

**The t(4;11) fusion protein MLL/AF4 regulates
telomerase reverse transcriptase (*TERT*) expression**

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1 Introduction

1.1 Haematopoiesis

The human blood consists of many different kinds of blood cells. There are the red blood cells (erythrocytes) and the white blood cells (leukocytes), further subdivided into blood cells like lymphocytes, granulocytes, monocytes etc. Those cell types have all different purposes ranging from transportation, wound healing or support of the immune system. The erythrocytes are for transportation and carry the oxygen or carbon dioxide through the blood stream. White blood cells or leukocytes include various cell types divided into four sub groups. Group one are the granulocytes containing neutrophils, eosinophils and basophils which destroy invading bacteria or parasites, secrete histamine and play a role in modulating allergic inflammation reactions. Group two consists of the monocytes which infiltrate the blood vessel surrounding tissue and differentiate to macrophages to fight pathogens or destroy damaged cells. The third group are the lymphocytes which consist of B-cells, mainly antibody producers, T-cells which play an important role in inflammation and regulating other immune cells. The last cell type in this group is the natural killer cell (NK-cell). NK cells kill virus infected cells and have been reported to kill gastric tumour cells (Miller, 2001). There are also thrombocytes in the blood which are small cell fragments produced by megakaryocytes in the bone marrow. On the other hand they support wound healing and coagulation.

All of these blood cells are derived from pluripotent haematopoietic stem cells (HSC) in the bone marrow. Pluripotent stem cells create progenitor cells with a predetermined fate (Figure 1-1).

HSCs are generated by a process called haematopoiesis and have been classified depending on their capacity to self-renew. In the beginning there are long-term self renewing HSCs which generate then other more short living HSCs resulting in pluripotent progenitors without self-renewal capacity (Reya et al., 2001).

HSCs occupy niches in the bone marrow. A niche is a microenvironment which consists of supporting cells providing necessary cytokines for self renewal and differentiation ability (Yin and Li, 2006).

The differentiation process of a HSC can result in a myeloid or a lymphoid progenitor cell. Following the lineage determination a series of differentiation steps leads first to the progenitor cell and then to a precursor cell. Progenitor cells still divide at a high rate, although numbers of cell divisions are limited. Continued differentiation of the precursors finally produces the fully differentiated and mature blood cell (Alberts, 2002).

In summary it can be stated that the haematopoiesis of HSCs is a delicate and a critical sequence of incidents with highly and tightly regulated processes. These processes are proliferation, maturation and differentiation. The tight regulation is necessary as for example many leukocytes are very short living and need to be replaced constantly. The half life of neutrophils in the peripheral blood for example lies between 8 and 10 hours which means there have to be ensured strictly controlled mechanisms for proper differentiation during the 10 to 14 days of their maturation. This maintains a constant level of neutrophils in the peripheral blood (Speck, 2001).

Disruption of this process can lead to a disordered form of haematopoiesis called leukaemia, the cancer of the blood.

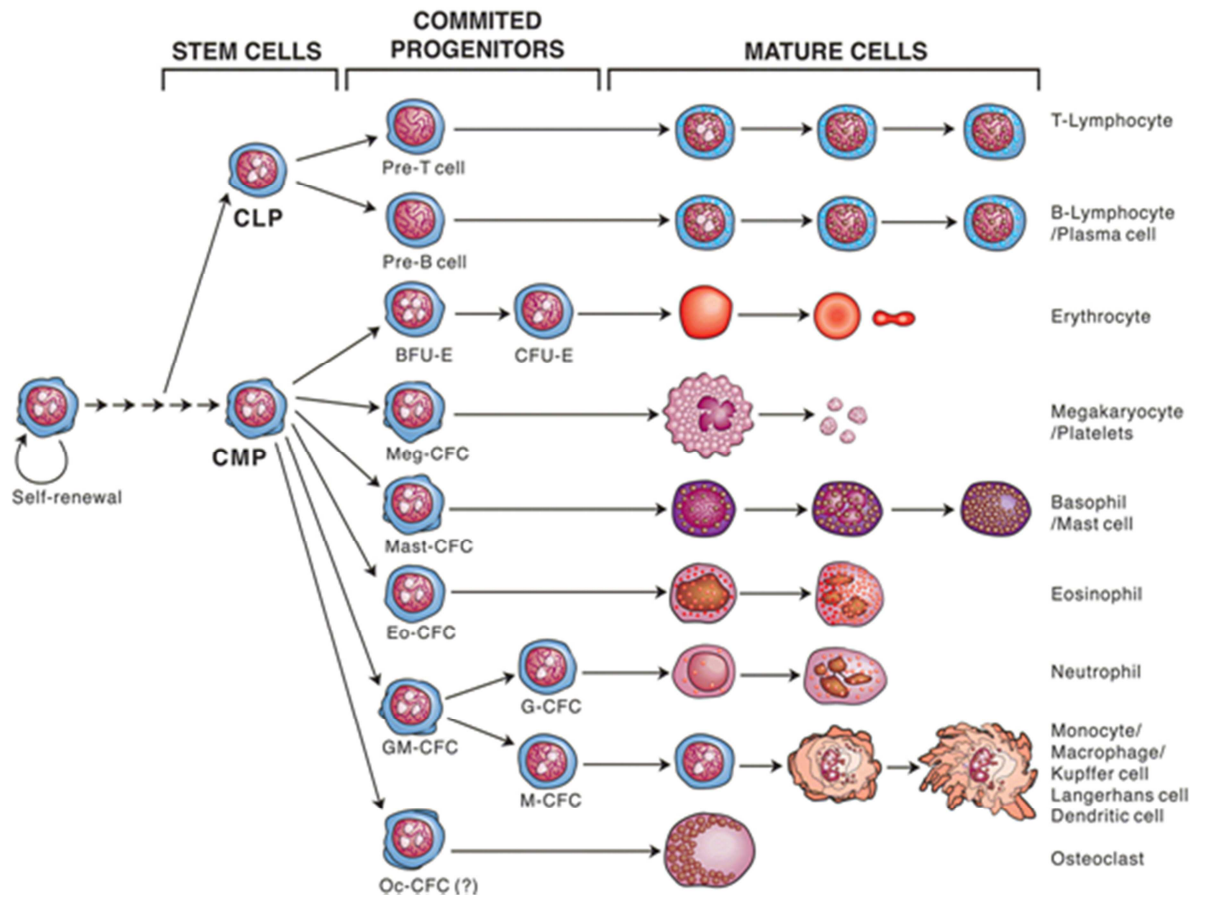


Figure 1-1 Overview of haematopoiesis

An overview of the haematopoiesis; Self-renewing haematopoietic stem cells produce common lymphoid (CLP) or common myeloid (CMP) progenitors which then differentiate into the different subtypes. (adapted from www.bloodlines.stemcells.com)

1.2 Leukaemia

Leukaemia [greek: *λευχαιμία*; *λευκος* (leukos): white and *αίμα* (aima) blood] is a disease of the blood. Characteristic for the disease is an abnormal increase of leukocytes and their precursors the leukoblasts in bone marrow and blood. Further characteristics are the ability of self renewal and impaired ability in haematopoietic differentiation (Speck, 2001). Leukaemia is classified in two major subgroups, the acute form and the chronic form of leukaemia.

Characteristic for the chronic leukaemias is the massive production of relatively mature but malignant leukoblasts and the disease is commonly diagnosed in adults. While acute forms have to be treated immediately, chronic forms can be monitored for some time before treatment to reach the highest efficiency of the therapy. Due to the slow progression of the chronic form of leukaemia it is difficult to diagnosis them in early stages. But once diagnosed it is possible to monitor the disease and apply the right form of therapy at its most effective time. The ten years event-free-survival lies currently between 55 and 70 percent. Nevertheless new forms of treatment are currently under investigation. For example a new agent, called imatinib (Gleevec ©, Novartis) has been primarily developed for treatment of t(9;22) *BCR/ABL* positive CML (Table 1) and is currently being monitored in clinical trials. Imatinib is an inhibitor of the ABL kinase. Actual studies show a 5 year event-free survival of about 90 % (Fausel, 2007).

This work is focused on acute forms of leukaemia where a rapid production and bloodstream infiltration with immature blood cells is characteristic for this progressive disease. In acute leukaemia the lymphoblasts inundate the bone marrow and the blood thereby displacing healthy cells. Other infiltrated organs are liver, spleen and lymph nodes. As consequence, the function of the blood system is severely disturbed which if left untreated leads to death within several weeks or months. While chronic forms of leukaemia are generally detected in adults, acute form, especially acute lymphoblastic leukaemia is also often diagnosed in young people and infants (Figure 1-2).

To further determine the type of leukaemia a second classification is necessary. It is important to know the lineage of cells from where the leukaemia appears. On the one hand it can be the lymphoid lineage, affecting mainly B and T cells or on the other, it can be the myeloid lineage, affecting e.g. granulocytes and monocytes.

