemic blasts (Altman, 1990; Bagg, 2007; McCulloh, 1987).

1.3 Hematopoiesis

Hematopoiesis is a dynamic process. When a hematopoietic stem cell divides it can result in the production of another hematopoietic stem cell (HSC) or progenitor cells. The progenitors include cells with restricted differentiation potential which finally mature into a fully functional blood cell. The main cellular components of blood are red blood cells (RBCs), white blood cells (WBCs) and platelets. The RBCs transport respiratory gases, platelets help in blood coagulation and WBCs play an important role in inflammation, phagocytosis and immunity.

Humans produce approximately 10^{16} blood cells of different types in their lifetime (Dick, 2003a). The production of of so many blood cells without a high rate of malignancy is likely due to the hierarchical organization of hematopoietic system. In human beings, hematopoiesis starts in the yolk sac and then moves to the fetal liver and spleen during development. In adults bone marrow is the major hematopoietic organ. In mouse local hematopoiesis occurs in the yolk sac during development, while lifelong hematopoiesis occurs in the bone marrow (Morrison *et al*., 1995; Weissman, 2000). In an adult mouse, hematopoiesis produces 2.4×10^{8} RBCs and 4×10^{6} non-lymphoid peripheral blood cells each day (Cheshier *et al*., 1999).

1.3.1 Hematopoietic Stem Cells (HSCs)

HSCs are the best characterized stem cell population in comparison to stem cells in other organs like the skin or the gut (Weissman, 2000). The self renewal and multipotency property of a HSC was proven in experiments in the 1950s. These experiments demonstrated that transfer of bone marrow from a healthy donor to a myeloablative recipient can regenerate

myelo-erythroid colonies in the lethally irradiated recipient (Becker *et al*., 1963; Till and McCulloch, 1961; Wu *et al*., 1968).

1.3.1.1 HSC hierarchy

Constant production of HSCs, which are capable of indefinite self-renewal, are able to produce different types of mature blood cells (Passegue *et al*., 2003). These self-renewing HSCs are termed long term repopulating hematopoietic stem cells (LT-HSCs). The LT-HSCs generate the short term repopulating hematopoietic stem cells (ST-HSCs) which are short lived, have limited self-renewal property and increased proliferation capability. The murine ST-HSCs can reconstitute hematopoiesis in a mouse for approximately 8 weeks (Passegue *et al*., 2003). The ST-HSCs then give rise to multipotent progenitors (MPPs) that have the potential to generate committed progenitors.

The committed progenitors can be of different lineages, either common myeloid progenitors (CMPs) for myelo-erythroid lineage, or common lymphoid progenitors (CLPs) for lymphoid lineage (Fig. 1.3.1.1). Thus, in this stem cell hierarchy, there is a gradual decrease in multipotency and self-renewal capability and an increased cell cycle activity (Lemischka, 1997).

HSCs maintain a balance between self-renewal and differentiation (Bonnet, 2002). HSCs are quiescent and divide slowly under stable conditions. In this state the division is asymmetrical, in that one HSC divides to give rise to HSC and a ST-HSC or a progenitor cell.. After HSC transplantation, HSC division is mostly symmetrical to regenerate the stem cell population for a certain period of time and then revert back to asymmetrical division (Warner *et al*., 2004). Stem cells are considered to reside in microenvironmental niches, which are required for the maintenance of stemness (Weiss and Geduldig, 1991; Wolf, 1979).

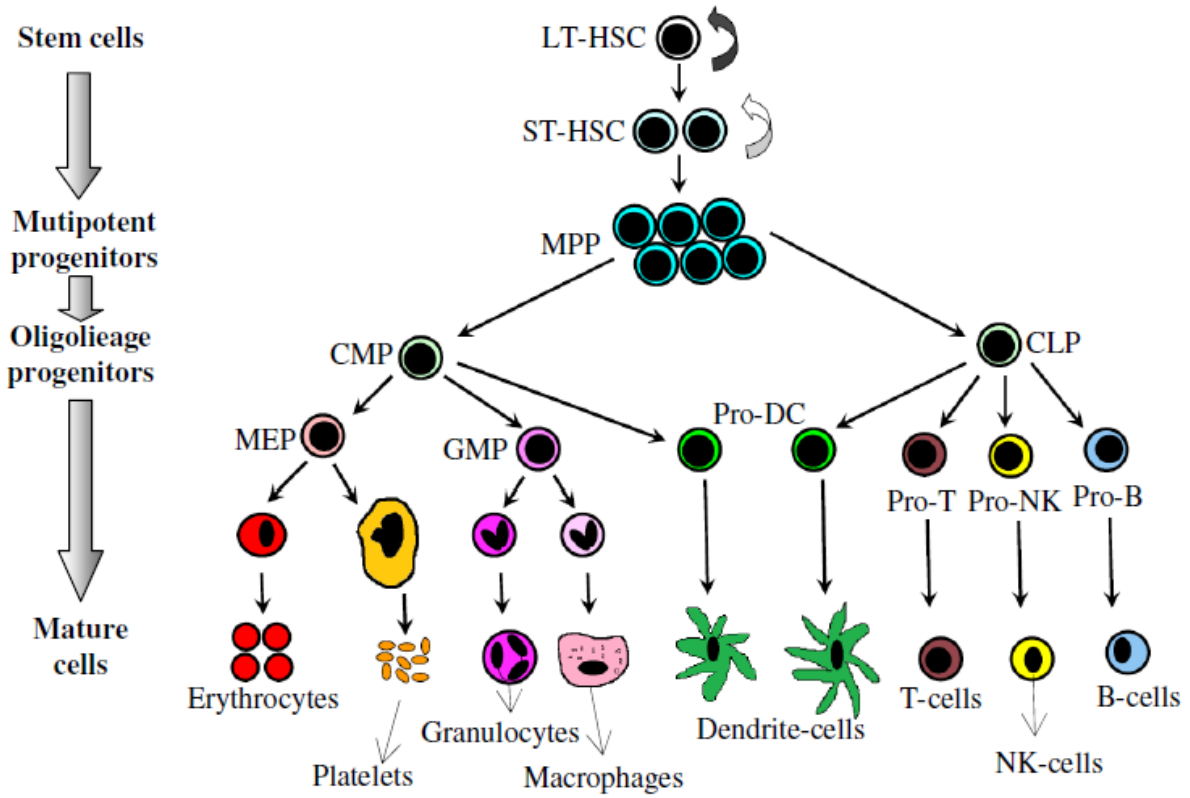


Fig. 1.3.1.1 Hematopoietic stem and progenitor cells: The hematopoietic stem and progenitor cell lineage comprises the long term hematopoietic stem cells (LT-HSCs), short term hematopoietic stem cells (ST-HSCs), multipotent progenitor (MPP) and further downstream oligolineage progenitors; common myeloid progenitor (CMP) and common lymphoid progenitor (CLP). These give rise to more mature progenitors, which finally differentiate into mature hematopoietic cells. (Adapted from Passague *et al*., 2003)

1.3.1.2 Properties of a HSC

Multipotency: Multipotency or multilineage differentiation is the capability of the HSCs to produce all mature blood cell types important in hematopoietic function (Kondo *et al*., 2003; Lemischka, 1997). A single HSC can produce at least eight to ten different lineages of mature cells (Bonnet, 2002; Cheshier *et al*., 1999).

Self-renewal: The property of self-renewal can be defined as the ability of HSCs to produce daughter cells with the exact same stem cell properties as the parent cell (Warner *et al*., 2004). Self-renewal can be symmetrical leading to production of two daughter HSCs or asymmetrical resulting in production of one HSC and one downstream progenitor with reduced self-renewal capability. The decision of the HSCs to enter the self-renewal process is

determined by several developmental regulators like the Wnt family members (Reya *et al*., 2003), Notch genes (Karanu *et al*., 2000), Sonic Hedgehog (Shh) (Bhardwaj *et al*., 2001), Hox family genes (Antonchuk *et al*., 2002; Buske *et al*., 2002; Thorsteinsdottir *et al*., 2002) and Polycomb group genes (Kajiume *et al*., 2004; Lessard and Sauvageau, 2003). The self-renewal property of a stem cell is also dependent on telomerase activity. As the cell differentiate from a HSC into MPPs, the telomerase activity is reduced (Morrison *et al*., 1996).

Cell cycle and HSC: The cell cycle is tightly regulated in HSC. The HSCs enter the G0 or quiescent phase to avoid stem cell exhaustion (Cheng *et al*., 2000). HSCs are rare among peripheral blood cells, 1 in 10000 to 100000 (Bonnet, 2002). It has been reported that approximately 8% to 10 % of LT-HSCs enter the cell cycle per day in an adult young mice (Passegue *et al*., 2003). The majority of HSCs remain inactive and are slow cycling in the adult hematopoietic system. The LT-HSCs are considered to be most primitive and reside in a quiescent state (Lemischka, 1997). Various homologues of cyclins, cyclin-dependent kinases and retinoblastoma (Rb) family members are differentially expressed within the hematopoietic system in mammals (Passague *et al*., 2005).

Apoptosis: Apoptosis is an actively regulated process throughout hematopoiesis (Opferman, 2007) and plays an important role in regulating the size of the HSC pool (Domen, 2001). For example, the ectopic expression of the anti-apoptotic protein BCL2 in transgenic mice resulted in an increase in the steady-state hematopoietic stem and progenitor cells in the bone marrow. In addition to this, there competitive repopulating potential of these cells was increased. (Domen, 2000). Murine HSCs do not express CD95 (Fas), an apoptotic triggering death receptor (Aguila and Weissman, 1996), and that Fas deficiency does not affect bone marrow hematopoiesis (Schneider *et al*., 1999).

HSC migration: Both homing, the migration of HSCs from peripheral blood to bone marrow and mobilization, when HSCs leave the bone marrow, are conserved through evolution (Kondo *et al*., 2003). The ability of HSCs to migrate appears to be useful in developing fetus, during blood loss, during bone marrow transplantations and also in making cell fate decisions by relocating the daughter HSCs to distinct bone marrow niches (Fig. 1.3.1.2).

HSC plasticity: The HSCs have the potential to give rise to other cell types including neural cells (Brazelton *et al*., 2000; Eglitis and Mezey, 1997; Mezey *et al*., 2000), skeletal muscle

(Bittner *et al*., 1999; Ferrari *et al*., 1998; Gussoni *et al*., 1999), cardiac muscle (Jackson *et al*., 2001; Orlic *et al*., 2001a; Orlic *et al*., 2001b), hepatic cells (Alison *et al*., 2000; Lagasse *et al*., 2000; Petersen *et al*., 1999; Theise *et al*., 2000) and also lung, skin, kidney and gut epithelia (Perez *et al*., 2001).

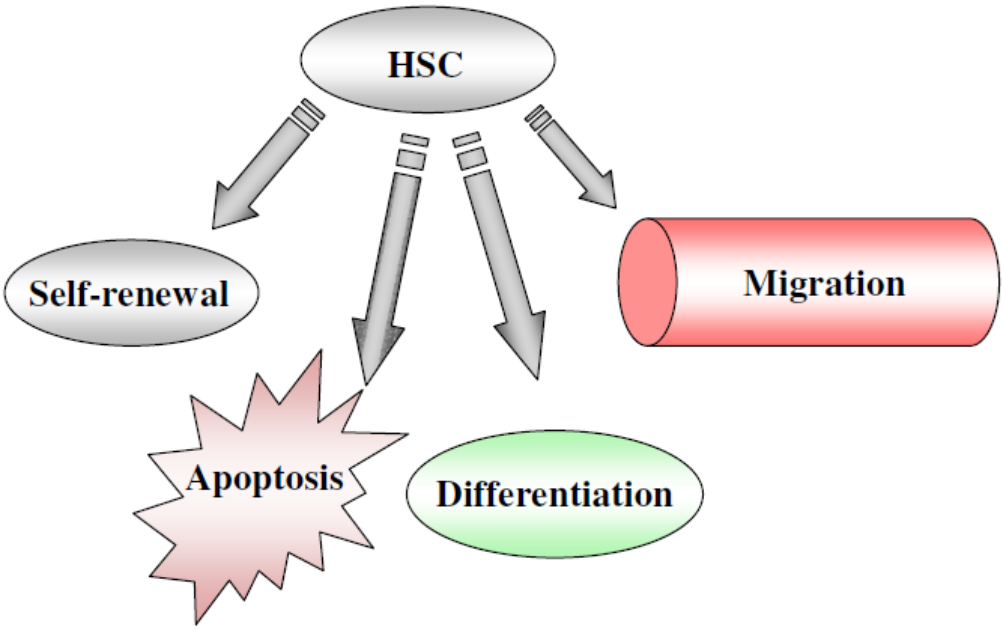


Fig. 1.3.1.2 Different fates of a HSC: After cell division, the daughter hematopoietic stem cell can self-renew, differentiate, undergo programmed cell death (apoptosis) or can acquire the property of migration under certain conditions and seed other organs. (Adapted from Weissman, 2000)

1.3.2 Leukemia

Leukemia occurs due to perturbance in the well orchestrated hematopoietic system by the acquisition of mutations. This leads to increased proliferation, block in differentiation, reduced apoptosis and prolonged survival. Leukemia develops from the clonal expansion of a transformed blast cell (Fialkow *et al*., 1987; McCulloch *et al*., 1979), is a multistep process (Hanahan and Weinberg, 2000) and is sustained by a leukemic stem cell (LSC). It has been hypothesized that at least two classes of genetic mutations are required for leukemic transformation – Class I mutations that result in increased cellular proliferation and/or survival advantage to hematopoietic progenitors and Class II mutations which result in

impaired differentiation of hematopoietic progenitors (Kelly and Gilliland, 2002). Mutations affecting tyrosine kinases that are involved in signal transduction such as *FLT3*, *RAS*, *KIT* are examples of Class I mutations. Whereas, alterations of transcription factors such as the *PML-RARα* and *AML1-ETO* fusion genes are examples Class II mutations (Fig. 1.3.2).

Several animal models have demonstrated that Class II fusion proteins alone are not sufficient to induce a full blown leukemia. For example, *PML-RARα* caused AML only in 30% of transgene expressing mice after a long latency period (Grisolano *et al*., 1997). *AML1-ETO* expressing animals do not develop leukemia but exhibit many abnormalities in hematopiesis that are also observed in leukemia patients (Guzman *et al*., 2002). However, when *AML1-ETO* was co-expressed with the tyrosine kinase *FLT3* length mutation (*FLT3-LM*), it was able to induce AML in a murine bone marrow transplantation model (Schessl *et al*., 2005). In addition to this, *AML1-ETO* is also known to collaborate with *Wilms tumor* (*WT1*), which is a proto-oncogene (Nishida *et al*., 2006). It has been reported that *TEL/TDGFRβ* fusion gene and *AML1-ETO* collaborate and induces AML in mice (Grisolano *et al*., 2003). Similarly, *PML-RARα* is known to co-operate with *FLT3-ITD* (Kelly *et al*., 2002; Reilly, 2002) and also with *BCL2* (Wuchter *et al*., 1999) to induce leukemia in mice.

Though perturbed proliferation and maturation arrest are the important events in leukemogenesis, there are other mechanisms which are essential for leukemic transformations. These mechanisms include alterations in apoptosis, increased telomere maintenance, deregulation of self-renewal process (Warner *et al*., 2004) and genomic instability (Passegue *et al*., 2003).

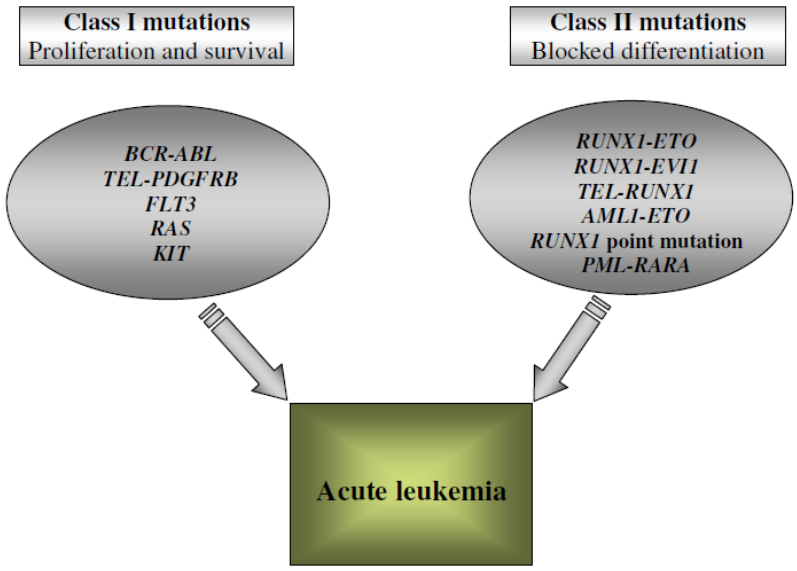


Fig. 1.3.2 Class I and Class II mutations: At least two classes of genetic mutations are required for leukemic transformation. Class I mutations result in increased proliferation and/or survival advantage and involve tyrosine kinases, and Class II mutations which lead to impaired differentiation involve the transcription factors. (Adapted from Speck and Gilliland, 2002)

1.3.2.1 Leukemia stem cells (LSCs) in AML

Earlier transplantation experiments demonstrated that only a small fraction of murine lymphoma cells could generate disease in the recipients (Bruce and Van Der Gaag, 1963) and only 1% to 4% of leukemic cells could form colonies in the spleen. These clonogenic leukemic cells were first described as leukemia stem cells (LSCs). The concept of LSCs is based on AML studies which showed that only a small subset of cells within the leukemic bulk was able to proliferate *in vitro* and *in vivo* (Wantzin and Killmann, 1977). Similar observations were reported in brain tumors, breast and colon cancers and mammary adenocarcinoma (Deshpande and Buske, 2007; Mendelsohn, 1962). The clonogenic assays with solid carcinoma cells also demonstrated a small subset of cells with tumor initiating ability (Mackillop *et al*., 1983). These reports suggest that there is a clear functional heterogeneity within the cancer cell population. This heterogeneity can be explained by two theories: (a) Stochastic model and (b) Cancer stem cell (CSC) model.

Stochastic Model: According to this model, all cells within the tumor bulk have an equal but low probability to enter cell cycle and regrow the tumor (Korn *et al*., 1973; Till *et al*., 1964).

The cell which attains the property of extensive proliferation undergoes multiple divisions (Dick, 2003b; Reya *et al*., 2001).

CSC Model: This model hypothesizes the existence of a rare population of CSCs which are functionally different from other cells (Buick and Pollak, 1984; Mackillop *et al*., 1983). These cells have extensive proliferation and self-renewal capacity which is essential to initiate a new tumor and produce a hierarchy of phenotypically distinct downstream progenitors. The descendants of CSC have limited proliferation and self-renewal potential and display some remnants of normal differentiation.

In murine transplantation experiment the expression of *MLL-GAS7* fusion gene in early HSC or MPP led to mixed lineage leukemia with biphenotypic progenitors. But this was not the case when the gene was transduced into common myeloid progenitors (CMPs) or into common lymphoid progenitors (CLPs). These experiments show that HSC that have the potential to differentiate into MPP are targets for induction of mixed lineage leukemia by *MLL* (So *et al*., 2003). Interestingly, in contrast to the above result, when murine HSC, CMP and GMP were transduced with *MLL-ENL* the cells were arrested in their differentiation at the myelomonocytic stage and leukemia was initiated. These experiments suggest that *MLL* induced myeloid leukemias can also start in committed progenitors (Cozzio *et al*., 2003). In support of the *MLL-ENL* model, another fusion gene *MOZ-TIF2* was also shown to confer LSC property to committed progenitor (Huntly *et al*., 2004). However, studies using the *MLL-AF9* fusion gene illustrated that committed progenitor as well as more downstream lineage positive cells can gain the LSC properties (Krivtsov *et al*., 2006; Somervaille and Cleary, 2006). In a murine model of *CALM/AF10* it was demonstrated that $B220^{+}$/myeloid $marker^{-}$ cells could propagate AML and regenerate the heterogeneity of the original tumor (Deshpande *et al*., 2006).

In a nutshell, these studies in murine AML models show that CSCs can arise either from HSCs which acquire mutations for transformation event or from transformed precursor cells or downstream progenitors which re-acquire stem cell features.

1.3.2.2 Cell of Origin in AML

In addition to the stochastic and CSC model an intermediate model was proposed to explain the "cell of origin" and LSC in AML. In cancer biology, the cell of origin has been defined as a precancerous cell that gives rise to a CSC (Smith, 2006). According to this model, an initial event occurring in the stem cells would create a 'preleukemic' stem cell which has the ability to differentiate into downstream lineages and that additional oncogenic events or alterations occur in the downstream progenitors to create LSC (Reya *et al*., 2001). The observation from a study of *AML1-ETO* fusion genes suggests that the HSCs are not leukemic. The AML1-ETO expression resulted in preleukemic stem cell and additional mutations are acquired by more committed progenitors leading to the transformation event (Miyamoto *et al*., 2000). The study of the *CALM/AF10* model proposes that either a HSC, MPP, or a myeloid progenitor which attains the lymphoid properties due to *CALM/AF10*, or a rare subset of naturally occurring lympho-myeloid cell could be the cell of origin in this leukemia. The other possibility could be that initial target cells have a differentiation block at the lymphoid stage and CALM/AF10 induces myeloid differentiation in these cells (Deshpande and Buske, 2007). Recent report on $CD34^+$ AML samples suggest that LSC populations resemble a murine LMPP (lymphoid-primed multipotential progenitor) which is similar a normal progenitor rather than to stem cells or MPPs. They have also shown that the LMPP population coexists with a GMP-like population (Goardon *et al*., 2011).

1.4 Causes of Leukemia

It is well known that genetic aberrations such as chromosomal aberrations play a pivotal role in leukemia development (Rabbitts, 1994). Leukemia results from acquisition of mutations in hematopoietic precursor or stem cells. These mutations include point mutations (single base pair insertion, deletion or substitution), gross chromosomal rearrangements such as deletions, insertions, amplifications, translocations, and epigenetic changes (Lin and Aplan, 2004).

Point mutations: These mutations have a crucial role in pathogenesis of acute leukemia. Activating point mutations have been identified in *RAS* (20%), *FLT3* (30% - 35%) and *KIT* (5%). The loss-of-function mutations are frequent in *CEBPA*, *AML1* and *GATA1* (Lin and Aplan, 2004; Gilliland and Tallman, 2002).

Gene amplification: This category of mutation is rare in leukemia patients. However, a few gene amplifications have been observed in AML e.g. *MYC* amplification in AML cell lines (Graham *et al*., 1985) and *MLL* amplifications in AML patients (Ariyama *et al*., 1998).

Chromosomal deletions: Three common deletions found in acute leukemias are 5q-, 7q- and 20q-(Gilliland and Tallman, 2002; Pedersen and Kerndrup, 1986; Swolin *et al*., 1981). In addition to this, the deletion of the *p15* and *p16* genes on the short arm of chromosome is common in ALL (Batova *et al*., 1997).

Chromosomal translocations: This type of chromosomal rearrangement is found in up to 65% of the acute and chronic leukemias (Raimondi, 1993; Solomon *et al*., 1991). Detailed chromosomal translocation studies have been useful in understanding the pathogenesis as well as identifying therapeutic targets of hematologic malignancies (Rowley, 1999). Chromosomal translocations between non-homologous chromosomes are common occurrence in leukemias and translocations between homologous chromosomes are rare events as in t(14;14) in T-cell leukemias (Rabbitts and Stocks, 2003). A detailed study on balanced chromosomal translocation has revealed that genes encoding transcription factor important for hematopoietic differentiation or signalling pathway proteins like tyrosine kinases are frequently affected by the translocations (Lin and Aplan, 2004; Rabbitts, 1991; Rabbitts, 2001; Rowley, 2001). Chromosomal translocations either result in the generation of fusion proteins e.g. BCR-ABL (common in myeloid leukemias) or lead to deregulated expression of a gene close to the translocation breakpoint such as *SCL* or *LMO2* (common in T-cell leukemias). The resulting fusion genes are oncogenic and activate signal pathways which lead to increased proliferation or block in differentiation (Ayton and Cleary, 2001; Sternberg and Gilliland, 2004). In about 25% of AML cases, balanced chromosomal translocations result in fusion proteins which are important for the development of leukemia (Brown *et al*., 1997; Heisterkamp *et al*., 1990; Kogan *et al*., 1998).

Several studies have suggested possible causes of chromosomal translocations. These include illegitimate V(D)J or immunoglobulin class switch recombination, homologous recombination, non-homologous end joining and DNA topoisomerase II subunit exchange (Aplan, 2006).

1.5 The t(10;11)(p12;q14) translocation

The t(10;11)(p12;q14) is a rare but recurring chromosomal translocation (Bohlander *et al*., 2000) and is found mainly in undifferentiated AML or T-cell ALL and in malignant lymphoma (Dreyling *et al*., 1998; Kumon *et al*., 1999; Narita *et al*., 1999). It has also been identified in acute megakaryoblastic, monocytic and eosinophilic leukemias (Caudell and Aplan, 2008; Jones *et al*., 2001; Nakamura *et al*., 2003; Salmon-Nguyen *et al*., 2000). The t(10;11)(p12;q14) translocation was first found in a patient with diffused histiocytic lymphoma (Sundstroem and Nilsson, 1976). It was first cloned and characterized in the human monocytic cell line U937 (Dreyling *et al*., 1996). This translocation results in the fusion of *CALM* on chromosome 11 band q14 to *AF10* on chromosome 10 band p12 (Fig. 1.5). This leads to the expression of the CALM/AF10 and the reciprocal AF10/CALM fusion transcript. *CALM/AF10* patients are known to have bad prognosis (Dreyling *et al*., 1998). The translocation is observed in younger patients (Kobayashi *et al*., 1997). Interestingly, CALM/AF10 fusion is found in almost 30% cases of T-ALL patients with T-cell receptor (TCR) γ/δ rearrangement (Asnafi *et al*., 2003).

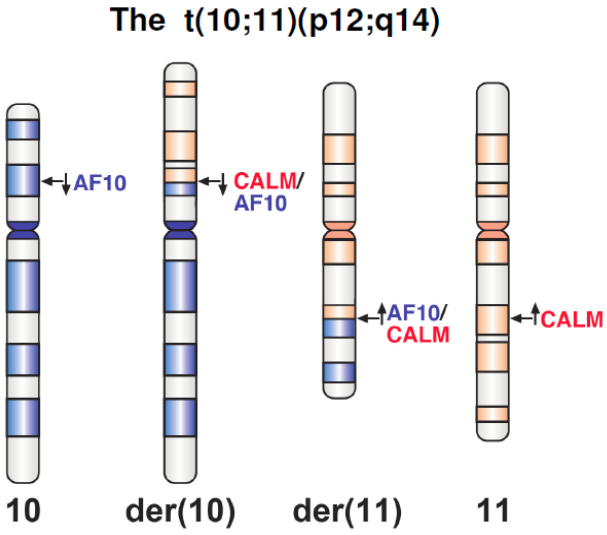


Fig. 1.5 t(10;11)(p12;q14) translocation: The t(10;11)(p12;q14) translocation results in the fusion of *CALM* gene on chromosome 11 and *AF10* gene on chromosome 10 to generate an in frame *CALM/AF10* fusion gene on derivative chromosome 10 and *AF10/CALM* fusion gene on derivative chromosome 11. (Diagram courtesy Prof. Dr. S.K. Bohlander).

1.5.1 *CALM*

The Clathrin Assembly Lymphoid Myeloid leukemia gene (*CALM* or *PICALM*) was first identified as a fusion partner of AF10 in t(10;11)(p12;q14) translocation. *CALM* is located on chromosome 11q14 and is a ubiquitously expressed protein. *CALM* encodes a 652 amino acid long protein containing an Epsin N-terminal homology (ENTH) domain and several other motifs such as DPF (ASP-Pro-Phe), NPF(Asn-Pro-Phe), and type I and II clathrin binding sequences (CBS I and II), which are involved in endocytosis (Klebig *et al*., 2003; Meyerholz *et al*., 2005; Tebar *et al*., 1999) (Fig. 1.5.1).

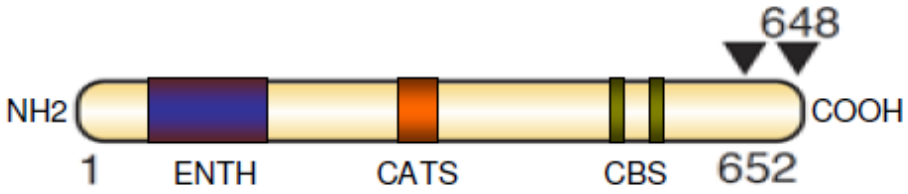


Fig. 1.5.1 Schematic representation of *CALM*: *CALM* encodes a 652 amino acid long protein and contains an Epsin N-terminal homology domain (ENTH), clathrin binding sequences (CBS), CATS (CALM interacting protein expressed in thymus and spleen) binding domain and several motifs such as DPF and NPF. (Diagram courtesy Prof. Dr. S.K. Bohlander)

There is a homology between the CALM protein and the clathrin assembly protein AP180 (Morris *et al*., 1993). The CALM protein moves clathrin to the membrane by interacting with calthrin heavy chain through its C-terminal CBS and with phosphoinositides through its N-terminal ENTH domain (Ford *et al*., 2001; Ford *et al*., 2002). Deregulation of *CALM* is associated with inhibition of receptor-mediated endocytosis and impairment of endosome trafficking in the trans golgi network (TGN) (Meyerholz *et al*., 2005; Tebar *et al*., 1999).

N-ethyl-*N*-nitrosourea (ENU) induced point mutation in the mouse homologue *Picalm* gene resulted in perturbed hematopoiesis, reduced growth and improper iron metabolism in mice harboring this mutation (Klebig *et al*., 2003). Using *CALM*-deficient mice, it was recently demonstrated that *CALM* plays an essential role in maturation of erythroid precursor and

transferrin incorporation. Moreover, another important observation was that *CALM* deficient mice had shortened life span along with retarded growth *in utero* (Suzuki *et al*., 2012). Using a yeast two-hybrid screen, two CALM interacting protein, CATS (CALM interacting protein expressed in thymus and spleen) and FHL2 (four and a half LIM domain protein 2) were identified (Archangelo *et al*., 2006; Pasalic *et al*., 2011). The CATS protein is expressed in thymus, spleen and colon. The CATS interacting domain in CALM is positioned from 221-335 amino acid of CALM and this domain is retained in the CALM/AF10 fusion protein. The CATS protein increases the nuclear localization of CALM as well as of the CALM/AF10 fusion protein and interacts with CALM *in vitro* and *in vivo* (Archangelo *et al*., 2006). The FHL2 interacting domain in CALM is mapped to amino acid 294-335 of CALM. FHL2 interacts with β-integrin (Samson *et al*., 2004) which in concert with clathrin has been shown to be involved in the endocytosis process of CALM protein (Tebar *et al*., 1999). FHL2 play a vital role in Wnt signaling (Labalette *et al*., 2004; Wei *et al*., 2003) and also influences several major cellular processes like transcriptional regulation, DNA replication and signal transduction pathways. *CALM* but not *CALM/AF10* reduces the transcriptional activation potential of FHL2 (Pasalic *et al*., 2011).

Altogether these findings suggest an important role of CALM in hematopoisis and basic cellular processes.

1.5.2 *AF10*

AF10 (ALL-1 fused gene from chromosome 10, also known as *MLLT10*) was first identified as a fusion partner of *MLL* in a recurring t(10;11)(p12;q23) translocation in AML (Chaplin *et al*., 1995a; Chaplin *et al*., 1995b). The ubiquitously expressed *AF10* is located on chromosome 10p12 and encodes a 109-kD protein of 1027 amino acids (Fig. 1.5.2). As reported in murine studies, AF10 expression is highest in testis but also expressed in ovary, thymus, colon, peripheral blood, brain and kidney (Chaplin *et al*., 1995b; Linder *et al*., 1998).

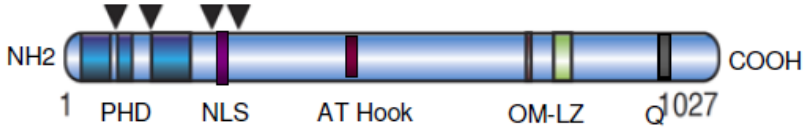


Fig. 1.5.2 Schematic representation of *AF10*: AF10 encodes a 109-kD protein of 1027 amino acid and contains N-terminal (NH2) plant homeodomain (PHD) zinc fingers, AT (adenine-thymine) rich hook, a bipartite nuclear localization signal (NLS) and a highly conserved octapeptide motif-leucine zipper (OM-LZ) domain. At the C-terminus (COOH) there is a glutamine rich (Q) domain. (Diagram courtesy Prof. Dr. S.K. Bohlander)

The domains of the AF10 protein include a plant homeodomain (PHD), an extended PHD finger (also known as leukemia-associated protein or LAP), AT-rich hook motif, a bipartite nuclear localization signal (NLS), an octapeptide motif and leucine zipper domain (OM/LZ) and a C-terminal glutamine-rich region. AF10 is a member of a highly conserved protein family, which includes AF17, BR140 and CEZF (Chaplin *et al*., 1995a; Linder *et al*., 2000). AF10 is a putative transcription factor due to its similarity in the structurally conserved PHD domain with other known transcription factors such as CBP, MLL, TRX and CCL (Aasland *et al*., 1995). The PHD and LAP domains of AF10 are highly conserved. The LAP domain is involved in homooligomerization and the AT-hook tends to bind to cruciform DNA (Aravind *et al*., 1998). The LZ domain of AF10, AF17 and CEZF is also reported to be conserved (Chaplin *et al*., 1995a). The LZ domain of the *Drosophila* homologue of AF10 *Alhambra* has been shown to deregulate the activity of PRE-mediated transcriptional silencing (Perrin *et al*., 2003). *Alhambra* interacts with heterchomatin protein1 (HP1) and suppresses position effect variegation (DiMartino *et al*., 2002).

Interestingly, it could be shown that the OM together with LZ domain contributes to the oncogenicity of AF10 in a *MLL/AF10* transformation model. The small OM/LZ motif interacts with YEATS4 (glioma amplified sequence 41, GAS41), which in turn interact with the INI1 (integrase interactor 1), a component of the SWI/SNF complex (Debernardi *et al*., 2002). Another important function of the OM/LZ motif is its capability of interacting with the histone H3K79 methyltransferase DOT1L. This interaction is critical for both *MLL/AF10* and *CALM/AF10* mediated leukemogenesis (Okada *et al*., 2005; Okada *et al*., 2006).

1.5.3 The *CALM/AF10* Fusion

The recurring t(10;11)(p12;q14) translocation in most cases generates a *CALM/AF10* fusion and also the reciprocal *AF10/CALM* fusion transcript. However, the *AF10/CALM* fusion transcript unlike *CAML/AF10* can not be detected in all patients carrying this translocation. Thus it seems that *CALM/AF10* is critical for malignant transformation (Dreyling *et al*., 1996) because in several patients this translocation is the only chromosomal abnormality and the AF10/CALM fusion transcript is not expressed (Abdou *et al*., 2002; Bohlander *et al*., 2000; Carlson *et al*., 2000).

The *CALM/AF10* fusion comprises almost the complete open reading frames (ORFs) of both *CALM* and *AF10* genes except for the last four amino acids of the C-terminal *CALM* gene and the N-terminal PHD domain of the *AF10* gene (Fig. 1.5.3). In contrast, the *AF10/CALM* fusion only generates a truncated AF10 protein (Dreyling *et al*., 1996).

At least four different breakpoints in *AF10* and three breakpoints in *CALM* have been reported in patients with a CALM/AF10 fusion (Bohlander *et al*., 2000). However, there seems to be no correlation between the breakpoint locations and the type of disease observed in the patients (AML or ALL). *CALM/AF10* causes global hypomethylation of H3K79 and increased genomic instability (Lin *et al*., 2009). It has also been reported that *CALM/AF10* causes H3K79 hypermethylation at the HOXA5 promoter (Okada *et al*., 2006).

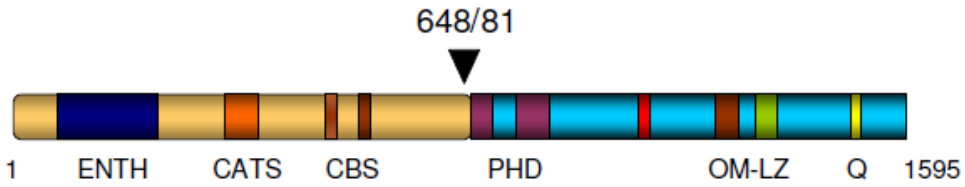


Fig. 1.5.3 Schematic representation of *CALM /AF10* fusion gene: The CALM/AF10 fusion comprises almost the complete open reading frames (ORFs) of both *CALM* and *AF10* genes except the last four amino acids of the C-terminal *CALM* gene and the N-terminal PHD domain of the *AF10* gene. (ENTH: Epsin N-terminal homology domain; CATS: CALM interacting protein expressed in thymus and spleen; CBS: clathrin binding sequences; PHD: plant homeodomain; OM-LZ: octapeptide motif and leucine zipper; Q: glutamine rich domain)

1.6 Mouse models of *CALM/AF10* leukemia

Several mouse models of *CALM/AF10* leukemia have been established. The mouse model have provided valuable tools to understand the process of leukemogenesis initiated by chromosomal translocation as well as to study other human diseases *in vivo* (Rabbitts, 2001). Due to the fact that mice and humans are similar at the genomic level, the mouse has become a standard animal model for several studies related to human diseases. Chromosomal translocation studies in mouse models have been possible using a variety of techniques such as conditional or inducible knock-ins, transgenic models, targeted and random *in vivo* gene disruption and retrovirally transduced bone marrow transplantation.

Several mouse models of the CALM/AF10 fusion protein have been generated.

1.6.1 Classical transgenics

Transgenic mice are created by introducing a foreign DNA (the transgene) into the male pronucleus which is then stably integrated into the genome (Gassmann and Hennet, 1998). The transgenic as well as genetically engineered mouse models are helpful in analyzing gene function, the identification of novel oncogenes, understanding the molecular and cellular basis of tumorigenesis and also for providing better clinical model for improved therapeutic strategies (Cheon and Orsulic, 2011). Since 1980s, several methods have been developed to generate mouse models of cancer. The most common ones are activation of oncogenes and inactivation of tumor-suppressing genes using transgenic, knock-out and knock-in mice. The transgenic and knock-in mice are used for gain-of-function studies and knock-out mice are employed in loss-of-function studies. The transgenic approach has led to a better understanding of the mechanisms of development and developmental genes, action of oncogenes, the cellular basis of the immune system (Hanahan, 1984) and also the pre-neoplastic state (Adams *et al*., 1999). The proper selection of regulatory elements in these models is crucial to study the impact of an oncogene in the most relevant cell type(s) (Adams *et al*., 1999).

The classical transgenic model involves the microinjection of the transgene into the male pronuclei of fertilized mouse oocytes. The resulting viable embryos are implanted into pseudo-pregnant foster mothers (Gassmann and Hennet, 1998). In 1980 this technique was first developed by Gordon and coworkers (Jaenisch, 1988). The advantages of the transgenic

approach are the straightforward assessment of the *in vivo* oncogenic functions of a gene, the short period of time to generate these mice compared to gene-targeting strategies (Cheon and Orsulic, 2011) and the capability to express most genes in a predictable manner (Jaenisch, 1988). Since this method employs the injection of the transgene into a fertilized oocyte, the transgene is incorporated into the host chromosomes at random positions; thereby the transgenic mouse obtains the gene in all its tissues. The expression of the transgene is dependent on several factors in spite of being present in all cells such as the selected promoter and enhancer element, the number of copies stably integrated and the locus of integration. Therefore, the regulatory elements have an impact on the tissue specific gene expression (Palmiter and Brinster, 1985).

This approach also has several disadvantages such as the inability to control the level and pattern of transgene expression, which vary among the transgenic founders lines due to random integration sites and different copy number of the transgene (Cheon and Orsulic, 2011; Gassmann and Hennet, 1998). The random integration might lead to silencing of the transgene expression due to positional effects or might result in unexpected phenotype due to the disruption of an important gene by the transgene. Another drawback of this approach is the limited availability of tissue-specific promoter.

However, several strategies have been employed to overcome these limitations such as using embryonic stem cell based transgenic mouse models which can partially overcome the problems with the level and pattern of expression of the transgene (Novak *et al*., 2000). The use of insulated DNA sequence elements at the boundaries of the transgene may prevent position effects (West *et al*., 2002). The copy number of the transgene can be controlled by employing site-specific integration of the transgene in embryonic stem cells (Beard *et al*., 2006) and single-copy transgenesis through long interspersed element type 1 (An *et al*., 2008).

1.6.2 The IgH-CALM/AF10 and pLck-CALM/AF10 transgenic models

Two classical transgenic models for *CALM/AF10* were established in our group using the immunoglobulin heavy chain enhancer/promoter (IgHE/P) and the proximal murine *Lck* promoter (pLck) (Krause, 2006). To avoid death during early embryonic development due to the early transgene expression and to express the CALM/AF10 transgene in appropriate cells, the transcription of *CALM/AF10* fusion gene was restricted to B and T cells using the above

mentioned promoters. The transgenic lines were established in FVB mice. Two lines were generated for the IgH-CALM/AF10 and three lines were generated with the *CALM/AF10* fusion gene under the control of proximal *Lck* promoter.

IgH enhancer promoter

The murine immunoglobulin heavy chain gene was the first cellular eukaryotic enhancer to be identified. It is located between the J_H gene segment cluster and μ constant region coding sequences. The variable region (V) promoter is controlled by the *IgH* intron enhancer (*EIgH*) post V region assembly and the *EIgH* is of interest for its tissue specificity (Eckhardt, 1992). Additionally, it has been reported in transgenic mice study that the microinjection of *Igμ* gene led to specific expression of the functionally rearranged heavy chain gene in lymphoid tissues. The tissue specific expression is probably due to the cis-acting DNA sequences present in the introduced *Igμ* gene (Grosschedl *et al*., 1984). Another study showed that Ig heavy chain enhancer driven *c-myc* expression in B-cells resulted in lymphoid malignancy in transgenic mice (Harris *et al*., 1988). These studies suggested that tissue specificity in these mice is due to the *IgH* enhancer, and the immunoglobulin promoter is responsible for the increased level of specific expression.

Lck promoter

The *Lck* gene encodes a lymphocyte-specific protein tyrosine kinase ($p56^{Lck}$), a member of the s*rc* family. The *src* family kinases are responsible in regulating cellular growth (Marth *et al*., 1988). *Lck* is expressed in T cells, most B cells and also in colon adenocarcinoma and small cell lung carcinoma derived human cell lines (Adler *et al*., 1988). The *Lck* gene has been implicated in the development of lymphoid malignancy (Garvin *et al*., 1988). Interestingly, in mammals, the *Lck* gene expression is regulated by two independent promoters, the proximal and the distal promoter. The proximal promoter is responsible for transcription of type I mRNA and is active in immature thymocytes. On the other hand, the distal promoter which transcribes type II mRNA, is active in mature T-cells (Brenner *et al*., 2002; Reynolds *et al*., 1990). These two promoters can function independently (Allen *et al*., 1992).

The IgH-CALM/AF10 and pLck-CALM/AF10 transgenic animals did not show any leukemic phenotype even after an observation period of over 15 months. There was no

detectable clinical, hematological, immunological, immunohistopathological and flow cytometry difference between transgenic and FVB wild type mice (Krause, 2006).

The reason for the lack of leukemia development in the IgH-CALM/AF10 transgenic mice could be because the expression of the CALM/AF10 fusion occured in mature B-cells which might not be susceptible to CALM/AF10 mediated transforamtion. There was also no leukemia development in the pLck-CALM/AF10 mice even though the proximal Lck promoter is known to be active in early immature thymocytes. Overall these observations suggest that the target cell for leukemic transformation by CALM/AF10 is a quite early cell in the hematopoietic hierarchy. This would explain the absence of leukemia development in these transgenic mice.

1.6.3 The Vav-CALM/AF10 transgenic mouse model

Interestingly, another CALM/AF10 transgenic mouse model was established, in which the CALM/AF10 expression is driven by *Vav* promoter (Caudell *et al*., 2007). The *Vav* promoter is a potent pan-hematopoietic promoter and is active throughout the hematopoietic component but is silent in non-hematopoietic cell types (Ogilvy *et al*., 1999). The *Vav* gene expression is first found in the fetal liver and then in all hematopoietic cell types including progenitor cells and their precursors (Adams *et al*., 1999). *Vav* is crucial for full lymphocyte development and function (Turner *et al*., 1997; Zhang *et al*., 1995).

Prior experiments were performed on *Vav* transgenic mice using a mammalian reporter, which is a biologically inert form of the human CD4 cell surface antigen. The data from these experiments demonstrated that the *vav-CD4* transgenes were actively expressed exclusively in the hematopoietic compartment in almost 80% of mice. As expected, the transgene expression was absent in non-hematopoietic tissues (Ogilvy *et al*., 1999). The hematopoietic cell types, which showed vav-CD4 transgene expression, included neutrophils, monocytes, megakaryocytes, eosinophils, B and T lymphocytes and nucleated erythroid cells.

In the Vav-CALM/AF10 transgenic model, the *Vav* regulatory elements directed CALM/AF10 expression in the hematopoietic compartment including thymus, spleen and bone marrow. The Vav-CALM/AF10 transgenic mice developed acute leukemia after a latency period of 12 months and at a penetrance of only 40% to 50%. Immunophenotypic analysis revealed that more than 50% of the leukemic mice were either $Mac1^{+}/B220^{+}$ or were

$MPO^+/B220^+$ cells within the tumor infiltrates. B220 'bright' and 'dim' populations suggested that B220 is expressed on non-B cell progenitor and that this population might represent the leukemic clone. In addition to this, around half of the leukemic mice had clonal *IgH* gene rearrangements. Thus, this model showed that CALM/AF10 is very leukemogenic. Aplan and colleagues discuss that additional collaborating genetic events are required for the CALM/AF10 fusion gene for complete leukemogenesis.

1.6.4 A Murine bone marrow transplantation model of *CALM/AF10* leukemia

This model employs retroviral transduction of primary hematopoietic cells followed by transplantation into lethally irradiated syngeneic recipient mice. In this model it is possible to assay the oncogenic potential of a gene of interest faster than with a classical transgenic mouse model.

In contrast to the *CALM/AF10* transgenic model, mice transplanted with retrovirally transduced bone marrow cells expressing *CALM/AF10* developed an acute leukemia with a 100% penetrance and after a median latency period of just 110 days. The leukemic mice were anemic, had circulating blasts and myeloid infiltration in different organs. The leukemic cells were positive for myeloid markers i.e. Gr1 (for granulocytes) and Mac1 (for macrophages), and also for lymphoid marker B220. Moreover, these cells had clonal DH-JH rearrangements. These observations led to the speculation that the target cell for CALM/AF10 was a multipotent progenitor cell with lymphoid features. This cell is different from a normal HSC and is capable of giving rise to AML. The leukemia inititating cell in this model was shown to reside in the $Mac1^-/B220^+$ compartment by the serial transplantation (Deshpande *et al*., 2006).

The murine bone marrow transplantation (mBMT) model is ideal to study the heterogeneity of a leukemic clone and also the behavior of leukemia initiating cells. The clonality analysis using Southern blotting demonstrated that the CALM/AF10 leukemia was of oligoclonal origin. This implies that transformation occurred only in a small fraction of the retrovirally transduced cells. These findings suggest that additional collaborative events are necessary for leukemia development also in the CALM/AF10 bone marrow transplantation model.

Different CALM/AF10 deletion mutants were generated and a detailed structure-function analysis of these mutants were performed in order to study the role of different domains of CALM and AF10 for leukemia development (Deshpande *et al*., 2011). The fusion of the C-terminal 248 amino acids of CALM, which includes the clathrin domain to the OM/LZ domain of AF10, generates a fusion protein which was named CALM/AF10-minimal fusion (CALM/AF10-MF) (Fig. 1.6.4). The CALM/AF10-MF protein has enhanced transformation capabilities in *in vitro* colony forming cell assays. Bone marrow transplantation studies with this mutant protein however resulted in a similar phenotype like the CALM/AF10 full length fusion.

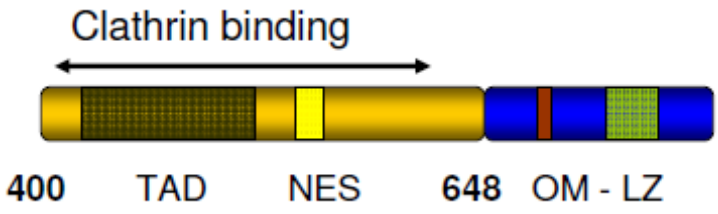


Fig. 1.6.4 Schematic representation of *CALM/AF10*-Minimal fusion gene: CALM/AF10-Minimal fusion (CALM/AF10-MF) is a deletion mutant and it consists of C-terminal 248 amino acids of CALM which includes CBS (clathrin binding sequences) and the OM-LZ (octapeptide motif and leucine zipper) domain of AF10. This protein has been shown to possess enhanced transformation capability *in vitro*. (TAD: trans-activating domain; NES: nuclear export signal) (Adapted from Deshpande *et al*., 2011)

1.7 CALM/AF10 target genes – *HOXA* cluster

HOX gene expression is an important in embryogenesis, organogenesis and also hematopoiesis (van Oostveen *et al*., 1999). The *HOX* genes are members of homeodomain family of genes that encode for transcription factors. The mammalian *HOX* genes are organized in clusters on four different chromosomes (Garcia-Fernandez, 2005; Pearson *et al*., 2005). The HOX clusters A, B and C have been reported to be essential for normal hematopoiesis (Abramovich and Humphries, 2005).

Clinical data and studies from experimental mouse models suggest the *HOX* gene involvement in leukemic transformation. Deregulated expression of *HOX* genes, due to

translocation event is associated with leukemias. The nuclear pore complex protein 98 (NUP98) fuses with several *HOXA* cluster genes including *HOXA9*, *HOXA11*, *HOXA13*, *HOXC11*, *HOXC13*, *HOXD11* and *HOXD13* in AML and myelodysplastic syndrome (MDS) patients (Lam and Aplan, 2001). Studies from mouse models demonstrated that overexpression of *Hoxa5*, *Hoxa9* or *Hoxa10* led to myeloid expansion and when the Hox cofactor *Meis1* was co-expressed AML was observed (Abramovich and Humphries, 2005; Grier *et al*., 2005). Similarly, mBMT studies expressing *Nup98-Hox* fusions resulted in abnormal myeloid differentiation, but in coexpression of the Nup98-Hox fusions with *Meis1* led to AML (Kroon *et al*., 2001; Pineault *et al*., 2003; Pineault *et al*., 2004). *Meis1* also collaborates with *Hoxb3* to induce leukemia (Thorsteinsdottir *et al*., 2001). *NUP98-HOXD13* transgenic mice showed upregulated *Hoxa7*, *Hoxa9* and *Hoxa10*, and developed a severe myelodysplastic syndrome that progressed to acute leukemia (Lin *et al*., 2005).

Chromosomal translocations resulting in *MLL* fusions, exhibit deregulated *HOX* gene expression in AML as well as in T-cell ALL patients (Hess, 2004). Gene expression profile studies have revealed that *HOX* genes are consistently overexpressed in AML (Armstrong *et al*., 2003; Mullighan *et al*., 2007). Murine BMT model of MLL fusions have clearly shown that *Hoxa9* and *Hoxa7* can transform myeloid progenitors in these models (Ayton and Cleary, 2003). *HOXA* cluster upregulation has been a common observation in case of *CALM/AF10* and *MLL* leukemias (Soulier *et al*., 2005).

Like MLL fusions, leukemias with an CALM/AF10 fusion also show upregulation of *HOXA* cluster genes. In pre-T-LBL *CALM/AF10* patients, *HOXA5*, *HOXA9* and *HOXA10* were shown to be upregulated when compared to pre-T-LBL patients without CALM/AF10 (Dik *et al*., 2005). *Vav-CALM/AF10* transgenic mice (Caudell *et al*., 2007) demonstrated an eightfold upregulation of *Hoxa5*, *Hoxa7*, *Hoxa10*, *Hoxa11* and *Meis1* in hematopoietic tissues (bone marrow, spleen and thymus) compared with clinically healthy *CALM/AF10* mice and an up to 500 fold upregulation of these *Hox* genes in *CALM/AF10* mice with myeloid leukemias (Caudell *et al*., 2007). Gene expression studies of *CALM/AF10* patients compared to other leukemic subgroups showed that the *HOX* cofactor *MEIS1* and the *HOX* cluster genes *HOXA3*, *HOXA5*, *HOXA7*, *HOXA9* and *HOXA10* were notably upregulated in *CALM/AF10* positive samples. Therefore, the upregulation of *HOXA* cluster genes and *MEIS1* in both CALM/AF10 and MLL-fusion positive leukemias suggests a similar leukemogenic mechanism in these leukemias (Mulaw *et al*., 2012).

1.8 Myeloid ecotropic insertion site1 (*Meis1*)

It is well known that *Hox* genes encode homeodomain (HD) containing transcription factors that play an important role during animal development. The Hox proteins interact with other DNA-binding proteins that direct the HOX activity and specificity towards distinct domains along the body axes (Moens and Selleri, 2006). These DNA-binding proteins are called Hox cofactors which include primarily the PBC (PBX and CEH-20) and MEIS classes. The Hox cofactors belong to the three amino acid loop extension (TALE) homeobox gene family (Burglin, 1997). The TALE classes of HD proteins have an additional three amino acids in the loop between helix 1 and helix 2. The PBC subclass of TALE HD protein includes fly Extradenticle (EXD) and vertebrate PBX homeoproteins. The MEIS subclass includes fly Homothorax (HTH) and vertebrate MEIS and PREP homeoproteins. The PBX proteins collaborate with HOX proteins from paralog groups 1 to 10 (Chang *et al*., 1996; Shen *et al*., 1997) and MEIS proteins interact with HOX paralogs 9 to 13 (Shen *et al*., 1997). This interaction between Hox and Hox cofactors increases the stability of the complex with DNA and the specificity for the target sequence. Meis1 has two α-helicase motifs in its N-terminal region referred to as Meinox domains M1 and M2 (Fig. 1.8). This region is called as Pbx-interacting motif (PIM) and binds with Pbx at this site (Mann and Affolter, 1998). At its C-terminal, Meis1 has a HD and downstream to it there lies a transactivation domain composed of 49 residues. This region is highly conserved among Meis1, Meis2 and Meis3 but is not found in the closely related family member Prep1 (Huang *et al*., 2005; Mamo *et al*., 2006; Wang *et al*., 2005). Meis family members interact with Pbx (Chang *et al*., 1997) and form stable heterodimers in a DNA-dependent as well as independent manner (Huang *et al*., 2005; Jacobs *et al*., 1999; Shanmugam *et al*., 1999; Shen *et al*., 1999). Meis interacts with Pbx and induces nuclear localization of Pbx by preventing its nuclear export (Abu-Shaar *et al*., 1999; Berthelsen *et al*., 1999) and promoting its nuclear localization (Huang *et al*., 2003; Saleh *et al*., 2000). Moreover, it has been shown that Meis and Hox proteins can interact in an indirect manner in the Hox-Pbx-Meis heterotrimeric complexes (Shen *et al*., 1999).

Meis1 was first identified as a common viral integration site in myeloid leukemic cells of BXH-2 mice and is located on proximal mouse chromosome 11 (Moskow *et al*., 1995). *Meis1* plays a central role in normal hematopoiesis. *Meis1* knock-out mouse embryos die by embryonic day 14.5 due to lack of megakaryocytes and intense reduction of myeloerythroid progenitors. Curiosly, the fetal liver cells from these mice were unable to radioprotect lethally

irradiated recipient mice and could not compete well in repopulation assays (Azcoitia *et al*., 2005; Hisa *et al*., 2004). These observations strongly suggest an important role for *Meis1* in self renewal and proliferation. Meis1 is highly expressed in the most primitive hematopoietic subpopulations and is down-regulated in later stages following differentiation (Imamura *et al*., 2002; Pineault *et al*., 2002). Reduced expression of *MEIS1* has been linked to the restless leg syndrome (Winkelmann *et al*., 2007; Xiong *et al*., 2009). Whereas, overexpression of *MEIS1* results in leukemia (Argiropoulos *et al*., 2007).

Fig. 1.8 Schematic representation of Meis1: Meis1 encodes a 390 amino acid protein and contains two alpha helicase motifs M1 (Meinox domain 1) and M2 (Meinox domain 2) in its N-terminal, and a nuclear localization signal (NLS) and Homeodomain (HD) in its C-terminal. PIM represents the Pbx-interacting motif. (Adapted from Mamo *et al*., 2006)

1.8.1 The Role of *Meis1* in leukemogenesis

The significance of *MEIS1* in human leukemogenesis was emphasized because of its frequent upregulation in primary AML and ALL samples (Imamura *et al*., 2002; Kawagoe *et al*., 1999; Rozovskaia *et al*., 2001). Murine BMT studies have revealed that *Meis1* synergizes with several *Hox* genes and *NUP98-HOX* fusion genes to accelerate the onset of AML. For instance, *Meis1* have been shown to collaborate with *NUP98-HOXA9* (Kroon *et al*., 2001) and *NUP98-HOXD13* (Pineault *et al*., 2003) resulting in AML. *MEIS1* also collaborates with *HOXB6* and considerably shortens the onset of AML (Fischbach *et al*., 2005). *Meis1* seems to collaborate with *AML1-ETO* leading to induction of AML (Naidu, 2009). Proviral insertional co-activation of *Hoxa7* and *Hoxa9* is associated with upregulation of *Meis1* (Nakamura *et al*., 1996). *Meis1* cooperates with *Hoxa9 leading to* rapid AML development in mice (Wang *et al*., 2005). In this model, *Meis1* induced the expression of *FLT3* and *CD34* which are associated with ST-HSCs. These findings suggested that the Meis1-Pbx complex regulates

the expansion of leukemia-initiating progenitors. *Meis1* also plays a role in the regulation of apoptosis in the caspase dependent pathway. Transient overexpression of *Meis1* in human and murine cell lines resulted in massive apoptosis. Moreover, it was shown that HD and PIM of *Meis1* are necessary for apoptosis induction (Wermuth and Buchberg, 2005). Overexpression of *Meis1* alone does not lead to leukemia development (Kroon *et al*., 1998; Thorsteinsdottir *et al*., 2001; Wang *et al*., 2005). However, when Meis1 is fused to transactivating domain of Vp16 transformation is observed (Mamo *et al*., 2006; Wang *et al*., 2006).

The collaborative effect of *Meis1* in the *Hoxa9* models of AML is associated with the expresion of several leukemic signature genes such as *Flt3* (Wang *et al*., 2005), *Cd34*, *Erg*, *Msi2h* (Wang *et al*., 2006) and *c-Myb* (Hess *et al*., 2006). In addition to this, coexpression of *Meis1* with *NUP98-HOXA10* or *NUP98-HOXD13* has also been linked to the upregulation of *Flt3* (Palmqvist *et al*., 2006). *CyclinD3* has been identified as a direct downstream target of *MEIS1* (Argiropoulos *et al*., 2010). The growth promoting activities of *Meis1* is linked to the *cyclinD-pRb* cell cycle control pathway. Several candidate *Meis1* upregulated genes as identified by microarray analysis include *Platelet factor 4* (*Pf4* or *Cxcl4*), *Flt3* as described above, *Delta-like homolog 1* (*Dlk1*), the oncogene *Tribbles 2* (*Trib2*), *Abcb1a* (*Mdr1*), *Ccl3* (*Mip1-α*), *Ccl4* (*Mip-1ß*) and *Rgs1*. Two genes were significantly downregulated by *Meis1*: *interferon consensus sequence-binding protein* (*ICSBP1*), a tumor suppressor gene, also known as *Irf8,* and *Notch1,* which plays an important role in cellular growth, survival and differentiation (Argiropoulos *et al*., 2008).

The core molecular mechanism responsible for the oncogenicity of *Meis1* is not known yet. However, numerous studies have suggested that *Meis1* collaborates with *Hox* genes for example *Hoxa9* through MEIS1-PBX-HOX complex. MEIS1 uses its PIM, HD and transactivating C-terminal domain to collaborate with *Hoxa9* (Mamo *et al*., 2006; Wang *et al*., 2005; Wong *et al*., 2007). This hypothesis, however, cannot explain the collaboration of *MEIS1* with *NUP98-HOXD13* in which both PBX1 and MEIS1 interacting domains of HOXD13 are are missing; nevertheless NUP98-HOXD13 strongly collaborates with *Meis1* in leukemogenesis (Pineault *et al*., 2003). These observations suggest homeodomain-dependent and independent activities of MEIS1.

In *MLL* leukemogenesis, *Meis1* plays a key role in induction and maintenance of the leukemia (Wong *et al*., 2007). It was shown that *Meis1* regulates the differentiation block, cycling activity, *in vivo* progression and self-renewal property of *MLL* leukemia cells. Thus, *Meis1* is the important and rate-limiting factor of LSC potential. Studies from murine *Mll-Af9* leukemia models have also demonstrated that *Meis1* is essential for the maintenance of MLL associated leukemias, and that lentivirus short hairpin RNA (shRNA) mediated inhibition of Meis1 induces cell-cycle arrest and cell death in these leukemias (Kumar *et al*., 2009). Besides this, it was shown that MEIS1 is required in leukemogenicity of MN1 (Heuser *et al*., 2011).

1.9 Aim of the study

CALM/AF10 fusion has been reported to be strongly leukemogenic. *CALM/AF10* patients are associated with a bad prognosis. Therefore, it is critical to understand the pathogenesis of *CALM/AF10* leukemias. *CALM/AF10* patients show upregulation of the *HOXA* cluster and *MEIS1* genes, suggesting possible roles for these genes in *CALM/AF10*-mediated leukemogenesis. *MEIS1* strongly collaborates with several *HOX* as well as *NUP98-HOX* genes, and this collaborative effect has been associated with several leukemic signature genes.

Classical transgenic mice expressing the *CALM/AF10* fusion under the control of immunoglobulin heavy chain enhancer-promoter or under the control of proximal *Lck* promoter did not develop leukemia even after an observation period of 15 months. Vav-*CALM/AF10* transgenic mice developed leukemia with a 50% penetrance. These results can only be explained if one assumes that additional collaborating factors or genetic events are required for CALM/AF10-mediated leukemogenesis. The overexpression of Meis1 might be one such collaborating factor. Therefore, the aim of this study is to analyze *Meis1* as a cooperating factor of *CALM/AF10* in leukemogenesis using the IgH-CALM/AF10 transgenic model. We used a murine BMT model to overexpress *Meis1* in these transgenic mice and analyzed the resulting leukemia.

In summary we have shown that CALM/AF10 and Meis1 could collaborate and induce acute myeloid leukemia when expressed in bone marrow cells. The disease was transplantable and represented a lympho-myeloid biphenotypic population. Hence, *Meis1* could be a potential therapeutic target in *CALM/AF10* leukemias.

2 Materials

2.1 Reagents and equipment for mouse work

5-Fluorouracil: 50 mg/ml stock solution Medac, Hamburg, Germany. The working solution was 300 µl of 5-FU stock solution mixed with 700 µl of phosphate buffered saline (3:7).

Formalin: 4% Formalin was prepared using Sodium hydrogen phosphate monohydrate ($NaH_2PO_4.H_2O$), Disodium hydrogen phosphate dihydrate ($Na_2HPO_4.2H_2O$) and 37% Formaldehyde (CH_2O) [Merck] in water.

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

Sterile syringes: BD Plastipak 1 ml syringe (BD Biosciences, Palo Alto, CA, USA) for tail vein injection in mice and Kendall Monoject 3 ml syringes (Tyco Healthcare, UK) for plating of CFCs. The stubs of 3 ml syringes were used to mash the spleens of mice.

Sterile needles: 0.4 mm × 19 mm for intravenous injection of 5-FU and cells in mice. 16 inch × 1.5 inch blunt end needles for dispensing and plating Methocult (CFC) media (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)

Cell Strainer: 40 µm Nylon cell strainer for mashing the spleen and filtering the tissue (BD Falcon, Franklin Lakes, NJ, USA)

2.2 Mammalian cell lines

GP+E86: Mouse fibroblast cell line

293T: Human embryonic kidney cell line

NIH-3T3: Mouse fibroblast cell line
32D myeloid: Mouse myeloid cell line

All cell lines were procured from the American Type Culture Collection (ATCC), Manassas, VA, USA.

2.3 Plasmids

MSCV-IRES-GFP/YFP (MIG/MIY): This is modified form of the MSCV (murine stem cell virus) vector. A bi-cistronic vector with GFP/YFP expression cassette and an internal ribosomal entry site (IRES)

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

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

2.4 Reagents, media and apparatus

2.4.1 Molecular biology

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

LB Medium: LB-broth and LB-agar (Carl Roth GmbH, Germany)

DNeasy Blood and Tissue Kit: Genomic DNA extraction kit (Qiagen GmbH, Hilden, Germany)

EndoFree Plasmid Maxi Kit: For extraction of plasmid from bacteria (Qiagen GmbH, Hilden, Germany)

Gel Extraction Kit: Qiaquick gel extraction kit (Qiagen GmbH, Hilden, Germany)

PCR Purification Kit: Qiaquick PCR purification kit (Qiagen GmbH, Hilden, Germany)

RNeasy Mini Kit: Total RNA extraction kit (Qiagen GmbH, Hilden, Germany)

DNAzol Reagent: Genomic DNA isolation reagent (Invitrogen, Carlsbad, CA, USA)

Trizol: Total RNA isolation reagent (Invitrogen, Carlsbad, CA, USA)

Molecular weight markers: 1 kb plus DNA ladder, 100 bp DNA ladder and 50 bp DNA ladder (Fermentas GmbH, St. Leon-Rot, Germany)

Dyes: 6X Orange DNA loading dye (Fermentas GmbH, St. Leon-Rot, Germany), SYBR Safe DNA Gel Stain (Invitrogen, Carlsbad, CA, USA)

Enzymes: T4 DNA Ligase, *Eco*RI, *Xho*I, *Pst*I from New England Biolabs (NEB, Beverly, MA, USA)

PCR: Taq DNA polymerase, Thermo Pol buffer from New England Biolabs (NEB, Beverly, MA), 0.2 ml PCR tubes (Biozym Scientific GmbH, Hess. Oldendorf, Germany)

Semi-quantitative RT-PCR: ThermoScript RT-PCR System for First-Strand cDNA Synthesis, DNase I DNA inactivating enzyme-Amplification Grade, Platinum Taq DNA Polymerase (Invitrogen, Carlsbad, CA, USA)

dNTP mix: 2 mM dNTP mix (Fermentas GmbH, St. Leon-Rot, Germany

Western blot: ECL Western blotting analysis system (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 fat free milk and 2.0 g dextran sulfate were dissolved in 17 ml water. 6 ml 20X SSC, 2 ml formamide, 1 ml 20% SDS and 80 µl of 500 mM EDTA were added to the above mixture. (The mentioned chemicals were obtained separately from Sigma-Aldrich, St. Louis, MO, USA)

Denaturation solution: 1.5 M NaCl and 0.5 N NaOH in water.

20X SSC: 175.3 g sodium chloride and 88.2 g sodium citrate were dissolved in 800 ml deionized water and the pH was adjusted to neutral (7.0). The final volume was adjusted to one litre.

DNA Crosslinking: DNA was cross-linked using GS Gene linker UV chamber (BIO-RAD Laboratories, Hercules, CA, USA)

2.4.2 Tissue culture

Methylcellulose media: Methocult GF M3434 (myeloid specific) methylcellulose medium with recombinant cytokines for the culture of CFCs (Stem Cell Technologies, Vancouver, Canada)

Media: Dulbecco's Modified Eagle Medium (DMEM) 4.5 g/l Glucose, L-Glutamine, Sodium pyruvate and 3.7 g/l $NaHCO_3$ (PAN biotech GmbH, Aidenbach, Germany).
Roswell Park Memorial Institute (RPMI - 1640) medium with L-Glutamine, 2.0 g/l $NaHCO_3$ (PAN Biotech GmbH, Aidenbach, Germany)

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

Fetal Bovine Serum (FBS): FBS Superior mycoplasma and endotoxin tested (Biochrom AG, Berlin, Germany)

Trypsin: EDTA: 0.05% Trypsin – EDTA (1X) (Gibco, Invitrogen, Carlsbad, CA, USA)

Penicillin/Streptomycin: Antibiotic solution with 10,000 µg/ml Pen G sodium and 10,000 µg/ml Streptomycin sulfate in 0.85% saline. (Gibco, Invitrogen, Carlsbad, CA, USA)

Murine cytokines: mIL3, mIL6, mSCF (lyophilized) (ImmunoTools GmbH, Friesoythe, Germany)

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

Propidium iodide: Propidium iodide solution (Invitrogen, Carlsbad, CA, USA) 10 mg/ml stock solution.

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

Cell Scrapers: 30 cm sterile cell scrapers (TPP, Switzerland)

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

Cell culture plates and dishes: Sterile 96 well, 24 well, 6 well plates (Sarstedt, Numbrecht, Germany) 100 mm × 20 mm dishes for adherent cells (Corning Inc., Corning, NY, USA), and 100 mm × 20 mm tissue culture dish for suspension cells (Sarstedt, Numbrecht, Germany)

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

2.4.3 Miscellaneous

Giemsa's solution: Azur-eosine-methylene blue solution for microscopy (Merck KGaA, Darmstadt, Germany)

May-Gruenwald's solution: Eosine-methylene blue solution modified (Merck KGaA, Darmstadt, Germany)

Cytospin apparatus: Cytospin 2 Shandon Apparatus (Thermo Fisher Scientific, Waltham, MA, USA)

Cytospin slides: Menzel-Glaeser superfrost microscope slides for fixing single cell suspensions and preparing blood smears (Gerhard Menzel GmbH, Braunschweig, Germany)

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

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

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

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

Mice: Parental mice strain were bred and maintained at the Helmholtz and Biocenter animal facility. The donor mice (FVB/N) for primary bone marrow cells were between 10 to 20 weeks old and recipients (FVB/N) were between 8 to 16 weeks old.

Two CALM/AF10 transgenic constructs were used to generate 5 transgenic lines in FVB mice. The two constructs differ in their promoters. One construct has the immunoglobulin heavy chain enhancer promoter (IgHE/P) which expresses CALM/AF10 in the late B-cell compartment. The other construct has the proximal murine *LcK* promoter (pLcK) which drives the CALM/AF10 expression in early T-cell compartment. Two transgenic lines were established with IgHCALM/AF10 construct and three transgenic lines were established using the pLcKCALM/AF10 (Krause, 2006). FVB wild type mouse and IgHCALM/AF10 transgenic line 1 were used as donors. FVB wild type mouse were used as recipients.

2.4.4 Software

Flow cytometry and FACS sorting: CellQuest Pro Version 3.1(f) (BD Biosciences, Palo Alto, CA, USA)

Kaplan-Meier Curves: SigmaPlot Version 12.0 (Systat Software Inc., San Jose, CA, USA)

Colony morphology and identification: Openlab software 3.0.8 (Improvision Deutschland, Tuebingen, Germany)

Primer designing: Primer3 program, Whitehead Institute, Massachusetts Institute of Technology (MIT), MA, USA (*http://frodo.wi.mit.edu/)*

2.5 Oligonucleotides

All the oligonucleotides were synthesized by Metabion AG, Martinsried, Germany.

Primers for DJ_H recombination

Oligonucleotide	Sequence 5' to 3'
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

Cloning primers for HA tagged Meis1

Oligonucleotide	Sequence 5' to 3'
HAMeis1for1	ACGTCCCAGACTACGCTATGGCGCAAAGGTAC
HAMeis1for2	ATGGTCTACCCATATGACGTCCCAGACTAC
HAMeis1for3	GACGAATTCCACCATGGTCTACCCATATG
HAMeis1rev	GGCTCGAGTTACATGTAGTGCCACTGCCCCT

Primers/Oligos for LM-PCR

Oligonucleotide	Sequence 5' to 3'
GFP-A	ACTTCAAGATCCGCCACAAC
GFP-B	ACATGGTCCTGCTGGAGTTC
Vectorette primer 224	CGAATCGTAACCGTTCGTACGAGAATCGCT
Nested Linker Primer B	TACGAGAATCGCTGTCCTCTCCTT
PstI Linker Top	CTCTCCCTTCTCGTCCTCTCCTTCCTGCA
PstI Linker Bottom	GGAAGGAGAGGACGCTGTCTGTCGAAGGTAAGGAACGGAC GAGAGAAGGGAGAG

Primers for semi-quantitative RT-PCR

Oligonucleotide	Sequence 5' to 3'
Meis1_FL_For	ATGGAGTAGGCATCCCCTCCACG
Meis1_FL_Rev	CATGCCCATATTCATGCCCATTCC
β-2microglobin_M_For	TGCTATCCAGAAAACCCCTC
β-2microglobin_M_Rev	CGGCCATAGTGTCATGCTTA
Meis1_ecto_For	TATGAGTGGAATGGGCATGA
Meis1_ecto_Rev	ACATTCAACAGACCTTGCAT
Meis1_endo_For	TATGAGTGGAATGGGCATGA
Meis1_endo_Rev	TGAGGGTGTCCAGGAATGTA

2.6 Antibodies

Name	Company	Label	Dilutions used
Gr-1	BD Pharmingen, Heidelberg	PE/APC	1:200
CD11b (Mac1)	BD Pharmingen, Heidelberg	PE/APC	1:200
Ter119	BD Pharmingen, Heidelberg	PE	1:200
B220	BD Pharmingen, Heidelberg	PE/APC	1:200
Sca-1	BD Pharmingen, Heidelberg	PE	1:200
CD117 (c-kit)	BD Pharmingen, Heidelberg	APC	1:200
CD4	BD Pharmingen, Heidelberg	PE	1:200
CD8	BD Pharmingen, Heidelberg	APC	1:200
Meis1/2 (C-17)	Santa Cruz Biotech. Inc., CA	-	1:200
Donkey Anti-Goat	Invitrogen, Carlsbad, CA	HRP	1:3000

3 Methods

3.1 Mouse Work

3.1.1 Background of Constructs

The murine stem cell virus (MSCV) vector was used for retroviral bone marrow transduction experiment. The MSCV vector has flanking long terminal repeat (LTR) sequences, an internal ribosomal entry site (IRES) and a green or yellow fluorescent protein gene (GFP/YFP). The IRES aids co-expression of the fluorescent protein. The MSCV vector is a gene vector and is replication defective i.e. within the cell it is not able to replicate and infect other cells. Retroviral vectors are used for making stable packaging cells. The advantage of retroviral vector is its long term expression through integration. The gene of interest was sub-cloned into the multiple cloning site of the MSCV vector. MSCV IRES GFP (MIG) empty vector was used as control for the experiment (Fig. 3.1.1).

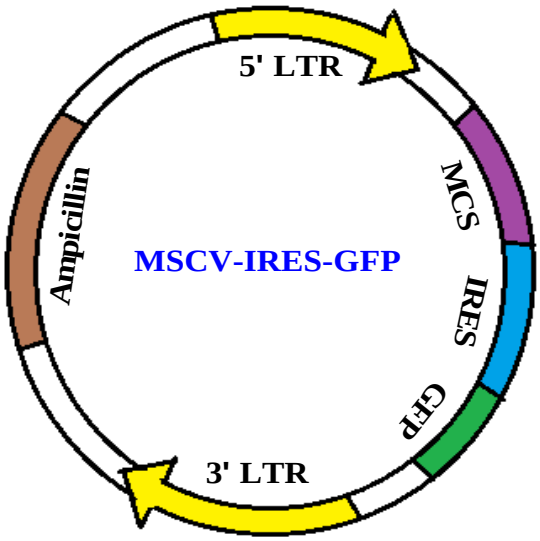


Fig 3.1.1 A schematic representation of the MIG (MSCV IRES GFP) empty vector control used for bone marrow transplantation experiments: MCS: multiple cloning site; IRES: internal ribosomal entry site; GFP: green fluorescent protein; LTR: long terminal repeat sequences.

3.1.2 Cloning details

The 1.2 kb *Meis1* gene was sub-cloned into the multiple cloning site of the MIG vector using the enzymes *Eco*RI and *Xho*I (Vegi, 2009). The 5.2 kb *CALM/AF10* full length gene was sub-cloned into the *Hpa*I site in the multiple cloning site of the MIG vector by blunt end ligation (Deshpande, 2006). The 1.0 kb *CALM/AF10-MF* gene was sub-cloned into the multiple cloning site of the MIG vector using the enzymes *Eco*RI and *Bam*HI (Deshpande, 2006). These constructs were made by Dr. Naidu Vegi (MIY-Meis1) and Dr. Deshpande (MIG-CALM/AF10).