The combination of this classification gives four major types of leukaemia. (Table 1)

Table 1 The four major types of leukaemia

LINEAGE/^{FORM}	Acute	Chronic
Lymphoid	Acute lymphoblastic leukaemia	Chronic lymphoblastic leukaemia
Myeloid	Acute myeloid leukaemia	Chronic myeloid leukaemia

Age specific incidence of leukaemia (USA 1997-2001)

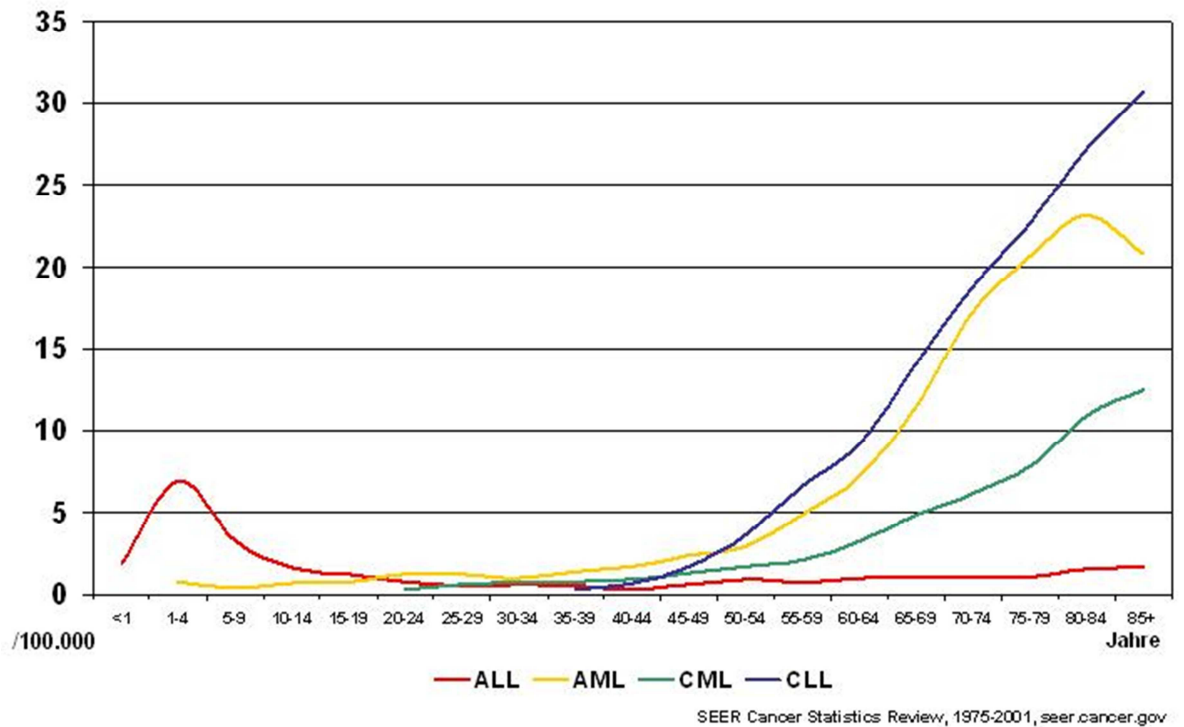


Figure 1-2 Age specific incidence of leukaemia in the US

This SEER statistics show the incidence of leukaemia in the US between 1997 and 2001. While acute leukaemias (ALL, AML) do also occur in young patients, chronic leukaemias are diagnosed in adults only. Of note is the ALL peak between the age of 1 and 5, a hallmark of this form of leukaemia. (www.cancer.gov)

1.3 The leukaemic stem cell

One so far unanswered question in leukaemia is whether there is one single malignant cell, the so called leukaemic stem cell (LSC), that is responsible for the onset of leukaemia or if every cell is able to initiate leukaemia. Over the past years several cancer stem cell models have been proposed and more and more data keeps the discussion alive.

For AML a few stem cell models have been established in the past. The first appearance of an AML model is based on pioneering work from the lab of John Dick in the early 1990's. It has been shown that xenotransplantations of a rare

subpopulation of human AML cells with similar immunophenotype characteristics of human HSC could successfully be engrafted in immunodeficient SCID mice (Lapidot et al., 1994). From that point on more experiments led to the formulation of two leukemic heterogeneity models, the stochastic model and the tumour hierarchy model (Figure 1-3). The hierarchy model suggests that the development of a leukaemic stem cell is probably induced by a mutation which transforms a HSC into a LCS. In xenotransplantation experiments leukaemic stem cells were detected with variable leukaemia initiation potential. Those leukaemic cells are named after their ability to induce leukaemia in immunodeficient SCID mice (SCID-leukaemia initiating cells, SL-IC). Three different types are described. The short-term SL-IC does induce leukaemia in a recipient but is not able to sustain the leukaemia in secondary recipients. Long-term SC-ILs can initiate leukaemia in the first recipient and harvested cells from that individual will induce leukaemia in serial transplantations. The third SC-IL type is the quiescent long-term SC-IL which induce leukaemia not in the primary recipient but in following recipient cohorts (Dick, 2008).

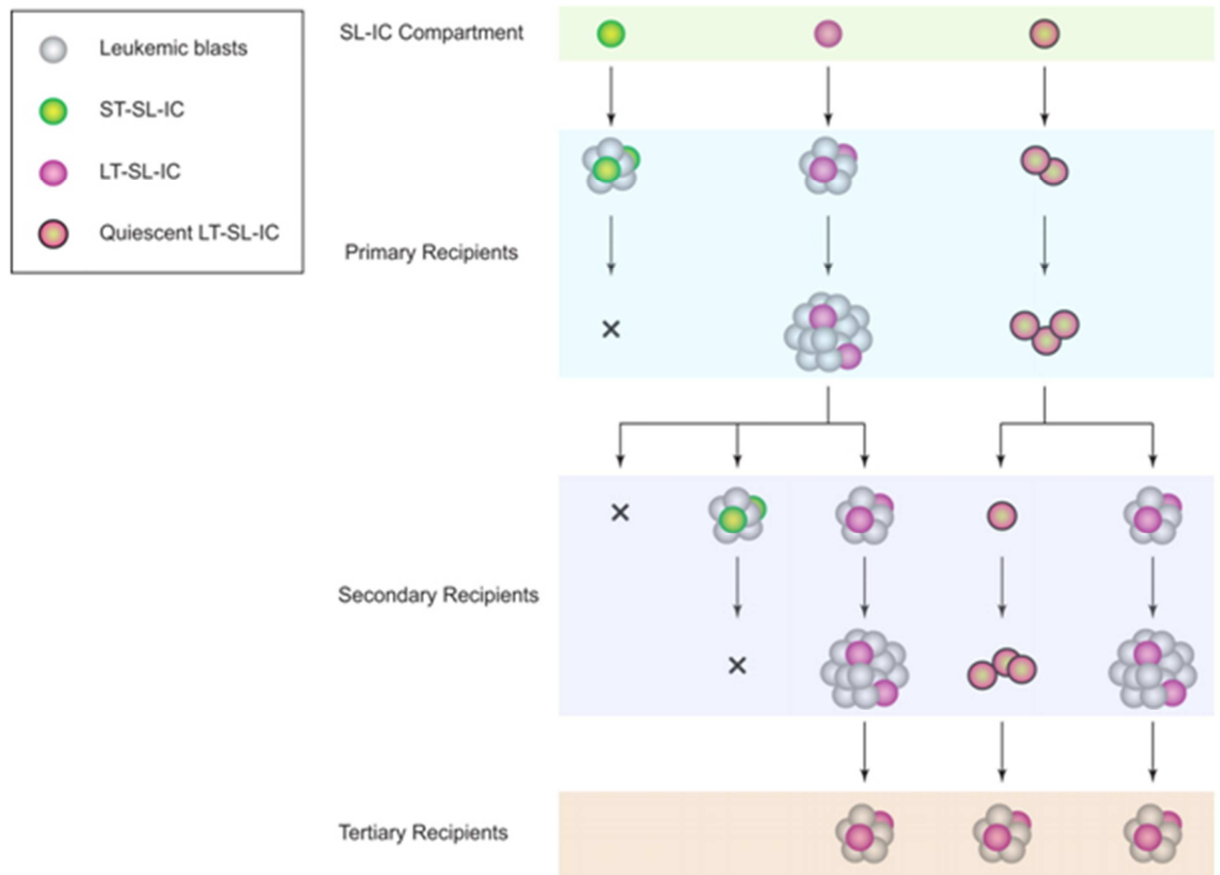


Figure 1-3 The leukaemic stem cell hierarchy

AML organised in a hierarchy. Short-term SCID-leukaemia initiating cells (ST-SL-IC) in green are only able to induce leukaemia in their first recipient. Long-term SCID-leukaemia initiating cells (LT-SL-IC) are able to sustain their leukaemia initiating ability in multiple transplantations. Quiescent LT-SL-ICs remain silent in the first recipient but may get active in second or further instance. Picture adapted from Dick., Blood 2008 (Dick, 2008).

On the other hand there is the stochastic model of tumour heterogeneity. This basically says that every malignant cell has a low but equal ability of forming a tumour. The heterogeneity within the cell subpopulation is necessary to initiate the malignancy. Isolated subgroups lack this ability (Wang and Dick, 2005).

Other questions are where the leukaemic cell of origin comes from or what the initial event for transforming a haematopoietic stem cell into a self renewing immortal malignant cell is. Is there more than one event necessary? Currently two models are discussed for the initiation and progression of leukaemia. The first model suggests that in the normal HSC pool a single cell gets a hit. This hit then causes a genetic

mutation which leads to the malignant transformation and expansion of the affected cell. During the now deregulated maturation process, additional mutations can occur and cause further severe disruptions mainly blocking the differentiation program and inducing uncontrolled proliferation. These mutations lead then to the infiltration of immature progenitor cells into the blood stream and in the end to the most prominent leukaemic phenotype.

The second model suggests a two-hit incident (Greaves, 2005). The first hit a HSC receives does not initiate expansion but immortalises the affected cell. The cell still differentiates but during early differentiation a second hit initiates expansion and blocks further differentiation steps. Additionally this transformation event(s) trigger the self-renewal program in order to generate LSCs. In the early stages of differentiation the self renewal programs are still deactivated, but the earlier the malignant transformation takes place, the easier for the cell to restart the self renewal program. Without activation of the self-renewal program it is likely that the committed progenitor due to the limited life span will die or undergo terminal differentiation (Wang and Dick, 2005). When the cell overcomes this differentiation bottleneck it is already transformed into the malignant LSC the expansion is uncontrollable.

A third hypothesis was formulated in the early 70s. The Knudson hypothesis stated that multiple mutations within the DNA of a malignant cell lead to the cancerous phenotype although his hypothesis is based on Retinoblastoma studies (Knudson, 1971). However his hypothesis could also be applied to the leukaemic background and would concord with the two-hit hypothesis.

In contrast for ALL no such model exists. Many research groups set up transplantation experiments with different subsets of leukaemic lymphoblasts but results are conflicting as the recent controversial discussion in Blood shows (Heidenreich and Vormoor, 2009). Within this dispute the results of Cox et al. (Cox et al., 2009) are discussed compared to results by le Viseur et al. (le Viseur et al., 2008). Each research group used different subsets of lymphoblasts deriving from human ALL patients for their transplantations in immunodeficient mice but showed heterogeneous success rates. The discussion is also based on methods and technical issues as well as the minimal necessary cell number to induce leukaemia. One

important point in the discussion is the choice of the mouse model. In the debate it was mentioned, that studies for rare leukaemic stem cells used more conventional irradiated NOD/scid mice whereas a wider variety of LSC populations were studied in newer genetically altered mouse strains like NSG (NOD/scid gamma) or NK cell depleted NOD/scid mice. In his recent review, Vormoor even asked the question whether the stem cell concept for ALL is dead (Vormoor, 2009). The answer can only be found in additional experiments. There are many publications from experiments which show that not all xenotransplanted ALL cells cause leukaemia (Uckun et al., 1998, Kong et al., 2008). Again others used different subsets of sorted leukaemic blasts to induce leukaemia in mice (le Viseur et al., 2008).