3.1.3 Preparation of high titre stable virus producing cell lines

High titre stable virus producing cell lines E86-Meis1 and E86-MIG were provided by Naidu (Vegi, 2009). The E86-CALM/AF10 full length and E86-CALM/AF10-MF cell lines were kindly provided by Aniruddha (Deshpande, 2006).

3.1.3.1 Methodology

On the first day, 1.2×10^6 293T cells were seeded on a 15 cm dish and used for transient transfection the following day. The medium was changed 4 hours prior to transfection. 30 µg of plasmid DNA of the gene of interest and the retroviral packaging construct Ecopac were added to sterile water to make up the volume to 1 ml. To the above water-DNA mixture, 100 µl of 2.5 M $CaCl_2$ was added drop wise. This mixture was then added slowly into a tube containing 1 ml sterile HBS pH 7.2. The tube was gently mixed and incubated at room temperature for 3-4 minutes. Then the mixture was added drop wise to a 15 cm dish plated with 293T cells. The dish was carefully placed in a 37ºC incubator. The medium was changed after 12 hours. The virus conditioned medium (VCM) was collected after every 12 hours from the cells. The VCM was then filtered with a 0.45 µm Millipore filter and stored at -80ºC for later use or used directly to transduce GP+E86 fibroblasts or murine bone marrow (Schessl *et al*., 2005)

5×10^4 GP+E86 fibroblasts were plated into 6 well plates. On the next day retroviral transduction was performed. For the transduction, 500 µl or 1 ml of fresh or frozen VCM from transfected 293T cells was layered on top of GP+E86 cells along with 10 µg/ml protamine sulfate. Fresh medium was added to the cells after 4 hours and the transduction procedure was repeated every 12 hours for four times. The cells were expanded for two days

after final transduction to allow GFP expression. The cells expressing GFP were sorted using fluorescence activated cell sorter (FACS) and propagated. These cells were used as stable virus producing cell lines to transduce murine bone marrow (Schessl *et al*., 2005).

3.1.3.2 Viral titre of GP+E86 cell lines

Titration was performed for the E86 cell lines, which were transduced with a specific virus, to estimate the virus production capacity of the cell line. If the viral titres of bulk cell lines were low after transduction, single cells were sorted into 96 well plates for expansion. After expansion, their viral titres were determined on NIH-3T3 cells. Clones producing highest titres were expanded, frozen and used for experiments.

3.1.3.3 Procedure

On the day zero, 1×10^5 NIH-3T3 cells were plated per well in 6 well plates. On the following day, one well was layered with 50 µl VCM from the E86 Meis1 cell line**,** the second well with 500 µl VCM and the third well was used as control containing the NIH-3T3 without VCM. 10 µg/ml Protamine sulfate was added along with VCM. After 4 hours fresh medium was added. The cells were incubated for 48 hours post transduction. After incubation the cells were analyzed for GFP expression at the FACS Calibur.

The NIH3T3 cells which were transduced with VCM were GFP positive cells. The percentage of GFP positive cells transduced with 50 µl VCM as well as 500 µl VCM was 15 % as determined by FACS Calibur. This indicates that out of 1×10^5 NIH-3T3 cells plated, only 15000 cells could be transduced with virus. However, the usual titre during the experiments ranged from 40 % to 70 % for E86 Meis1.

3.1.4 Retroviral transduction of primary bone marrow

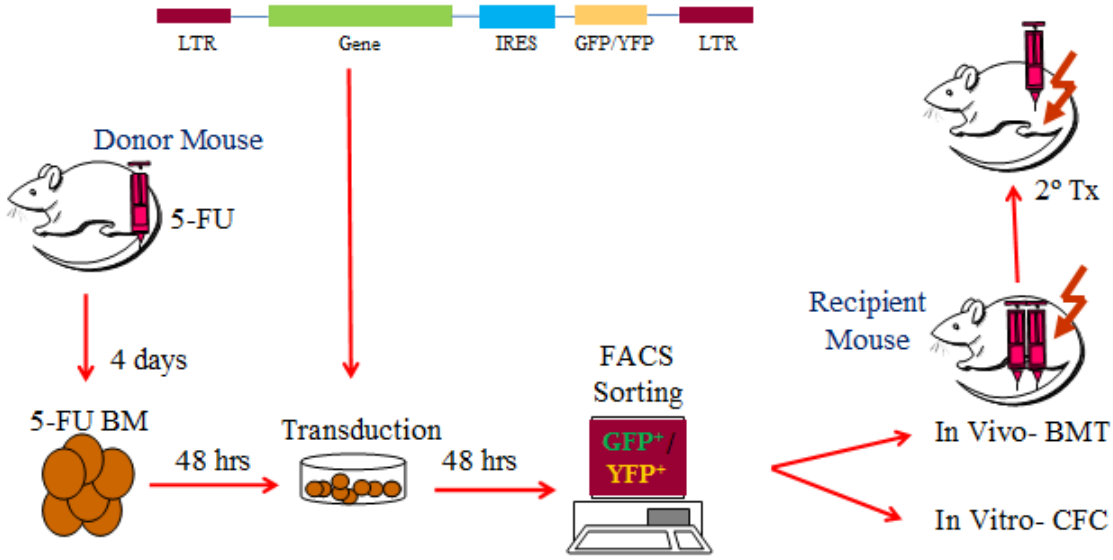


Fig 3.1.4 Schematic representation of bone marrow transplantation model: 5-FU (5-Fluorouracil) treated bone marrow cells were transduced with the gene of interest and sorted after 48 hours for GFP/YFP positive cells using Fluorescence activated cell sorting. The positive cells were either injected into lethally irradiated recipient mice or used for functional assay like colony forming cell assay. Bone marrow cells from primary leukemic mice were further transplanted into secondary recipients.

3.1.4.1 Bone Marrow Transplantation Model

The murine bone marrow transplantation model employs *ex vivo* retroviral gene transfer of primary hematopoietic cells followed by transplantation into lethally irradiated syngeneic mouse recipients. The purpose of this model is to directly assess the oncogenic potential of the targeted gene. The application of this model extends to identification of new proto-oncogenes and mechanisms of leukemic transformation.

The FVB/N mouse strain was used for our experiments. These mice were maintained at the Haematologikum animal house and Biocenter animal (Martinsried) facility. The mice were provided with autoclaved chow and supplied with drinking water containing ciprofloxacin and acetic acid. The bone marrow donor mice were between 10 and 20 weeks old. Donor mice were injected with 90 milligrams of 5-Fluorouracil (5-FU) per kg of body weight to eliminate the cycling cells and to enrich for the hematopoietic progenitor cells. Four days

after 5-FU treatment, the bone marrow cells were extracted from these mice by the crushing method. In this method, the bones were crushed in serum supplemented phosphate buffered saline using a pestle and mortar to extract the bone marrow cells. The cells were sieved twice in 0.45 micron filters to remove cell debris. After sieving, the bone marrow cells were treated with ammonium chloride for red blood cell lysis. The lysed cells were then washed with serum supplemented phosphate buffered saline and used for experiments. The bone marrow cells were cultured for two days with a cytokine cocktail (10 ng/ml mIL6, 6 ng/ml mIL3 and 100 ng/ml mSCF) in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 15% FBS.

The GP-E86 ecotrophic packaging cell lines were irradiated with 4000 cGy one day prior to the retroviral transduction. On the third day, the bone marrow cells were overlaid on gamma-irradiated GP-E86 cell lines containing the retroviral construct (co-culture). 10 µg/ml protamine sulfate was added as a crosslinker to the medium during viral transduction. The transduced bone marrow cells were removed carefully without disturbing the adherent monolayer of GP-E86 cell line 36 hours post transduction. On the seventh day, the GFP/YFP positive cells were sorted by FACSVantage and used for bone marrow transplantation or for *in vitro* assays (Fig. 3.1.4).

3.1.4.2 Bone marrow transplantation and assessment of mice

Strategy:

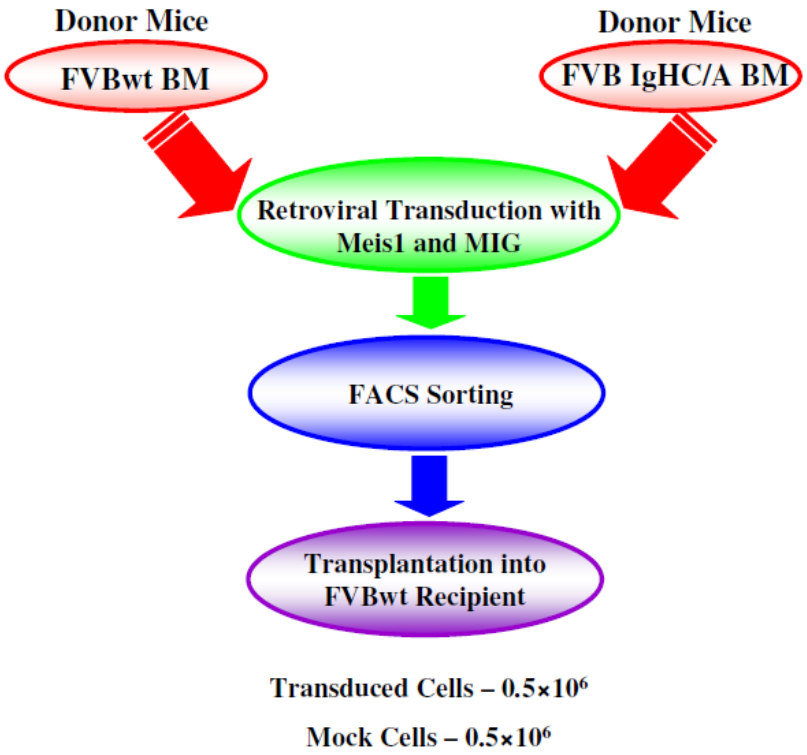


Fig 3.1.4.2 Schematic representation of BMT strategy: FVBwt mice and IgHCALM/AF10 transgenic line1 mice were used as donors for the experiment. The donor bone marrow (BM) cells were retrovirally transduced with E86 cell lines containing MSCV-IRES-YFP-Meis1 (MIY-Meis1 ; experimental arm) or MSCV-IRES-GFP (MIG ; control arm) containing retroviral particles. The transduced cells were sorted using flow cytometry. 0.5 × 10^6 of GFP/YFP positive cells and 0.5 × 10^6 of mock cells were injected into lethally irradiated syngeneic recipient mice.

The recipient mice were between 8 and 16 weeks old FVB wild type mice. These mice were lethally irradiated with 800 cGy prior to transplantation. Retrovirally transduced bone marrow cells were injected together with mock transduced cells intravenously into the tail vein of the recipient mice using a sterile 0.4 mm × 19 mm needle (Fig. 3.1.4.2). For secondary and tertiary transplantations, bone marrow cells from leukemic mice were injected the same way with or without irradiation. The transplanted mice were kept in individually vented cage (IVC) systems. The mice were assessed at regular intervals for leukemic symptoms by blood withdrawal from the tail vein using sterile scalpels or by the observance

of symptoms that included crouching, frizzled body hair, paleness of the feet, heavy breathing and disturbed gait. Mice were considered moribund when one of these symptoms was observed.

Moribund mice were sacrificed by CO_2 asphyxiation. Peripheral blood was drawn with a sterile 0.4 mm × 19 mm needle by puncturing the heart immediately after sacrificing the mouse. The femurs, tibia and spleens were taken from these mice. The bones were crushed to obtain bone marrow cells. Spleens were macerated to produce single cell suspensions. The white blood cells (WBC) and red blood cells (RBC) counts were made per ml of peripheral blood and peripheral blood smears were prepared. Ammonium chloride buffer was used to lyse red blood cell (RBC) for peripheral blood, bone marrow and spleen cells by incubating the cells in this buffer for 20 minutes at 4ºC. After lysis, the cells were washed in serum supplemented phosphate buffered saline and used for cytospin and flow cytometric analysis. The remaining cells were frozen at -80ºC for later use.

3.1.5 Flow cytometric analysis of murine cells

Bone marrow, spleen and peripheral blood cells were immunostained with various fluorescence-conjugated antibodies. Unstained cells were used as control. Staining was performed in PBS with the fluorescence-conjugated antibodies using a 1:200 dilution for each antibody. After incubation at 4ºC for 20 minutes, the samples were washed with PBS to remove excess antibody. The cells were finally resuspended in FACS buffer (2% fetal bovine serum and 5 µg/ml propidium iodide in phosphate buffered saline). Antibodies used for flow cytometry were labeled with phycoerythrin for Gr-1, CD11b (Mac1), Ter119, Sca-1, CD4 and allophycocyanin for CD11b (Mac1), B220, CD117 (c-kit) and CD8. Fluorescence was detected using a FACS Calibur flow cytometer and analyzed using the CellQuest Pro software. Dead cells were gated out using PI staining and forward scatter (FSC).

3.1.6 *In vitro* assay (Colony Forming Cell assay)

The Colony Forming Cell (CFC) assay is an *in vitro* assay, which is used to quantify different multi-potential and lineage-restricted progenitors from primary bone marrow cells. The assay is based on the ability of hematopoietic progenitors to proliferate and differentiate into colonies in semi-solid media in response to cytokine stimulation. Semi-solid media allow the clonal progeny of a single progenitor cell to stay together and thus to be recognized as

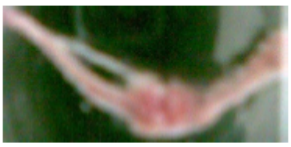
distinct colonies. The colonies formed can be enumerated and characterized according to their unique morphology.

Methylcellulose has several advantages over other semi-solid media such as it is an inert polymer, has good optical clarity, provides better growth for erythroid colonies and multipotent progenitors can be assayed simultaneously in the same culture dish. Methylcellulose supplemented with cytokines 10 ng/ml mIL-6, 6 ng/ml mIL-3 and 100 ng/ml mSCF (Methocult M3434) was used to analyse the differentiation of clonogenic progenitors by plating the primary cells in this media. Methocult M3434 is myeloid specific and optimized for the detection and quantification of mouse hematopoietic progenitors in bone marrow, spleen, peripheral blood and fetal liver samples. M3434 supports the optimal growth of erythroid progenitors (CFU-E, BFU-E), granulocyte-macrophage progenitors (CFU-GM, CFU-G, CFU-M) and multipotential granulocyte, erythroid, macrophage, megakaryocyte progenitors (CFU-GEMM). Bone marrow cells transduced with different retroviral constructs expressing various genes and oncogenes were assayed for their colony forming capacity.

The bone marrow cells were obtained from 5-FU injected donor mouse. The cytokine supplemented bone marrow cells were then retrovirally transduced with several genes of interest. The transduced cells were then sorted for GFP or YFP positivity using FACS. 500 sorted cells were seeded for primary plating and 1000 cells for secondary and tertiary replating. The methylcellulose plates were incubated at 37ºC in presence of 5% CO_2 and humidity for 10 days. Colony identification, counting and replating was performed on the 10^{th} day in appropriate dilutions followed by cytospin preparations and FACS staining (Fig. 3.1.6a; Fig. 3.1.6b). The experiments were performed in triplicates.

CFC Assay

5 fluorouracil injected mouse



Bone marrow

step 1

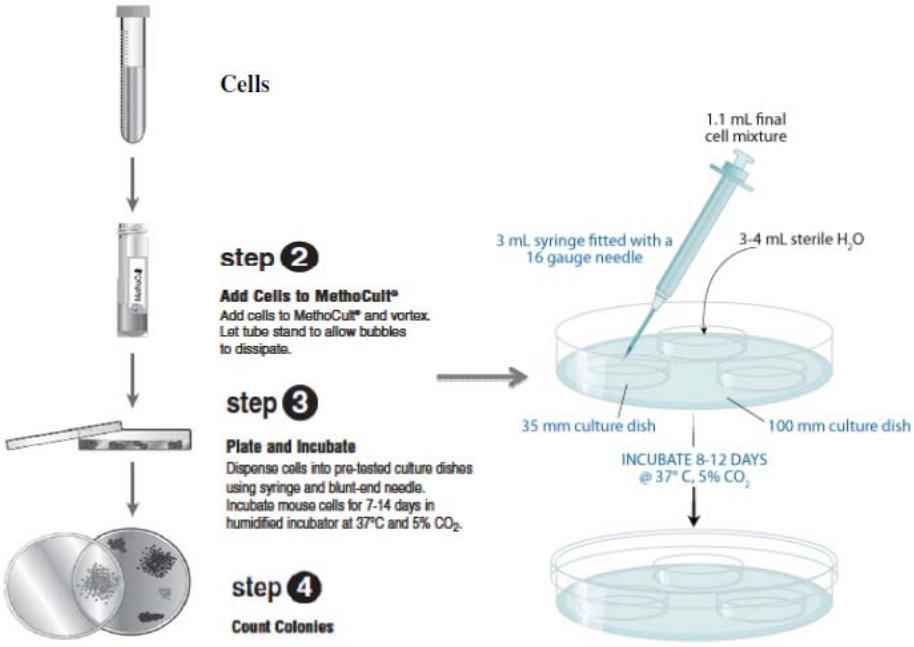
Prepare Cells

FACS

BM cells + cytokines

FACS Sorting

Screened for their transformed phenotype by CFC assay



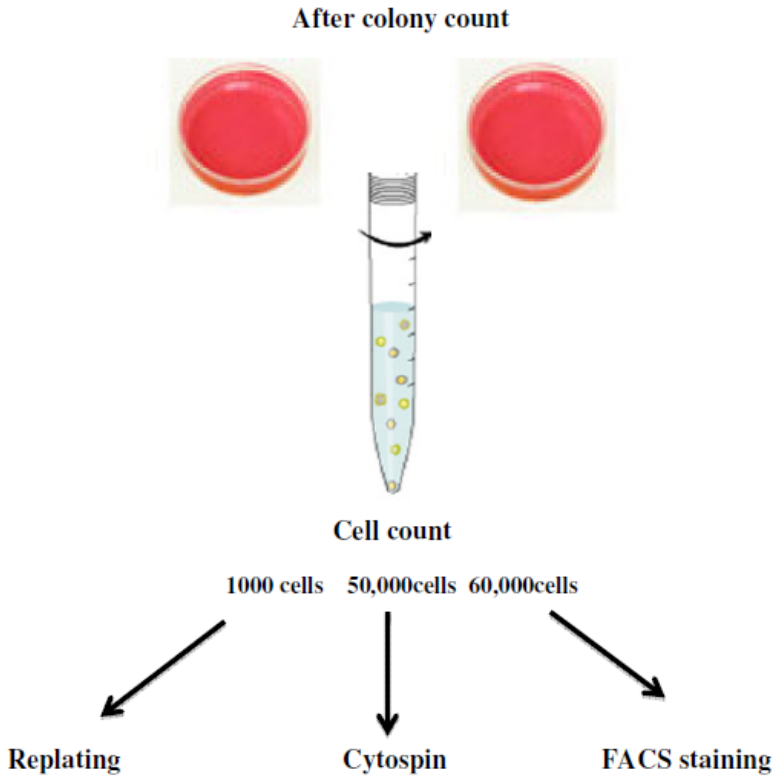


Fig. 3.1.6a Colony Forming Cell assay: The 5-fluorouracil injected donor bone marrow cells were retrovirally transduced with the gene of interest and sorted using FACS. The sorted cells were then added to methocult, mixed by vortexing and plated on culture dishes using syringe and blunt-end needle. The plates were then incubated for 10 days in humidified incubator at 37ºC and 5% CO_2. After 10 days the colonies were identified and counted. The culture dishes containing the methylcellulose were washed thoroughly with pre-warmed phosphate buffered saline. The individual cells were then harvested by centrifugation. 1000 cells were used for replating, 50,000 cells for cytospin preparations and remaining cells were stained with fluorescence-conjugated antibodies and analysed using FACS Calibur.

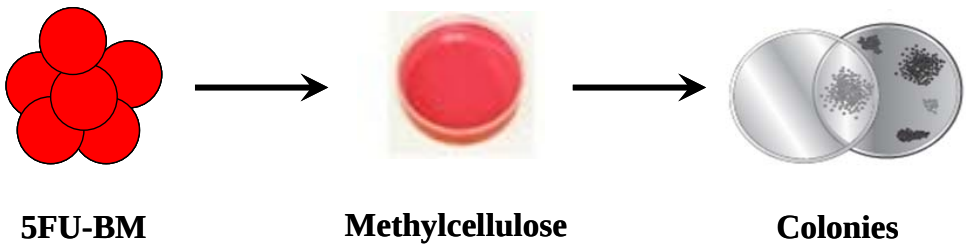


Fig. 3.1.6b Schematic representation of the *in vitro* CFC assay: 5-FU (5-Fluorouracil) treated bone marrow (BM) cells were transduced with the gene of interest. The transduced cells were FACS sorted and plated in

methylcellulose media. 500 cells were seeded for primary plating and incubated at 37ºC in humidified CO_2 incubator. The colonies were identified and enumerated after an incubation period of 10 days.

3.1.7 Different types of colonies were visible in primary CFC assay

Primary CFC assay for all the above mentioned genes led to formation of different types of colonies in the methylcellulose plates. The different types of colonies include colony forming unit-granulocyte (CFU-G), colony forming unit-macrophage (CFU-M), colony forming unit-granulocyte/macrophage (CFU-GM), burst forming unit-erythroid (BFU-E) and colony forming unit-granulocyte / erythroid / macrophage / megakaryocyte (CFU-GEMM).

3.1.7.1 Salient properties of different colony forming units

CFU-G: A CFU-G contains at least 20 granulocyte cells. The colony consists of mature, lineage committed progenitors and the cells are round, bright, smaller and uniform in size (Fig. 3.1.7.1a).

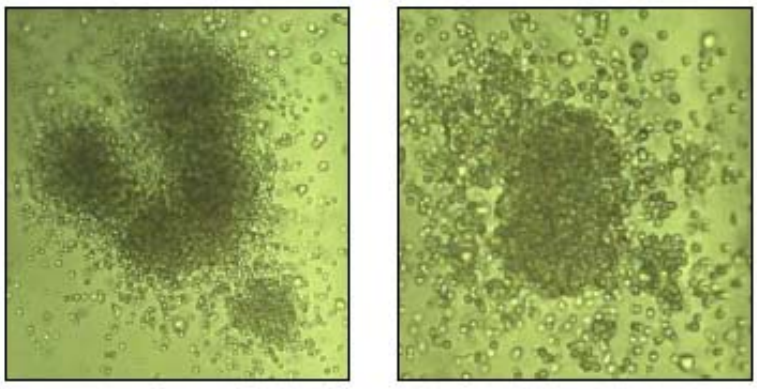


Fig. 3.1.7.1a Colony forming unit – Granulocyte: Distinct, small and uniform sized granulocytes are clearly visible in the centre as well as in the periphery.

CFU-M: A CFU-M contains at least 20 macrophage cells. This type of colony also consists of mature, lineage committed progenitors and the cells are large with an oval to round shape and appear to have a grainy or grey centre (Fig. 3.1.7.1b).

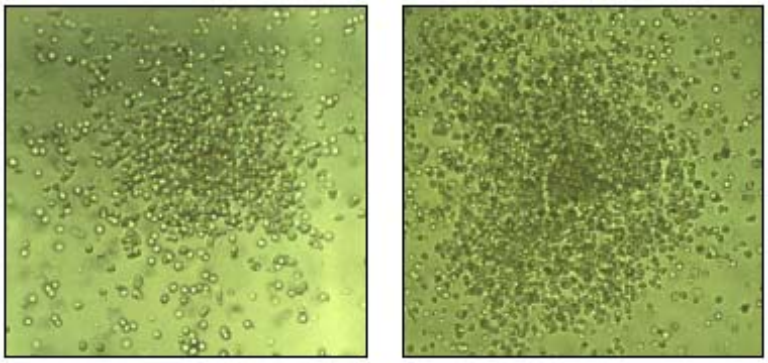


Fig. 3.1.7.1b Colony forming unit – Macrophage: Large, refractile and well separated macrophage cells with grey centre.

CFU-GM: A CFU-GM contains at least 30 granulocyte and macrophage cells. These colonies have a dense core surrounded by cells and often contain multiple cell clusters. The individual cell can be indentified and are easy to distinguish. Large, round and refractile macrophages as well as small, round, uniform granulocytes are clearly visible at the periphery (Fig. 3.1.7.1c).

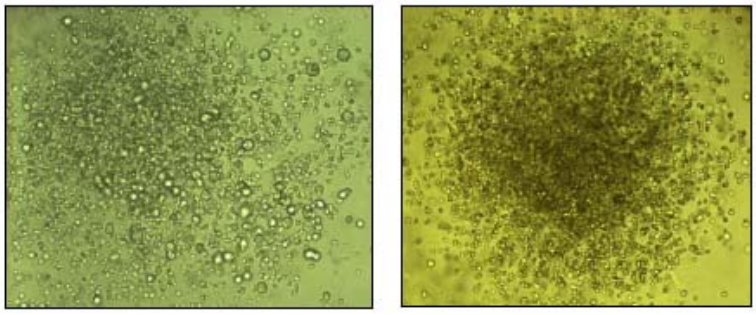


Fig. 3.1.7.1c Colony forming unit – Granulocyte/Macrophage: Granulocyte and macrophage cells are clearly visible and appear in a stardust pattern.

BFU-E: BFU-E colonies are immature and require erythropoietin (EPO), Interleukin 3 (IL-3) and stem cell factor (SCF) for their optimal growth. The colony contains a minimum of 30 cells in a cluster. The cells are tiny and difficult to distinguish as they appear to be fused together (Fig. 3.1.7.1d).

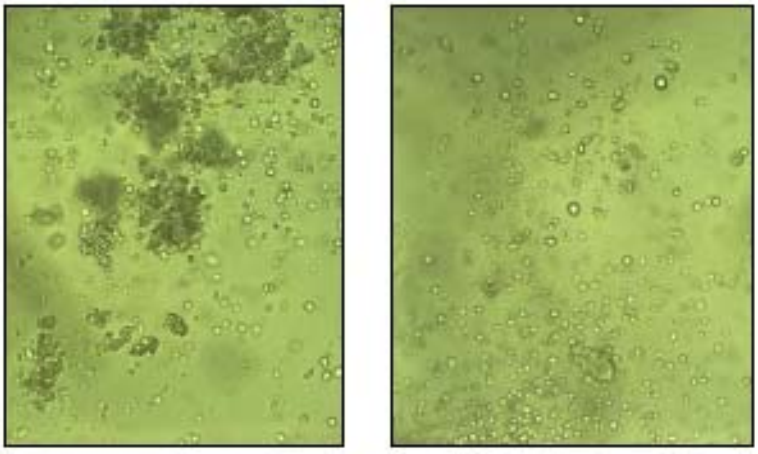


Fig. 3.1.7.1d Burst forming unit – Erythroid: The cells are small and fused together in a grape-like structure. Unlike granulocyte the BFU-E cells are difficult to distinguish.

CFU-GEMM: CFU-GEMM represents the multi-potential progenitor that consists of erythroid and myeloid cluster. The colonies are generally large and consist of more than 500 cells. CFU-GEMM colonies usually have a dense core with an indistinct border between the core and the peripheral cells (Fig. 3.1.7.1e).

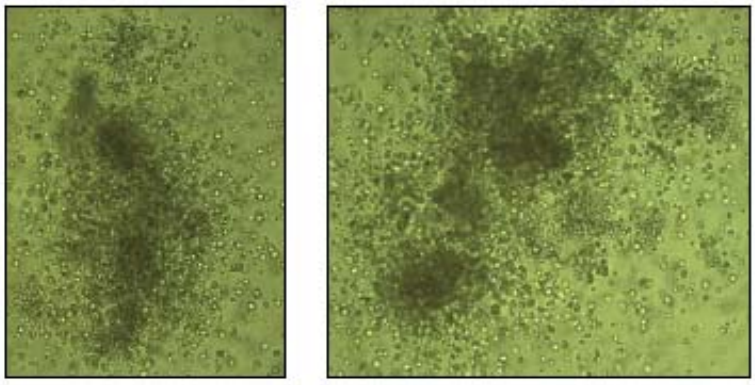


Fig. 3.1.7.1e Colony forming unit – Granulocyte/ Erythroid/Macrophage/Megakaryocyte: Both the myeloid and erythroid cells are present and has a compact centre. The CFU-GEMM colonies might have more than one cluster. The erythroid cells are present in the centre and the myeloid cells are clearly visible in the periphery.

CFU-Blast: CFU-Blast represents the early immature precursor cells in the hematopoietic lineage. The blast colonies are very compact and are mostly visible in secondary and tertiary CFC (Fig. 3.1.7.1f).

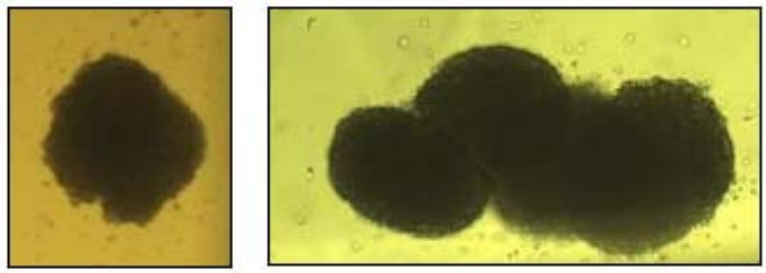


Fig. 3.1.7.1f Colony forming unit - Blast: Blast cells represent the precursor cells which are immature. The blast cells are morphologically are very compact.

3.1.7.2 CFC Replating

The proliferative potential of the primary colony was tested by replating the primary colonies into secondary and tertiary CFC. After identification and enumeration of the primary CFC colonies, the primary plates containing the methylcellulose were washed thoroughly two to three times with pre-warmed phosphate buffered saline in order to get rid of the methylcellulose. The individual cells were then harvested and resuspended in 1 ml phosphate buffered saline. The number of living cells were counted using trypan blue. 1000 cells were used for secondary replating. The remaining cells were used for cytospin preparations and for staining with fluorescence-conjugated antibodies and analysed using FACS Calibur. The same method was used to harvest the cells from secondary and tertiary replatings (Fig 3.1.7.3).

3.1.7.3 Strategy:

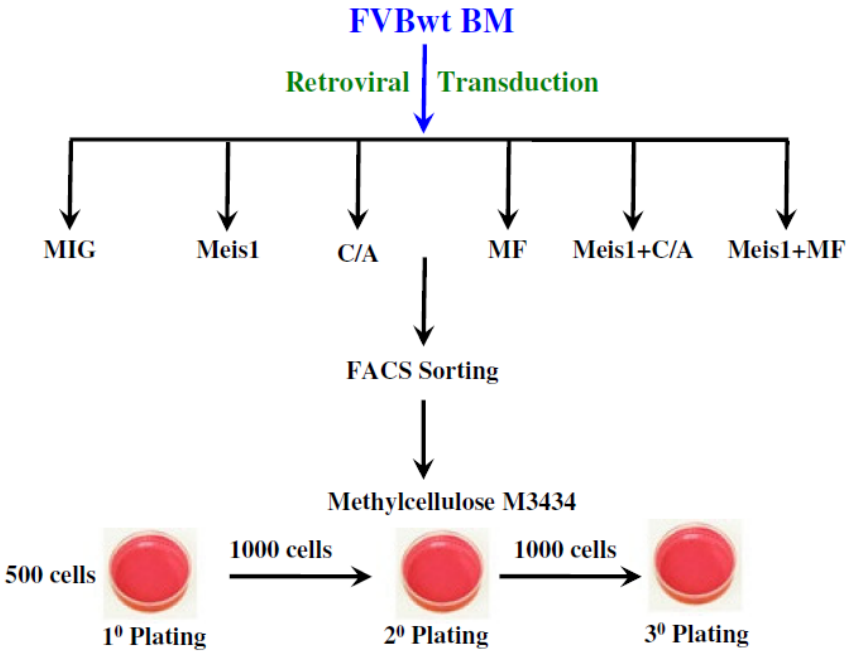


Fig 3.1.7.3 Schematic representation of CFC Assay: BM cells were extracted from 5-FU treated FVBwt donor mice. The primary BM cells were transduced with different retroviral constructs taking MSCV-IRES-GFP (MIG) as control arm, MSCV-IRES-YFP-Meis1, MSCV-IRES-GFP-CALM/AF10 full length (C/A), MSCV-IRES-GFP-CALM/AF10-Minimal Fusion (MF), Meis1+C/A and Meis1+MF. The transduced cells were FACS sorted and plated in methylcellulose media. 500 cells were seeded for primary plating and 1000 cells for secondary and tertiary replating.

3.1.8 Cytospin preparations and Wright Giemsa staining

The cytospin technique uses low-centrifugal force to separate and deposit a monolayer of cells onto a defined area of a slide. Thus, the cells are concentrated for good nuclear presentation and proper identification.

Cytospins of single cell suspensions were performed by resuspending the cells in DMEM medium at a concentration of 1×10^5 cells per 300 µl. This was introduced into the cytospin apparatus and centrifuged at 450 rpm for 10 minutes. The cells were permanently fixed on glass slides and the slides were air-dried overnight.

In order to carry out the Wright Giemsa staining, the slides were immersed in an undiluted May-Gruenwald's stain for 3 minutes. In the next step the slides were rinsed in water for 5

minutes in order to remove excess stain. Then the slides were immersed in freshly prepared 1:50 diluted Giemsa stain for 1 hour. The slides were again rinsed in water by dipping in and out several times and keeping in water for 5 minutes. After the rinsing process, the slides were air-dried overnight and observed under a light microscope for morphology.

3.1.9 Histopathological analysis of sick mice

The sick mice were sacrificed by CO_2 asphyxiation and cervical dislocation. The sacrificed mice were sprayed with 70% ethanol. The peritoneal cavity of sacrificed leukemic mice was dissected to expose all the organs. Most of the blood was drained by cutting the peritoneal artery and absorbed with a tissue paper. The dissected mice along with a cut portion of their spleens were fixed in 4% formalin. The fixed mice were sent for histopathological analysis to Dr. Leticia Quintanilla-Fend, Institute for Pathology, Tuebingen.

3.2 Microbiology Techniques

3.2.1 Bacterial Cultures and glycerol stocks

Bacterial cells from glycerol stock were streaked on a LB-agar plate containing the required antibiotic. After incubation for approximately 12-14 hours at 37ºC, a single colony was picked from the agar plate and inoculated in LB medium containing the required antibiotic. The medium containing the inoculum was incubated for 12-14 hours at 37ºC in a shaker at 200 rpm.

For preparation of glycerol stocks, 850 µl of bacterial culture was mixed with 150 µl of glycerol and immediately stored at -80ºC.

3.2.2 Electrocompetent bacteria

Electrocompetent bacterial cells of *E. coli* strain XL-1 blue (Stratagene) were prepared according to Sambrook and Russel, 2001. A single colony was picked and transferred to 10 ml of LB medium. The medium containing the inoculum was incubated overnight in a shaking incubator at 37ºC and 200 rpm. This primary culture was diluted into 400 ml LB medium and incubated for approximately 2-3 hours at 37ºC until the OD_{600} reached 0.5-0.6.

The culture was transferred on ice in order to stop the bacterial growth. The cells were centrifuged at 4500 rpm for 5 minutes at 4ºC. Then the cells were washed twice with 40 ml of ice cold water (double distilled). In the next step, the cells were washed twice with 20 ml of 10% glycerol and finally resuspended in 800 µl of 10% glycerol. The competent cells were aliquoted (50 µl) and snap frozen in liquid nitrogen. The cells were then stored at -80ºC.

3.2.3 Electroporation

10 pg of DNA was used for electroporation. 50 µl of electrocompetent bacteria were thawed on ice for 2-3 minutes. In the meantime, cuvette was kept on ice. The DNA and electrocompetent bacteria were mixed carefully by flicking the tube. After one minute incubation on ice, the DNA and bacteria were transferred to the electroporation cuvette (2 mm electrode gap). The cuvette was placed in an electroporator (Easyjet Prima, Equibio) and the cells were electroporated at 2.5 KV (12.5 kV/cm, 15 µF, 335 Ω, 5 ms pulse duration). After electroporation, 1 ml of LB medium was added immediately to the cells and the content was transferred to a fresh 1.5 ml eppendorf tube from the cuvette. The 1.5 ml eppendorf tube was incubated at 37ºC shaker at 200 rpm for 1 hour. After 1 hour incubation, the bacteria were plated on LB agar plates containing the appropriate antibiotic. The LB agar plates were incubated at 37ºC overnight for selection of transformed bacteria.

3.3 Molecular biology

3.3.1 RNA and genomic DNA isolation and cDNA preparation

The RNA extraction was performed using RNeasy Mini Kit (Qiagen). The kit includes a denaturing guanidine isothiocyanate containing buffer for cell lysis and a silica gel based membrane for RNA isolation. A maximum of 1×10^7 cells were used for RNA extraction. The RNA was extracted as per the manufacturer's instructions. The concentration of RNA was quantified with a spectrophotometer and also by running on an agarose gel.

For cDNA synthesis, Thermoscript RT-PCR Kit from Invitrogen was used. The RNA samples were treated with Deoxyribonuclease I to remove genomic DNA contamination. The cDNA was prepared according to manufacturer's instructions for semi-quantitative PCRs.

Genomic DNA was extracted using DNeasy Mini kit for the DJ_H rearrangement PCRs, and DNAzol was used to extract genomic DNA for Southern blotting and LM-PCR as described by the manufacturer. Genomic DNA was quantified using a QUBIT Fluorimeter.

3.3.2 Plasmid DNA extraction

Plasmid DNA was extracted from bacteria by alkaline lysis method using Qiagen Endonuclease-free Maxi kit as per the manufacturer's instructions.

3.3.3 Agarose gel electrophoresis

To determine the size of DNA fragments, estimate DNA concentration, DNA fragment extraction or analysis of PCR reaction products, horizontal agarose gel electrophoresis was performed. The agarose concentration of the gel was between 0.8 to 1.5% depending on the expected size of the DNA bands. SYBR Safe DNA Gel Stain was added to the agarose to allow DNA visualization in the gel under UV light. The electrophoresis was carried out in 0.5X TAE buffer at room temperature and at a voltage range of 70 to 100 V.

3.3.4 Extraction of DNA fragments from agarose gel

For gel extraction of PCR products or enzymatically digested DNA, the desired DNA band from the gel was cut out under UV light using a sterile surgical blade. The gel extraction was performed using QIAquick Gel Extraction kit according to the manufacturer's instructions.

3.3.5 PCRs

3.3.5.1 PCR for D-J recombination status

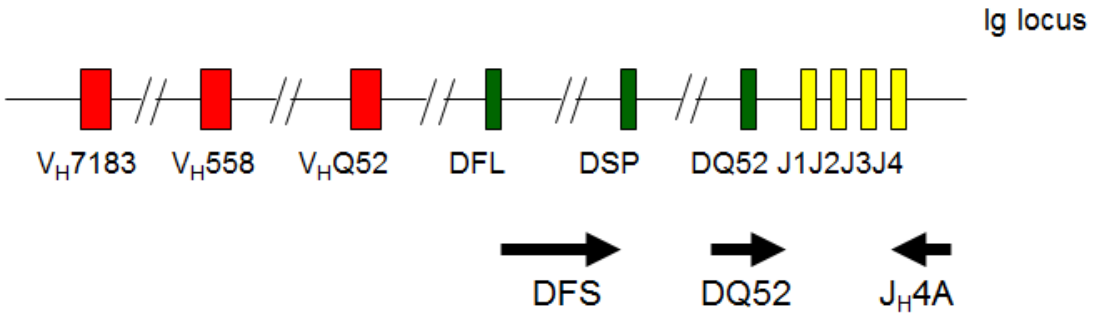


Fig 3.3.5.1 Diagrammatic representation of immunoglobulin heavy chain locus: DJ_H rearrangement in the Immunoglobulin locus (Ig locus) was detected by multiplex PCR strategy. In this PCR, two degenerate forward

primers DFS and DQ52 and one reverse primer J_H4A are used. With this strategy the most common DJ_H rearrangement events are detected.

DJ_H rearrangements in the Immunoglobulin locus were detected by a multiplex PCR strategy. This strategy employs 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 J_H4 segment. All the three primers mentioned were used in a single PCR reaction. For the germline configuration, the DQ52 and J_H4A primers were used to amplify a 2.15 kb germline fragment. DJ_H1, DJ_H2, DJ_H3 and DJ_H4 rearrangements involving DFL, DSP or DQ52 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 94ºC for 1 minute followed by 35 cycles of 1 minute at 94ºC, I minute at 60ºC and 1 minute 45 seconds at 72ºC. Final extension was carried out at 72ºC for 10 minutes. One µl of 300 ng/µl genomic DNA was used as template in this PCR reaction.

3.3.5.2 PCR to evaluate gene expression in murine tissues

Semi-quantitative reverse transcriptase PCR was performed on leukemic bone marrow, spleen and peripheral blood RNA to confirm the expression of ectopic (proviral) and endogenous *Meis1* and the *CALM/AF10*. The mouse β-2 microglobin housekeeping gene was used for normalization.

3.3.5.3 LM-PCR (Linker-mediated PCR)

The LM-PCR is used to identify the retroviral integration sites. This was adapted to allow amplification of the 3' end of integrated MIG virus from the GFP gene through the 3' LTR into the adjacent genomic DNA to the next *Pst*I site, which was ligated to the *Pst*I bubble linker. The genomic DNA (1 µg) from leukemic mice was digested with *Pst*I and the fragments were ligated to the bubble linker at room temperature. In the following step a PCR was performed (PCR A) on 10 µl of the ligation product using Vectorette primer 224 and a GFP primer (GFP-A). The bubble linker contains a 30-nucleotide non-homologous sequence in the middle which prevents binding of the Vectorette primer in the absence of the minus strand generated by the GFP primer. 1 µl of the PCR A reaction product (one-fifteenth) was used as template for a second nested PCR (PCR B) using a primer GFP-C and a Nested

Linker Primer B. 10 µl (one-half) of the final PCR B product was then separated by electrophoresis using 2% agarose gel. Individual bands were then excised, purified and then cloned into pGEM-T Easy vector and sequenced using Nested Linker Primer B for the integration site of the retrovirus (Riley *et al*., 1990; Schessl *et al*., 2005).

3.4 Western Blotting

3.4.1 Sample preparation and cell lysis (total cell extract)

Ice-cold PBS was added to the cultured cells on 10 cm dish after removing the media. The cells were then scraped off and transferred to a microcentrifuge tube and centrifuged at 2000 rpm for 5 minutes at 4ºC. The pellet was washed with ice-cold PBS. The cells were lysed using 100 µl of RIPA buffer (1X PBS, 1% TritonX 100, 0.5% sodium deoxycholate, 0.1% SDS) with protease inhibitor cocktail (100 mM PMSF, 10 mg/ml Aprotinin, 10 mg/ml Leupeptin, 10 mg/m, Pepstatin) by mixing them in a roller for 30 minutes at 4ºC. After the lysis, the sample was centrifuged at 14000 rpm for 30 minutes at 4ºC. The resulting supernatant was transferred to a new microcentrifuge tube and either frozen at -80ºC or kept on ice for the determination of the protein concentration.

3.4.2 Determination of protein concentration

The Bradford method was used for measuring the protein concentration. The assay is based on the shift of absorbance maxima for an acidic solution of Coomassie Brilliant Blue G-250 from 465 nm to 595 nm. This shift is due to protein binding. Both hydrophobic and anionic interactions stabilize the anionic form of the dye, causing a visible color change. In this assay, the extinction coefficient of a dye-albumin solution is constant over a 10-fold concentration range. Dilutions of Bovine Serum Albumin (BSA) of known concentrations were used to establish a standard curve. The linear range of the assay was from 1 µg to 25 µg per ml. Six different BSA concentrations were prepared by diluting 1 µg, 5 µg, 10 µg, 15 µg, 20 µg, 25 µg in 800 µl of distilled water. One microliter of protein extract was diluted in distilled water to make a final volume of 800 µl. 200 µl of Bradford reagent was added to the tubes and mixed well by vortexing. The contents were transferred to polystyrol cuvettes. A determination of the standard curve of the spectrophotometer with distilled water and the

protein standards was performed using the specific program for protein in the spectrophotometer. The samples were measured following the standard curve determination.

3.4.3 SDS PAGE

Total cell extract proteins were separated on a denaturing gel consisting of 10% resolving gel and 5% stacking gel. The percentage of resolving gel was selected based on the molecular weight of protein. The samples were diluted 1:1 with 2X Laemmli buffer and incubated at 95ºC for 5 minutes. 20 µg of protein was loaded on each lane. The electrophoresis was initially performed at 70 V for three hours in the cold room at 4ºC.

3.4.4 Wet transfer

The wet transfer system was used for protein blotting. A PVDF membrane was used for transfer. The membrane was wetted in methanol for 30 seconds, rinsed in distilled water for 5 minutes and equilibrated in transfer buffer for 10 minutes. The system was assembled putting a sponge at both ends of the sandwich, 1.5 mm Whatman paper in contact with the sponge and the gel over the paper towards the negative pole. A pipette was rolled over the gel to remove air bubbles. The membrane was placed carefully on the gel and the cassette was closed. The PVDF membrane was towards the positive pole to permit the protein (negatively charged) to migrate from the gel to the membrane. The transfer was performed overnight at 100 mA at 4ºC with ice pack and constant stirring of the transfer buffer for keeping the system homogeneously cool. The observation of high molecular weight proteins of the pre-stained protein standard on the membrane was an indicator of successful transfer.

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

After the transfer, the antibody detection of protein was performed as per the instructions of the supplier (Santa Cruz Biotech. Inc., CA, USA; Invitrogen, Carlsbad, CA, USA). The membrane was blocked with 5% milk powder (blocking solution) to prevent non-specific binding of antibodies by incubating in a roller for one hour at room temperature. The membrane was then rinsed with TBST and incubated with primary antibody at 1:200 dilution in milk for 1 hour at room temperature. After incubation with primary antibody, the membrane was washed three times with TBST (0.1% Tween-20). The secondary antibody

conjugated to Horse Radish Peroxidase (HRP) was diluted 1:3000 in milk and put on the membrane for one-hour incubation at room temperature. The membrane was washed three times again with TBST. To detect the antibodies on the membrane, the ECL Plus Western Blotting Detection Kit was used according to the manufacturer's instructions. After washing, the ECL detection solution was placed on the membrane for 3 minutes. Two solutions (Solution A and Solution B) from this kit were used in a ratio of 1:40 for detection of protein on the membrane. The membrane was then covered with plastic film and put in a cassette for exposure of the film. The film was exposed to the membrane in a dark room with different exposure times of between 5 seconds to 1 minute depending on the strength of the signal observed.

3.5 Cell culture techniques

3.5.1 Culture of cells

The mammalian cells were cultivated in CO_2 incubators at 37ºC, 5% CO_2 and 95% relative air humidity. The culture media were supplemented with 15% fetal bovine serum (FBS) and penicillin-streptomycin (final concentration of Penicillin: 100 U/ml and Streptomycin: 100 µg/ml).

The adherent cell lines were grown in complete Dulbecco's Modified Eagle Medium (DMEM). The cells were harvested with Trypsin-EDTA to detach the cells from the surface of the plate. The trypsinized cells were either used for subculturing or for preparation of frozen stocks.

4 Results

4.1 Protein expression of Meis1 in GP+E86 (GP+E86 Meis1) retroviral producer cell line

In order to determine the integrity and proper expression of the Meis1 protein, Western blotting was performed on the whole cell lysate of the GP+E86 Meis1 cell line. This retroviral producer cell line was used for the transduction of murine bone marrow cells. The GP+E86 MIG and native E86 cell lines were used as negative controls for Western blotting. A protein of 53 kDa molecular weight, the expected size for Meis1, was clearly observed in the GP+E86 Meis1 cell line only, confirming the expression of Meis1 in this cell line (Fig. 4.1).

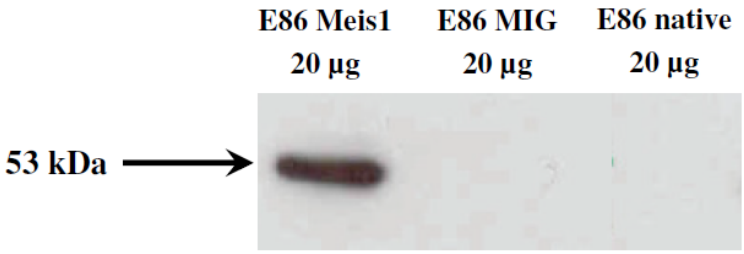


Fig. 4.1 Expression of Meis1 in the retroviral producer cell line GP+E86 Meis1: The expression of Meis1 was observed by Western blotting of the whole cell lysate from the GP+E86 Meis1 cell line, which was used for the transduction of murine bone marrow cells in the experiments described in this work. The retroviral producer cells line containing the empty retrovirus (E86 MIG) or the parent cell line (E86 native) did not express the Meis1 protein.

4.2 Determining whether Meis1 expression cooperates with CALM/AF10 in the transformation of hematopoietic cells

Since IgH-CALM/AF10 transgenic mice do not develop leukemia, our goal was to determine whether certain factors might cooperate with CALM/AF10 to induce leukemia development. We selected Meis1 as a potential co-operating factor of CALM/AF10 because Meis1 is known to collaborate with Hox fusion gene and because Meis1 is highly expressed in

CALM/AF10-positive human leukemia cells. In order to determine the collaborative effect, we performed *in vitro* CFC assay and developed *in vivo* mouse models. For our experiments we used stable retroviral producer cell lines GP+E86 expressing different genes under the control of the strong viral LTR promoter. We established a CALM/AF10 + Meis1 model by transplanting lethally irradiated recipient mice with IgH-CALM/AF10 transgenic bone marrow cells transduced with the Meis1 expressing retrovirus. We complemented these models with CFC assays, in which wild type bone marrow cells were co-transduced with CALM/AF10, CALM/AF10 minimal fusion (MF) and Meis1 expressing retroviruses.

4.2.1 *In vitro* – Colony Forming Cell (CFC) Assay

The CFC assay determines the colony forming capabilities of hematopoietic cells on methylcellulose. Cells transduced with CALM/AF10 alone do not behave differently in the CFC assay from cells transduced with an empty retrovirus (Deshpande *et al.*, 2011). Therefore, our hypothesis was that co-expression of Meis1 with CALM/AF10 might transform hematopoietic cells *in vitro*. Thus we performed the CFC assays with cells transduced with Meis1 alone, CALM/AF10 alone, CALM/AF10-minimal fusion (MF), CALM/AF10 with Meis1 and MF with Meis1 containing retroviruses. As control we have used cells transduced with empty vector (MIG) containing retroviruses. The viruses which were produced from several GP+E86 cell lines are schematically presented in Fig. 4.2.1.

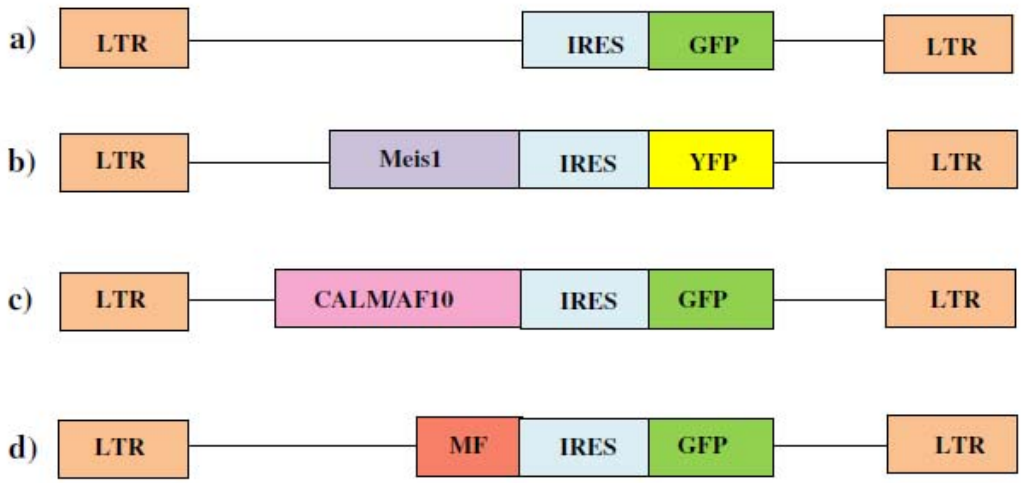


Fig. 4.2.1 Schematic representation of the retroviral constructs used for bone marrow transplantation experiments: a) empty vector control (MIG), b) *Meis1*, c) full length *CALM/AF10* and d) *CALM/AF10-minimal*

fusion (MF). LTR: Long terminal repeat sequences. IRES: Internal ribosome entry site, GFP: Green fluorescent protein, YFP: Yellow fluorescent protein.

Mouse bone marrow cells were retrovirally co-transduced with the Meis1 retroviruses (yellow fluorescence) and one of the other two retroviruses (CALM/AF10 and MF; green fluorescence) produced by these different cell lines. The retrovirally transduced cells were double-sorted for GFP and YFP positivity and 500 GFP/YFP double-positive cells were plated in myeloid specific methylcellulose based semi solid media (Methocult M3434). In case of bone marrow cells retrovirally transduced with Meis1 alone were sorted for YFP positivity, and the bone marrow cells transduced with CALM/AF10 alone, MF alone and empty vector (MIG) were sorted for GFP positivity. After an incubation period of 10 days at 37ºC in a humidified CO_2 incubator, the colonies were identified and enumerated.

4.2.1.1 Primary CFC assay

After 10 days, the colonies were analyzed and enumerated. Since we used myeloid specific methocult media, the growth of cells belonging to the myeloid compartment such as granulocytes, macrophages and erythroid colonies was supported.

In these primary CFC assays, the mean total colonies for CALM/AF10 was 168 (±58), Meis1 184 (±54) and Meis1+CALM/AF10 198 (±52) CFU/500 input cells as compared to MIG 108 (±9) CFU/500 input cells (the experiments were performed in triplicates). Whereas, the mean frequency of colonies for the MF and Meis1+MF arms were 164 (±7) and 144 (±45) CFU/500 input cells, respectively (n=3). The total number of colonies in all the experimental arms was higher than the MIG control arm, but the difference was not significant (Fig. 4.2.1.1a).

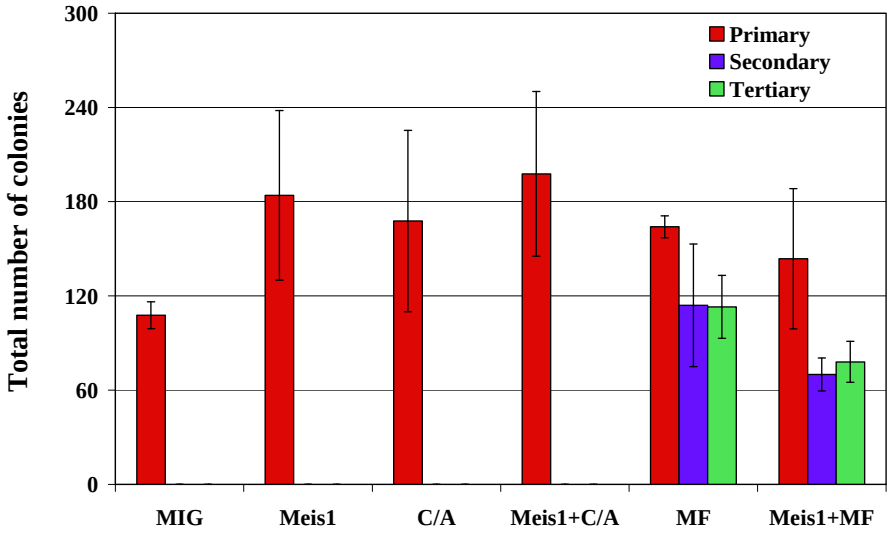


Fig. 4.2.1.1a Graphical representation of total number of colonies in CFC assay: Bar graph showing the total number of colonies observed in methylcellulose plates using different retroviral constructs: empty vector control EGFP (MIG), Meis1, CALM/AF10 (C/A), Meis1 in combination with CALM/AF10 (Meis1+C/A), CALM/AF10-minimal fusion (MF) and Meis1 in combination with MF (Meis1+MF). MF and Meis1+MF could be replated into secondary and tertiary CFC. The experiments were performed in triplicates (n=3).

In the primary CFC assay cells transduced with viruses expressing Meis1 alone, Meis1 in combination with CALM/AF10 or in combination with the CALM/AF10-minimal (MF) fusion showed a slightly higher number of CFU-GM than empty vector control (MIG). However the difference was not statistically significant. There was no difference in the proportion of G, M, GEMM, BFU-E or blast like colonies under these experimental conditions. Interestingly, only the co-expression of Meis1 and the MF resulted in a significant increase in the number of CFU-blast colonies 37 (±20) in the primary CFC assay. This increase in blast colonies was not seen when either of the genes was expressed alone hinting at a collaboration of Meis1 with the CALM/AF10 minimal fusion protein in the transformation of primary hematopietic cells in this assay system. (Fig. 4.2.1.1b; Fig. 4.2.1.1c).

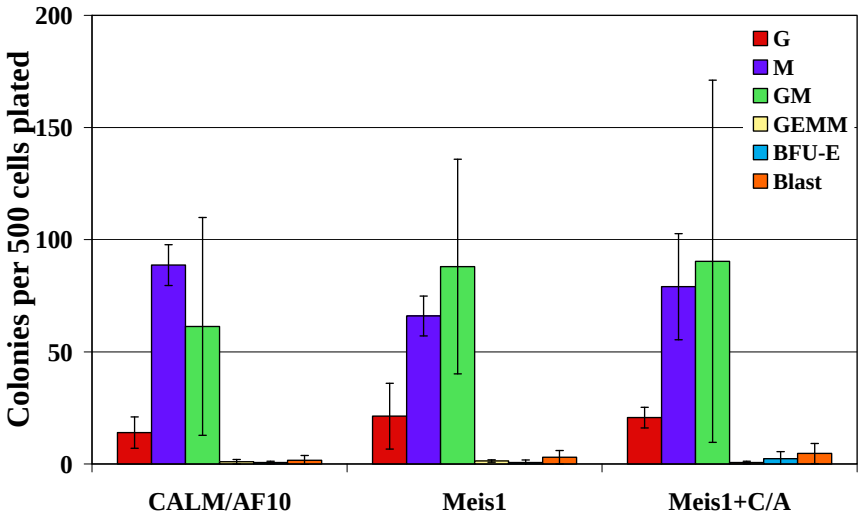


Fig. 4.2.1.1b Graphical representation of different types of colonies enumerated per 500 cells plated in primary CFC assay: Bar graph showing the different types of colonies observed in CALM/AF10, Meis1 and Meis1 along with CALM/AF10 (Meis1+C/A) primary transduced bone marrow cells. G: Granulocyte, M: Macrophage, GM: Granulocyte/Macrophage, GEMM: Granulocyte / Erythroid / Macrophage / Megakaryocyte, BFU-E: Burst Forming Unit–Erythroid, Blast: Precursor cells. The experiments were performed in triplicates (n=3).

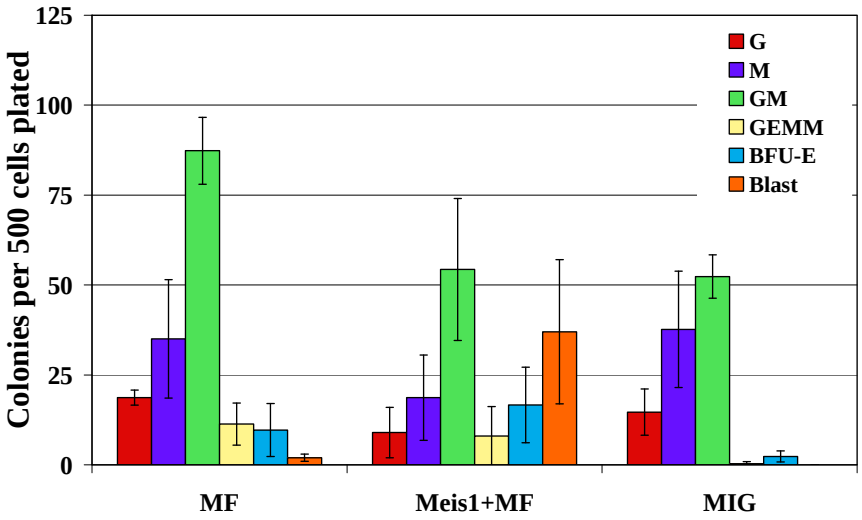


Fig. 4.2.1.1c Graphical representation of different types of colonies enumerated per 500 cells plated in primary CFC assay: Bar graph showing the different types of colonies observed in CALM/AF10-MF (MF), Meis1 along with MF (Meis1+MF) and empty vector control MIG primary transduced bone marrow cells. G: Granulocyte, M: Macrophage, GM: Granulocyte/Macrophage, GEMM: Granulocyte / Erythroid / Macrophage / Megakaryocyte, BFU-E: Burst Forming Unit–Erythroid, Blast: Precursor cells. The experiments were performed in triplicates (n=3).

4.2.1.2 Secondary and tertiary CFC assay (Replating)

The proliferative potential of the primary colony was tested by replating the primary colonies into secondary CFC. The colonies from the primary plates were collected into single cell suspensions by multiple washings with pre-warmed phosphate buffered saline. The washed cells were then counted and 1000 cells were used per plate in the secondary plating. The same procedure was performed for the third replating.

Surprisingly, secondary colonies were visible only in the MF and Meis1+MF experimental arms. Primary colonies from the other experimental arms, which included CALM/AF10, Meis1, Meis1+CALM/AF10, and the MIG control, did not replate. Immature blast cells (CFU-blast) were clearly visible in the secondary replating of MF (37 (±8)) and Meis1+MF (40 (±7)) transduced bone marrow cells (Fig. 4.2.1.2a). The cells transduced with MF and Meis1+ MF did also replate a third time. The morphology of the colonies visible in the second and third replating was blast-like. There were 44 (±12) CFU-blast colonies per plate in the MF and 51 (±4) CFU-blast colonies in the Meis1+MF arm in the third replating. Thus, the blast cell counts were higher in the third than in the second replating of MF and Meis1+MF transduced bone marrow cells (Fig. 4.2.1.2b). There was no increase in the total number of colonies in secondary and tertiary CFC replating (Fig. 4.2.1.1a), but there was a slight increase in proportion of blast like colonies, especially in the Meis1+MF transduced cells and a reduction in colonies with a GM morphology in the third replating.

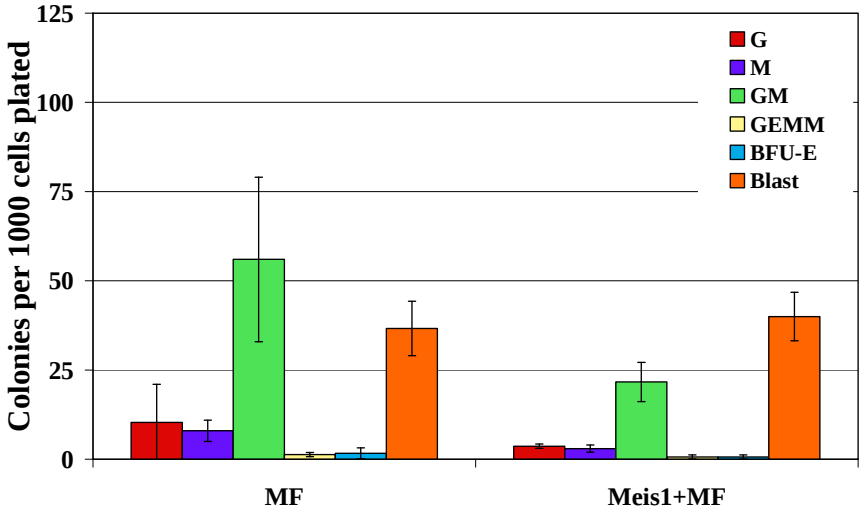


Fig. 4.2.1.2a Graphical representation of different types of colonies enumerated per 1000 cells plated in the second replating assay: Bar graph showing the different types of colonies observed in secondary replating

of CALM/AF10-MF (MF) and Meis1 along with MF (Meis1+MF). G: Granulocyte, M: Macrophage, GM: Granulocyte/Macrophage, GEMM: Granulocyte / Erythroid / Macrophage / Megakaryocyte, BFU-E: Burst Forming Unit–Erythroid, Blast: Precursor cells. The experiments were performed in triplicates (n=3).

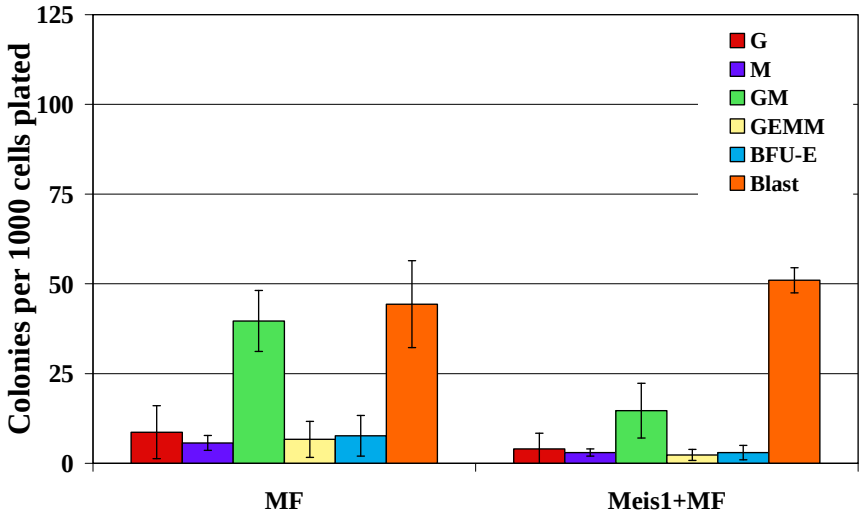


Fig. 4.2.1.2b Graphical representation of different types of colonies enumerated per 1000 cells plated in third replating assay: Bar graph showing the different types of colonies observed in tertiary replating of CALM/AF10-MF (MF) and Meis1 along with MF (Meis1+MF). G: Granulocyte, M: Macrophage, GM: Granulocyte/Macrophage, GEMM: Granulocyte / Erythroid / Macrophage / Megakaryocyte, BFU-E: Burst Forming Unit–Erythroid, Blast: Precursor cells. The experiments were performed in triplicates (n=3).

4.2.1.3 Flow cytometric analyses of cells obtained from CFC assays

The cells were harvested from primary, secondary and tertiary CFC plates and immunostained with various fluorescence-conjugated antibodies such as Gr1 (granulocyte), Mac1 (macrophage), Ter119 (erythroid), B220 (B-cells), Sca1 and cKit (stem cell marker) to determine the surface marker of on these cells. The cells were then analyzed in a flow cytometer (FACS Calibur).

The mature marker Gr1 was more prominent in the cells transduced with CALM/AF10 (52% (±16%)) and MIG (53% (±4%)) (Fig. 4.2.1.3a). Cells derived from CFC plates with MF transduced cells were positive for Sca1 50% (±24%) and cKit 45% (±14%) (Fig. 4.2.1.3b). The B220 staining was slightly higher on Meis1 transduced cells (12% (±7%)) than on MIG transduced cells or in the other experimental arms (Fig. 4.2.1.3c).

The Sca1/cKit stem cell marker was most prominent in MF and Meis1+MF transduced cells with 40% (±8%) and 36% (±7%), respectively, compared to the other experimental arms in the secondary replating (Fig. 4.2.1.3d). In tertiary replating the proportion of Sca1/cKit positive cells increased both in the MF and Meis1+MF transduced cells with 47% (±8%) and 45% (±7%) being positive for these markers, respectively. (Fig. 4.2.1.3e). However, there was not significant difference in the Sca1/cKit staining pattern between MF and Meis1+MF transduced cells.

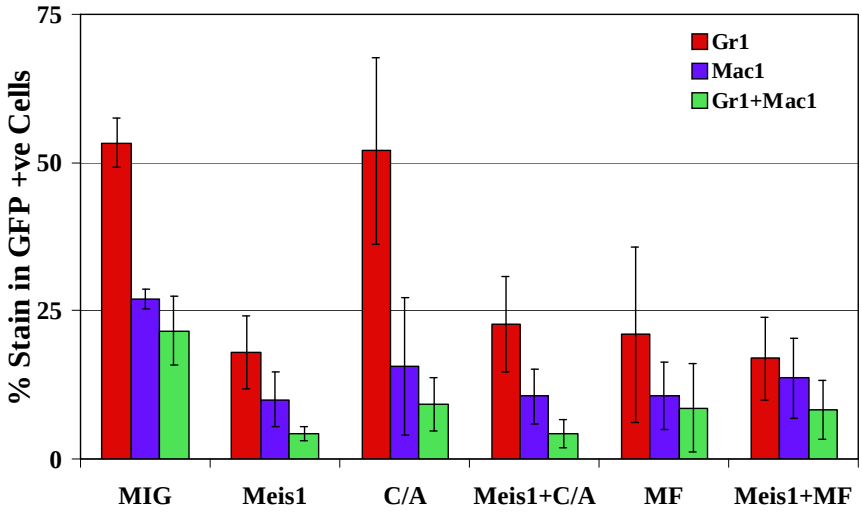


Fig. 4.2.1.3a Graphical representation of granulocyte and macrophage staining for different experimental arms in primary CFC assay: Bar graph showing the percentage stains for primary CFC of empty vector control MIG, Meis1, CALM/AF10 (C/A), Meis1 in combination with CALM/AF10 (Meis1+C/A), CALM/AF10-minimal fusion (MF) and Meis1 in combination with MF (Meis1+MF) for granulocyte (Gr-1), macrophage (Mac-1) and granulocyte/macrophage (Gr-1+Mac-1) in GFP positive cells. The data presented here are from three independent experiments (n=3).

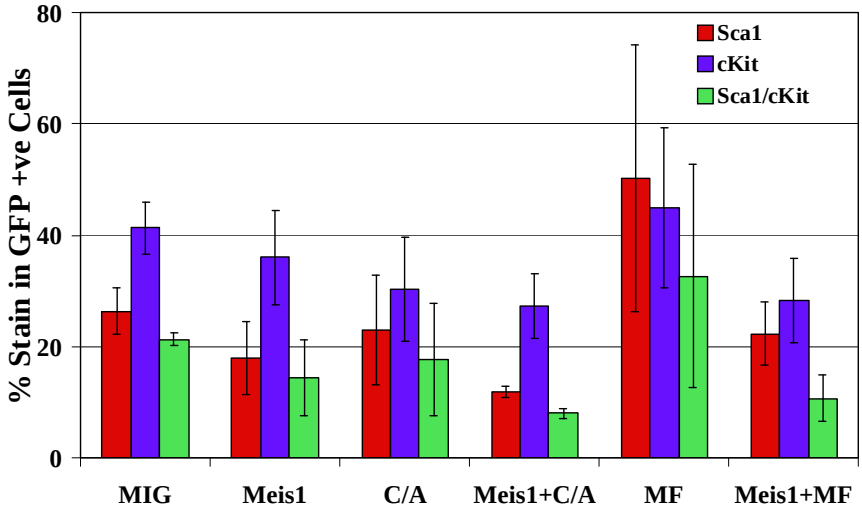


Fig. 4.2.1.3b Graphical representation of Sca-1 and cKit staining for different experimental arms in the primary CFC assay: Bar graph showing the percentage stains for primary CFC of empty vector control MIG, Meis1, CALM/AF10 (C/A), Meis1 in combination with CALM/AF10 (Meis1+C/A), CALM/AF10-minimal fusion (MF) and Meis1 in combination with MF (Meis1+MF) transduced cells for Sca-1, c-Kit and Sca-1/c-Kit. The transduced cells were identified by GFP fluorescence. The data presented here are from three independent experiments (n=3).

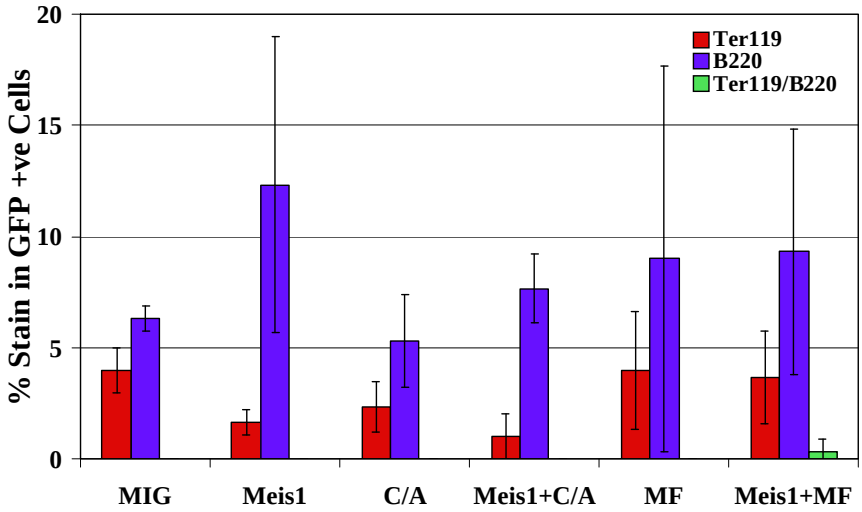


Fig. 4.2.1.3c Graphical representation of Ter-119 and B220 staining for the different experimental arms in the primary CFC assay: Bar graph showing the percentage stains for primary CFC of empty vector control MIG, Meis1, CALM/AF10 (C/A), Meis1 in combination with CALM/AF10 (Meis1+C/A), CALM/AF10-minimal fusion (MF) and Meis1 in combination with MF (Meis1+MF) transduced cells for Ter-119, B220 and Ter-119/B220. The transduced cells were identified by GFP fluorescence. The data presented here are from three independent experiments (n=3).

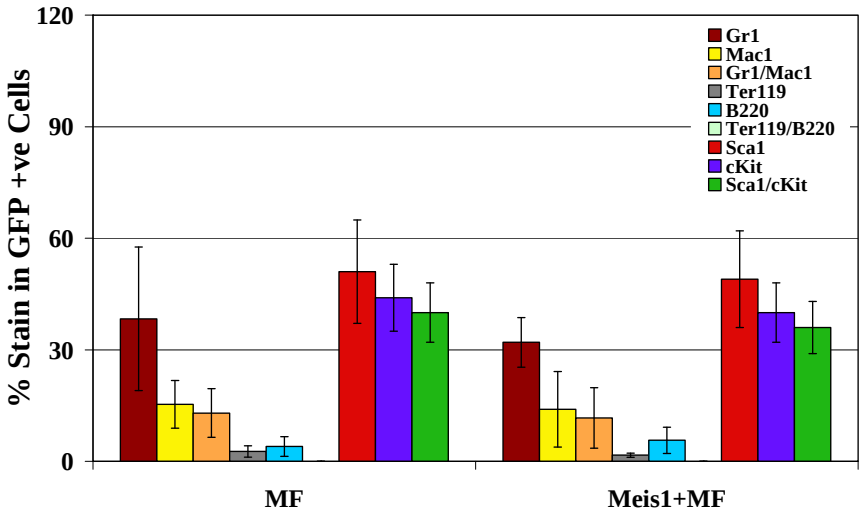


Fig. 4.2.1.3d Graphical representation of different surface markers present on cells of MF and Meis1+MF in the secondary CFC assay: Bar graph showing the percentage stains for secondary CFC of CALM/AF10-minimal fusion (MF) and Meis1 in combination with MF (Meis1+MF) for granulocyte (Gr1), macrophage (Mac1), granulocyte/macrophage (Gr1/Mac1), erythrocytes (Ter119), B cells (B220), Ter119/B220, stem cell markers (Sca1, cKit and Sca1/cKit) in GFP positive cells. The data presented here are from three independent experiments (n=3).

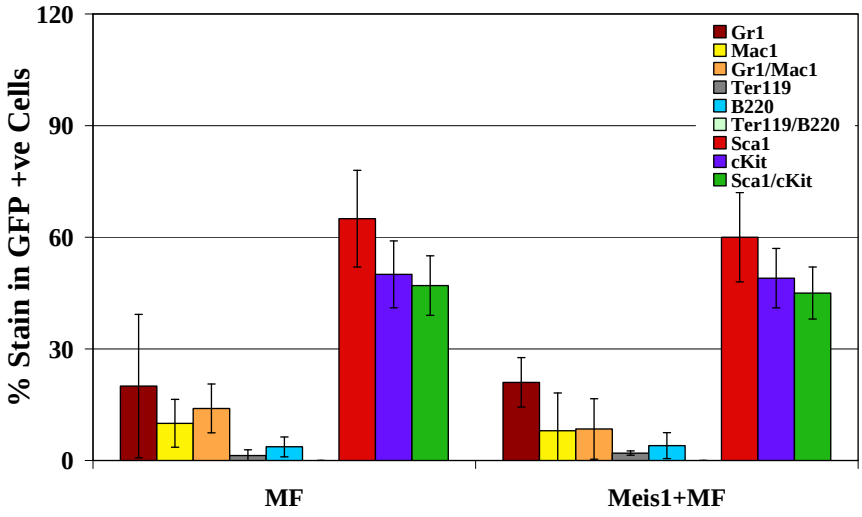


Fig. 4.2.1.3e Graphical representation of different surface markers present on cells of MF and Meis1+MF in tertiary CFC assay: Bar graph showing the percentage stains for tertiary CFC of CALM/AF10-minimal fusion (MF) and Meis1 in combination with MF (Meis1+MF) for granulocyte (Gr1), macrophage (Mac1), granulocyte/macrophage (Gr1/Mac1), erythrocytes (Ter119), B cells (B220), Ter119/B220, stem cell markers (Sca1, cKit and Sca1/cKit) in GFP positive cells. The data presented here are from three independent experiments (n=3).