The contribution of rearranged oncogenes involving the *MLL* gene (*MLL/AF4* t(4;11) (Krivtsov et al., 2008), *MLL/AF9* t(9;11) (Chen et al., 2008) or *MLL/ENL* t(11;19) (Bach et al., 2010)) led to a malignant transformation of haematopoietic progenitor cells and created AML as well as ALL phenotypes in transplanted mice. A recent publication showed for the translocation t(4;11) that also the AF4/MLL fusion product is able to induce ALL in mice (Bursen et al., 2010). Thus developing or creating a LSC is dependent on chromosomal changes within the cell. The successful engraftment of the malignant haematopoietic progenitor cells showed stem cell abilities like self renewal and rapid expansion of blasts.

The *in utero* detection of translocation events (Greaves, 2005) and the later onset of the leukaemia strongly suggests the two-hit model in accordance with the multiple-hit hypothesis of Knudson (Knudson, 1971).

Ultimately knowledge about the mechanisms which cause leukaemia are still unclear. It may depend on the state of differentiation or if there are different subsets, at which time point the leukaemic hit occurs, the number of necessary hits or which mutations are involved. Clearly more experiments are necessary to answer these questions or to support one of the LSC hypotheses in ALL.

1.4 Acute leukaemia

1.4.1 Acute myeloid leukaemia

This form of leukaemia is the most common form of myeloid neoplasm in adults accounting for ~25 % of leukaemia. In children it accounts for 15 – 20 % (Deschler and Lubbert, 2006). The systemic haematologic disease arises from the clonal proliferation of myeloid precursors characterised by a block of differentiation, an abnormal high rate of proliferation and a decrease rate in apoptosis. Apoptosis is programmed cell death which is initiated from a cell itself in response to a variety of stimuli and often the trigger for this suicide mechanism derives from a failure in the repair mechanisms of the genetic material of the cell. Once this cell death mechanism does not work properly it gives rise to abnormal proliferation of the blood cells and is therefore one reason for causing leukaemia.

The incidence of AML in adults is between 3 to 5 per 100,000 cases per year representing about 3 % of all malignant diseases. The median age of adult patients is 68 years, although the incidence of people over 65 years lies at 17 of 100,000 compared to an incidence of 1.8 of 100,000 cases considering people under 65 years (Deschler et al., 2006) (NCI – SEERS database).

In 1976 AML was morphologically classified by introducing the French-American-British (FAB) classification (Bennett et al., 1976) (Table 2). The FAB classification is based on cytohistochemistry of bone marrow cells dependent on morphological and cytochemical characterisation of the leukoblasts which allows a classification into eight subgroups (M0-M7). The majority of AML cases are de novo diseases, meaning they occur without obvious reason. However, one has to distinguish between these diseases and secondary AML cases where specific risk factors can be identified. An increased incidence of AML was found in patients after exposure to radioactive material, benzene and cytostatics (Infante and White, 1983). Especially after treatment with intensive combined chemotherapies the onset of therapy-induced AML (t-AML) became clear. Mainly, treatment with alkylating compounds leads within 2 to 7 years to a t-AML. Those t-AMLs often show cytogenetic changes like aneuploidies, like monosomy 5 or 7, which have a poor prognosis. t-AML can occur after exposition to topoisomerase-II inhibitors within a shorter time period of 1 to 5

years which is then characterised by a translocation involving the mixed lineage leukaemia (*MLL*) gene (Estey and Dohner, 2006).

Table 2 The FAB classification of AML

The French-American-British (FAB) Classification of AML		
Class	Definition	Frequency
M0	Acute myeloid leukaemia with minimal differentiation	2-5 %
M1	Acute myeloid leukaemia without maturation	10-15 %
M2	Acute myeloid leukaemia with maturation	25-30 %
M3	Acute promyelocytic leukaemia	10-15 %
M4	Acute myelomonocytic leukaemia	25-30 %
M5	Acute monocytic leukaemia	10-15 %
M6	Acute erythroleukaemia	3-4 %
M7	Acute megakaryocytic leukaemia	1 %

1.4.2 Acute lymphoblastic leukaemia

Acute lymphoblastic leukaemia (ALL) is the most common leukaemia in children and young adolescents. There is a peak age of developing this disease between 2 and 5 years (Pui et al., 2008) as well as a gradual increase from the age of 45 on (www.cancer.gov, Figure 1-2). Acute lymphoblastic leukaemia in children is the major type of leukaemia with about 80 % to 85 % of all leukaemia cases (Chowdhury and Brady, 2008, Shah et al., 2008). Depending on the disease subtype, with current forms of treatment the chances of curing the disease are fairly high although a range from 10 % to 90 % demonstrates the biological diversity of the disease (Greaves, 2002). In an actual case study of the Berlin-Frankfurt-Muenster group (BFM group) the estimated 6 year event free survival of children with ALL was 80 % (ALL-BFM 95) (Moricke et al., 2008). However, children with AML have only a cure rate of about 45 % (Pui et al., 2004b). In recent years, a growing number of methods to investigate malignant haematopoietic cells on a molecular level have been used to further characterise and define subsets of leukaemia dependent on chromosomal and genetic abnormalities.

Acute lymphoblastic leukaemia is caused by haematopoietic precursors which are committed to differentiate into B or T cells. Those then malignant cells carry chromosomal mutations which allow them to self renew unlimited and at the same time they stop any further differentiation.

In terms of prognosis, age is also exceptionally important. Children between the age of 1 and 9 have a better prognosis than either adolescents (10 years and over) or infants (younger than one year) (Pui et al., 2001). The five year event free survival (EFS) rate for the former is 88 %, adolescents between 10 and 15 years is estimated at 73 % (Vitale et al., 2006, Kinlen, 2004). Older adolescents have a 69 % 5 year EFS and infants only 44 % (Kinlen, 2004).

In addition biological factors also play a role for prognosis and outcome of a patient. An important prognostic marker is the occurrence of cytogenetic or chromosomal aberrations like translocations. This results in good prognosis for patients with hyperdiploidy (more than 50 chromosomes) or the fusion gene *TEL/AML1* t(12,21)

(p13,q22). On the other hand patients with *BCR/ABL* t(9;22) or *MLL/AF4* t(4;11) have a very poor prognosis. Five year EFS for hyperdiploidy was estimated at 91 %, 89 % for *TEL/AML1* and only 37 % for *BCR/ABL* – positive patients as well as only 32 % of the *MLL/AF4* diagnosed ones (Greaves, 2006).

1.4.3 Diagnosis and treatment of acute leukaemia

The first method of diagnosis is a complete blood count. This will give the first indication for leukaemic development. It is normally followed by characterisation of the immunophenotype by FACS analysis. From this result conclusions can be drawn about the presents of immature blasts in the blood and also which type of cell is affected like B cell or T cell ALL and classification of AML by WHO standards (Vardiman et al., 2002). Further morphological investigations are done to classify AML according to the FAB classification system. Molecular diagnostic examinations of the malignant cells reveal often cytogenetic aberrations which, due to global trials and microarray studies, have led to individual therapy. Molecular diagnostics include fluorescent in situ hybridisation (FISH) analysis for detection of chromosomal aberrations like reoccurring translocations, inversions or loss of chromosomal segments. More sensitive methods like PCR or DNA sequencing are needed to detect mutations which have been associated with the onset of certain type of leukaemia. For example a commonly mutated gene has been detected in *FLT3*, a tyrosine kinase receptor, which has been reported to be constitutively active in AML (Reilly, 2003). All those examinations make it possible to risk assess the patients and set up the therapy according to the risk group.

According to the type of leukaemia and age of the patient, patients are risk assessed. This risk assessment determines the risk of the patient relapsing after therapy. This gives the clinician the opportunity to choose the therapy conditions according to the patient. Low to standard risk patients therefore need a less intensive therapy with less side effects than a patient with high or even very high risk of relapse.

Treatment of AML has two goals. The first is to induce the remission of the malignant cells, while the second is to maintain the remission. For induction of remission two drug types are used namely standard dose of cytarabine (AraC) and anthracycline drugs, like daunorubicin (Tallman et al., 2005) (Table 3). A successful

induction of remission is defined by complete remission (CR) of leukaemic cells in the blood and a presents of less than 5 % blasts in the bone marrow. In adults remission rates of 60 – 70 % are currently achieved. Within a few weeks normal bone marrow cells will start to build new blood cells. To prevent a relapse further treatment is needed. Those therapies are normally multiple treatments with high doses of AraC (Mayer et al., 1994). Although good results have been achieved so far by induction and consolidaton therapy still 50 – 70 % of patients are expected to relapse within 4 years. The chances of success of a CR after relapse are associated with the time of CR after the primary therapy. Generally if CR lasts for one year or less, chances of a secondary CR are between 10 – 15 %. If primary therapy achieved a time of CR for more than one year, chances of a second CR raise to 40 – 60 % (Robak and Wierzbowska, 2009). Currently allogenic stem cell transplantation has been the most effective therapy for AML patients in first or subsequent remission (Vicente et al., 2007). Several new therapeutic approaches for AML therapy are currently being investigated in clinical studies. A new drug called gemtuzumab ozogamicin is an anti-CD33 monoclonal antibody conjugated with calicheamicin which breaks DNA double strands (Hamann et al., 2002a, Hamann et al., 2002b, Pui et al., 2008). In combination with standard therapy this agent showed promising results (Feldman et al., 2005). Further agents currently used in trials are hypomethylating agents like azacytidin (Vidaza ®), new nucleoside analogous like Clofarabine which inhibit ribonucleotide reductase and DNA polymerase leading to apoptosis or tyrosine kinase (FMS-like tyrosine kinase 3 FLT₃) inhibitors like tandutinib which inhibit tyrosine phosphorylation and thereby induce apoptosis (Robak and Wierzbowska, 2009).