4.2.2 Meis1 collaborates with the CALM/AF10 fusion gene in a murine bone marrow transplantation leukemia model

The murine bone marrow transplantation model employs *ex vivo* retroviral gene transfer of primary hematopoietic cells followed by transplantation of the transduced cells into lethally irradiated syngeneic mouse recipients. The purpose of this model is to assess the oncogenic potential of a gene of interest. Using this model it is also possible to identify new proto-oncogenes and understand the detailed mechanism of leukemic transformation. The advantage of this model is that the leukemia develops in an intact organism in the presence of growth factors and the proper micro-environment. The bone marrow transplantation model is widely used to increase our understanding of leukemogenesis Moreover, because of its longer duration, in the bone marrow transplantation model additional mutational events (e.g. point mutations or the consequences of the retroviral integration) can occur which might be required for full leukemia development. Thus, in comparison with the CFC assay the bone marrow transplantation model is a more realistic model to study the effect of oncogenes in the hematopoietic compartment. In this work, we combined the bone marrow transplantation model with a transgenic model in order to study the collaborative effects of Meis1 overexpression with the CALM/AF10 fusion gene in leukemia development.

To analyze whether *Meis1* is a collaborating factor for the *CALM/AF10* fusion gene in leukemia development, lethally irradiated recipient mice were transplanted with IgH-CALM/AF10 transgenic bone marrow cells transduced with a Meis1 expressing retrovirus. As controls lethally irradiated recipient mice were transplanted with IgH-CALM/AF10 transgenic bone marrow cells transduced with the empty retrovirus (MIG, expressing EGFP (enhanced green fluorescent protein) only) or with wildtype bone marrow cells transduced with a Meis1 expressing retrovirus. As additional controls, mice transplanted with wildtype bone marrow cells transduced with the empty retrovirus (MIY, expressing EYFP (enhanced yellow fluorescent protein) only) were used. These mice were transplanted by Sayantanee Dutta as a common control arm for our group. The data for these mice were kindly provided by her. The FVB IgH–CALM/AF10 transgenic line 1 and FVB wild type mice were used as bone marrow donors. The donor mice were injected with 5-fluorouracil (5-FU) 5 days prior to bone marrow harvest. 5-FU is a pyrimidine analogue and it affects rapidly dividing cells. Thus, the donor bone marrow cells are enriched with stem cells and long term repopulating cells. The 5-FU injected bone marrow donor cells were transduced with Meis1 expressing

retrovirus or with empty retrovirus MIG, and injected into lethally irradiated syngeneic recipient mice (Fig. 4.2.2a).

19 mice were transplanted with IgH-CALM/AF10 transgenic bone marrow cells transduced with the Meis1 expressing retrovirus, 17 mice were transplanted with wildtype bone marrow cells transduced with Meis1 retrovirus, 8 mice were transplanted with IgH-CALM/AF10 transgenic bone marrow cells transduced with empty retrovirus EGFP and 8 mice were transplanted with wildtype bone marrow cells transduced with the empty retrovirus MIY (Fig. 4.2.2b; Table A.1 (Appendix); Table A.2 (Appendix); Table A.3 (Appendix); Table A.4 (Appendix)). The lethally irradiated recipient mice will die of hematopoietic crisis if the injected cells fail to engraft the marrow. Therefore, non-transduced or mock cells were used as rescuer cells. 0.5×10^6 of retrovirally transduced cells and 0.5×10^6 of mock-transduced cells were injected into lethally irradiated FVB wildtype mice (Fig. 4.2.2a). The transplanted mice were monitored closely and examined for the engraftment of the transplanted bone marrow cells. The actual transplantation, that is the injection of the retrovirally transduced bone marrow cells into the tail vain of lethally irradiated mice, was performed by my colleagues Naresh Koneru and Sayantanee Dutta.

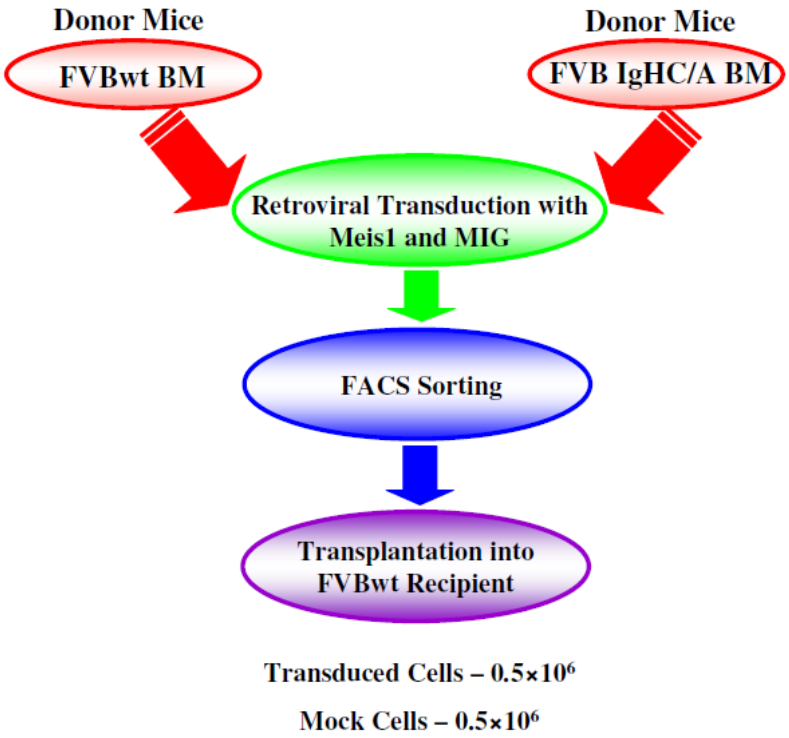


Fig 4.2.2a Schematic representation of experimental strategy: FVB wildtype (FVBwt) mice and FVB IgH-CALM/AF10 transgenic line1 mice were used as donors for the experiment. The donor bone marrow (BM) cells

were retrovirally transduced with E86 cell lines containing MSCV-IRES-YFP-Meis1 (MIY-Meis1; experimental arm) or MSCV-IRES-GFP (MIG ; control arm) containing retrovirus. The transduced cells were sorted using flow cytometry. 0.5×10^6 of GFP/YFP positive cells and 0.5×10^6 of mock cells were injected into lethally irradiated syngeneic FVB mice.

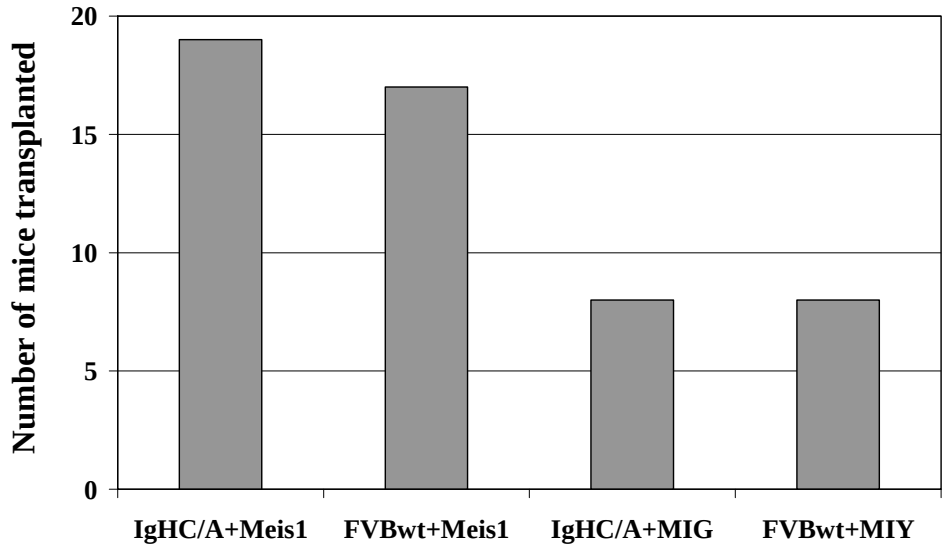


Fig 4.2.2b Graphical representation of total number of mice transplanted for experimental and control arms: A total of nineteen lethally irradiated recipient mice (n=19) were transplanted with IgH-CALM/AF10 transgenic bone marrow cells transduced with Meis1 expressing retrovirus (IgHC/A+Meis1); a total of seventeen lethally irradiated recipient mice (n=17) were transplanted with FVB wildtype bone marrow cells transduced with Meis1 expressing retrovirus (FVBwt+Meis1). As control for our experiments, a total of eight lethally irradiated recipient mice (n=8) were transplanted with IgH-CALM/AF10 transgenic bone marrow cells transduced with MIG empty retrovirus (IgHC/A+MIG); other control arm for our experiments was the mice (n=8) transplanted with FVB wildtype bone marrow cells transduced with MIY empty retrovirus (FVBwt+MIY) These mice were transplanted as a common control arm by Sayantanee Dutta from our group and the data for these mice were kindly provided by her.

4.3 Meis1 expression in IgH-CALM/AF10 transgenic bone marrow cells increases engraftment

0.5×10^6 of retrovirally transduced cells and 0.5×10^6 of non-transduced mock cells were injected into lethally irradiated recipient mice. The transplanted hematopoietic stem and progenitor cells thus provide short-term and long-term engraftment in the recipient mice. The

engraftment was measured at 4 and 8 weeks post transplantation. Peripheral blood samples from transplanted mice were analyzed for their engraftment percentage at 8 weeks post transplantation by flow cytometry using GFP fluorescence as an indicator for retrovirally transduced cells (Fig. 4.3).

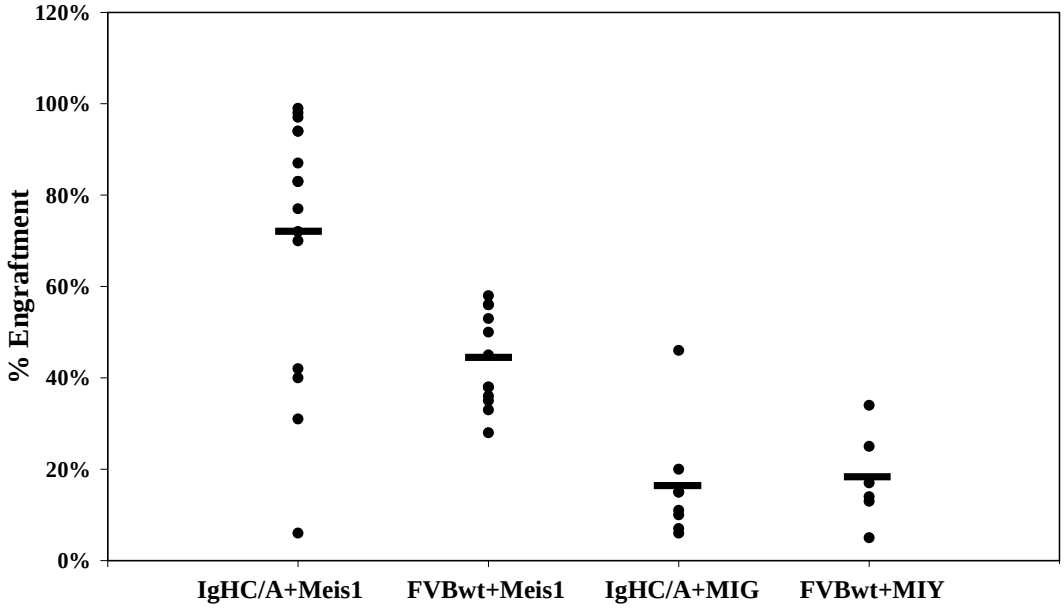


Fig. 4.3 Engraftment percentages of mice transplanted with IgH-CALM/AF10 transgenic bone marrow cells transduced with Meis1 expressing retrovirus (IgHC/A+Meis1), mice transplanted with wildtype bone marrow cells transduced with Meis1 expressing retrovirus (FVBwt+Meis1), mice transplanted with IgH-CALM/AF10 transgenic bone marrow cells transduced with empty retrovirus EGFP (IgHC/A+MIG) and mice transplanted with wildtype bone marrow cells transduced with empty retrovirus EYFP (FVBwt+MIY) at 8 weeks post transplantation: The engraftment percentages from peripheral blood were detected for these mice using flow cytometry to measure the proportion of cells expressing the green or yellow fluorescent protein (GFP or YFP). IgHC/A+Meis1 mice showed an average of 72% (±29%), FVBwt+Meis1 mice showed 44% (±10%), IgHCA+MIG mice showed 16% (±13%) and FVBwt+MIY mice showed 18% (±10%) engraftment in the peripheral blood. The FVBwt+MIY mice were transplanted as a common control arm for our group by Sayantanee Dutta and the data for these mice were kindly provided by her.

Mice transplanted with IgH-CALM/AF10 transgenic bone marrow cells transduced with the Meis1 expressing retrovirus showed a higher engraftment than the mice transplanted with wildtype bone marrow cells transduced with the Meis1 virus or the MIY empty retrovirus and the mice transplanted with IgH-CALM/AF10 transgenic bone marrow cells transduced with

the empty retrovirus (MIG). Thus, Meis1 seems to confer a growth advantage *in vivo* in the mice transplanted with IgH-CALM/AF10 transgenic bone marrow cells transduced with Meis1 expressing retrovirus. Please note that the MIG and MIY empty retroviruses can be considered identical for the purpose of these experiments. They differ only in a few amino acids in the fluorescent protein.

4.4 Meis1 expression collaborates with CALM/AF10 in leukemia development *in vivo* in a combined transgenic/bone marrow transplantation model

Mice transplanted with IgH-CALM/AF10 transgenic bone marrow cells transduced with Meis1 expressing retrovirus (IgHC/A+Meis1; n=19) died of aggressive acute leukemia with a 100% penetrance ranging from 77 to 357 days after transplantation with a median latency of 187 days. Four of 19 mice were found dead in their cages and could not be analyzed. Hence these mice were censored in the survival curve plot. The remaining 15 mice were analyzed and characterized to be leukemic. To prove that these mice had indeed developed leukemia we performed secondary and tertiary transplants using the leukemic cells from these mice. For secondary and tertiary transplantations 1×10^6 leukemic cells were injected into recipient mice All the IgHC/A+Meis1 secondary mice (n=4) developed acute myeloid leukemia and died within a range of 21 to 28 days (median latency of 25 days). All the IgHC/A+Meis1 tertiary transplanted mice (n=4) also developed an aggressive acute myeloid leukemia by 15 days post transplantation. The Kaplan-Meier survival curves for the different experimental arms are shown in Fig. 4.4.

Surprisingly, some mice transplanted with wildtype bone marrow cells transduced with Meis1 expressing retrovirus (FVBwt+Meis1; n=17) also developed acute leukemia with a median latency of 210 days (range 84 to 518 days). However, the penetrance of leukemia development was only 29% in this experimental arm. These 29% of Meis1 transplanted mice (5 out of 17) had clear symptoms of leukemia. On the other hand, 10 out of 17 mice either died due to some unknown reason and had no symptoms of leukemia, or were found dead in their cages and could not be analyzed. Hence these mice were censored in the survival curve plot. The remaining 2 out of 17 Meis1 transplanted mice remained healthy till the current

observation point i.e. 518 days post transplantation. Since we do not know the fate of these two mice after the current observation point, therefore these two mice were censored in the survival curve plot (Fig. 4.4).

All the secondary mice injected with cells from the primary FVBwt+Meis1 leukemic mice (n=4) developed acute myeloid leukemia and died with a latency period of 28 to 63 days post transplantation (median latency of 44 days). However, as expected all the secondary recipient mice injected with cells from primary non-leukemic FVBwt+Meis1 mice (n=2) remained healthy and did not develop leukemia up to current observation period of 122 days post transplantation. Therefore, these two mice were also censored in this survival curve analysis. The tertiary mice transplanted with leukemic cells from secondary leukemic FVBwt+Meis1 mice (n=4) developed aggressive acute myeloid leukemia and died within a range of 21 to 28 days post transplantation (median latency of 25 days) (Fig. 4.4).

The Kaplan-Meier survival curves for primary, secondary and tertiary transplanted mice of different experimental and control arms were plotted (Fig. 4.4) using SigmaPlot Version 12.0. Most of the mice transplanted with IgH-CALM/AF10 transgenic bone marrow cells transduced with empty retrovirus EGFP (IgHC/A+MIG; n=8) remained healthy and did not develop leukemia up to current observation period of 392 days post transplantation. Three (3) out of these 8 IgHC/A+MIG mice were old and died after one year post transplantation. These mice did not have any symptoms of leukemia. Therefore, the mice which were followed up till the current observation point (5 mice) and the mice which were old and died (3 mice), were censored in this survival curve analysis (Fig. 4.4).

Most of the mice transplanted with wildtype bone marrow cells transduced with empty retrovirus EYFP (FVBwt+MIY; n=8) remained healthy and were observed up to 224 days post transplantation. Three (3) out of 8 FVBwt+MIY mice died due to some unknown reason and were non-leukemic. The remaining five mice were healthy and followed up till the current observation point (224 days). Hence, all of the FVBwt+MIY mice were censored in the survival plot (Fig. 4.4).

In summary, the following mice were censored in the survival curve analysis: (1) Mice which were found dead in their cages and could not be analyzed, (2) the mice which died due to some unknown reason other than leukemia, and (3) the mice which remained alive and

followed up till the current observation point . Since we do not know the fate of the living mice after the current observation point, therefore these mice were also censored (Table 4.4).

Even though about 30% of the mice transplanted with wildtype bone marrow cells transduced with the Meis1 expressing retrovirus developed leukemia, this is in stark contrast to the 100% of mice which developed leukemia after being transplanted with CALM/AF10 transgenic bone marrow cells transduced with the Meis1 expressing retrovirus. It is thus pretty obvious from these data that Meis1 strongly collaborates with CALM/AF10 in the induction of leukemia.

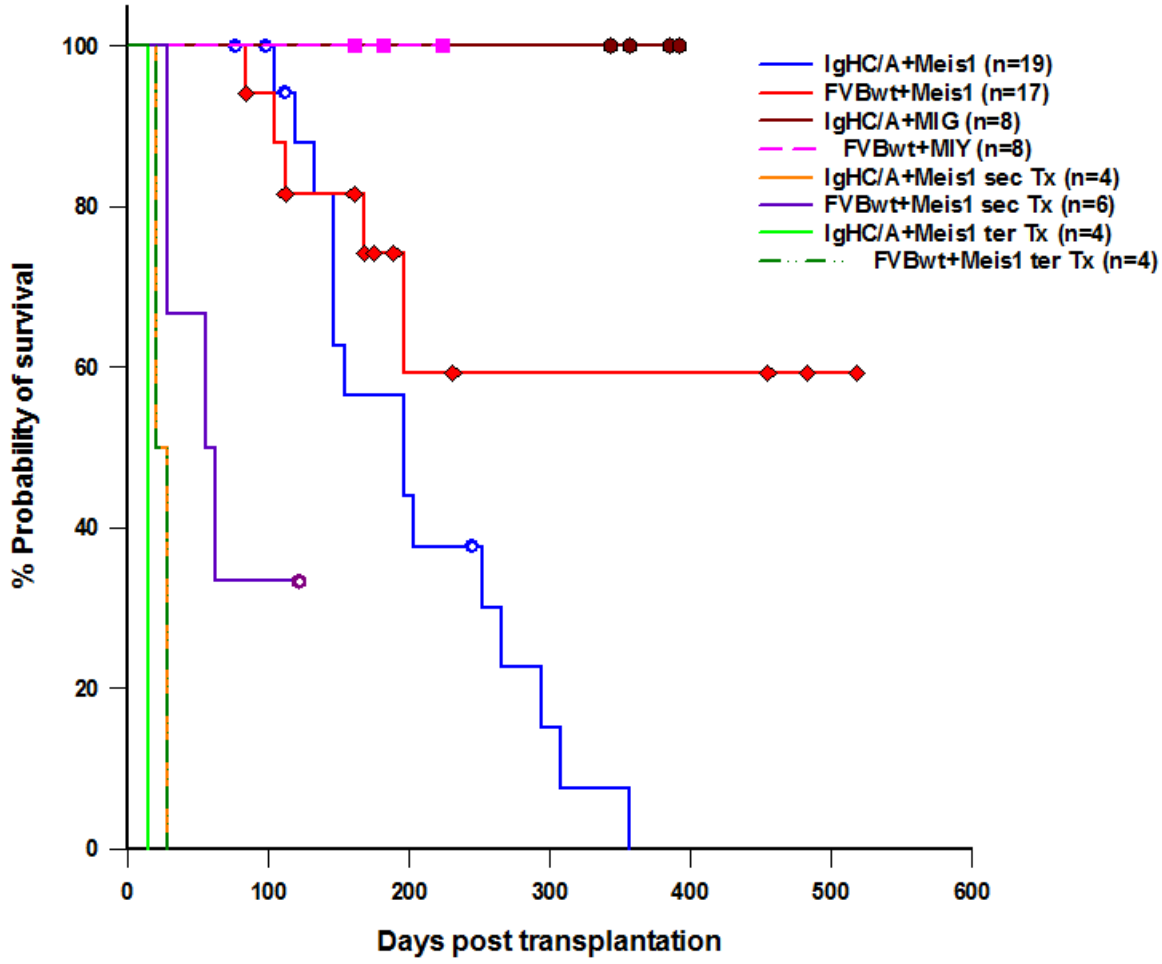


Fig. 4.4 Kaplan-Meier survival curves of primary, secondary and tertiary mice transplanted with IgH-CALM/AF10 transgenic bone marrow cells transduced with Meis1 expressing retrovirus (IgHC/A+Meis1), wildtype bone marrow cells transduced with Meis1 expressing cells (FVBwt+Meis1), IgH-CALM/AF10 transgenic bone marrow cells transduced with empty vector MIG expressing EGFP (IgHC/A+MIG) and wildtype bone marrow cells transduced with empty vector MIY expressing EYFP (FVBwt+MIY): Kaplan Meier survival curve analysis showing the percentage of survival for different

experimental and control mice against number of days post transplantation. The different experimental mice include IgHC/A+Meis1 (n=19), FVBwt+Meis1 (n=17), IgHC/A+MIG (n=8) and FVBwt+MIY (n=8). In addition to the primary transplanted mice, this graph also includes the curves for secondary and tertiary transplanted mice (IgHC/A+Meis1 secondary transplanted mice (n=4); FVBwt+Meis1 secondary transplanted mice (n=6); IgHC/A+Meis1 tertiary transplanted mice (n=4); FVBwt+Meis1 tertiary transplanted mice (n=4)). The primary transplanted IgHC/A+Meis1 mice died within a range of 77 to 357 days (median 187 days) and primary transplanted FVBwt+Meis1 mice died within a range of 84 to 518 days (median 210 days). The IgHC/A+MIG control mice remained healthy and are still under observation for 392 days post transplantation. The FVBwt+MIY control mice also remained healthy and are under observation for 224 days post transplantation. All the IgHC/A+Meis1 secondary transplanted mice (n=4) died within a range of 21 to 28 days (median 25 days) and IgHC/A+Meis1 tertiary transplanted mice (n=4) died within 15 days post transplantation. The FVBwt+Meis1 secondary transplanted leukemic mice (n=4) died within a range of 28 to 63 days (median 44 days). The FVBwt+Meis1 secondary mice transplanted from primary non-leukemic mice (n=2) remained healthy and were observed up to 122 days post transplantation. All the FVBwt+Meis1 tertiary transplanted mice (n=4) from secondary leukemic FVBwt+Meis1 mice died within a range of 21 to 28 days (median 25 days). The mice which were found dead in the cages and could not be analyzed, the mice which died due to some unknown reason other than leukemia, and the mice which remained alive and followed up to the current observation point are censored in this survival curve analysis. Different shaped dots on the lines denote the censored mice. (sec: secondary; ter: tertiary; Tx: transplantation).

Gene	No. of mice transplanted	Days of survival	No. of Leukemic mice	No. of censored mice		
				Died in cage	Non-leukemic	Remained alive
IgHC/A+Meis1	19	77-357	15	4	0	0
FVBwt+Meis1	17	84-518	5	5	5	2
IgHC/A+MIG	8	343-392	0	0	3	5
FVBwt+MIY	8	161-224	0	0	3	5
IgHC/A+Meis1 sec. Tx	4	21-28	4	0	0	0
FVBwt+Meis1 sec. Tx	6	28-122	4	0	-	2
IgHC/A+Meis1 ter. Tx	4	15	4	0	0	0
FVBwt+Meis1 ter. Tx	4	21-28	4	0	0	0

Table 4.4 Summary of Kaplan Meier survival curve for different primary, secondary and tertiary transplanted mice

4.5 Mice transplanted with IgH-CALM/AF10 transgenic bone marrow cells transduced with a Meis1 expressing retrovirus develop an aggressive acute myeloid leukemia

A total of 19 FVB wildtype mice were transplanted with IgH-CALM/AF10 transgenic bone marrow cells transduced with the Meis1 expressing retrovirus. The transplanted mice were monitored for leukemic symptoms such as frizzled body hair, paleness of the foot pads, breathing problems and lethargy. Moribund mice were sacrificed and further analyzed for leukemia (Table 4.5). The parameters considered for leukemic mice included measurement of WBC and RBC counts from peripheral blood, spleen weight and length and peripheral blood smears. The sacrificed and dissected mice were then fixed in 4% formalin and sent for histopathological examination.

Summary of IgHC/A+Meis1 mice	
No. of transplanted mice	19
No. of leukemic mice analyzed	15
No. of mice not analyzed	4
Remaining mice	0
Median latency (days)	187

Table 4.5 Summary of mice transplanted with IgH-CALM/AF10 transgenic bone marrow cells transduced with Meis1 expressing retrovirus (IgHC/A+Meis1).

The sacrificed, leukemic mice showed a median bone marrow engraftment levels of 92% (±12%), peripheral blood engraftment levels of 78% (±17%) and spleen engraftment levels of 79% (±27%) (Table A.5 (Appendix)).

4.5.1 Analysis of leukemic mice transplanted with IgH-CALM/AF10 transgenic bone marrow cells transduced with Meis1 expressing retrovirus

4.5.1.1 WBC and RBC counts

The peripheral blood of sacrificed mice was characterized by a dramatic increase in the number of WBCs (hyperleukocytosis) ranging from 12×10^6 to 440×10^6 of WBCs per milliliter as compared to control mice ranging from 3×10^6 to 8×10^6 WBCs per milliliter (Fig.4.5.1.1a; Table A.6 (Appendix)). The leukemic mice also had a decreased red blood cell (RBC) counts (anemia) ranging from 1×10^9 to 4×10^9 per milliliter as compared to control mice (n=4) with RBC counts ranging from 8×10^9 to 10×10^9 RBCs per milliliter (Fig. 4.5.1.1b; Table A.6 (Appendix)).

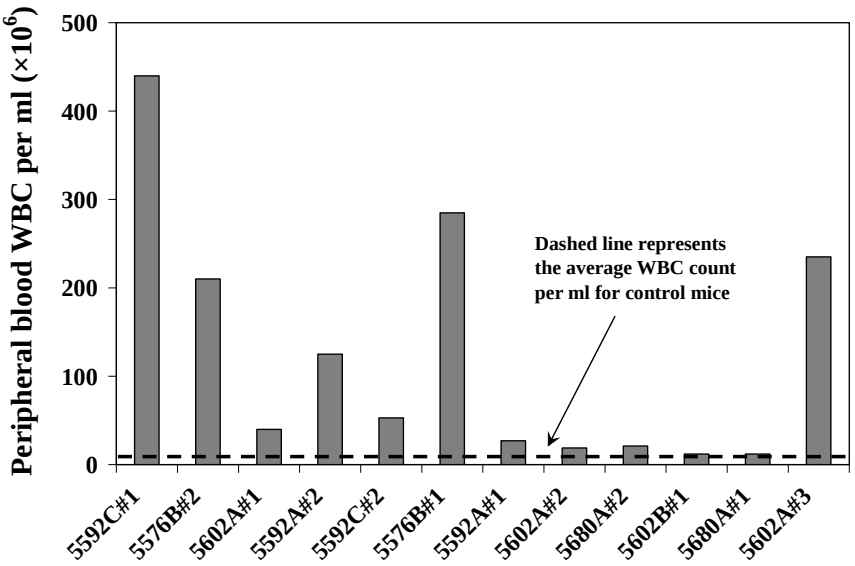


Fig. 4.5.1.1a Graphical representation of WBC counts in the peripheral blood of leukemic mice transplanted with IgH-CALM/AF10 transgenic bone marrow cells transduced with Meis1 expressing retrovirus (IgHC/A+Meis1): The leukemic mice demonstrated increase in WBC counts (hyperleukocytosis). The average WBC count per ml for IgHC/A+Meis1 mice is 123×10^6 and the average WBC count per ml for control mice is 6×10^6.

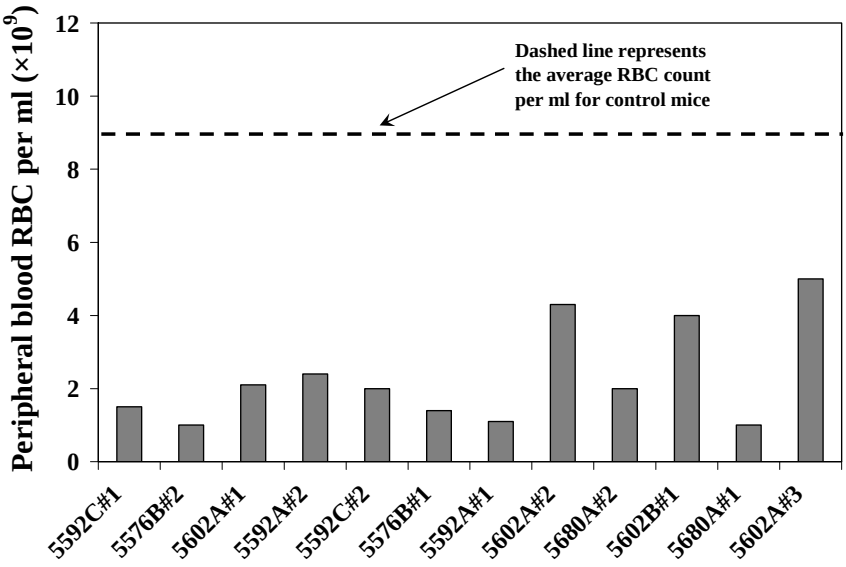


Fig. 4.5.1.1b Graphical representation of RBC counts in the peripheral blood of leukemic mice transplanted with IgH-CALM/AF10 transgenic bone marrow cells transduced with Meis1 expressing retrovirus (IgHC/A+Meis1): The leukemic mice demonstrated decrease in RBC counts (anemia). The average RBC count per ml for IgHC/A+Meis1 mice is 2×10^9 and the average RBC count per ml for control mice is 9×10^9.

4.5.1.2 The leukemic mice were characterized by splenomegaly

Enlargement of the spleen was a common feature of these leukemic mice (Fig. 4.5.1.2a; Fig. 4.5.1.2b; Fig. 4.5.1.2c; Table A.7 (Appendix)).

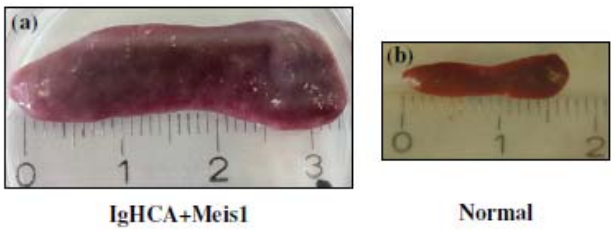


Fig. 4.5.1.2a Comparison of the spleen from a mouse transplanted with IgH-CALM/AF10 transgenic bone marrow cells transduced with Meis1 expressing retrovirus (IgHC/A+Meis1) mouse with the spleen of a control mouse: The spleens of IgHC/A+Meis1 diseased mice were larger **(a)** compared to the control mouse **(b)**. The average spleen weight of IgHC/A+Meis1 leukemic mice was 475 mg as compared to an average of 126 mg in control mice.

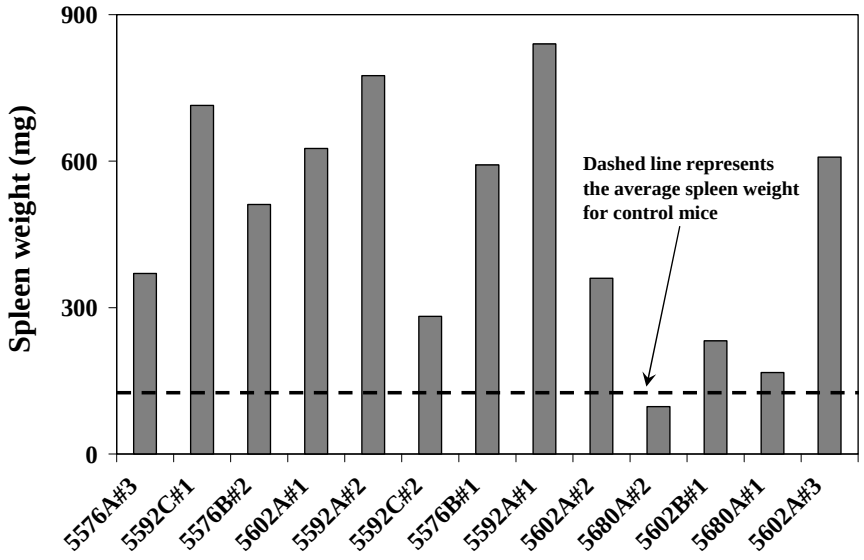


Fig. 4.5.1.2b Graphical representation of spleen weight of leukemic mice transplanted with IgH-CALM/AF10 transgenic bone marrow cells transduced with Meis1 expressing retrovirus (IgHC/A+Meis1): The leukemic mice demonstrated enlargement of the spleen (splenomegaly). The average spleen weight of IgHC/A+Meis1 leukemic mice was 475 mg as compared to an average of 126 mg in control mice.

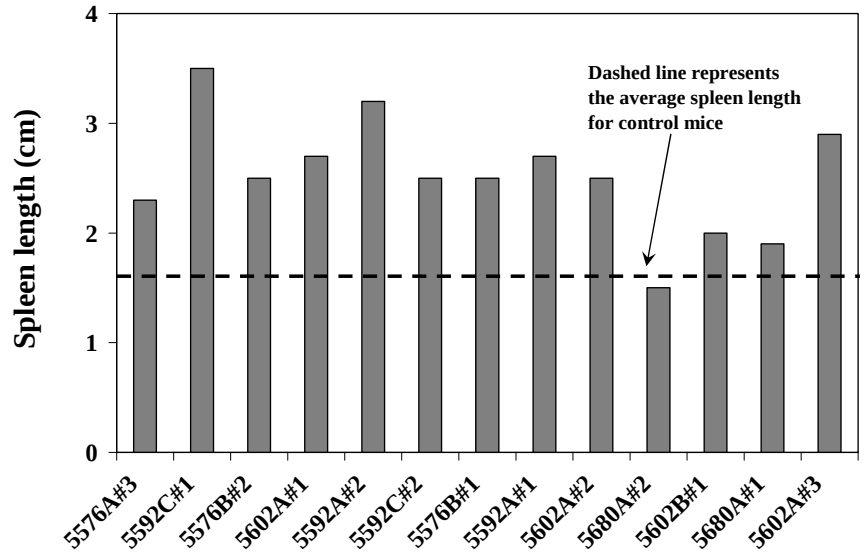


Fig. 4.5.1.2c Graphical representation of spleen length of leukemic mice transplanted with IgH-CALM/AF10 transgenic bone marrow cells transduced with Meis1 expressing retrovirus (IgHC/A+Meis1): The leukemic mice demonstrated enlargement of the spleen (splenomegaly). The average spleen length of IgHC/A+Meis1 leukemic mice was 2.5 cm as compared to an average of 1.6 cm in control mice.

4.5.1.3 Histopathology demonstrated leukemic blast infiltration in multiple organs

Hematoxylin and eosin (H&E) staining was performed in histology. The hematoxylin stains the nuclei of cells blue and eosin stains the cytoplasm red. Histopathological analyses of multiple organs including spleen, liver, lungs, kidneys, thymus and lymph nodes showed blast infiltration (Fig. 4.5.1.3). Leukemic blasts are characterized by the presence of large nucleus, prominent nucleolus and moderate amount of cytoplasm. The infiltration of leukemic blasts in non-hematopoietic organs emphasized the aggressive nature of the disease.

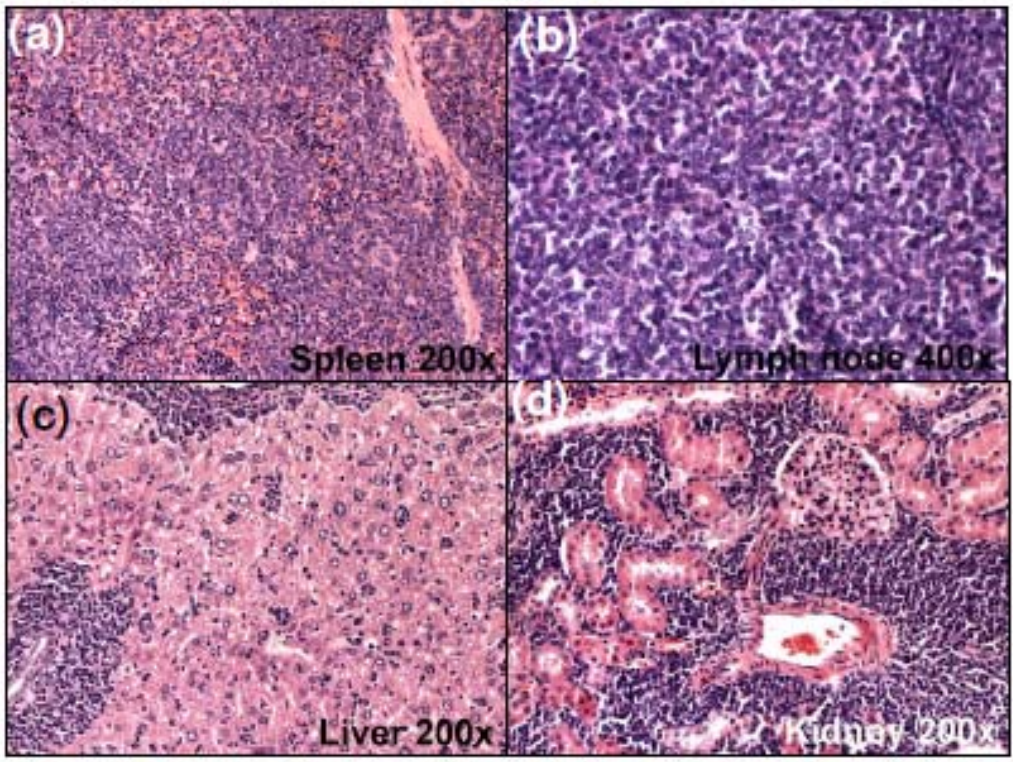


Fig. 4.5.1.3 Immunohistopathology of diseased mice transplanted with IgH-CALM/AF10 transgenic bone marrow cells transduced with Meis1 expressing retrovirus: (a) Histological study demonstrated infiltration of myeloid blasts in multiple organs. **(b and c)** In the spleen the blastic cells were predominantly in the red pulp. **(d)** The lymph node and liver also showed infiltration of blast cells. Diffuse infiltrations of blast cells were found in kidney.

4.5.1.4 The leukemic cells from mice transplanted with IgH-CALM/AF10 transgenic bone marrow cells transduced with Meis1 expressing retrovirus were positive for myeloid markers on immunohistochemical analyses

Several histochemical and immunohistochemical stains are used to identify the type and stage of different cells, and also to differentiate between myeloid and lymphoid leukemias. The myeloperoxidase (MPO) stain is used as a myeloid marker in the diagnosis of AML. MPO staining is negative in case of acute lymphoid leukemia (ALL). Thus MPO staining is used to distinguish between AML and ALL. Another stain called choloro-acetate esterase (CAE) is also used to confirm the myeloid nature of AML cells. B220 and CD3 stainings differentiate between B and T lymphoid cells, respectively.

Immunohistochemical stainings demonstrated MPO positive blasts and thus the myeloid nature of the leukemia (Fig. 4.5.1.4).

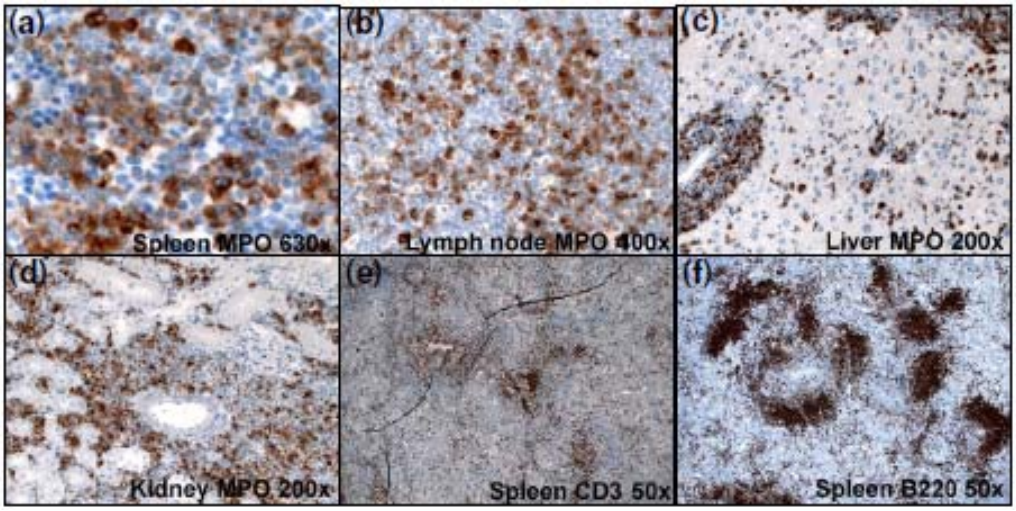


Fig. 4.5.1.4 Histochemical and immunohistochemical staining of leukemic blasts for diseased mice transplanted with IgH-CALM/AF10 transgenic bone marrow cells transduced with Meis1 expressing retrovirus: (a, b, c and d) Immunohistochemical analyses demonstrated the presence of blasts positive for myeloperoxidase (MPO) in high number in spleen, lymph node, liver and kidney. **(e)** Immunohistochemical staining in the spleen for CD3 showed the residual reactive T-cells around the central arteries. **(f)** The B220 staining shows the residual B-cells**.**

4.5.1.5 Morphological analysis of cells from hematopoietic organs of leukemic mice transplanted with IgH-CALM/AF10 transgenic bone marrow cells transduced with Meis1 expressing retrovirus revealed their myeloid nature and a high number of infiltrating blast like cells

Cytospin slides of cells from different hematopoietic organs including bone marrow, spleen and peripheral blood of leukemic mice were prepared and stained with May-Grunwald-Giemsa stain (Fig. 4.5.1.5). Cytological studies of the leukemic mice revealed differentiated myeloid cells and a large number of blast cells (Table 4.5.1.5a; Table 4.5.1.5b).

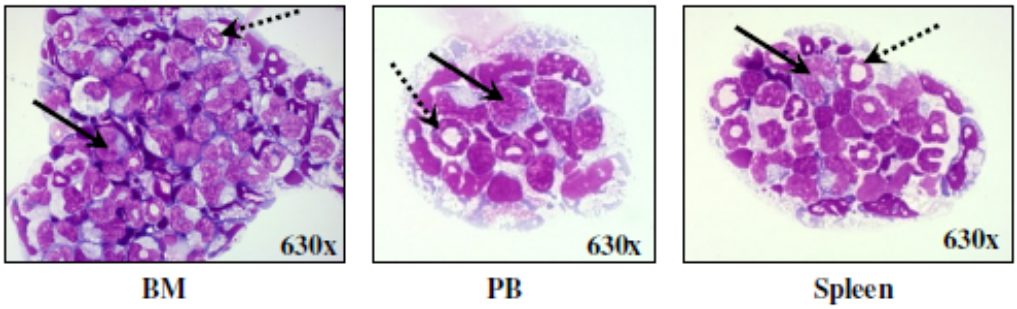


Fig. 4.5.1.5 Blast like cells from organs of leukemic mice transplanted with IgH-CALM/AF10 transgenic bone marrow cells transduced with a Meis1 expressing retrovirus: May-Grünwald-Giemsa stained cytospins of bone marrow (BM), peripheral blood (PB) and spleen from mice transplanted with IgH-CALM/AF10 transgenic bone marrow cells transduced with Meis1 expressing retrovirus showed differentiated myeloid cells (dotted arrows) and a number of blast cells (firm arrows).

Experiment no.	% Blast like cells BM	% Blast like cells Spleen	% Blast like cells PB	Diagnosis
5576B#1	85	56	56	AML
5576B#2	55	57	47	AML
5592A#2	74	59	68	AML
5592C#1	48	53	39	AML
5602A#1	55	51	62	AML
5602A#3	57	51	32	AML
5680A#2	29	47	49	AML

Table 4.5.1.5a Percentage of blast like cells from different organs of mice transplanted with IgH-CALM/AF10 transgenic bone marrow cells transduced with the Meis1 expressing retrovirus: 100 cell differential counts from bone marrow (BM), spleen and peripheral blood (PB) showed a very high percentage of blast cells. The cytospin slides revealed accumulation of myeloid blasts with an average of 58% in BM, 53% in spleen and 50% in PB. Staining of cytospin preparations from the BM, spleen and PB of control mice showed absence of blast like cells.

Experiment no.	% myeloid PB	% lymphoid PB	Lymphoid/Myeloid ratio
5576B#1	98	2	0.02:1
5576B#2	96	4	0.04:1
5592A#2	96	4	0.04:1
5592C#1	98	2	0.02:1
5602A#1	88	12	0.14:1
5602A#3	87	13	0.15:1
5680A#2	91	9	0.09:1

Table 4.5.1.5b Percentage myeloid and lymphoid cells in peripheral blood (PB) of mice transplanted with IgH-CALM/AF10 transgenic bone marrow cells transduced with the Meis1 expressing retrovirus

(IgHC/A+Meis1): There was a reversal of the lymphoid to myeloid ratio in the PB of IgHC/A+Meis1 leukemic mice with an enormous myeloid proliferation and decrease in lymphoid growth in this compartment. The lymphoid to myeloid ratio in PB of control mice was 2:1.

The morphological and immunohistochemical findings in the leukemic mice were diagnostic of an AML.

4.6 Mice transplanted with wildtype bone marrow cells transduced with the Meis1 expressing retrovirus also developed acute myeloid leukemia

A total of 17 mice transplanted with FVB wildtype bone marrow cells transduced with the Meis1 expressing retrovirus were monitored for leukemic symptoms. The engraftment of these mice was analyzed at 4 and 8 weeks (Fig. 4.3) post transplantation. The sacrificed mice showed a median bone marrow engraftment levels of 80% (±26%), peripheral blood engraftment levels of 66% (±19%) and spleen engraftment levels of 62% (±15%) (Table A.8 (Appendix)).

Out of the total of 17 mice transplanted (n=17), only 29% of them (5 out of 17) were diagnosed to be leukemic based on several parameters including WBC count, spleen weight, immunohistochemical stainings and morphological analysis. On the other hand, 10 out of 17 mice either died due to some unknown reasons and had no symptoms of leukemia (5 mice), or were found dead in their cages and could not be analyzed (5 mice). The remaining 2 out of 17 Meis1 transplanted mice remained healthy and were followed up till 518 days post transplantation (Table 4.6). The engraftment percentages from peripheral blood of these two mice were 33% and 36% at 60 days post transplantation. However, the engraftment percentages decreased to 12% and 17% respectively at 200 days post transplantation.

Summary of FVBwt+Meis1 mice	
No. of transplanted mice	17
No. of leukemic mice analyzed	5
No. of non-leukemic mice analyzed	5
No. of mice could not be analyzed	5
No. of mice remained alive	2 (518 days post Tx)
Median latency (days)	210

Table 4.6 Summary of mice transplanted with FVB wildtype bone marrow cells transduced with the Meis1 expressing retrovirus (FVBwt+Meis1) (Tx= transplantation)

4.6.1 Characterization of mice transplanted with wildtype bone marrow cells transduced with Meis1 expressing retrovirus

4.6.1.1 WBC and RBC counts

The peripheral blood of the leukemic mice was characterized by an increase in the number of WBCs ranging from 20×10^6 to 175×10^6 per milliliter (Fig. 4.6.1.1a; Table A.9 (Appendix)) and a decrease in the RBC counts ranging from 1×10^9 to 6×10^9 per milliliter (Fig. 4.6.1.1b; Table A.9 (Appendix)). On the other hand, the non-leukemic mice exhibited normal WBC counts ranging from 2×10^6 to 5×10^6 per milliliter (Fig. 4.6.1.1a; Table A.10 (Appendix)) and normal RBC counts ranging from 2×10^9 to 7×10^9 per milliliter (Fig. 4.6.1.1b; Table A.10 (Appendix)).

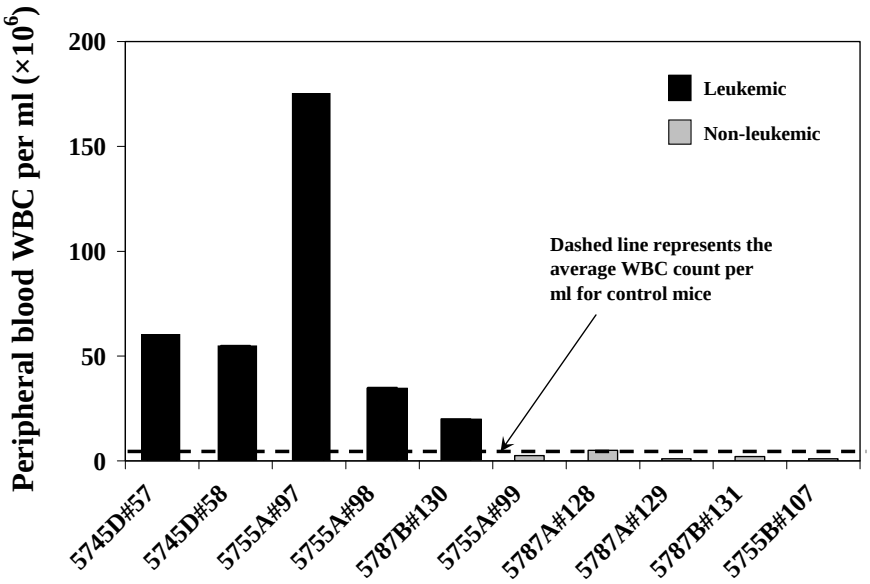


Fig. 4.6.1.1a Graphical representation of WBC counts in the peripheral blood of mice transplanted with FVB wildtype bone marrow cells transduced with the Meis1 expressing retrovirus (FVBwt+Meis1): The leukemic mice demonstrated an increase in WBC counts (hyperleukocytosis). The average WBC count per ml for leukemic FVBwt+Meis1 mice is 69×10^6. On the other hand, the non-leukemic mice demonstrated normal WBC counts. The average WBC count per ml for non-leukemic FVBwt+Meis1 mice is 2×10^6. For control mice, the average WBC count per ml is 6×10^6 and is shown as a dashed line in the graph.

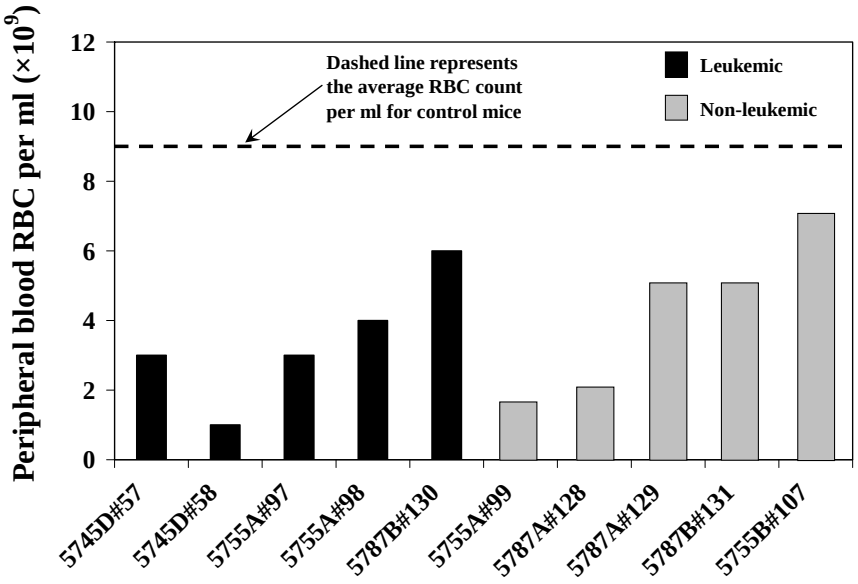


Fig. 4.6.1.1b Graphical representation of RBC counts in the peripheral blood of mice transplanted with FVB wildtype bone marrow cells transduced with the Meis1 expressing retrovirus (FVBwt+Meis1): The leukemic as well as the non-leukemic mice demonstrated a decrease in RBC counts (anemia). The average RBC

count per ml for leukemic FVBwt+Meis1 mice is 3×10^9 and for non-leukemic mice is 4×10^9. The average RBC count for control mice is 9×10^9/ml and is shown as a dashed line in the graph.

4.6.1.2 Mice transplanted with wildtype bone marrow cells transduced with the Meis1 expressing retrovirus were characterized by splenomegaly

All the leukemic mice had enlarged spleen (Fig. 4.6.1.2a; Fig. 4.6.1.2b; Fig. 4.6.1.2c; Table A.11 (Appendix)). Surprisingly, 2 out of 5 non-leukemic mice from this experimental arm also had enlarged spleen (Fig. 4.6.1.2b; Fig. 4.6.1.2c; Table A.12 (Appendix)). Therefore, detailed analyses were performed for these mice.

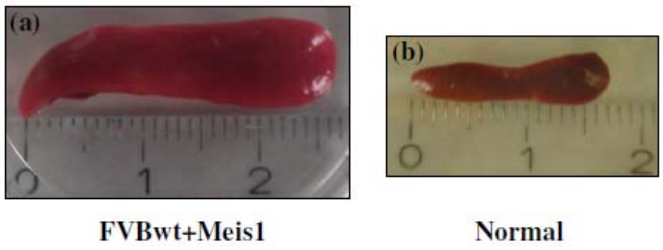


Fig. 4.6.1.2a Comparison of spleen from mice transplanted with wildtype bone marrow cells transduced with the Meis1 expressing retrovirus (FVBwt+Meis1) and a control mouse: The spleens of FVBwt+Meis1 mice **(a)** were larger compared to the control mouse **(b)**. The average spleen weight of FVBwt+Meis1 mice was 384 mg as compared to an average of 126 mg in control mice.

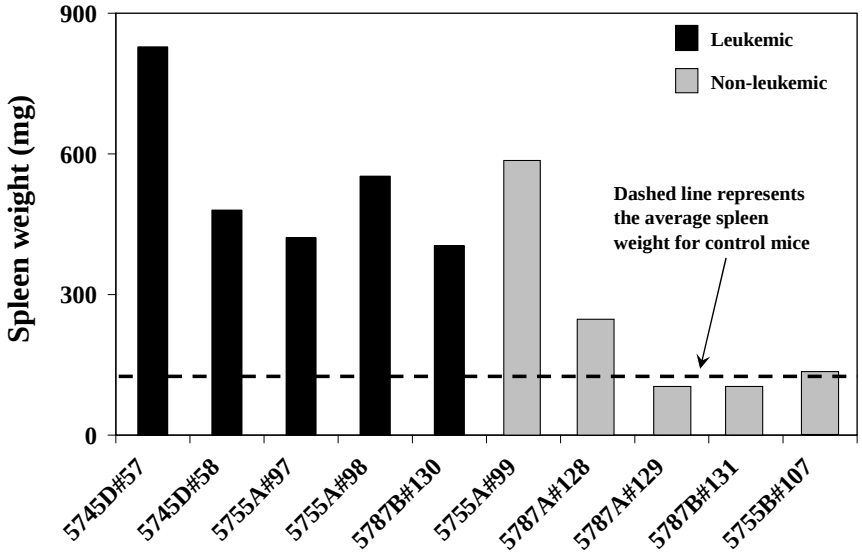


Fig. 4.6.1.2b Graphical representation of spleen weight of mice transplanted with FVB wildtype bone marrow cells transduced with the Meis1 expressing retrovirus (FVBwt+Meis1): The leukemic mice

demonstrated enlargement of the spleen (splenomegaly). The average spleen weight of FVBwt+Meis1 leukemic mice was 537 mg. Two of the non-leukemic mice demonstrated enlargement of the spleen and other three non-leukemic mice had normal spleens. The average spleen weight of FVBwt+Meis1 non-leukemic mice was 232 mg. The average spleen weight for control mice is 126 mg and is shown as a dashed line in the graph.

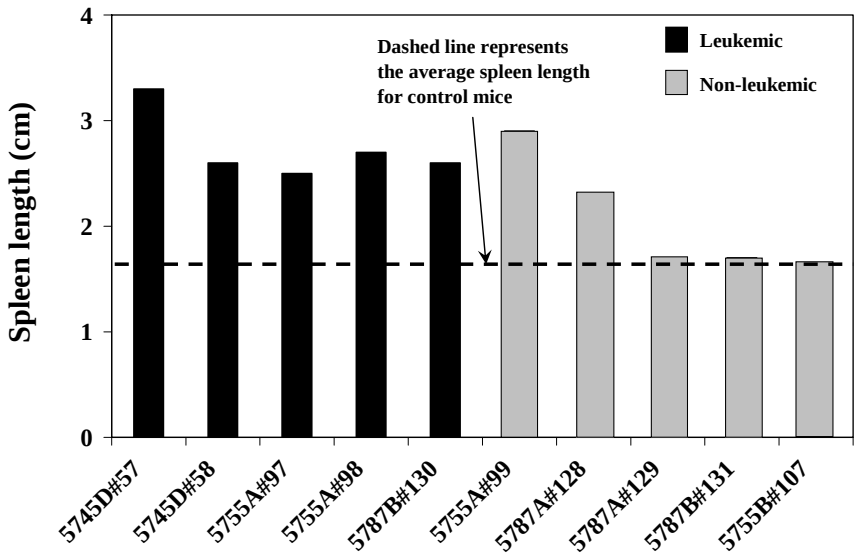


Fig. 4.6.1.2c Graphical representation of spleen length of mice transplanted with FVB wildtype bone marrow cells transduced with the Meis1 expressing retrovirus (FVBwt+Meis1): The leukemic mice demonstrated enlargement of the spleen (splenomegaly). The average spleen length of FVBwt+Meis1 leukemic mice was 2.7 cm. Two of the non-leukemic mice also demonstrated enlargement of the spleen and other three non-leukemic mice had normal spleen. The average spleen length of FVBwt+Meis1 non-leukemic mice was 2.0 cm. The average spleen length of control mice is 1.6 cm and is shown as a dashed line in the graph.

4.6.1.3 Histopathology demonstrated leukemic blast infiltration in multiple organs of leukemic mice transplanted with wildtype bone marrow cells transduced with the Meis1 expressing retrovirus

Histologically the spleen, liver, lungs, kidneys, heart and pancreas showed diffuse infiltrations characterized by the presence of large tumor cells with blastic chromatin, one or more prominent nucleoli and moderate amount of cytoplasm (Fig. 4.6.1.3).

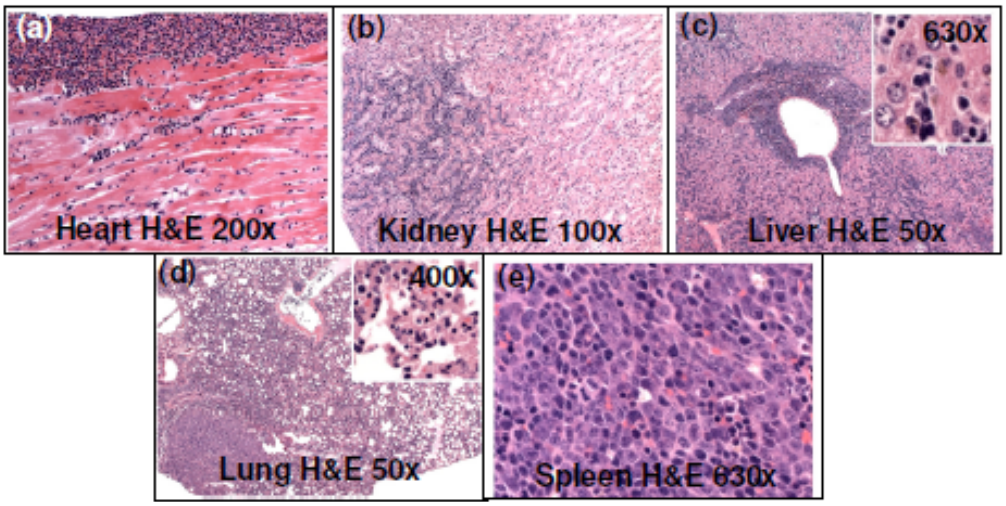


Fig. 4.6.1.3 Immunohistopathology of leukemic mice transplanted with wildtype bone marrow cells transduced with the Meis1 expressing retrovirus (FVBwt+Meis1): Histological study demonstrated infiltration of myeloid blasts in multiple organs. **(a)** In the heart the neoplastic cells were observed between the muscle fibers. **(b)** In the kidneys blast infiltration was predominatly subcpasular. **(c)** In the liver the blastic cells occupied the periportal regions and sinusoidal spaces. **(d and e)** The lung and spleen were also infiltrated with blast cells. H&E: Hematoxylin and eosin stain

4.6.1.4 Immunohistochemical analysis of leukemic mice transplanted with wildtype bone marrow cells transduced with the Meis1 expressing retrovirus showed positivity for myeloid markers

Immunohistochemical staining demonstrated the presence of MPO and CAE positive blasts, thus confirming the myeloid nature of the disease (Fig. 4.6.1.4).

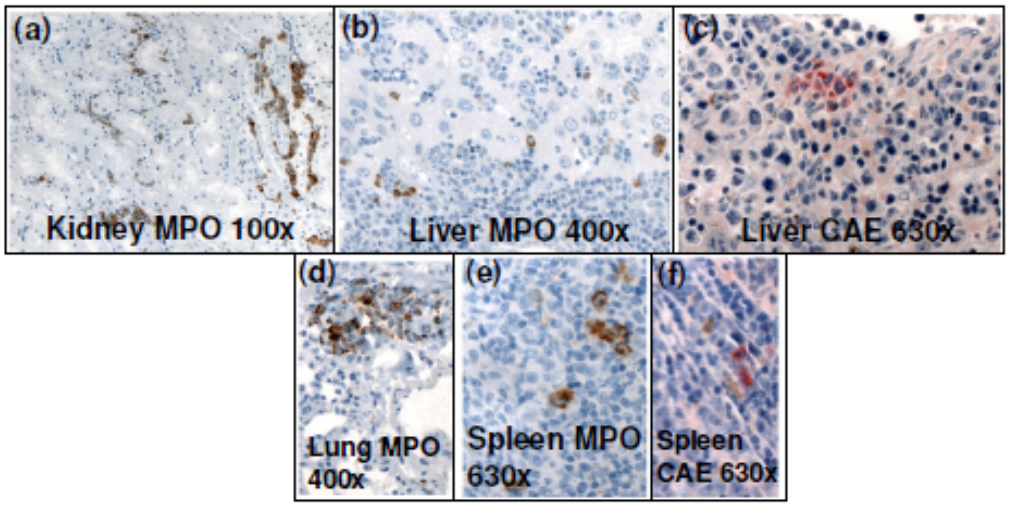


Fig. 4.6.1.4 Histochemical and immunohistochemical staining of leukemic blasts for leukemic mice transplanted with wildtype bone marrow cells transduced with the Meis1 expressing retrovirus

(FVBwt+Meis1): (a, b, d and e) Immunohistochemical analysis demonstrated the presence of blasts positive for myeloperoxidase (MPO) in high numbers in kidney, liver, lung and spleen. **(c and f)** Immunohistochemical staining in the liver and spleen for CAE (chloro-acetate esterase) also demonstrated the presence of myeloid blasts.

4.6.1.5 Morphological analysis of cells from hematopoietic organs of mice transplanted with wildtype bone marrow cells transduced with Meis1 expressing retrovirus

Cytospin preparations from different hematopoietic organs including bone marrow, spleen and peripheral blood of leukemic mice transplanted with wildtype bone marrow cells transduced with the Meis1 expressing retrovirus (FVBwt+Meis1) showed differentiated myeloid cells and high numbers of blast cells (Fig. 4.6.1.5a; Table 4.6.1.5a; Table 4.6.1.5b). Thus the morphological and immunohistochemical analyses of FVBwt+Meis1 leukemic mice are diagnostic of AML.

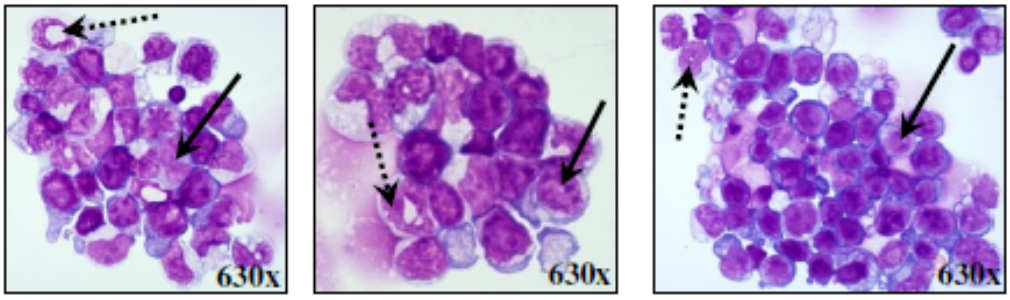


Fig. 4.6.1.5a Blast like cells from organs of leukemic mice transplanted with wildtype bone marrow cells transduced with the Meis1 expressing retrovirus (FVBwt+Meis1): May-Grünwald-Giemsa stained cytospin preparations of bone marrow (BM), peripheral blood (PB) and spleen from leukemic FVBwt+Meis1 mice showed differentiated myeloid cells (dotted arrows) and a number of blast cells (firm arrows).

Experiment no.	% Blast like cells BM	% Blast like cells Spleen	% Blast like cells PB	Diagnosis
5745D#57	26	57	35	AML
5745D#58	62	75	34	AML
5755A#97	63	30	38	AML
5755A#98	50	34	36	AML
5787B#130	21	23	22	AML

Table 4.6.1.5a Percentage of blast like cells from leukemic FVBwt+Meis1 mice bone marrow (BM), spleen and peripheral blood (PB): 100 cell differential counts from BM, spleen and PB showed a high percentage of blast cells. The cytospin slides revealed accumulation of myeloid blasts with an average of 44% in BM, 44% in spleen and 33% in PB. Staining of cytospin preparations from BM, spleen and PB of control mice showed no blast like cells.

Experiment no.	% myeloid PB	% lymphoid PB	Lymphoid/Myeloid ratio
5745D#57	90	10	0.11:1
5745D#58	86	14	0.16:1
5755A#97	98	2	0.02:1
5755A#98	97	3	0.03:1
5787B#130	70	30	0.43:1

Table 4.6.1.5b Percentage myeloid and lymphoid in peripheral blood (PB) of from leukemic FVBwt+Meis1 mice: The reversal in the lymphoid to myeloid ratio in PB of FVBwt+Meis1 leukemic mice showed an enormous myeloid proliferation and a decrease in lymphoid cells in this compartment. The lymphoid to myeloid ratio in PB of control mice is 2:1.

However, cytological studies from non-leukemic mice transplanted with wildtype bone marrow cells transduced with the Meis1 expressing retrovirus (FVBwt+Meis1) revealed a normal phenotype (Fig. 4.6.1.5b; Table 4.6.1.5c; Table 4.6.1.5d).

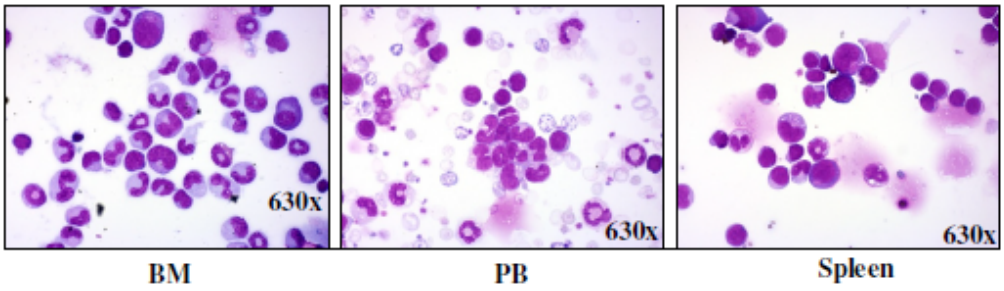


Fig. 4.6.1.5b Cytospin analysis of FVBwt+Meis1 non-leukemic mice: Cytological studies of May-Grünwald-Giemsa stained bone marrow (BM), peripheral blood (PB) and spleen from non-leukemic FVBwt+Meis1 mice were normal.

Experiment no.	% Blast like cells BM	% Blast like cells Spleen	% Blast like cells PB	Diagnosis
5755A#99	NA	0	NA	No disease
5787A#128	0	0	0	No disease
5787A#129	0	0	0	No disease
5787B#131	0	0	0	No disease
5755B#107	0	0	0	No disease

Table 4.6.1.5c Percentage of blast like cells from non-leukemic FVBwt+Meis1 non-leukemic mice bone marrow (BM), spleen and peripheral blood (PB): 100 cell differential counts from BM, spleen and PB showed absence of blast cells. The cytospin slides revealed a normal phenotype (NA= not available).

Experiment no.	% myeloid PB	% lymphoid PB	Lymphoid/Myeloid ratio
5755A#99	NA	NA	NA
5787A#128	52	48	0.92:1
5787A#129	47	53	1.13:1
5787B#131	42	58	1.38:1
5755B#107	40	60	1.50:1

Table 4.6.1.5d Percentage myeloid and lymphoid in peripheral blood (PB) from non-leukemic FVBwt+Meis1 mice: The lymphoid to myeloid ratio in PB of FVBwt+Meis1 non-leukemic mice was normal (NA= not available).

4.7 Flow cytometric analyses of transplanted mice

In order to characterize the leukemia based on different surface markers present on the leukemic cells, cell suspensions were prepared from the hematopoietic organs of sacrificed mice and stained with different lineage specific markers including Gr-1 and Mac-1 for the myeloid lineage, B220 for B-cells, Ter-119 for erythroid cells, Sca-1 and c-Kit for stem cells and CD4 and CD8 for T-cells.

The leukemic cells of the mice transplanted with IgH-CALM/AF10 transgenic bone marrow cells transduced with the Meis1 expressing retrovirus, showed expression of myeloid the markers Gr-1 and Mac-1 in bone marrow, spleen and peripheral blood (BM: 50% (±32%); spleen: 40% (±27%); PB: 46% (±33%)) (Fig. 4.7a; Fig. 4.7c; Fig. 4.7e; Fig. 4.7g). In addition to this, the B220 cell surface marker in leukemic bone marrow cells was consistently observed on about half of the cells (54% (±25%)) (Fig. 4.7c; Fig. 4.7g). The leukemia was also characterized by the co-expression of myeloid and lymphoid markers ($Gr1^+Mac1^+/B220^+$) which is a typical feature of CALM/AF10-associated leukemias (Fig. 4.7a; Fig. 4.7c; Fig. 4.7e; Fig. 4.7i). The myeloid-lymphoid double positive population was observed in all the leukemic mice with an average of 41% (±21%) in bone marrow, 37%

(±26%) in the peripheral blood and 39% (±24%) in the spleen. Interestingly, these mice had a very low percentage of Sca-1/c-Kit positive cells (2% (±2%)) and were negative for the T-cell lineage markers CD4/CD8 (Fig. 4.7b; Fig. 4.7d; Fig. 4.7f).

In contrast, on the leukemic cells of the mice transplanted with wildtype bone marrow cells transduced with Meis1 expressing retrovirus, the lymphoid-myeloid $Gr1^{+}Mac1^{+}/B220^{+}$ co-expression was absent or very low (BM: 5% (±5%); spleen: 7% (±7%); PB: 6% (±5%)) (Fig. 4.7a; Fig. 4.7c; Fig. 4.7e; Fig. 4.7j). Cells obtained from all organs from the Meis1 transplanted mice were highly positive for Gr-1/Mac-1 co-staining (BM: 68% (±20%); spleen: 36% (±27%); PB: 45% (±23%)) (Fig. 4.7a; Fig. 4.7c; Fig. 4.7e; Fig. 4.7h). The leukemic cells from these mice were also negative for the T-cell markers CD4/CD8 and the stem cell markers Sca-1/c-Kit (Fig. 4.7b; Fig. 4.7d; Fig. 4.7f).

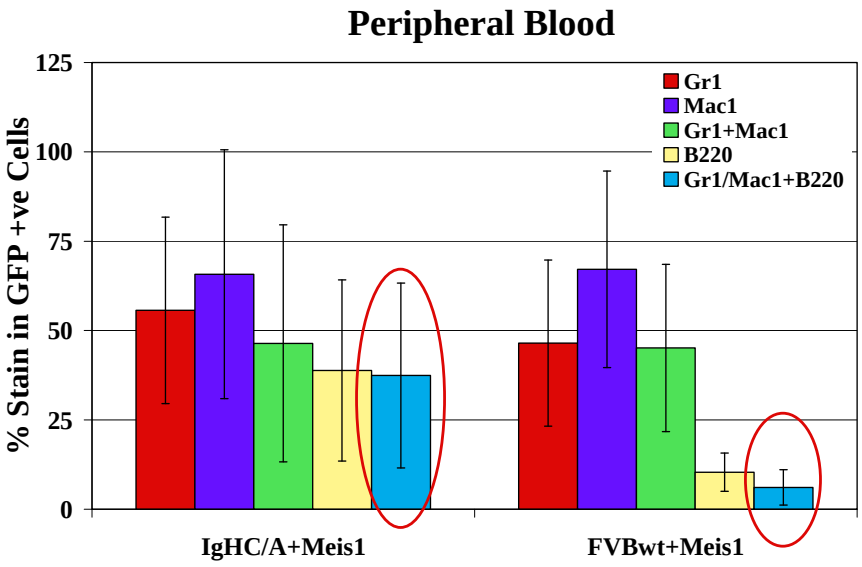


Fig. 4.7a Graphical representation of the percentage of cells staining positive for various markers in the peripheral blood of IgHC/A+Meis1 and FVBwt+Meis1 mice: The cells from the peripheral blood of IgHC/A+Meis1 mice showed a marked increase in Gr1/Mac1+B220 myeloid-lymphoid double positive cells (37% (±26%)) as compared to FVBwt+Meis1 mice (6% (±5%)). The percentage of Gr1+Mac1 population is almost same in the peripheral blood of IgHC/A+Meis1 (46% (±33%)) and FVBwt+Meis1 (45% (±23%)) leukemic mice. IgHC/A+Meis1: Mice transplanted with IgH-CALM/AF10 transgenic bone marrow cells transduced with Meis1 expressing retrovirus. FVBwt+Meis1: Mice transplanted with FVB wildtype bone marrow cells transduced with Meis1 expressing retrovirus.

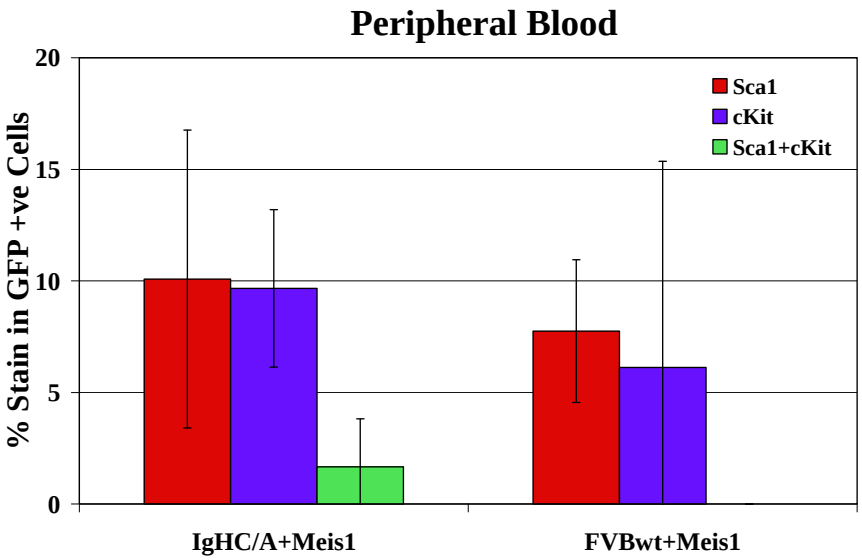


Fig. 4.7b Graphical representation of Sca-1/cKit staining in peripheral blood of IgHC/A+Meis1 and FVBwt+Meis1 mice: The Sca1+cKit double positive population is present at a very low percentage in the peripheral blood of IgHC/A+Meis1 2% (±2%) while it is absent in FVBwt+Meis1. IgHC/A+Meis1: Mice transplanted with IgH-CALM/AF10 transgenic bone marrow cells transduced with the Meis1 expressing retrovirus. FVBwt+Meis1: Mice transplanted with FVB wildtype bone marrow cells transduced with the Meis1 expressing retrovirus.