Therapy for acute lymphoblastic leukaemia normally consists of an induction therapy to achieve remission of the immature leukaemic cells in the blood and bone marrow. This is followed by an intensification therapy with the goal to remove residual leukaemic cells. Subsequently there a continuation therapy follows to further remove any residual malignant cells and to minimise the risk of relapse. An exception is made with mature B cell ALL that needs to be treated with a short term intensive chemotherapy including high doses of methotrexate, cytarabine and cyclophosphamide (Patte et al., 2001).

For standard risk patients the induction therapy is carried out by applying at least three different chemotherapy drugs. Current treatment protocols suggest a combination with a glucocorticoid like prednisone or dexamethasone, vincristine and asparaginase or anthracycline. To patients with a higher risk of relapse a fourth or even more drugs are applied. The final goal is to achieve 100% remission of malignant haematopoietic cells in the blood system although it is known that this could be hard to achieve. The actual clinical remission currently reach is between 96 and 99 % in children and 78 – 93 % in adults (Pui and Evans, 2006).

Table 3 Chemotherapeutic drugs

Chemotherapeutic drug	Effect
Methotrexate	Inhibits metabolism of folic acid, thereby inhibiting all purine synthesis
Cytarabine	DNA damage and DNA and RNA polymerase inhibition
Cyclophosphamide	Causes DNA crosslinks and leads to apoptosis
Glucocorticoid (Prednisone, Dexamethasone)	induction of apoptosis by binding to the glucocorticoid receptor in sensitive lymphoblasts
Vincristine	Interferes with tubulin and causes cell cycle arrest.
L-asparaginase	Serum depletion of asparagine, inhibiting protein synthesis
Anthracycline (Doxorubicin, Daunorubicin)	inhibits RNA and DNA synthesis; inhibits topoisomerase II; creates free oxygen radicals that damage DNA and cell membrane
6-mercaptopurine	purine analogue; inhibits purine nucleotide synthesis, thereby altering RNA and DNA synthesis

In the following consolidation phase there is no standard therapy for the second treatment phase. Post-remission treatments with combinations of corticosteroids, vincristine, methotrexate and mercaptopurine have so far given good results. Also here the pheno- and genotype of the leukaemic cells are linked to the outcome of the therapy. The most radical treatment is allogeneic stem cell transplantation. Especially in the group of high risk patients this form of treatment shows the most benefit for the patient. For so far unknown reasons even after the consolidation therapy the risk of relapse still remains (Pui et al., 2008).

The subsequent continuation therapy should further minimise the risk of relapse as remaining cells are quiescent, chemotherapy resistant and self renewing dormant LSCs. Although 60 % of childhood cases can be treated within 12 months the chance of relapse is still present. The maintenance therapy continues for 2 to 3 further years. In different randomised trials a therapy with mercaptopurine turned out to be the most favourable drug as other drugs had unacceptable rates of side effects mainly liver defects or even an increased risk of death during remission (Pui et al., 2008).

A new treatment protocol for infants was introduced in the Interfant-99 trial. 482 patients between 0 and 12 months diagnosed with ALL have entered the trial. For the induction phase four drugs have been administered, dexamethasone, vincristine, daunorubicin and L-asparaginase. Following the induction phase the consolidation phase, called MARAM, was initiated. MARAM consisted of 6-mercaptopurine, methotrexate, leucovorin rescue, prednisone, cytarabine and L-asparaginase. After the MARAM phase the OCTADD phase has been initiated with a combination of dexamethasone, 6-thioguanine, vincristine, daunorubicin, cytarabin, prednisone and cyclophosphamide. Following these treatment phases intensification phases or maintenance phases then follow. If a donor was available some high-risk patients received bone-marrow transplantation. For the outcome of the study 445 patients in complete remission have been analysed. 163 patients had relapsed from which 146 died during relapse treatment. This resulted in an overall survival rate of 55.3 % which was better compared to other treatment protocols like the BFM (48 % survival) or CCG-1953 (45 %). In this study MLL rearrangement and age younger than 6 months were strong indicators for poor outcome. Their 4-year event free survival was below 40 %. Especially for this group of patients new forms of treatment are

necessary (Pieters et al., 2007, van der Linden et al., 2009, Van der Velden et al., 2009)

1.5 The molecular genetics of childhood ALL

Acute lymphoblastic leukaemia (ALL) is the most common form of malignancy in childhood representing about 25 % of all cases. The B cell precursor ALL is the most common form of ALL in children (Armstrong and Look, 2005). Chromosomal abnormalities are a hallmark of ALL but also give insight into the molecular biology behind the disease. Cytogenetic anomalies in acute lymphoblastic leukaemia occur with different frequencies in children and adults. Figure 1-4 gives an overview of current cytogenetic anomalies in childhood ALL. 25 % of ALL positive diagnosed children show in cytogenetic examinations a so called hyperdiploidy, which means more than 46 chromosomes (Paulsson and Johansson, 2009). Gene expression studies demonstrated, that ALL with hyperdiploidy represent a separate subset of B-precursor ALL. Although being a subset, further analyses did not show any specific genes involved in the development of hyperdiploid pre-B All. Recently, the first evidence of a specific gene mutation was found within the receptor tyrosine kinase *FLT3* gene in about 20 % of the investigated hyperdiploid pre-B ALL cases (Armstrong et al., 2004). This finding is important as it would be the first gene associated with hyperdiploid ALL and this kinase would be a good target for therapy. As patients with hyperdiploid ALL have a very good prognosis, the addition of FLT3 inhibitory treatment may further improve patient outcome. The loss of chromosomes, called hypodiploidy, is a marker for poor prognosis and is diagnosed in ca. 1 % of childhood ALL patients. One big group of abnormalities is the chromosomal rearrangement or translocation group. The reciprocal translocation, which is the common form of translocation in leukaemia, is the exchange of chromosomal material. This translocation event often creates two fusion genes, which then lead to abnormal function of the involved genes. The most frequent diagnosed translocation is t(12;21) (p13;q22) fusing the genes *TEL* and *AML1*. This translocation cannot be diagnosed by normal karyotyping but with molecular techniques this translocation is found in 22 % childhood ALL cases. Interestingly this translocation can be found in the blood of newborn babies up to 5 to 10 years before the leukaemia development (Greaves and Wiemels, 2003). The mechanisms of leukaemogenesis induction by

TEL/AML1 are still not fully understood. *TEL* and *AML1* have been shown to be key players in normal haematopoiesis, thus suggesting that the *TEL/AML1* fusion protein severely disrupts haematopoietic development (Wang et al., 1997). As mentioned before the expression of *TEL/AML1* has a good prognosis and the 5-year event free survival rate is around 90 % not depending on the age and leukocyte count. This means that these patients are candidates for a less-intensive therapy

Translocations involving the *MLL* gene are diagnosed in about 8 % of childhood ALL cases and will be described in section 1.6.

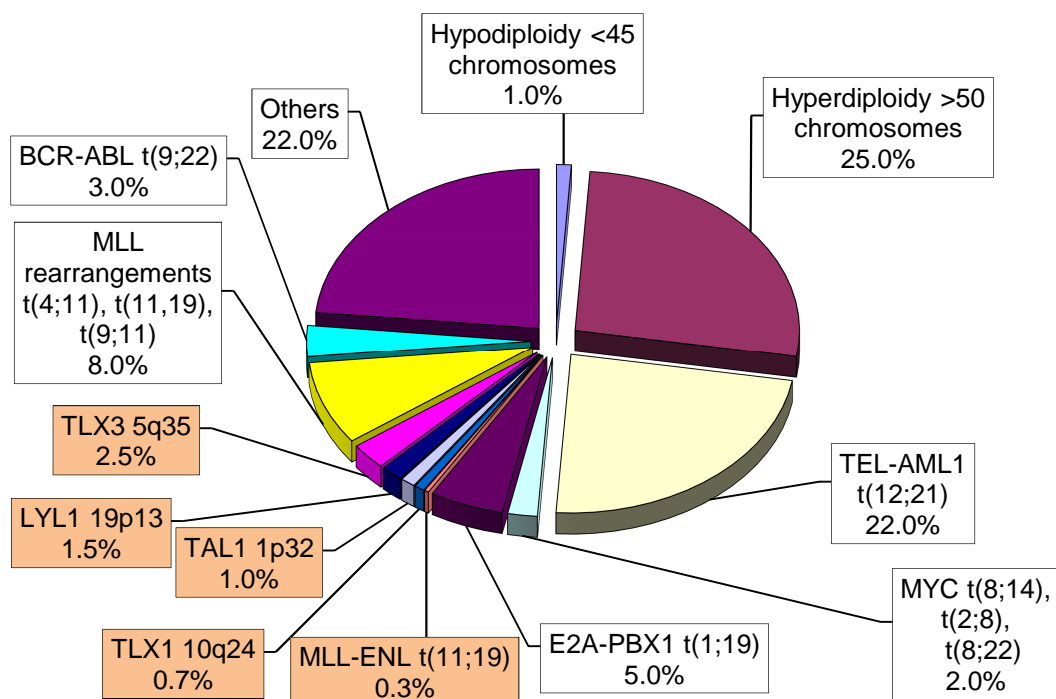


Figure 1-4 Cytogenetic aberrations in childhood ALL

The picture represents an overview of cytogenetic abnormalities in Childhood ALL for patients between 1 and 18 years. The coloured names represent cytogenetic aberrations in T-ALL, all other abnormalities are diagnosed in B-ALL. Adopted from Pui et al, 2004 (Pui et al., 2004a)

1.5.1 Infant ALL

Infants younger than one year with acute lymphoblastic leukaemia represent a subgroup of childhood ALLs as their characteristics and also prognosis differ from that of patients older than one year. At diagnosis, infants with ALL often present high risk leukaemia features like CD10 negativity, an immature pro-B phenotype and

a high leukocyte count (Bhojwani et al., 2009). A genetic hallmark of infant ALL is translocation involving the *MLL* gene which is diagnosed in about 80 % of all infant ALL cases. This translocation is a marker for a very aggressive form of leukaemia with a poor prognosis. Infants with *MLL* rearranged ALL treated according to the Interfant-99 protocol had an estimated 4 year survival rate of about 40 % (Pieters et al., 2007).