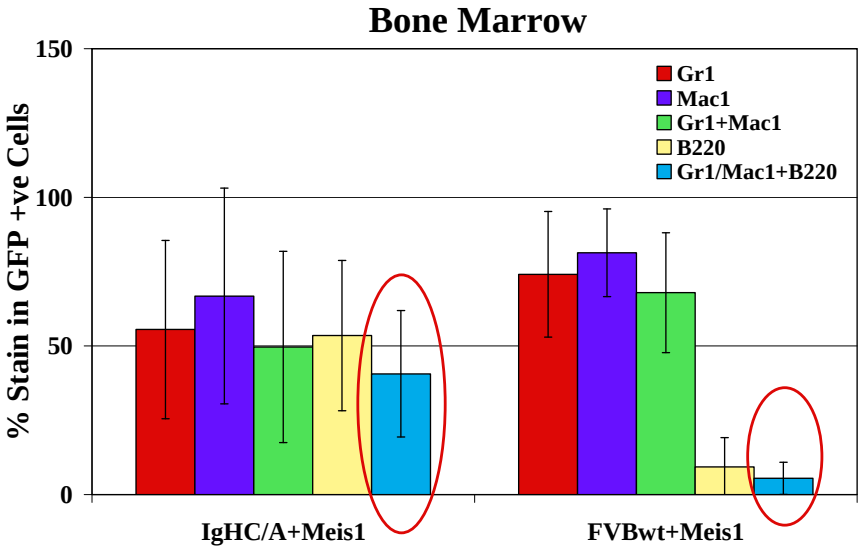


Fig. 4.7c Graphical representation of myeloid-lymphoid double positive population in the bone marrow of IgHC/A+Meis1 and FVBwt+Meis1 mice: Bone marrow cells from the IgHC/A+Meis1 mice showed a marked elevation of Gr1/Mac1+B220 myeloid-lymphoid co-staining with 41% (±21%) compared to cells from FVBwt+Meis1 mice (5% (±5%)). The % of Gr1+Mac1 population is higher in the bone marrow of FVBwt+Meis1 (68% (±20%)) than IgHC/A+Meis1 (50% (±32%)) leukemic mice. IgHC/A+Meis1: Mice transplanted with IgH-CALM/AF10 transgenic bone marrow cells transduced with the Meis1 expressing

retrovirus. FVBwt+Meis1: Mice transplanted with FVB wildtype bone marrow cells transduced with the Meis1 expressing retrovirus.

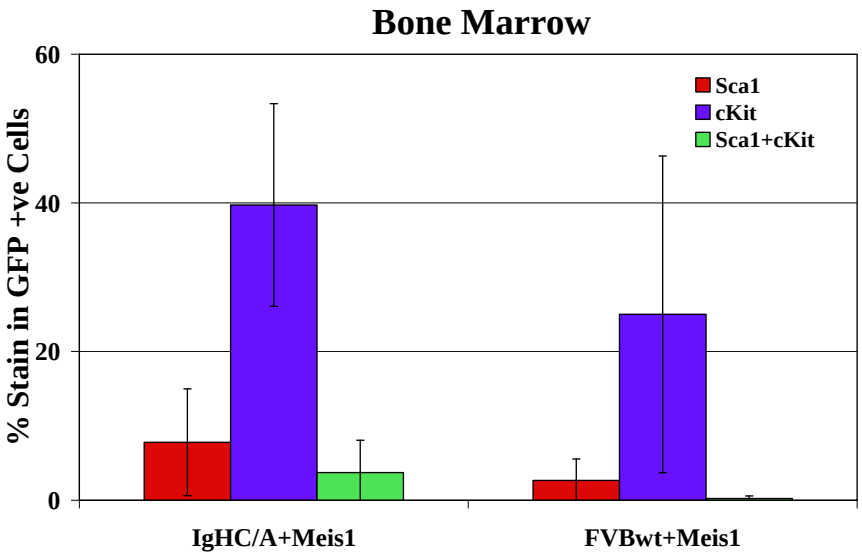


Fig. 4.7d Graphical representation of Sca-1/cKit staining in the bone marrow of IgHC/A+Meis1 and FVBwt+Meis1 mice: The Sca1+cKit double positive population is present in the bone marrow of IgHCA+Meis1 4% (±4%) while it is absent in FVBwt+Meis1. IgHC/A+Meis1: Mice transplanted with IgH-CALM/AF10 transgenic bone marrow cells transduced with the Meis1 expressing retrovirus. FVBwt+Meis1: Mice transplanted with FVB wildtype bone marrow cells transduced with the Meis1 expressing retrovirus.

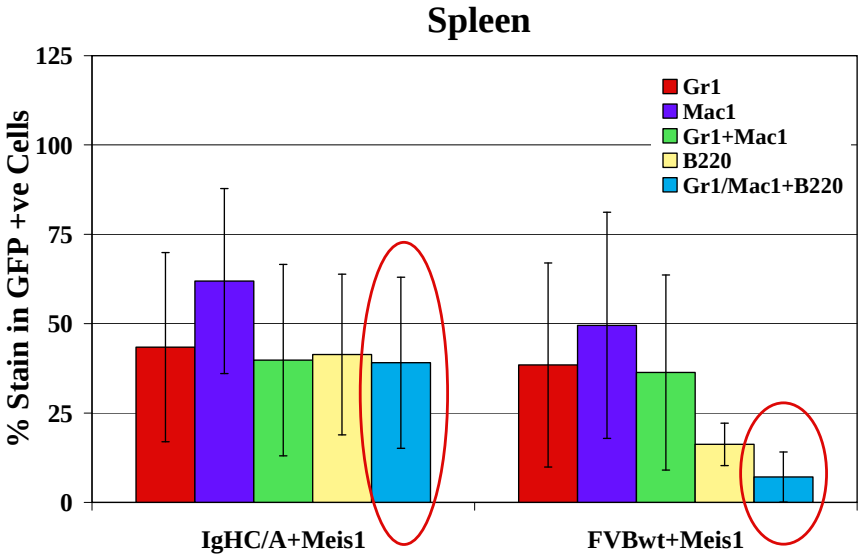


Fig. 4.7e Graphical representation of myeloid-lymphoid double positive population in the spleens of IgHC/A+Meis1 and FVBwt+Meis1 mice: Speen cells from the IgHC/A+Meis1 mice showed a significantly higher Gr1/Mac1+B220 double positive population (39% (±24%)) than spleen cells from FVBwt+Meis1 mice

(7% (±7%)). The proportion of Gr1+Mac1 postive cells is almost same in the spleen of IgHC/A+Meis1 (40% (±27%)) and FVBwt+Meis1 (36% (±27%)) leukemic mice. IgHC/A+Meis1: Mice transplanted with IgH-CALM/AF10 transgenic bone marrow cells transduced with the Meis1 expressing retrovirus. FVBwt+Meis1: Mice transplanted with FVB wildtype bone marrow cells transduced with the Meis1 expressing retrovirus.

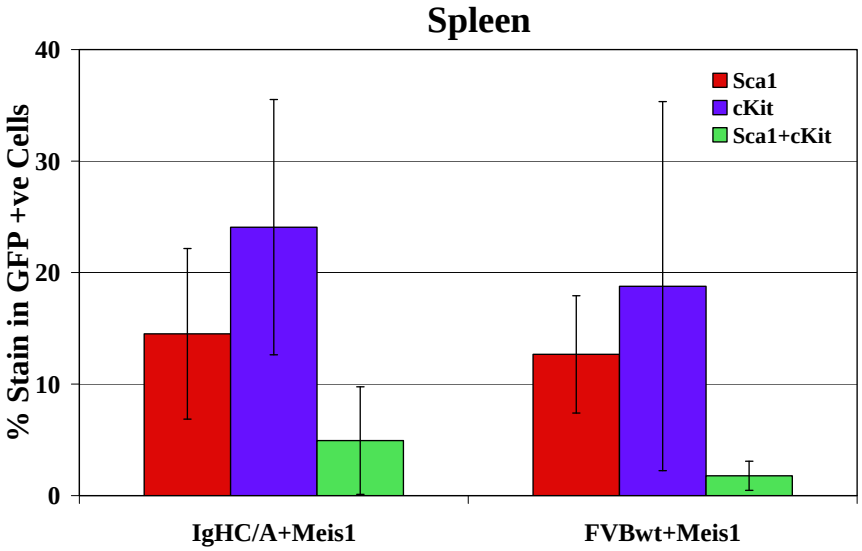


Fig. 4.7f Graphical representation of Sca-1/cKit staining in the spleen of IgHC/A+Meis1 and FVBwt+Meis1 mice: The Sca1+cKit double positive population in cells from the spleen of IgHC/A+Meis1 mice is slightly higher (5% (±5%)) than in the spleen cells from FVBwt+Meis1 mice (2% (±1%)). IgHC/A+Meis1: Mice transplanted with IgH-CALM/AF10 transgenic bone marrow cells transduced with the Meis1 expressing retrovirus. FVBwt+Meis1: Mice transplanted with FVB wildtype bone marrow cells transduced with the Meis1 expressing retrovirus.

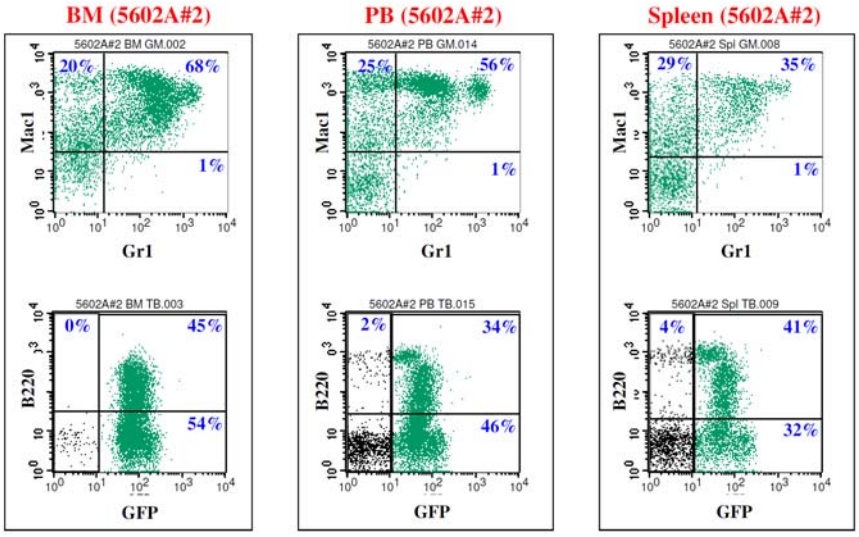


Fig. 4.7g Scatter plot of flow cytometric analyses of BM, PB and spleen cells from IgHC/A+Meis1 leukemic mouse 5602A#2: The majority of cells from the leukemic bone marrow (BM), peripheral blood (PB)

and spleen stained positive for myeloid markers Gr-1 and Mac-1, and also for lymphoid marker B220. The staining percentages are indicated in the quadrants of the plots. IgHC/A+Meis1: Mice transplanted with IgH-CALM/AF10 transgenic bone marrow cells transduced with the Meis1 expressing retrovirus.

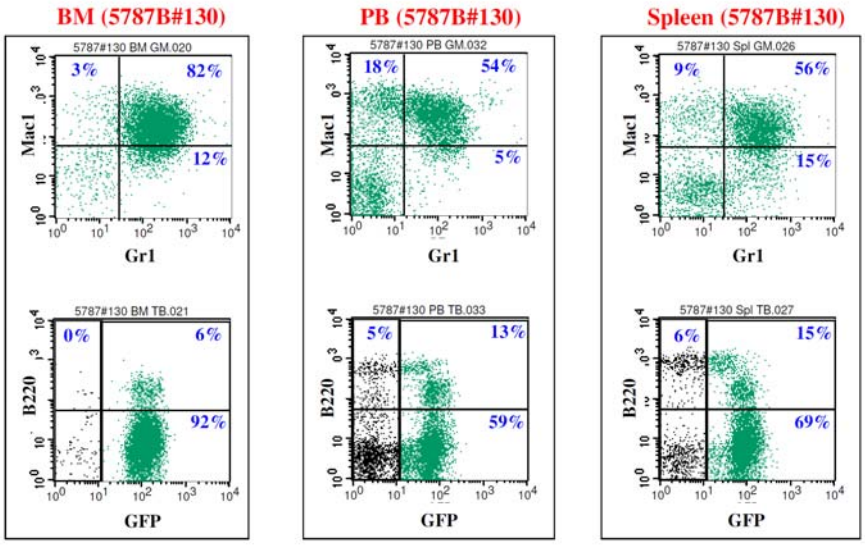


Fig. 4.7h Scatter plot of flow cytometric analyses of BM, PB and spleen cells from FVBwt+Meis1 leukemic mouse 5787B#130: The majority of cells from the leukemic bone marrow (BM), peripheral blood (PB) and spleen stained highly positive for myeloid markers Gr-1 and Mac-1, but less so for the lymphoid marker B220. The staining percentages are indicated in the quadrants of the plots. FVBwt+Meis1: Mice transplanted with FVB wildtype bone marrow cells transduced with the Meis1 expressing retrovirus.

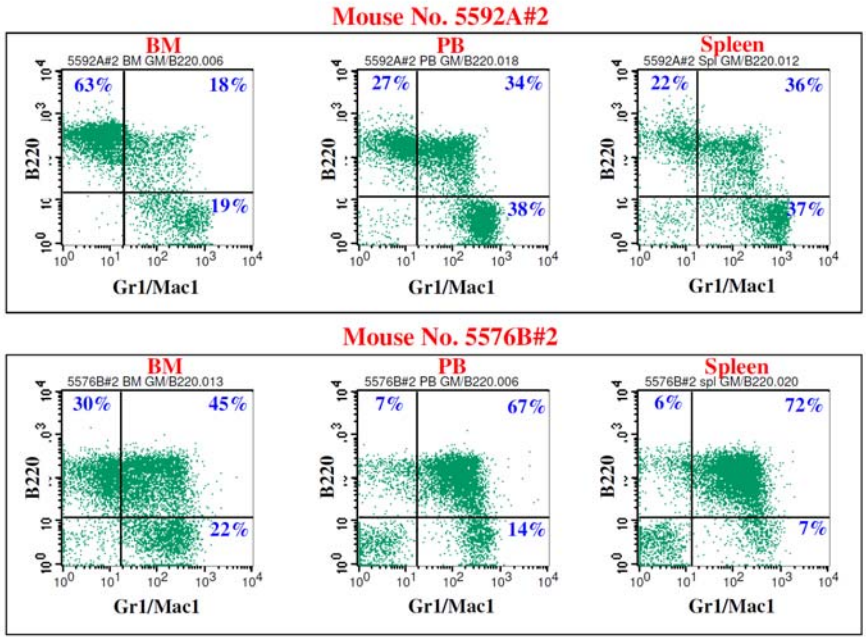


Fig. 4.7i Co-staining of myeloid and lymphoid markers (Gr-1/Mac-1+B220) on cells from IgHCA+Meis1 leukemic mice: Scatter plot of BM, PB and spleen cells showing a Gr-1/Mac-1+B220 double positive cell

population which is typical of CALM/AF10-associated leukemias. The staining percentages are indicated in the quadrants of the plots. IgHC/A+Meis1: Mice transplanted with IgH-CALM/AF10 transgenic bone marrow cells transduced with the Meis1 expressing retrovirus.

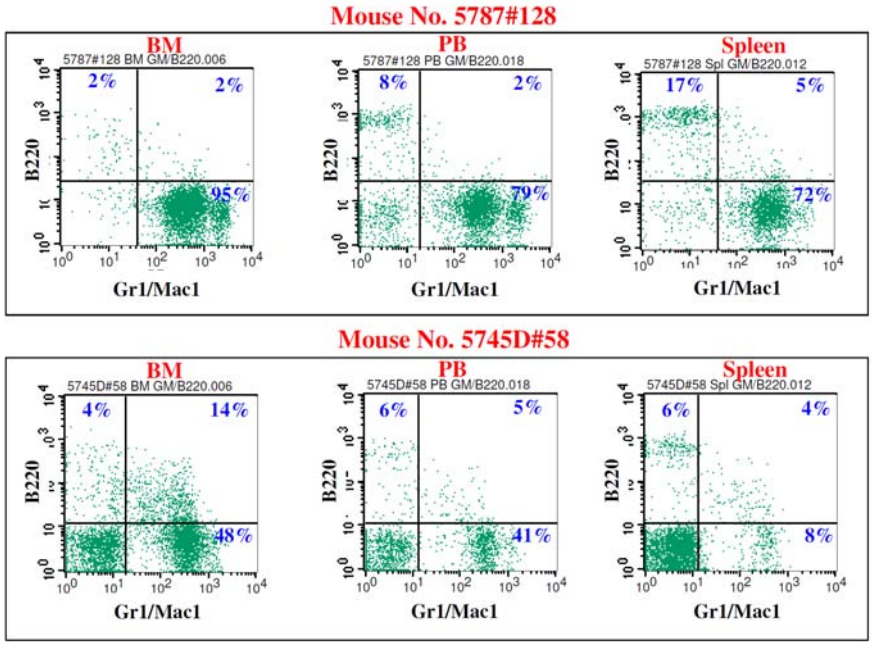


Fig. 4.7j Co-staining of myeloid and lymphoid markers (Gr-1/Mac-1+B220) for FVBwt+Meis1 leukemic mice: Unlike IgHCA+Meis1 leukemic mice, FVBwt+Meis1 mice showed a very small myeloid-lymphoid double positive population in the bone marrow, peripheral blood and spleen. The staining percentages are indicated in the quadrants of the plots. FVBwt+Meis1: Mice transplanted with FVB wildtype bone marrow cells transduced with the Meis1 expressing retrovirus.

4.8 Transplantation of secondary and tertiary recipient mice

According to Bethesda proposals, leukemia can be distinguished from other, less severe hematopoietic disorders by the fact that leukemia can be transplanted into secondary recipients and is able to lead to leukemia in these secondary recipients (Kogan *et al*., 2002). Therefore, to test whether the primary leukemic cells could repopulate and outnumber the normal hematopoietic compartment, secondary transplantations were performed. We injected the leukemic cells from the bone marrow of primary leukemic mice into the tail vein of secondary recipient mice. The secondary recipient mice were either lethally irradiated and then injected with primary leukemic cells along with mock cells, or they were directly injected with primary leukemic cells without irradiation. To further examine the

aggressiveness of the leukemia, tertiary transplantations were also performed. In tertiary transplantation the bone marrow cells from sacrificed secondary mice were injected directly into the tail vein of tertiary recipient mice.

Some of the secondary recipient mice were injected with 1×10^6 primary leukemic cells without irradiation. The rest of the mice were myeloablated using 800 cGy total body irradiation and injected with 1×10^6 primary leukemic cells and 2×10^6 mock cells. All the tertiary recipient mice were injected with 1×10^6 of secondary leukemic cells without irradiation.

4.8.1 Secondary and tertiary transplantations of primary leukemic mice transplanted with IgH-CALM/AF10 transgenic bone marrow cells transduced with the Meis1 expressing retrovirus (IgHC/A+Meis1)

All the IgHC/A+Meis1 secondary mice (n=4) developed acute myeloid leukemia and died within a range of 21 to 28 days (median latency = 25 days, Table A.13 (Appendix)). The disease phenotype was similar to that seen in the primary leukemic mice. All the IgHC/A+Meis1 tertiary transplanted mice (n=4) also developed an aggressive acute myeloid leukemia by 15 days post transplantation (Table A.14 (Appendix)). The tertiary leukemic mice had the same leukemia phenotype as the primary and secondary transplanted mice (Fig. 4.4).

4.8.2 Secondary and tertiary transplantations of primary leukemic and non-leukemic mice transplanted with FVB wildtype bone marrow cells transduced with the Meis1 expressing retrovirus (FVBwt+Meis1)

Cells from FVBwt+Meis1 primary leukemic as well as non-leukemic mice were injected into secondary recipients. All the secondary mice injected with primary leukemic cells (n=4) developed acute myeloid leukemia and died within a latency period of 28 to 63 days post transplantation (median latency = 44 days, Table A.15 (Appendix)). The disease phenotype was similar the one seen in the primary leukemic mice. However, as expected, all the secondary recipient mice injected with cells from primary non-leukemic mice (n=2) remained healthy and did not develop leukemia even up to 122 days post transplantation. These mice are still under observation (Table A.15 (Appendix)). The FVBwt+Meis1 tertiary transplanted

mice (n=4) from secondary leukemic mice developed an aggressive acute myeloid leukemia and died within a range of 21 to 28 days post transplantation (median latency = 25 days, Table A.16 (Appendix)). The tertiary leukemic mice had the same leukemia phenotype as the primary and secondary transplanted mice.

All the secondary and tertiary mice showed the same disease as the primary leukemic mice (Table 4.8.2a; Table 4.8.2b). Therefore, the disease was retransplantable and fulfilled to criteria for leukemia.

Gene	No. of transplanted mice	Average latency period (days)
IgHC/A+Meis1	4	25
FVBwt+Meis1	4 (leukemic mice)	44
FVBwt+Meis1	2 (non-leukemic mice)	Remained healthy up to an observation period of 122 days

Table 4.8.2a Summary of IgHC/A+Meis1 and FVBwt+Meis1 secondary transplanted mice: IgHC/A+Meis1: Mice transplanted with IgH-CALM/AF10 transgenic bone marrow cells transduced with the Meis1 expressing retrovirus; FVBwt+Meis1: Mice transplanted with wildtype bone marrow cells transduced with the Meis1 expressing retrovirus.

Gene	No. of transplanted mice	Average latency period (days)
IgHC/A+Meis1	4	15
FVBwt+Meis1	4	25

Table 4.8.2b Summary of IgHC/A+Meis1 and FVBwt+Meis1 tertiary transplanted mice: IgHC/A+Meis1: Mice transplanted with IgH-CALM/AF10 transgenic bone marrow cells transduced with the Meis1 expressing retrovirus; FVBwt+Meis1: Mice transplanted with wildtype bone marrow cells transduced with the Meis1 expressing retrovirus.

4.9 DJ_H rearrangement PCR from the leukemic bulk

Some of the leukemic cells of the primary leukemic mice transplanted with IgH-CALM/AF10 transgenic bone marrow cells transduced with the Meis1 expressing retrovirus (IgHC/A+Meis1) stained positive for both the B-cell marker B220 and myeloid markers ($Gr1^+Mac1^+/B220^+$), which is a characteristic feature of CALM/AF10-associated leukemia (Deshpande et al., 2006). Therefore, we thought that these leukemic cells might have the properties of B cells such as *IgH* gene rearrangement.

Genomic rearrangements of the diversity (D) and joining (J) segments in the immunoglobulin heavy chain locus are markers of lymphoid cells. Our multiplex PCR strategy detects the most common DJ_H rearrangements. In this PCR, wild type mouse spleen cells served as the positive control with the rearranged bands DJ_H3 and DJ_H4. The 32D murine myeloid cell line was used as the negative control. There were bands indicating a rearrangment in the multiplex PCR when DNA from the 32D murine myeloid cells was used as template. In this PCR, only the 2.1 kb germline band was visible.

The DNA obtained from the leukemic cells of the IgHC/A+Meis1 mice was positive for clonal DJ_H rearrangements. Since the multiplex PCR was performed from unsorted bulk bone marrow, in addition to a major bright band, which is indicative of the clonal rearrangement, other faint bands were also visible indicating that normal B cells contaminated the leukemic cells. Moreover, different leukemias had different rearrangement patterns indicating the different clonalities of the different leukemias. DNA from a secondary leukema showed a similar rearrangement pattern as the primary leukemia it was derived from (Fig. 4.9a).

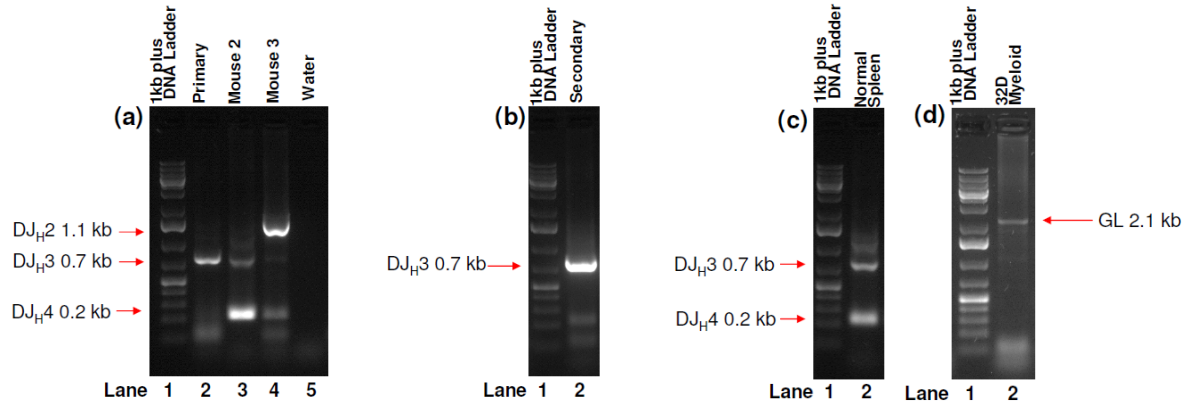


Fig. 4.9a Analysis of IgH DJ rearrangements in the bone marrow from IgHC/A+Meis1 leukemic mice: PCR analysis of genomic DNA extracted from unsorted bone marrow of leukemic IgHC/A+Meis1 mice showed different bands. The PCR template in the different PCR reactions is given in the following description. **(a)** Lane 1: DNA marker, lane 2: DNA from IgHC/A+Meis1 mouse 1 with band of 0.7 kb corresponding to a DJ_H3 rearrangement; lane 3: DNA from IgHC/A+Meis1 mouse 2 with bands of 0.7 kb and 0.2 kb corresponding to a DJ_H3 or DJ_H4 rearrangement, respectively; lane 4: DNA from IgHC/A+Meis1 mouse 3 with bands of 1.1 kb and 0.2 kb corresponding to a DJ_H2 or DJ_H4 rearrangement, respectively; lane 5: water control. **(b)** Lane 1: DNA marker; lane 2: DNA from a IgHC/A+Meis1 secondary mouse with band of 0.7 kb corresponding to a DJ_H3 rearrangement (This secondary mouse was transplanted from the primary leukemic mouse 1). **(c)** Lane 1: DNA marker; lane 2: DNA from wild type normal mouse spleen as positive control with bands of 0.7 kb and 0.2 kb corresponding to a DJ_H3 or DJ_H4 rearrangement respectively. **(d)** Lane 1: DNA marker; lane 2: DNA from the 32D murine myeloid cell line as negative control with the germline (GL) band of 2.1 kb and without any rearranged bands.

Mice transplanted with FVB wildtype bone marrow cells transduced with the Meis1 expressing retrovirus (FVBwt+Meis1) mice were also positive for DJ_H rearrangements. However, the pattern of rearrangement was different from IgHC/A+Meis1 leukemic mice. Unlike IgHC/A+Meis1 mice, all the FVBwt+Meis1 mice had similar pattern of bands after PCR analyses. Three major rearrangements (DJ_H2, DJ_H3 and DJ_H4) were clearly visible in all the mice and were almost similar to the wild type murine spleen. The secondary mouse showed the similar rearrangement as the primary mouse (Fig. 4.9b). This polyclonal rearrangement pattern seen after our multiplex PCR is most likely due to the contamination of the DNA from mature B cells. The germline amplification product, which should be visible

from the DNA of the leukemia, was not visible probably due the fact that this 2.1 kb fragment was not amplified as efficiently as the smaller fragments, which are derived from rearranged loci.

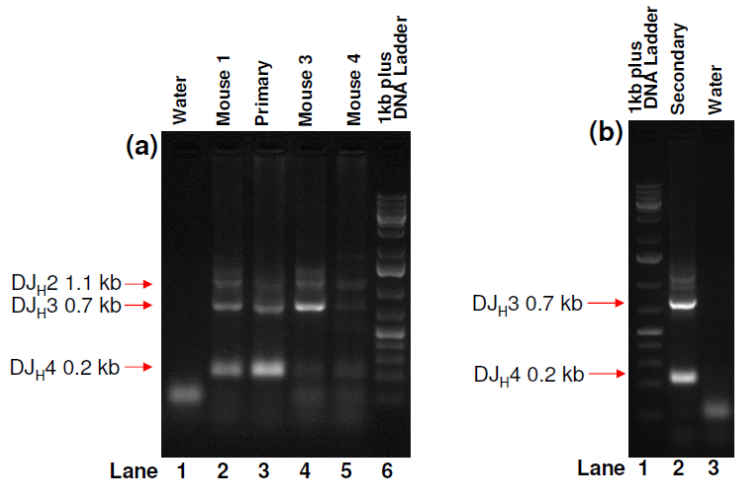


Fig. 4.9b Analysis of IgH DJ rearrangement in the bone marrow of FVBwt+Meis1 mice: PCR analysis of genomic DNA extracted from bone marrow of all FVBwt+Meis1 mice showed a similar pattern of rearranged bands, which is probably due to the presence of DNA from normal B cells with DJ_H rearrangements. **(a)** Lane 1: water control, lane 2: DNA from FVBwt+Meis1 mouse 1 with bands of 1.1 kb, 0.7 kb and 0.2 kb corresponding to a DJ_H2, DJ_H3, or DJ_H4 rearrangement, respectively lane 3: DNA from FVBwt+Meis1 mouse 2 with bands of 0.7 kb and 0.2 kb corresponding to a DJ_H3 or DJ_H4 rearrangement respectively; lane 4: DNA from FVBwt+Meis1 mouse 3 with bands of 1.1 kb and 0.7 kb corresponding to a DJ_H2 or DJ_H3 rearrangement respectively; lane 5: DNA from FVBwt+Meis1 mouse 4 with bands of 1.1 kb, 0.7 kb and 0.2 kb corresponding to a DJ_H2, DJ_H3 or DJ_H4 rearrangement, respectively; lane 6: DNA marker. **(b)** Lane 1: DNA marker; lane 2: DNA from FVBwt+Meis1 secondary mouse with bands of 0.7 kb and 0.2 kb corresponding to a DJ_H3 or DJ_H4 rearrangement, respectively (This secondary mouse was transplanted from the primary leukemic mouse 2). Normal mouse spleen DNA was taken as positive control (Fig. 4.8.a (c)) and DNA from the 32D murine myeloid cell line was taken as the negative control (Fig. 4.8.a (d)).

5 Discussion

Chromosomal translocations are frequently found in human leukemias. Some of these translocations result in the formation of fusion genes. The fusion proteins play an important role in leukemogenesis. To dissect the various factors necessary for the development of leukemia, we concentrated on the CALM/AF10 fusion protein as a model for our study (Dreyling *et al*., 1996). The CALM/AF10 results from the recurring t(10;11)(p12;q14) translocation which is rare and is associated with a poor prognosis (Bohlander *et al*., 2000). The t(10;11)(p12;q14) translocation has been observed in acute leukemias of several lineages including myeloid, lymphoid, megakaryocytic, eosinophilic and undifferentiated leukemias. It has also been described in malignant lymphomas.

Several studies on patients bearing the t(10;11)(p12;q14) translocation revealed this to be the only chromosomal abnormality. This strongly suggests CALM/AF10 to be the key event leading to malignant transformation of the hematopoietic cells (Bohlander *et al*., 2000). This is in line with a murine retroviral transduction and bone marrow transplantation model of CALM/AF10 (Deshpande *et al*., 2006), in which the expression of *CALM/AF10* after retroviral transduction of bone marrow cells results in the development of an aggressive acute leukemia with relatively short latency period of 110 days. This suggests that only a few additional mutations might be required for *CALM/AF10*-mediated leukemogenesis. Gilliland and colleagues suggested that at least two genetic events are required for leukemic transformation – increased cellular proliferation (Class I mutations) and block in differentiation (Class II mutations) (Kelly and Gilliland, 2002). However, the concept of just two classes of mutations does not reflect reality accurately. It is also often difficult to classify a given mutation into any one of the two classes.

This is also seen in recent studies on the molecular pathways involved in leukemogenesis. The development of AML is a multistep process and requires more than the two classes of mutations described above. Recently two approaches were used to decipher more genetic events involved in leukemogenesis. The first approach involves the karyotyping and DNA hybridization onto oligonucleotide arrays like SNP-arrays and array-CGH. The second approach involves the identification of mutations using classical Sanger sequencing or more advanced next generation sequencing (Murati *et al.*, 2012). Thus, several studies using these techniques have suggested that leukemogenic alterations affect as many as five different

classes of genes. The five different classes of proteins encoded by these genes include signaling pathway components, transcription factors, epigenetic regulators, tumor suppressors and RNA maturation or DNA repair related factors (Fig. 5a) (Murati *et al.*, 2012; Thiede, 2012).

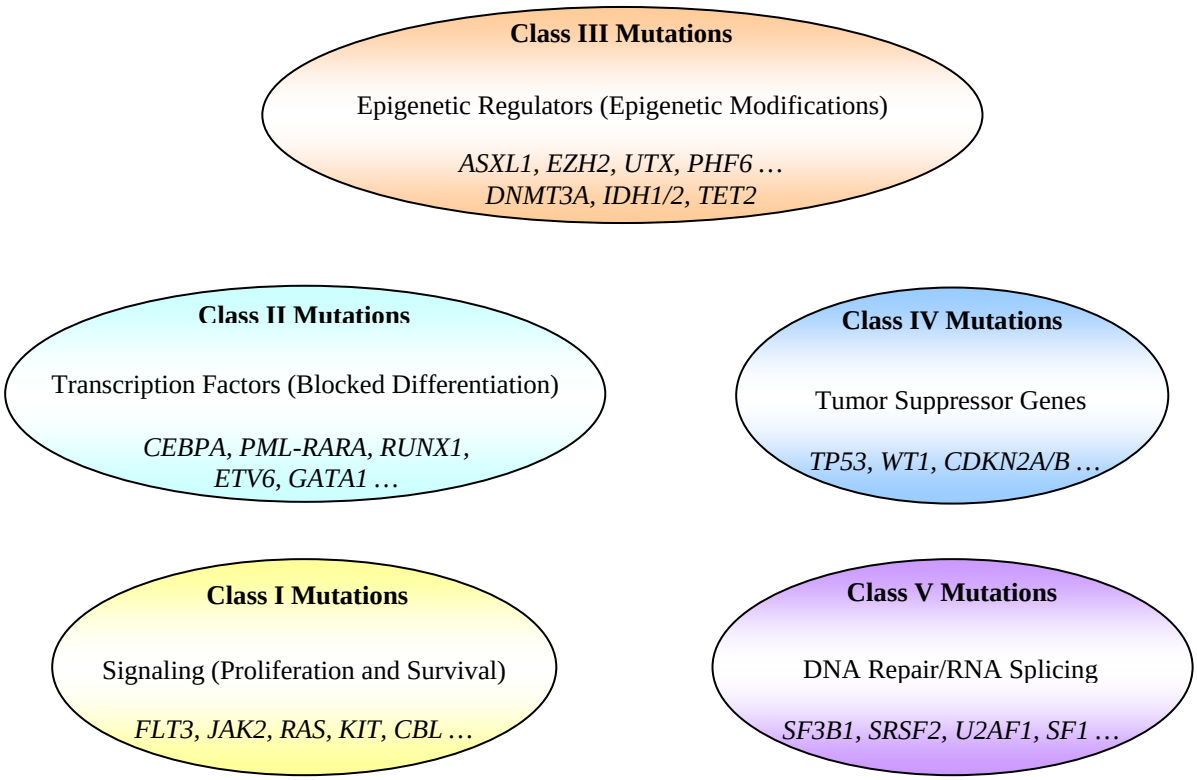


Fig. 5a Schematic representation of five classes of leukemogenic genes. (Adapted from Murati *et al.*, 2012 and Thiede, 2012)

Thus, the long latency period and incomplete penetrance observed in *CALM/AF10* transgenic mice under the control of *Vav* promoter can be explained by the requirement for additional genetic events affecting genes in the five classes described above (Caudell *et al.*, 2007). The requirement for additional mutations might also explain the long latency (median 187 days) observed in our mouse model.

As described earlier, IgHCALM/AF10 transgenic mice did not develop leukemia possibly due to the late expression of *CALM/AF10* fusion gene in mature B-cells, which might not be

susceptible to CALM/AF10 mediated transformation anymore. A finding that supports this view is the fact that *CALM/AF10* primarily targets lineage-uncommitted progenitors and that *CALM/AF10* patients develop hematologic malignancy of multi-lineages. This implies that CALM/AF10-driven leukemias arise from stem cells or progenitors with multi-lineage potential (Kobayashi *et al*., 1997). The upregulation of the *Hoxa* cluster including *Meis1* is a common phenomenon in *CALM/AF10*-positive leukemias (Mulaw *et al*., 2012; Dik *et al*., 2005; Caudell *et al*., 2007). Moreover, *Meis1* has been shown to collaborate with several *Hox* genes and the *NUP98-HOXD13* fusion gene to accelerate leukemia development (Thorsteinsdottir *et al*., 2001; Pineault *et al*., 2003).

Therefore, we sought to evaluate the role of *Meis1* as a collaborating factor of *CALM/AF10*. To achieve this, lethally irradiated recipient mice were transplanted with IgH-CALM/AF10 transgenic bone marrow cells transduced with a Meis1 expressing retrovirus. In these mice *CALM/AF10* is expressed late in the mature B-cell compartment. Mice transplanted with IgH-CALM/AF10 transgenic bone marrow cells transduced with the Meis1 expressing retrovirus showed rapid engraftment with GFP positive cells at 8 weeks post transplantation. All the mice succumbed to an aggressive acute leukemia with a median latency of 187 days and with 100% penetrance. This relatively long latency indicates that additional mutations were required for the development of the leukemia. The massive infiltration of leukemic blasts in non-hematopoietic organs of leukemic mice transplanted with IgH-CALM/AF10 transgenic bone marrow cells transduced with the Meis1 expressing retrovirus underscored the aggressive nature of the leukemia. The acute leukemias that developed in these mice were predominantly myeloid leukemias. The myeloid nature of the leukemia was confirmed by immunohistology and staining for myeloid markers. The leukemic cells also stained positive for the B-cell marker B220 in addition to myeloid markers. Indeed, a biphenotypic population of myeloid and lymphoid markers ($Gr1^+Mac1^+/B220^+$) was present, which is a typical characteristic feature of CALM/AF10-driven leukemias (Deshpande *et al*., 2006). These leukemic mice had clonal DJ_H rearrangements. This hints at the lymphoid identity of these cells.

Altogether, the myeloid nature of the leukemia, the positive staining for B-cell marker B220, the presence of clonal DJ_H rearrangements in the unsorted leukemic bone marrow and the presence of a myeloid-lymphoid biphenotypic population suggest that the target of transformation of CALM/AF10 might have be an early progenitor capable of both lymphoid

as well as myeloid differentiation. Another possibility could be that the leukemia is propagated by lymphoid progenitor (positive for $B220^+$ with IgH DJ rearrangement) which is impaired in its lymphoid development by the action of CALM/AF10 and which then enters a default myeloid differentiation program. In our model the IgH promoter driven *CALM/AF10* expression is late in the B-cell compartment. Therefore, *Meis1* seems to play an important role in leukemogenesis by still being able to initiate transformation of rather differentiated cells with the help of CALM/AF10.

The endogenous expression of *Meis1* and *Hox* genes is highest in the early hematopoietic compartment and is downregulated with differentiation (Pineault *et al*., 2002). However, in our model the expression of *Meis1* is driven by LTR retroviral promoter. Therefore, *Meis1* is expressed throughout the hematopoietic compartment and the expression is stronger than the expression of the endogenous *Meis1*. It could be possible that expression of *Meis1* (a transcription factor) in the early hematopoietic compartment is causing increased proliferation and thereby more replication resulting in occurrence of more mutations. In this way, *Meis1* promotes the accumulation of additional mutations. The acquisition of additional mutations and expression of CALM/AF10 in the B cell compartment is thus creating a differentiation block in these cells. In this scenario the default myeloid differentiation program is initiated in these cells leading to myeloid leukemias. In addition to this, CALM/AF10 expression is known to cause genomic instability (Lin *et al*., 2006). Thus, both CALM/AF10 and Meis1 expression might promote the acquisition of additional mutation, which are required for leukemia development. This hypothesis explains the relative long latency of leukemia development in our model.

To gain a better understanding of the leukemia models developed here, it will be important to perform transcriptional profiling of both IgH-CALM/AF10 transgenic mice as well as of leukemic mice transplanted with IgH-CALM/AF10 transgenic bone marrow cells transduced with the Meis1 expressing retrovirus to determine the downstream targets and the genes which are differentially regulated. Finally, sequencing the genome or exomes of these leukemias should directly reveal additional mutations that are driving the disease.

Surprisingly, mice transplanted with FVB wildtype bone marrow cells transduced with the Meis1 expressing retrovirus also died at a median latency of 210 days. Among the sacrificed mice only 29% were diagnosed with acute myeloid leukemia. This observation was very

surprising to us, since it is reported that the overexpression of *Meis1* alone has no transforming activity (Kroon *et al*., 1998; Thorsteinsdottir *et al*., 2001). It is crucial to note that the most important difference between our model and the ones reported in literature is the mouse strain. The bone marrow transplantation experiments with *Meis1* alone were conducted in the C57BL/6 background but we used FVB strain. In a recent report, the selection of the mouse strains was shown to have a great effect on the result of bone marrow transplantation experiments (Otsuru *et al.*, 2010). The FVB mouse strain is widely used for generating transgenic animals because of their high reproductive performance and large prominent pronuclei which facilitate micro injection of DNA (Taketo *et al.*, 1991). A detailed study on spontaneous lesions in aging FVB mice indicated that the incidence of tumors in these mice is higher than in other mouse strains. Especially, lung cancer is observed at an increased frequency in FVB mice (Mahler *et al.*, 1996). Another study has reported that keratinocytes from FVB mice are more susceptible to malignant progression than other strains suggesting an increased sensitivity of this strain (Woodworth *et al.*, 2004). The analysis of the sensitivity to chemical induction of squamous cell carcinomas in the skin showed that FVB mice are more likely to develop squamous cell carcinomas than other mouse strains (Hennings *et al.*, 1993).

From these observations a hypothesis could be drawn based on the mouse genetic background, retroviral insertion event and accumulation of rare events to explain the occurrence of leukemia in mice transplanted with FVB wildtype bone marrow cells transduced with the Meis1 expressing retrovirus (Fig. 5b). The “rare events” could be the activating oncogenes or inactivating tumor suppressor genes. At this point it is important to note that FVB wild type mice retrovirally transduced with the empty MIY vector remained healthy and were followed up to an observation period of 224 days post transplantation. In addition to this, mice transplanted with IgH-CALM/AF10 transgenic bone marrow cells transduced with the empty MIG retrovirus remained healthy and were kept under observation for 392 days post transplantation.

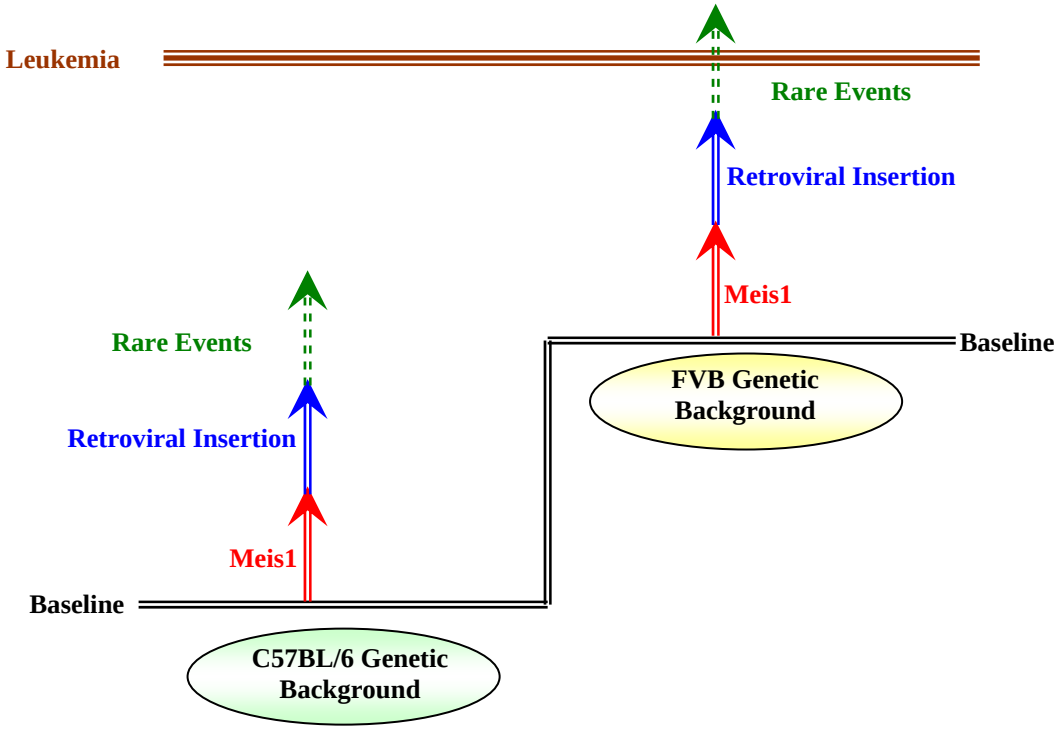


Fig 5b Diagram of the proposed differences in the genetic background of C57BL/6 and FVB mice: The base line level of the FVB strain is closer to the threshold level required for leukemic transformation than in the C57/BL6 strain. Therefore, in C57BL/6 mice *Meis1* retrovirally transduced bone marrow cells could not accumulate enough additional "rare events" to cross the threshold to leukemia. In contrast to this, the injection of FVB bone marrow cells overexpressing Meis1 leads to consistent leukemia development because the threshold is reached much faster than in the the C57/BL6 strain.

Therefore, these studies support the fact that the difference in mouse strain should be taken into consideration.

In summary, we could show in a combined transgenic bone marrow transplantation mouse model that Meis1 collaborates with CALM/AF10 to induce an acute myeloid leukemia. The cells from the primary mice were transplantable into secondary and tertiary recipients confirming their leukemic nature. This collaboration between Meis1 and CALM/AF10 was not seen in colony forming cell assays. In CFCs CALM/AF10 together with Meis1 failed to induce the transformation of hematopoietic progenitors. This difference between the two assay systems could either be due to the lack of required growth factors and conditions necessary for the proliferation of the leukemia initiating cell or lack of additional mutational

events required for transformation in the CFC setting. A detailed analyses of the leukemia generated in this model might uncover additional genetic events required for the development of CALM/AF10-induced leukemia.

6 Summary

Chromosomal translocations are common in human leukemias. Detailed studies of chromosomal translocation have been useful in understanding the pathogenesis and identifying therapeutic targets in hematologic malignancies. Some translocations result in the formation of fusion genes. These fusion proteins play an important role in leukemogenesis. The t(10;11)(p12;q14) translocation is rare but recurring and results in the formation of the CALM/AF10 fusion protein. Patients with this translocation have a bad prognosis.

To understand how CALM/AF10 leads to leukemia, various mouse models have been established. In a murine bone marrow retroviral transduction and transplantation model Deshpande *et al.* (2006) showed that mice expressing CALM/AF10 in their bone marrow cells developed an acute myeloid leukemia with a penetrance of 100% and a short latency period of 110 days. Using a transgenic mouse model, in which CALM/AF10 was under the control of Vav promoter, Peter Aplan and colleagues demonstrated that only 40% to 50% of mice developed leukemia after a long latency of 10 to 12 months. Two classical transgenic CALM/AF10 models were established in our group using the immunoglobulin heavy chain enhancer/promoter (IgH-CALM/AF10) and proximal murine LcK promoter (pLck-CALM/AF10) to drive CALM/AF10 expression. These transgenic mice did not show any leukemic phenotype even after an observation period of 15 months. Taken together these studies strongly suggest that additional collaborating factors are required for the *CALM/AF10* fusion gene to induce leukemia.

Meis1, a Hox cofactor, is known to collaborate with several Hox genes and Hox fusion genes such as *HOXA9* and *NUP98-HOXD13*. In these studies, *Meis1* played a critical role in accelerating the development of leukemia. It could also be shown that *MEIS1* is highly expressed in CALM/AF10 positive human leukemia cells. Therefore, I sought to determine whether the homeobox gene *Meis1* collaborates with CALM/AF10 in inducing leukemia.

In order to achieve this goal, lethally irradiated non-transgenic mice were transplanted with IgH-CALM/AF10 transgenic bone marrow cells transduced with a *Meis1* expressing retrovirus. The transplanted mice developed an acute leukemia with a penetrance of 100% and a median latency period of 187 days. The leukemia showed predominantly myeloid features such as the presence of myeloid marker positive cells. The myeloid blast cells

infiltrated in multiple hematopoietic as well as non-hematopoietic organs. The leukemic cells were also positive for the B-cell marker B220. Cells that were positive for both lymphoid and myeloid markers, a characteristic feature of CALM/AF10-induced leukemia, were also detected in all the mice. The leukemic cells had clonal DJ_H rearrangements. Overall, these data suggest that the transformed cell might be an early progenitor cell capable of lymphoid as well as myeloid differentiation or that the leukemia was initiated by a $B220^+$ IgH DJ rearranged cell with blocked lymphoid differentiation, which started a default myeloid differentiation program. By performing serial secondary and tertiary transplantations the leukemic nature of the disease could be confirmed. Colony forming cell assays showed that CALM/AF10 in collaboration with Meis1 failed to induce the transformation of hematopoietic progenitors *in vitro*. This could either be due to the lack of required growth factors and conditions necessary for the proliferation of the transformable cell or lack of additional events essential for progression towards leukemia development.

In conclusion, I have demonstrated that Meis1 collaborates with CALM/AF10 in inducing acute myeloid leukemia. Additional, detailed analyses of the leukemia initiating cell in these models would help to better understand the pathogenesis of CALM/AF10-induced leukemia.

7 Zusammenfassung

Translokationen treten bei humanen Leukämien sehr häufig auf. Die Analyse von Chromosomentranslokationen hat sowohl zum Verständnis der Pathogenese von Leukämien als auch zur Identifizierung von therapeutischen Zielen geführt. Manche Translokationen führen zur Bildung von Fusionsgenen. Diese Fusionsproteine spielen in der Leukämogenese eine wichtige Rolle. Das Fusionsprotein CALM/AF10 entsteht durch die seltene, aber wiederholt auftretende Translokation t(10;11)(p12;q14). Patienten mit dieser Translokation haben eine schlechte Prognose.

Um zu verstehen, wie es von der Expression des Proteins CALM/AF10 zum Ausbruch der Leukämie kommt, wurden verschiedene Mausmodelle etabliert. Deshpande *et al.* (2006) konnten in einem Mausknochenmarktransplantationsmodell nach retroviraler Transduktion zeigen, dass die Expression von CALM/AF10 zu einer akuten myeloischen Leukämie mit einer Penetranz von 100% und einer kurzen Latenzzeit von 110 Tagen führt. In einem transgenen Mausmodell, bei dem CALM/aF10 unter der Kontrolle des Vav-Promotors exprimiert wurde, wurden von Peter Aplan und Kollegen in lediglich 40 - 50% der Mäuse nach einer langen Latenzzeit von 10 bis 12 Monaten Leukämien beobachten. In unserer Gruppe wurden zwei klassische transgene CALM/AF10-Mausmodelle entwickelt, bei denen CALM/AF10 vom Immunglobulin Heavy-Chain Enhancer Promotor (IgH-CALM/AF10) bzw. vom proximalen murinen Lck-Promoter (pLck-CALM/AF10) gesteuert wurde. Diese transgenen Mäuse zeigten auch nach 15 monatiger Beobachtungszeit noch immer keinen leukämischen Phänotyp. Zusammenfassend zeigen diese Studien, daß neben dem Fusionsgene CALM/AF10 weitere Faktoren zur Induktion von Leukämie notwendig sind.

Von *Meis1*, einem Hox–Kofaktor, ist bekannt, dass es mit mit einigen Hox Genen und auch Hox Fusionsgenen, wie *HOXA9* und *NUP-HOXD13*, kollaboriert. In diesen Studien spielte *Meis1* eine wichtige Rolle in der Beschleunigtung der Leukämieentwicklung. Ebenfalls konnte gezeigt werden, daß *MEIS1* in CALM/AF10 positiven humanen Leukämiezellen sehr hoch exprimiert wird. Aufgrund dieser Beobachtungen beschloss ich nachzuweisen, ob das Homeoboxgen *Meis1* mit CALM/AF10 bei der Leukämieentwicklung kollaboriert.

Um dieses Ziel zu erreichen, wurden letal bestrahlte, nicht transgene Mäuse mit IgH-CALM/AF10 transgenen Knochenmarkszellen transplantiert, die mit Meis1 exprimierendem

Retrovirus transduziert wurden. Die transplantierten Mäuse entwickelten eine akute Leukämie mit einer Penetranz von 100% und einer mittleren Latenzzeit von 187 Tagen. Die Leukämie zeigte vorwiegend myeloische Eigenschaften mit myeloischen Oberflächenmarkern. Die Blasten infiltrierten sowohl hämatopoetische als auch in nicht hämatopoetische Organe. Die Leukämiezellen waren ebenfalls positiv für den B-Zellmarker B220. Auch Zellen, die sowohl für lymphoide als auch myeloische Marker positiv waren – dies ist ein charakteristisches Zeichen für CALM/AF10 induzierte Leukämie – wurden in allen Mäusen gefunden. Die Leukämiezellen hatten klonale DJ_H Umlagerungen. Insgesamt lassen diese Daten den Schluss zu, dass die transformierte Zelle eine frühe Vorläuferzelle sein könnte, die sowohl zur lymphatischen als auch zur myeloischen Differenzierung fähig ist oder daß die Leukämie in einer $B220^+$ IgH DJ rearrangierten Zelle mit blockierter lymphatischer Differenzierung entstanden ist, bei der das Standardprogramm der myeloischen Differenzierung abgerufen wurde. Durch Transplantation in sekundäre und tertiäre Rezipientenmäuse konnte bestätigt werden, dass es sich in der Tat um eine Leukämie handelte. Im Colony Forming Cell-Assay hingegen führte die Kollaboration von CALM/AF10 mit Meis1 nicht zur Transformation von hämatopoetischen Vorläuferzellen. Dies könnte zum einen daran liegen, dass notwendige Wachstumsfaktoren und Wachstumsbedingungen für die Proliferation der transformierbaren Zellen fehlten, oder, dass zusätzlichen genetische Ereignissen, die für die Leukämieentstehung essentiell sind, nicht vorhanden waren.

Zusammenfassend konnte ich zeigen, daß Meis1 mit CALM/AF10 bei der Induktion der akuten myeloischen Leukämie kollaboriert. Zusätzliche, detailliert Analysen der Leukämie induzierenden Zellen in diesem Modell würden helfen, die Pathogenese der CALM/AF10 induzierten Leukämie besser zu verstehen.

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APPENDIX: Tables

Serial no.	Experiment no.	Gene
1	5576A#1	IgHC/A+Meis1
2	5576A#2	IgHC/A+Meis1
3	5576A#3	IgHC/A+Meis1
4	5576B#1	IgHC/A+Meis1
5	5576B#2	IgHC/A+Meis1
6	5576C#1	IgHC/A+Meis1
7	5592A#1	IgHC/A+Meis1
8	5592A#2	IgHC/A+Meis1
9	5592B#2	IgHC/A+Meis1
10	5592C#1	IgHC/A+Meis1
11	5592C#2	IgHC/A+Meis1
12	5602A#1	IgHC/A+Meis1
13	5602A#2	IgHC/A+Meis1
14	5602A#3	IgHC/A+Meis1
15	5602B#1	IgHC/A+Meis1
16	5680A#1	IgHC/A+Meis1
17	5680A#2	IgHC/A+Meis1
18	5680A#3	IgHC/A+Meis1
19	5680B#1	IgHC/A+Meis1

Table A.1 0.5×10^6 of transduced bone marrow cells and 0.5×10^6 of mock cells were injected into lethally irradiated syngeneic recipient mice (n = 19). Mock cells indicate the mock transduced GFP negative bone marrow cells (IgHC/A+Meis1: Mice transplanted with IgH-CALM/AF10 transgenic bone marrow cells transduced with Meis1 expressing retrovirus)

Serial no.	Experiment no.	Gene
1	5745C#65	FVBwt+Meis1
2	5745C#66	FVBwt+Meis1
3	5745D#56	FVBwt+Meis1
4	5745D#57	FVBwt+Meis1
5	5745D#58	FVBwt+Meis1
6	5755A#96	FVBwt+Meis1
7	5755A#97	FVBwt+Meis1
8	5755A#98	FVBwt+Meis1
9	5755A#99	FVBwt+Meis1
10	5755A#101	FVBwt+Meis1
11	5755B#107	FVBwt+Meis1
12	5787A#127	FVBwt+Meis1
13	5787A#128	FVBwt+Meis1
14	5787A#129	FVBwt+Meis1
15	5787B#130	FVBwt+Meis1
16	5787B#131	FVBwt+Meis1
17	5787B#132	FVBwt+Meis1

Table A.2 0.5×10^6 of transduced bone marrow cells and 0.5×10^6 of mock cells were injected into lethally irradiated syngeneic recipient mice (n = 17). Mock cells indicate the mock transduced GFP negative bone marrow cells (FVBwt+Meis1: Mice transplanted with wildtype bone marrow cells transduced with Meis1 expressing retrovirus)

Serial no.	Experiment no.	Gene
1	5852A#187	IgHC/A+MIG
2	5852B#189	IgHC/A+MIG
3	5856A#191	IgHC/A+MIG
4	5856A#192	IgHC/A+MIG
5	5856A#193	IgHC/A+MIG
6	5856A#194	IgHC/A+MIG
7	5856B#190	IgHC/A+MIG
8	5856C#197	IgHC/A+MIG

Table A.3 0.5×10^6 of transduced bone marrow cells and 0.5×10^6 of mock cells were injected into lethally irradiated syngeneic recipient mice (n = 8). Mock cells indicate the mock transduced GFP negative bone marrow cells (IgHC/A+MIG: Mice transplanted with IgH-CALM/AF10 transgenic bone marrow cells transduced with empty retrovirus EGFP)

Serial no.	Experiment no.	Gene
1	5926#303	FVBwt+MIY
2	5939A#5	FVBwt+MIY
3	5939A#6	FVBwt+MIY
4	5939A#7	FVBwt+MIY
5	5939A#8	FVBwt+MIY
6	5939A#9	FVBwt+MIY
7	5939B#10	FVBwt+MIY
8	5939B#11	FVBwt+MIY

Table A.4 0.5×10^6 of transduced bone marrow cells and 0.5×10^6 of mock cells were injected into lethally irradiated syngeneic recipient mice (n = 8). Mock cells indicate the mock transduced YFP negative bone marrow cells (FVBwt+MIY: Mice transplanted with FVB wildtype bone marrow cells transduced with empty retrovirus EYFP)

Experiment No.	Engraftment		
	Peripheral blood	Bone marrow	Spleen
5576A#3	NA	96%	78%
5592C#1	98%	99%	94%
5576B#2	93%	98%	95%
5602A#1	50%	53%	46%
5592A#2	96%	97%	88%
5592C#2	96%	98%	89%
5576B#1	94%	96%	90%
5576A#1	77%	97%	84%
5592A#1	91%	97%	91%
5602A#2	80%	98%	74%
5680A#2	2%	77%	43%
5680A#3	4%	1%	6%
5602B#1	92%	95%	81%
5680A#1	82%	91%	64%
5602A#3	70%	95%	72%

Table A.5 Percentage engraftment of peripheral blood, bone marrow and spleen of leukemic mice transplanted with IgH-CALM/AF10 transgenic bone marrow cells transduced with Meis1 expressing retrovirus: The sacrificed mice showed a median peripheral blood engraftment levels of 78% (±17%), bone marrow engraftment levels of 92% (±12%) and spleen engraftment levels of 79% (±27%) (NA = not available)

Mouse no.	Retroviral construct	Peripheral blood RBC per ml×10^9	Peripheral blood WBC per ml×10^6
5576A#3	IgHC/A+Meis1	NA	NA
5592C#1	IgHC/A+Meis1	1.5	440
5576B#2	IgHC/A+Meis1	1.0	210
5602A#1	IgHC/A+Meis1	2.1	40
5592A#2	IgHC/A+Meis1	2.4	125
5592C#2	IgHC/A+Meis1	2.0	53
5576B#1	IgHC/A+Meis1	1.4	285
5592A#1	IgHC/A+Meis1	1.1	27
5602A#2	IgHC/A+Meis1	4.3	19
5680A#2	IgHC/A+Meis1	2.0	21
5602B#1	IgHC/A+Meis1	4.0	12
5680A#1	IgHC/A+Meis1	1.0	12
5602A#3	IgHC/A+Meis1	5.0	235

Table A.6 RBC and WBC counts in the peripheral blood of leukemic mice transplanted with IgH-CALM/AF10 transgenic bone marrow cells transduced with Meis1 expressing retrovirus (IgHC/A+Meis1) (NA = not available)

Mouse no.	Retroviral construct	Spleen weight (mg)	Spleen length (cm)
5576A#3	IgHC/A+Meis1	370	2.3
5592C#1	IgHC/A+Meis1	714	3.5
5576B#2	IgHC/A+Meis1	511	2.5
5602A#1	IgHC/A+Meis1	626	2.7
5592A#2	IgHC/A+Meis1	775	3.2
5592C#2	IgHC/A+Meis1	282	2.5
5576B#1	IgHC/A+Meis1	592	2.5
5592A#1	IgHC/A+Meis1	840	2.7
5602A#2	IgHC/A+Meis1	360	2.5
5680A#2	IgHC/A+Meis1	97	1.5
5602B#1	IgHC/A+Meis1	232	2.0
5680A#1	IgHC/A+Meis1	167	1.9
5602A#3	IgHC/A+Meis1	608	2.9

Table A.7 Splenomegaly in mice transplanted with IgH-CALM/AF10 transgenic bone marrow cells transduced with Meis1 expressing retrovirus (IgHC/A+Meis1)

Experiment No.	Engraftment		
	Peripheral blood	Bone marrow	Spleen
5745D#58	86%	91%	83%
5755A#97	91%	97%	70%
5745D#57	75%	92%	69%
5755A#99	55%	NA	53%
5787A#128	71%	82%	68%
5787B#130	71%	84%	76%
5755A#98	74%	91%	54%
5787B#131	35%	16%	39%
5787A#129	40%	90%	46%

Table A.8 Percentage engraftment of peripheral blood, bone marrow and spleen of mice transplanted with FVB wldtype bone marrow cells transduced with Meis1 expressing retrovirus: The sacrificed mice showed median peripheral blood engraftment levels of 66% (±19%), bone marrow engraftment levels of 80% (±26%) and spleen engraftment levels of 62% (±15%).

Mouse no.	Retroviral construct	Peripheral blood RBC per ml×10^9	Peripheral blood WBC per ml×10^6
5745D#57	FVBwt+Meis1	3	60
5745D#58	FVBwt+Meis1	1	55
5755A#97	FVBwt+Meis1	3	175
5755A#98	FVBwt+Meis1	4	35
5787B#130	FVBwt+Meis1	6	20

Table A.9 RBC and WBC counts in the peripheral blood of leukemic mice transplanted with FVB wildtype bone marrow cells transduced with Meis1 expressing virus (FVBwt+Meis1).

Mouse no.	Retroviral construct	Peripheral blood RBC per ml×10^9	Peripheral blood WBC per ml×10^6
5755A#99	FVBwt+Meis1	1.6	2.5
5787A#128	FVBwt+Meis1	2	5
5787A#129	FVBwt+Meis1	5	1
5787B#131	FVBwt+Meis1	5	2
5755B#107	FVBwt+Meis1	7	1

Table A.10 RBC and WBC counts in the peripheral blood of non-leukemic mice transplanted with FVB wildtype bone marrow cells transduced with Meis1 expressing virus (FVBwt+Meis1)

Mouse no.	Retroviral construct	Spleen weight (mg)	Spleen length (cm)
5745D#57	FVBwt+Meis1	828	3.3
5745D#58	FVBwt+Meis1	480	2.6
5755A#97	FVBwt+Meis1	421	2.5
5755A#98	FVBwt+Meis1	552	2.7
5787B#130	FVBwt+Meis1	404	2.6

Table A.11 Spleen details of mice transplanted with wildtype bone marrow cells transduced with Meis1 expressing retrovirus (FVBwt+Meis1) leukemic mice

Mouse no.	Retroviral construct	Spleen weight (mg)	Spleen length (cm)
5755A#99	FVBwt+Meis1	584	2.9
5787A#128	FVBwt+Meis1	242	2.3
5787A#129	FVBwt+Meis1	100	1.7
5787B#131	FVBwt+Meis1	100	1.7
5755B#107	FVBwt+Meis1	132	1.6

Table A.12 Spleen details of mice transplanted with wildtype bone marrow cells transduced with Meis1 expressing retrovirus (FVBwt+Meis1) non-leukemic mice

Experiment no.	Gene	w or w/o irradiation	Primary leukemic cells	Mock cells	Days of survival	Disease
5688A#1	IgHC/A+Meis1	w irradiation	1.0×10^6	2.0×10^6	28	AML
5688B#1	IgHC/A+Meis1	w/o irradiation	1.0×10^6		28	AML
5901C#268	IgHC/A+Meis1	w irradiation	1.0×10^6	2.0×10^6	21	AML
5901F#265	IgHC/A+Meis1	w/o irradiation	1.0×10^6		21	AML

Table A.13 IgHC/A+Meis1 secondary transplanted mice: The secondary recipient mice transplanted with primary leukemic cells developed aggressive acute myeloid leukemia with a median latency of 25 days post transplantation (w or w/o – with or without). IgHC/A+Meis1: Mice transplanted with IgH-CALM/AF10 transgenic bone marrow cells transduced with Meis1 expressing retrovirus.

Experiment no.	Gene	w/o irradiation	Secondary leukemic cells	Days of survival	Disease
5764A#61	IgHC/A+Meis1	w/o irradiation	1.0×10^6	15	AML
5764B#62	IgHC/A+Meis1	w/o irradiation	1.0×10^6	15	AML
5782A#93	IgHC/A+Meis1	w/o irradiation	1.0×10^6	15	AML
5782B#125	IgHC/A+Meis1	w/o irradiation	1.0×10^6	15	AML

Table A.14 IgHC/A+Meis1 tertiary transplanted mice: The tertiary recipients transplanted with secondary leukemic cells developed aggressive acute myeloid leukemia with a median latency of 15 days post transplantation (w/o – without). IgHC/A+Meis1: Mice transplanted with IgH-CALM/AF10 transgenic bone marrow cells transduced with Meis1 expressing retrovirus.

Experiment no.	Gene	w or w/o irradiation	Primary leukemic cells	Mock cells	Days of survival	Disease
5901B#257	FVBwt+Meis1	w irradiation	1.0×10^6	2.0×10^6	28	AML
5901E#258	FVBwt+Meis1	w/o irradiation	1.0×10^6		28	AML
5901A#256	FVBwt+Meis1	w irradiation	1.0×10^6	2.0×10^6	63	AML
5901D#252	FVBwt+Meis1	w/o irradiation	1.0×10^6		56	AML
6012#50	FVBwt+Meis1	w/o irradiation	1.0×10^6		under observation (122)	No Disease
6012#51	FVBwt+Meis1	w/o irradiation	1.0×10^6		under observation (122)	No Disease

Table A.15 FVBwt+Meis1 secondary transplanted mice: Four (4) of the secondary recipient mice transplanted with primary leukemic cells developed acute myeloid leukemia with a median latency of 44 days. Two (2) of the secondary recipient mice transplanted with cells from primary non-leukemic mice did not develop leukemia even after 122 days post transplantation (w or w/o – with or without). FVBwt+Meis1: Mice transplanted with FVB wildtype bone marrow cells transduced with Meis1 expressing retrovirus.

Experiment no.	Gene	w/o irradiation	Secondary leukemic cells	Days of survival	Disease
5969A#35	FVBwt+Meis1	w/o irradiation	1.0×10^6	28	AML
5969A#37	FVBwt+Meis1	w/o irradiation	1.0×10^6	28	AML
5969B#36	FVBwt+Meis1	w/o irradiation	1.0×10^6	21	AML
5969B#38	FVBwt+Meis1	w/o irradiation	1.0×10^6	21	AML

Table A.16 FVBwt+Meis1 tertiary transplanted mice: The tertiary recipients transplanted with leukemic cells from secondary leukemic mice developed acute myeloid leukemia with a median latency of 25 days post transplantation (w/o –without). FVBwt+Meis1: Mice transplanted with FVB wildtype bone marrow cells transduced with Meis1 expressing retrovirus.

ABBREVIATIONS

5-FU	5-Fluorouracil
µ	Micro (1 x 10^{-6})
µF	Microfarad
µg	Microgram
µl	Microlitre
µM	Micromolar
µm	Micrometer
Ω	Ohm
AF10	ALL 1 fused gene from chromosome 10 (MLL10)
ALL	Acute lymphoblastic leukemia
AML	Acute myeloid leukemia
Amp	Ampicillin
ANTH	AP180 N-terminal homology
APC	Allophycocyanin
APS	Ammonium persulfate
AT	Adenine-thymine
B220	B-cell marker
BFU-E	Burst forming unit-erythroid
BMT	Bone marrow transplantation
bp	base pairs
BSA	Bovine serum albumin
$CaCl_2$	Calcium chloride
CAE	Chloro-acetate esterase
cALL	Common acute lymphoblastic leukemia
CALM	Clathrin Assembly Lymphoid Myeloid Leukemia Gene
CBS	Clathrin binding sequences

CD3	Cluster of differentiation 3
CD4	Cluster of differentiation 4
CD8	Cluster of differentiation 8
cDNA	Complementary DNA
CFC	Colony forming cell
CFU	Colony forming unit
CFU-G	Colony forming unit-granulocyte
CFU-M	Colony forming unit-macrophage
CFU-GM	Colony forming unit-granulocyte/macrophage
CFU-GEMM	Colony forming unit-granulocyte / erythroid / macrophage /megakaryocyte
CGH	Comparative genomic hybridization
cGy	Centigray
CH_2O	Formaldehyde
CLL	Chronic myeloid leukemia
CLP	Common lymphoid progenitor
cm	Centimeter
CML	Chronic lymphocytic leukemia
CMP	Common myeloid progenitor
CO_2	Carbondioxide
CSC	Cancer stem cell
D-J	Diversity-Joining
DMEM	Dulbecco's Modified Eagle Medium
DMSO	Dimethylsulfoxide
DNA	Deoxyribonucleic acid
dNTP	Deoxyribonucleotide triphosphate
DPBS	Dulbecco's phosphate buffered saline
DPF	ASP-Pro-Phe
DTT	Dithiothreitol

e.g.	Example
ECL	Enhanced chemiluminescence
EDTA	Ethylenediaminetetraacetic acid
EGFP	Enhanced green fluorescent protein
ENTH	Epsin N-terminal homology
ENU	*N*-ethyl-*N*-nitrosourea
ext-PHD	Extended plant homeodomain
EYFP	Enhanced yellow fluorescent protein
FAB	French-American-British classification for acute leukemia
FACS	Fluorescence activated cell sorting
FBS	Fetal bovine serum
FVB	Friend virus B
FVBwt BM	FVB wildtype bone marrow
FVB IgHC/A	FVB wildtype IgH-CALM/AF10
g	gram
GF	Growth factor
GFP	Green fluorescent protein
GP+E86	3T3-based retroviral packaging cell line
Gr-1	Granulocyte marker
H&E	Hematoxylin and eosin
HBS	Hank's balanced salt
HD	Homeodomain
HR	High resolution
hr(s)	hour(s)
HRP	Horse radish peroxidase
HSC	Hematopoietic stem cell
Ig	Immunoglobulin
IgH	Immunoglobulin heavy chain
IRES	Internal ribosome entry site

IV	Intravenous
IVC	Individually vented cage
kb	kilobases
kD	kilodalton
KV	kilovolts
l	liter
LAP	Leukemia-associated protein
LB	Luria Bertani medium
Lck	Lymphocyte-specific protein tyrosine kinase
LMPP	Lymphoid-primed multipotential progenitor
LSC	Leukemic stem cell
LT-HSC	Long-term hematopoietic stem cell
LTR	Long terminal repeat sequences
M	molar
M1	Meinox domain 1
M2	Meinox domain 2
m	milli (1×10^{-3})
mM	millimolar
Mac-1	Macrophage marker
MCS	Multiple cloning site
MDS	Myelodysplastic syndrome
MEIS1	Myeloid Ecotropic viral Integration Site 1
mg	milligram
MIG	MSCV IRES GFP
min	minute(s)
ml	milliliter
mm	millimeter
MIY	MSCV IRES YFP
MPO	Myeloperoxidase

MPP	Multipotent progenitor
MRC	Medical Research Council classification for acute myeloid leukemia
mRNA	messenger RNA
ms	millisecond
MSCV	Murine stem cell virus
n	nano (1×10^{-9})
NaCl	Sodium chloride
$NaHCO_3$	Sodium bicarbonate
$Na_2HPO_4.2H_2O$	Disodium hydrogen phosphate dihydrate
$NaH_2PO_4.H_2O$	Sodium hydrogen phosphate monohydrate
NaOAc	Sodium acetate
NaOH	Sodium hydroxide
ng	nanogram
NH_4Cl	Ammonium chloride
NLS	Nuclear localization signal
NPF	Asn-Pro-Phe
nt	nucleotide
OM/LZ	Octapeptide motif and leucine zipper
O/N	overnight
ºC	degree Celsius
OD	Optical density
p	pico (1×10^{-12})
PAGE	Polyacrylamid Gel Electrophoresis
PB	Peripheral blood
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PE	Phycoerythrin
PHD	Plant homeodomain
pg	picogram

PIM	Pbx-interacting motif
PMSF	Phenylmethanesulfonylfluoride or phenylmethylsulfonyl fluoride
PtdIns(4,5)P2	Phosphatidylinositol-4,5-bisphosphate
PVDF	Polyvinylidene difluoride
RBC	Red blood cells
RIPA	Radio-Immunoprecipitation Assay
RNA	Ribonucleic acid
RNAse A	Ribonuclease A
rpm	revolutions per minute
RPMI	Roswell Park Memorial Institute medium
RT	Room temperature
RT-PCR	Reverse transcriptase PCR
Sca-1	Stem cell antigen 1
SCF	Stem cell factor
SDS	Sodium dodecylsulfate
sec	second(s)
SNP	Single-nucleotide polymorphism
Spl	Spleen
SSC	Saline sodium citrate buffer
ST-HSC	Short-term hematopoietic stem cell
TALE	Three amino acid loop extension
TBE	Tris-borate-EDTA
TBS	Tris-buffered saline
TBST	Tris-Buffered Saline and Tween 20
TCA	Tris-chloro-acetate
TCR	T-cell receptor
TE	Tris-EDTA buffer
Ter119	Erythroid marker
TGN	Trans golgi network

Tm	melting temperature
Tx	Transplantation
U	unit
UV	Ultraviolet
V	volts
VCM	Viral conditioned medium
V-D-J	Variable diversity joining
vol.	volume
WB	Western blot
WBC	White blood cells
WHO	World Health Organization
YFP	Yellow fluorescent protein

SINGLE LETTER CODES FOR AMINO ACIDS

A (Ala)	Alanine
M (Met)	Methionine
B	Asparagine or Aspartic acid
N (Asn)	Asparagine
C (Cys)	Cysteine
P (Pro)	Proline
D (Asp)	Aspartic acid
Q (Glu)	Glutamine
E (Glu)	Glutamic acid
R (Arg)	Arginine
F (Phe)	Phenylalanine S (Ser) Serine
G (Gly)	Glycine
T (Thr)	Threonine
H (His)	Histidine
V (Val)	Valine
I (Ile)	Isoleucine
W (Trp)	Tryptophan
K (Lys)	Lysine
Y (Tyr)	Tyrosine
L (Leu)	Leucine
Z	Glutamine or Glutamic acid

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