An interesting characteristic of childhood ALL and especially infant ALL is the *in utero* origins of the disease. Greaves and colleagues (Greaves, 2005) investigated Guthrie blood spots of diagnosed ALL patients for leukaemia associated chromosomal anomalies. In infant ALL patients the translocation t(4;11) coding for the *MLL/AF4* and *AF4/MLL* fusion gene was found in nearly 100 % of the investigated cases. Other chromosomal anomalies like the t(12;21) *TEL/AML1* in childhood ALL or t(8;21) *AML1/ETO* in childhood AML were also found but only in 75% and 50% respectively. As the disease for some patients did not show at birth but years later this supports the two-hit model of leukaemogenesis as secondary events are needed to accomplish the transition to AML or ALL. Also this would fit into the hierarchy model of leukaemia as the immortalised cells, leukaemic stem cells, stay quiescent until an event occurs that results in development of the malignancy. In case of t(4;11) already present in an ALL at birth, this would suggest that the second hit also occurs prenatal which indicates the rapid development of t(4;11) positive ALL. This second hit could be the contribution of both derivatives of the t(4;11) translocation. Each of the derivatives has been shown to induce leukaemia in mouse models, but together show highest transforming potential (Bursen et al., 2010, Gaussmann et al., 2007, Krivtsov et al., 2008).

1.6 MLL rearrangements

For *MLL* more than 100 translocation partners have been identified with about 64 characterised at the molecular level. The most common reciprocal translocation in ALL is the fusion of the *MLL* gene to the gene *AF4* creating the translocation t(4;11)(q31;q23) (66% of all cases), followed by *ENL* t(11;19) (q23;p13.3) (~15%) and *AF9* t(9;11)(p22;q23)(~8.5%).

MLL translocations play also a role in acute myeloblastic leukaemia (AML). Most common fusion partners here are *AF9* t(9;11)(p22;q23) (~30%), *ELL* t(11;19)(q23;p13.1), *AF10* ins(10;11)(p12;q23q13) and *AF6* t(6;11)(q27;q23) (~10% each) (Meyer et al., 2009).

In various knockout experiments it was demonstrated that *MLL* needs a fusion partner to form an active oncogene while truncated versions of the wild type gene did not lead to leukaemogenesis (Li et al., 2005).

As illustrated in figure 1-5 *MLL* has several important domains for modifying genetic regulation processes.

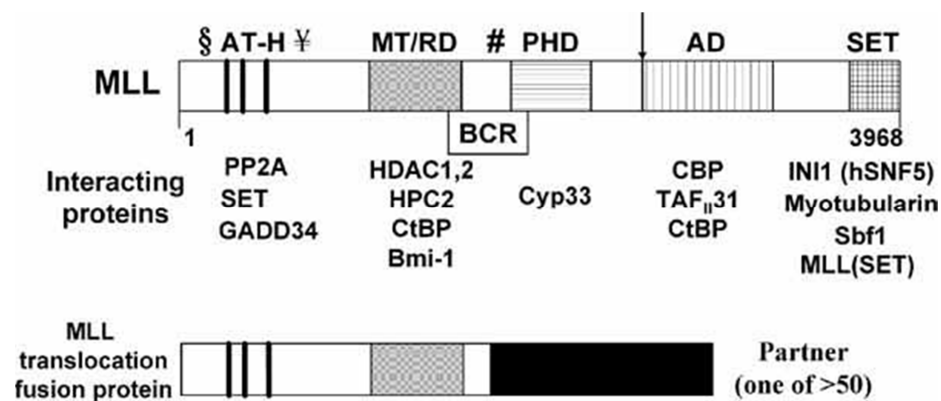


Figure 1-5 The *MLL* wild type and fusion protein

The schematic shows an overview of the main domains of the *MLL* wild type protein. These are:

AT-H, AT-hooks; MT/RD, DNA methyltransferase homology/repression domain; BCR, breakpoint cluster region; PHD, plant homeodomain; AD, transcriptional activation domain; SET, histone methyltransferase domain. Arrow indicates Taspase 1 cleavage site. Proteins that interact with those important domains are noted below the domain (Popovic and Zeleznik-Le, 2005).

The breakpoint cluster region (BCR) genetically lies between exon 5 and 11 in the *MLL* gene. It spans over 8.3 kb and indicates that the chromosomal rearrangement reallocates three important domains namely the plant homeodomain, the transcriptional activation domain and the histone methyltransferase domain (SET). More and more evidence shows, that especially the rearrangement of the SET domain has an important impact on the leukaemic cell. It seems that by the displacement of the SET domain, the whole epigenetic phenotype of the cell changes.

The exact mechanisms of leukaemogenesis caused by *MLL* translocations are not fully understood so far. The common finding is the involvement of the N-terminus of the *MLL* protein fused to a partner protein. Those partner proteins do not show any common characteristics. Rowley hypothesised in 1992 (Rowley, 1992) that the der(11), meaning the fusion gene coding for the N-terminal part of an *MLL* fusion, is the key oncogene for the onset of leukaemia. Previous studies demonstrated the potential of fusion genes to disrupt the development of stem and precursor cells (Cleary, 1991).

In animal models the oncogenic potential of *MLL* fusions has already been studied. In the year 2000, two independent groups reported (DiMartino et al., 2000, Lavau et al., 2000) the oncogenic characteristic of *MLL-ELL* transfected primary myeloid progenitors and the ability of those transformed progenitors in mice to develop acute myeloid leukaemia. It is still not clear whether the N-terminal *MLL* part is sufficient for the leukaemic transformation or if the fusion partner is necessary. It has been shown for fusion proteins like *MLL/AF9* (Hemenway et al., 2001) or *MLL/AF10* (DiMartino et al., 2002) that *MLL* and its fusion partner are necessary of the transformation. On the contrary Martin published in 2003 (Martin et al., 2003) the transformation ability of a truncated form of the *MLL* wild type gene. Investigations showed upregulation of *HOXA7*, *HOXA9* and *MEIS1*, which is a common observation in acute leukaemia. Despite those two models, the role of *MLL* fusion proteins and their contribution to leukaemogenesis still remain unanswered. The general question is whether a fusion partner is necessary to cause leukaemia or *MLL* alone is necessary to cause the malignant transformation.

1.7 The translocation t(4;11)

The reciprocal translocation t(4;11) creating the fusion genes *MLL/AF4* and *AF4/MLL* is detected in about 66 % of ALL cases with *MLL* gene fusions (Meyer et al., 2009) but remains very rare in AML. In about 80 % of infants with ALL *MLL* fusions are detected and in over 50 % of *MLL* involved translocations, *AF4* is the fusion partner gene. An important group of leukaemias connected with *MLL* translocations is the group of therapy induced secondary leukaemias. Especially after treatment with topoisomerase II inhibitors like etoposide, the occurrence of *MLL*

translocations is detected in about 25 % of treated adults (Pedersen-Bjergaard et al., 1998).

In patients with the translocation t(4;11), the fusion gene *MLL/AF4* is always detected. In a study published in 2007 Kowarz and colleagues report that the reciprocal counterpart *AF4/MLL* was only detected in 80 % of all studied t(4;11) positive cases (Kowarz et al., 2007). The remaining 20 % showed complex translocation events involving more than two genes like the three way translocation resulting in der(1) PBX1/MLL, der(4) AF4/PBX1 and der(11) MLL/AF4 or loss of the N-terminal part of the der(4) resulting in MLL/AF4 and Δ MLL.

1.8 The genes *MLL* and *AF4*

Like the *HOX* genes themselves also two groups of HOX regulators have been detected in *Drosophila melanogaster*. The genes of the polycomb group (pcG) have been identified as HOX gene expression inhibitors while genes of the trithorax group (trxG) act as activators for *HOX* genes (Grimaud et al., 2006). In recent years the mechanisms of gene expression regulation by those protein groups has been investigated. Both pcG and trxG proteins seem to be epigenetic gene regulators meaning that they alter transcription by changing the methylation status of histones and the resulting alteration of chromatin structure in the gene's promoter region (Simon and Tamkun, 2002). The histone methyltransferase MLL is the human homologue to *Drosophila melanogaster* trithorax and a member of the Su(var)9-3, Enhancer of Zeste and trithorax (SET) family (Gu et al., 1992, Djabali et al., 1993). The MLL gene codes for a multiple domains consisting protein with 3969 amino acids and a molecular weight of ~430 kDa. Important domains are 3 AT-hook DNA binding domains, a DNA methyltransferase homology domain (DNMT), a central zinc finger plant homeodomain (PHD) as well as a highly conserved SET domain, which has the catalytic histone methyltransferase activity. The MLL protein has been identified to control the gene expression of several HOX transcription factors, including HOXA7, HOXA9 and HOXA10 (Ernst et al., 2004b, Nakamura et al., 2002). In *mll* knockout mice it was shown that *mll* is essential for the correct initiation of the *hox* genes during embryonic development. *Mll* knockout lead to

lethal disruptions during mouse haematopoiesis (Ernst et al., 2004a, Ernst et al., 2004b, Hess et al., 1997).

Functional studies in human cells revealed that MLL in cooperation with other cofactors formed a stable super complex called SET1 to transfer methyl groups to lysine K4 of the histone H3 (H3K4) (Terranova et al., 2006, Nakamura et al., 2002). The double or triple methylation of H3K4 within the promoter region of the MLL target genes facilitates the transcriptional status of those promoters (Bernstein et al., 2002, Santos-Rosa et al., 2003). Direct promoter activation by binding and methylation of H3K4 was demonstrated for *HOXA9* in the human cell line HeLa and *hoxc8* in murine fibroblast cells (Milne et al., 2002, Nakamura et al., 2002). However, within the MLL complex other proteins have been identified which have the ability to modify histone methylation. The cofactor WDR5 has been shown to modify methylation of H3K4 (Wysocka et al., 2005).

Not only methylation but also acetylation activity has been identified for the MLL complex. A factor called MOF has been identified that is able to transfer acetyl groups to lysine K16 of histone H4 (Dou et al., 2005). The interaction of MLL with the tumour suppressor MENIN was also described as being very important (Yokoyama et al., 2004). A N-terminal Menin binding motif (MBM) enables the interaction of MENIN and MLL which allows recruitment of lens epithelium derived growth factor (LEDGF), a transcription factor essential for MLL mediated gene expression (Yokoyama and Cleary, 2008).

To function properly MLL has to be posttranslational cleaved which is catalysed by TASPASE1, a threonine-aspartase (Hsieh et al., 2003a). The cleaved products (MLLC and MLLN) will then reassociate and build the central part of the MLL complex (Hsieh et al., 2003b, Yokoyama et al., 2002). Taspase1 knockout mice have shown to be viable but show a significant lower histone H3K4 methylation status (Takeda et al., 2006). This leads to the assumption that MLL plays a very important role in general gene transcription regulation. This is backed up by the findings of Nakamura (Nakamura et al., 2002) who showed a connection between RNA modifying proteins as well as components of the RNA-polymerase II transcription machinery and MLL. Binding of MLL has been detected in about 40 % of all genes of which about 90 % show RNA-polymerase II binding (Guenther et al., 2005).

The gene *AF4* (*ALL-1 fused gene on chromosome 4*) has been identified as MLL fusion partner in 1992 (Gu et al., 1992). *AF4* is a generally expressed gene (Frestedt et al., 1996) of 300 kb length and belongs to the ALF gene family. The mRNA is 10.5 kb and is comprised of 23 exons coding for a 131 kDa protein localised in the nucleus. Five functional domains have been identified in the AF4 protein. From N-terminal to C-terminal these are a N-terminal homology domain (NHD), a conserved domain of the ALF protein family (ALF), a proline –serine rich region (pSer), a nuclear localisation sequence (NLS) and a C-terminal homology domain (CHD). The function of AF4 is still under investigation. It is known that it can bind DNA and has transcription initiating characteristics so AF4 is assumed to be a transcription factor (Ma and Staudt, 1996, Prasad et al., 1995).

AF4 has also been described as a critical factor for lymphocyte development. Isnard and colleagues investigated murine *af4* knockout models and described a defect in the differentiation of B and T lymphocytes (Isnard et al., 2000). Furthermore this leads to the assumption that AF4 translocations promote oncogenic development of haematopoietic cells. This has been investigated by Bursen and colleagues as they transfected murine embryonic fibroblasts (MEF) with AF4 wild type and AF4/MLL constructs clearly demonstrated a growth transforming ability and hence oncogenic potential (Bursen et al., 2004).

An important study to reveal AF4 function was published in 2007 by Bitoun, Oliver and Davies (Bitoun et al., 2007), here illustrated in figure 1-6. They demonstrate the involvement of AF4 in a protein complex containing RNA-polymerase II (Pol II). In this study it was demonstrated that AF4 has an important impact on the transcription activity of Pol II by enabling transcriptional elongation. AF4 together with the ENL/AF9 complex binds to the kinase p-TEFb and associates with the Pol II complex. P-TEFb then phosphorylates Pol II inhibitors thereby initiating transcription. AF4/ENL/AF9 are also phosphorylated, bind to Pol II and recruit AF10 and DOT1L. This enables methylation of histone H3 lysine K79 causing chromatin remodelling. A negative feedback mechanism has also been described. The phosphorylation of AF4 lowers its transactivation activity furthermore phosphorylation of ENL/AF9 leads to their degradation by a so far unknown mechanism. pAF4 then detaches from the complex and is mediated via the E3 ubiquitin ligase SIAH to 26S proteasomal degradation (Bursen et al., 2004).

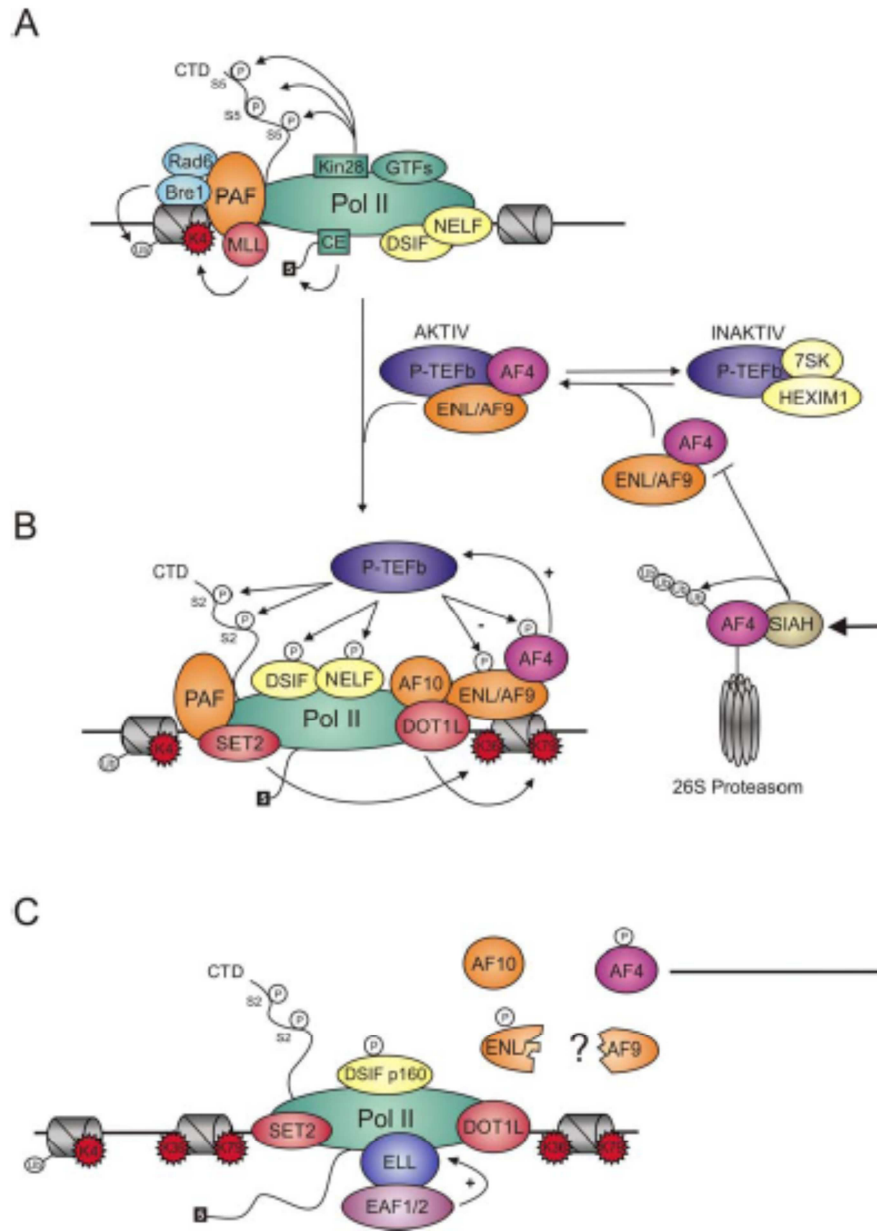


Figure 1-6 The role of the AF4 protein complex

The three stages of RNA polymerase II transcriptional elongation and chromatin remodelling mediated by AF4. The figure shows (A) the pre-initiation complex with Pol II being inhibited by NELF and DSIF. By binding to ENL/AF9 and P-TEFb AF4 mediates (B) the activation of Pol II by degradation of the inhibitory factors. Furthermore AF4-ENL/AF9 bind to the Pol II complex and recruit AF10 and DOT1L enabling H3K79 methylation. Phosphorylation of AF4 and ENL/AF9 by P-TEFb (C) initiates degradation of ENL/AF9 by an unknown mechanism and proteasomal degradation of AF4 via SIAH ubiquitin ligases. Amended from (Bitoun et al., 2007)

1.9 The fusion proteins MLL/AF4 (der11) und AF4/MLL(der4)

The protein MLL/AF4 (der (11); figure 1-7) is the fusion product of the MLL N-terminus with three domains (AT-Hook, DNA MT and two sub-nuclear localisation signal (SNL) domains) and the C-terminus of AF4 with four domains (ALF homology domain, pSer domain, NLS domain and CHD domain). In functional studies with MLL/AF4 it was shown, that the fusion protein did not have an effect on HOX gene expression (Bertrand et al., 2003). However, over expression of the fusion gene did implicate a role in the cell cycle and apoptosis. Interestingly affected cells showed a diminished proliferation and a G1 phase cell cycle arrest but also were more resistant to apoptosis mediating stimuli (Caslini et al., 2004, Gaussmann et al., 2007). In 2005 Thomas and colleagues published that siRNA mediated knockdown of MLL/AF4 in t(4;11) positive cell lines led to an increasing apoptosis rate (Thomas et al., 2005).

A more novel discovery is the influence of MLL/AF4 on the regulation of microRNAs. MicroRNAs have been reported to regulate gene expression by inhibiting the translation of mRNA or mediating degradation of the transcript (Novina and Sharp, 2004, Meister and Tuschl, 2004). Nakamura and colleagues report in 2007, that MLL fusion proteins target Drosha, a protein complex that is important for microRNA processing (Nakamura et al., 2007). In a recent publication the research team around Popovic discovered that the over expression of microRNA mir196b in bone marrow precursors leads to increasing proliferation and survivability as well as to an inhibition of differentiation (Popovic et al., 2009).

Three mouse models have been developed to perform *in vivo* studies to reveal the potential of MLL/AF4 to develop ALL or AML. In 2006 Metzler and colleagues published a study where they created knock in mice by using the cre-loxP recombinase system. Affected mice developed a B cell lymphoma but neither ALL or AML phenotype (Metzler et al., 2006). In the same year Chen and colleagues published an attempt to induce leukaemia by generating mice expressing a *mll-AF4* construct. Although the mice developed haematopoietic malignancies, mostly B-Cell lymphomas, no leukaemic phenotype was induced (Chen et al., 2006). Another

attempt to create a mouse model that develops ALL or AML upon MLL/AF4 expression was published in 2008. In this model the C-terminal part of AF4 was successfully been cloned into the endogenous murine *mll* gene thereby expression was controlled by the endogenous *mll* promoter. This approach successfully created mice which developed AML or ALL. Furthermore this study demonstrated the importance of the H3K79 methylation for maintaining the leukaemic phenotype (Krivtsov et al., 2008).

Another important study was published in 2008 by Guenther and colleagues. They investigated using microarray studies and CHIP on chip studies the effect of MLL/AF4 expression on global gene regulation. It was shown, that the chromatin structure in genes targeted by MLL/AF4 was heavily influenced by altered histone modification by trithorax group proteins and DOT1 methylases (Guenther et al., 2008).

The role of AF4/MLL (der (4); figure 1-7) in leukaemogenesis is still widely unknown. AF4/MLL consists of the NHD and ALF domain from the AF4 part and the BD, PHD, TAD, SET domain and the FYRN and FYRC interaction domains, furthermore the cleavage site for taspase 1. Two recent publications have investigated the fusion protein AF4/MLL and its role in leukaemia. So far it is known that AF4/MLL unlike the AF4 wild type is not subject to proteasomal degradation and thus accumulates in AF4/MLL positive cells. This is very likely the reason for the oncogenic effects and growth stimulation of cells (Bursen et al., 2004, Gaussmann et al., 2007).

Cotransfected cells with *AF4/MLL* and *MLL/AF4* demonstrated the highest growth rate and also the highest resistance against apoptosis. Also two genes which are described as stem cell genes Oct4 and Nanog showed an increase expression rate upon coexpression of both fusion genes. The combination of both stem cell genes has been described for the maintenance of pluripotency and self-renewal of embryonic stem cells (Gaussmann et al., 2007). To what degree this contributes to the leukaemic phenotype still has to be investigated.

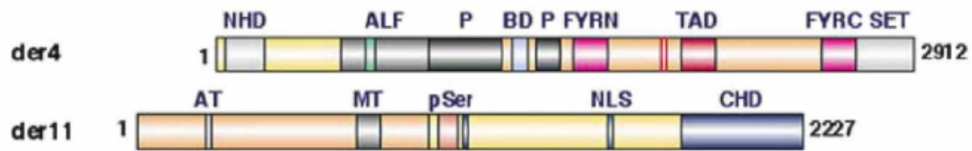


Figure 1-7 AF4/MLL der(4) and MLL/AF4 der(11)

The picture shows a schema of der(4) AF4/MLL and der(11) MLL/AF4. Numbers show the amino acids; der(4): NHD N-terminal homology domain, ALF ALF protein family homology domain, P PHD zincfinger domain, BD bromodomain, FYRN/FYRC internal interaction domain, TAD transactivation domain, SET Histone methyltransferase domain; the two lines next to TAD are the taspase cleavage site; der(11) AT AT hook, MT DNA methyltransferase domain, pSer proline-serine rich domain, NLS nuclear localisation sequence, CHD C-terminal homology domain; taken from (Bursen et al., 2004)

1.10 The HOX genes

With the discovery of the homeobox sequence, a DNA region of highly conserved homology in various species, a milestone was set.

Firstly those homeobox containing genes (or *HOX* genes) were discovered in flies (*Drosophila melanogaster*) but homologues have also been found in mammals. The homeobox genes are well studied in mice and humans. In humans a total of 39 *HOX* genes are clustered in four complexes distributed on four different chromosomes. These clusters are named HoxA located on chromosome 7, HoxB on chromosome 17, HoxC on chromosome 12 and HoxD on chromosome 2. Sequence similarities in the homeobox motive of the 39 *HOX* genes makes it possible to group those genes in 13 paralogous groups, where paralogous means that a gene has a copy located on another locus within the same genome. But none of the *HOX* clusters has the full set of 13 *HOX* genes (Figure 1-8).

Investigations have shown that *HOX* genes are regulated by their epigenetic status and they are regulated by their own state of expression. For example the HoxA cluster is regulated by a cascade which means that each *HOX* gene activates the expression of the next one while it causes repression of the previous *HOX* gene.

HOX genes are developmental genes. In flies and mice they tightly regulate the segment structure of the embryo and determine the body axis. *In vivo* knockout

experiments have demonstrated severe developmental changes proving the importance of the *HOX* genes.

The role of the *HOX* genes in the haematopoiesis and leukaemogenesis has also been in the focus of research. *HOX* genes, especially of the A, B and C cluster seem to be expressed in haematopoietic stem cells and immature progenitor cells while being less expressed at later differentiation states. In *HOX* over expression and knockout experiments in mice (characterized in Figure 1-8) results show that *HOX* genes play a crucial role in HSC self renewal as well as a block in lymphoblast development. Further investigations demonstrated that *HOX* genes have the potential to cause or at least influence the onset of myeloproliferative disorders and leukaemia.

In *Drosophila melanogaster* one of the key regulator of *HOX* genes are the genes of the trithorax group. The human homologue of those trithorax genes are the *mixed lineage leukaemia (MLL)* genes. In *MLL* $-/-$ double knockout experiments affected embryonic bodies were not capable to form haematopoietic populations. This defect could be overcome by over expression of *HOX* genes *A9* and *A10* furthermore by *B3* and *B4*. (Ernst et al., 2004b). The discovery of two major co-factors for HOX proteins gave more insight into the complex regulation mechanism of HOX in development. PBX1 and MEIS1 directly interact with the HOX transcription factors and are important for the DNA binding efficiency of the HOX proteins. It has been shown, that MEIS1 and PBX1 can enhance HOX binding specificity and affinity (Shen et al., 1997). Further MEIS1 in combination with PBX1 has been shown to increase transforming ability of MLL fusion gene transfected cells (Wong et al., 2007, Wang et al., 2006). Knockout experiments for both *PBX1* and *MEIS1* have demonstrated their important role in development and self renewal potential of HSCs.

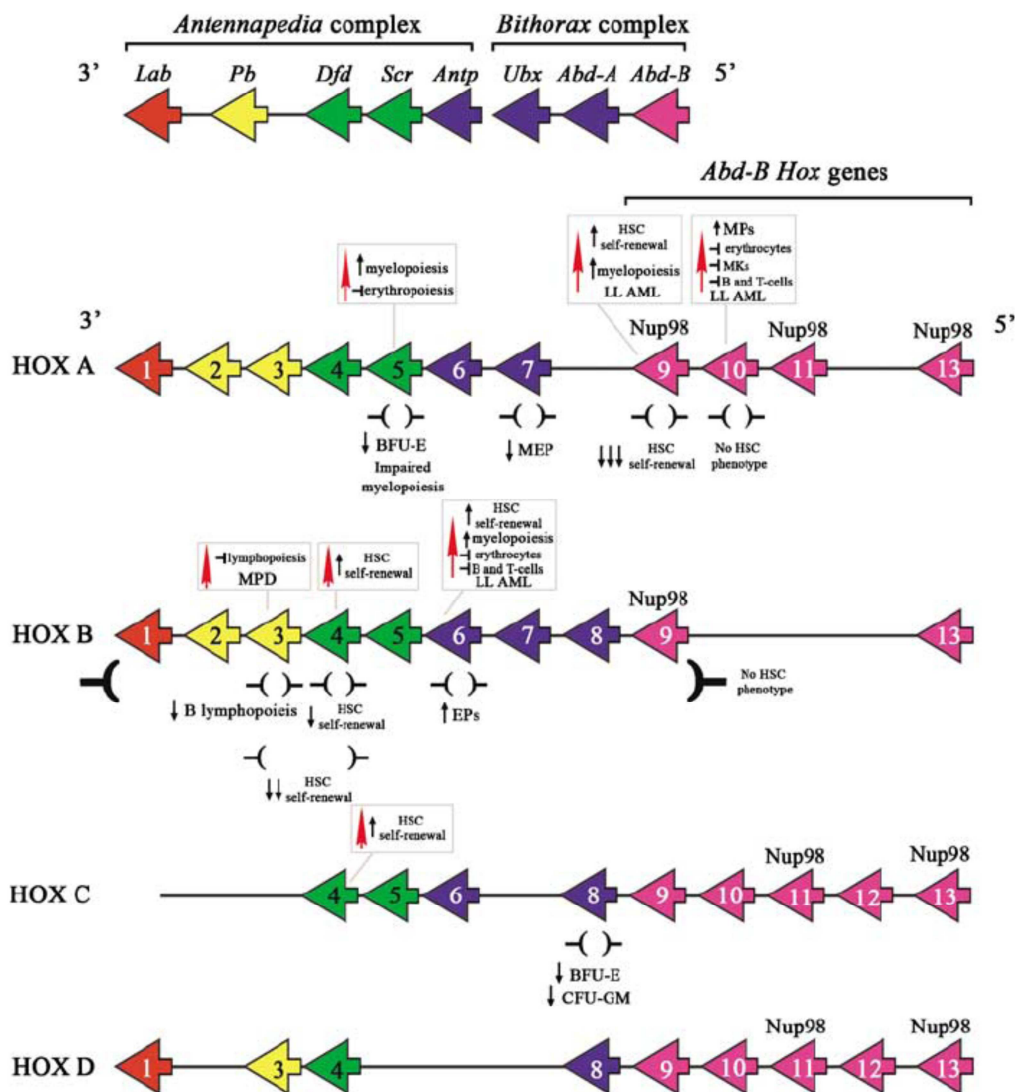


Figure 1-8 Overview of the Hox cluster in human

This figure demonstrates the simplified structure of the human Hox clusters (HoxA, HoxB, HoxC and HoxD) compared to the *Drosophila melanogaster* Hox complex on the top. Homologue genes are shown in the same colour. This picture also shows, that no Hox cluster contains all 13 HOX genes. Different effects on hematopoiesis are also shown. Red arrows indicated over expression of the Hox gene, while brackets show knockout mouse experiments. (Argiropoulos and Humphries, 2007)

Various research groups have concentrated their work on the correlation of the altered *HOX* gene expression profile and pro-leukaemogenic *MLL* rearrangements. As described before, *MLL* rearrangements have been detected in ALL and AML cases alike. In all studied cases of leukaemias with a *MLL* rearrangement a dysregulated *HOX* profile was detected. In addition to *HOX*, dysregulation of *MEIS1* is a frequent observation in haematopoietic malignancies. Elevation of *HOXA9*

expression levels for example is strongly correlated with a poor prognosis in acute myeloid leukaemia (AML). Further studies showed that especially *HOXA9* is essential for the survival of MLL rearranged acute leukaemias (Faber et al., 2009). Over expression of MLL fusion genes in mice showed upregulation of certain members of the clustered HOX genes, especially *HOXA9*.

But also the posterior HOXA genes *HOXA7* and *HOXA10* have a prominent leukaemogenic potential.

In a series of lymphoblastic leukaemias an elevated *HOX* gene expression profile, especially of the HOXA cluster, was monitored. In a recent publication (Bach et al., 2010) the oncogenic potential of every gene within the HOXA cluster was investigated. As expected by the authors *HOXA7*, *HOXA9* and *HOXA10* were demonstrated to have the ability to cause leukaemogenesis. A new finding was that also anterior *HOXA* genes like *HOXA1*, *HOXA4* and *HOXA6* were also able to transform haematopoietic cells. The transforming potential was not as potent as of the posterior *HOXA* genes, but it was shown, that those specific anterior *HOXA* genes have transforming ability. Apart from *HOXA5*, all other genes of the HOXA cluster had an effect on haematopoiesis, be it either transformation into a malignant cell or a delay in the differentiation process. As a conclusion the authors described HOXA proteins as rheostats that are necessary to adjust the complex haematopoietic differentiation depending on the varying environment.

1.11 Telomerase

The main function of telomerase is maintaining the chromosomal ends, the so called telomeres. The telomeres are a piece of double stranded DNA at the end of each chromosome. The telomeres harbour only non coding sequence of DNA and consist of nucleotide sequence TTAGGG repeats.

Normal somatic mammalian cells have a limited rate of proliferation *in vitro*. The maximum number of cell divisions is called the Hayflick limit (Hayflick, 1979). As soon as this limit has been reached, critical shortened telomeres trigger a permanent growth arrest, also called senescence (Shay et al., 1991). To overcome the senescence, human cells must inactivate the RB and p53 signalling pathway. Cell populations that overcome senescence will continue to divide until a second

proliferation check point is reached, called crisis. This state is characterised by very short telomeres, chromosomal end to end fusions and anaphase bridges which results in the activation of apoptosis or programmed cell death.

In very rare occasions cells are also able to overcome this stage by maintaining those short telomeres. Those cells are then termed immortal. Often it has been shown that a high telomerase activity is responsible for the telomere upkeep. Regulatory mechanisms behind this are still under investigation. In cells which have a telomere maintenance but no telomerase activity a second more uncommon way of telomere lengthening has been discovered, the so called ALT (alternative lengthening of telomeres) mechanism (Muntoni and Reddel, 2005, Bryan et al., 1997) where the telomeres are rebuilt by complex recombination events. More than 90 % of all types of cancer show a high telomerase activity to keep the telomeres up, while the telomerase independent ALT mechanism was only found in approximately 10 %. (Kim et al., 1994)

Only a few types of normal human cells show a high activity of telomerase. Those are stimulated lymphocytes, intestinal epithelium, basal keratinocytes, haematopoietic stem cells and germ cells in ovaries and testis (Kim et al., 1994, Hahn, 2005, Counter et al., 1995, Hiyama et al., 1995b).

Telomerase as a diagnostic and prognostic marker is useful for a broad spectrum of cancer types (Hiyama and Hiyama, 2003, Ulaner, 2004, Shay and Bacchetti, 1997). As the telomerase activity rises during the onset of the disease it is possible to monitor the tumour tissue growth by its level of telomerase activity. Depending on the tumour the telomerase activity can be high from the beginning or increase during the disease. Increasing telomerase activity may reflect increased tumour progression. (Shay and Bacchetti, 1997, Hiyama and Hiyama, 2002)

Also in cases when it is not possible to distinguish between benign and malignant cancer by morphological examinations, telomerase activity may aid in diagnosis. This group consist for example of brain tumours, adenocarcinoma, pancreatic endocrine tumours and intraepithelial neoplasms. In those tumours a correlation between telomerase activity and prognosis of the patient has been found. In neuroblastoma cells, a common tumour in children, high telomerase activity

correlated with a poor prognosis, while the outcome was much better when a low activity was determined (Hiyama and Hiyama, 2002, Hiyama et al., 1995a, Pearson et al., 2000, Falchetti et al., 1999, Poremba et al., 1999).

The correlation of telomerase activity and cancer prognosis led to the fact that telomerase or its components could be a valuable drug target (Shay and Wright, 2002). Multiple potential chemotherapeutic strategies based on telomere and telomerase biology are in development. This includes drugs that directly target the telomeres, the telomerase or telomerase associated regulatory mechanisms as well as telomerase immunotherapy and telomerase mediated tumour specific gene therapy. The telomerase consists of two major subunits. The protein subunit is named telomerase reverse transcriptase (TERT) and telomerase RNA component (TERC). The RNA component TERC is complimentary to the last telomeric repeat of the DNA, binds there and the reverse transcriptase (TERT) can add the complimentary nucleotides to the telomere DNA. DNA polymerases can then completely reconstruct the telomere. TERC will dissociate from the strand and target the next free 3' DNA telomeric repeat. The telomerase core components hTERT and TERC have been shown to be preferable drug targets (Pendino et al., 2006).

The RNA template or TERC was thought to be a good direct target as it is very important for the telomere lengthening (Corey, 2000). Inhibiting oligonucleotides targeting this template should have no difficulties in reaching the desired location. The challenge here is to establish a delivery system and to maintain the oligonucleotides stability meaning to protect the oligonucleotides until they reach their target without being degraded by endonucleases (Shay and Wright, 2006). Also antisense molecules have been tested to inhibit the RNA component. Those molecules do not target the template region. The most effective molecules were those which targeted sequences that are important for the formation of the telomerase enzyme. Another tested group of molecules were ribozymes which can induce telomere shortening and apoptosis in some cell line models (Yeo et al., 2005).

Negative side effects in telomerase inhibitory therapy are the effects on telomerase positive proliferative cells like stem cells, germ cells or different types of regenerative epithelial cells. Those cell populations normally show much longer telomeres than malignant cell populations. Although those normal proliferative cells

have telomerase activity, they have a much slower rate of proliferation than malignant cells. Considering the differences in the rate of proliferation an anti-telomerase therapy should affect the malignant highly proliferative cells much more than the slower proliferating normal cells.

1.11.1 The reverse transcriptase of the telomerase, TERT

Investigations in cancer biology and telomerase activation have shown that the protein subunit of the telomerase, TERT, plays a very important role (Nakamura and Cech, 1998). Over expression of TERT reactivated telomerase activity in various primary cell types but also immortalised those cells (Bodnar et al., 1998). TERC, the RNA component of the telomerase, was detected in malignant and normal cells equally (Feng et al., 1995, Yi et al., 2001).

Although TERT expression alone is sufficient for inducing immortalisation, the event itself is rare. This suggests a strict regulation of the TERT promoter. The research team of Tanaka (Tanaka et al., 1999) has shown that the promoter is GC rich and inhibited by DNA methylation. This type of inactivation is often detected in differentiated or senescent cells (Lopatina et al., 2003, Shin et al., 2003). Inhibition of DNA methylation on the other hand leads in human teratocarcinoma (HT) cells to reactivation of TERT expression. Another regulatory mechanism that has been investigated in normal and malignant cells is the acetylation of histones (Annunziato and Hansen, 2000). By acetylation and deacetylation of histones the transition from euchromatin (transcription activation) to heterochromatin (repression of transcription) is regulated. Inhibition of histone deacetylases (HDAC) in normal telomerase negative cells leads to telomerase activation.

Table 4 Transcription factors for TERT

factor	role	reference
c-Myc	activator	(Wu et al., 1999, Bazarov et al., 2009)
E2F1	repressor/activator	(Crowe and Nguyen, 2001, Crowe et al., 2001)
Mad1	repressor	(Oh et al., 2000)
Menin	repressor	(Lin and Elledge, 2003)
p53	repressor	(Toh et al., 2005, Kanaya et al., 2000, Shats et al., 2004)

Various transcription factors for TERT expression have been identified. One of them is the oncoprotein C-MYC which is an activator. It forms a sequence-specific DNA-binding protein complex with MAX and by binding of C-MYC to e-boxes which are short DNA sequences upstream of a gene in the promoter and binding sites for many transcription factors it activates the expression of TERT (Blackwood and Eisenman, 1991, Bazarov et al., 2009). Furthermore the protein MAD-1 has been identified as an antagonist to C-MYC (Zhou and Hurlin, 2001).

The transcription factor E2F1 has a dual role in the regulation of TERT. E2F1 is an important regulator of the cell cycle. Over expression of E2F1 is sufficient to initiate the expression of S-phase genes and stimulates proliferation of resting cells (Dimova and Dyson, 2005). E2F1 has a repressive role as complex E2F1-RB1-HDAC. (Dyson, 1998). During the cell cycle and cell aging the tumour suppressor retinoblastoma 1 RB1 is dephosphorylated and thereby active. Through RB1 activation formation of E2F1-RB1-HDAC complexes have a repressive effect on the expression of E2F1 mediated transcription (Narita et al., 2003). In tumour cells E2F1 acts as transcriptional repressor for TERT (Crowe and Nguyen, 2001, Crowe et al., 2001) while it acts as activator in normal cells.

The well-known tumour suppressor gene, p53 is also a potent suppressor of TERT. Over expression of p53 or activation of endogenous p53 results in a quick decrease of TERT expression levels. Further studies suggested that p53 interacts with the

transcription factor SP1 which binds to the TERT promoter and inhibits TERT expression (Kanaya et al., 2000). Additional studies indicate that p53 inhibits TERT expression via induction of the cyclin dependent kinase inhibitor p21 (Shats et al., 2004). P21 also induces formation of the E2F-RB-HDAC complex via dephosphorylation of RB, repressing TERT expression by binding of E2F to the relevant binding site within the TERT promoter. Therefore p53 manages, next to inhibition of SP1, to suppress expression of TERT via induction of p21 (Shats et al., 2004).

1.12 RNA Interference

In the beginning of 1990 two independent research teams made an unexpected discovery. They introduced genes into plants to give the blossoms a stronger colour. But the blossoms lost their colour in part or completely upon the gene over expression (van der Krol et al., 1990). A research group around Wassenegger published in 1994 that introducing genes into plant caused inactivation of the endogenous one resulting in loss of gene function (Wassenegger et al., 1994).

Four years later in 1998, Craig Mellow and Andrew Fire showed in *Caenorhabditis elegans*, a nematode, that genes can be knocked out by treating the worms with complementary short double stranded RNAs (dsRNA) (Fire et al., 1998).

Improvements and research in the following years provided a new and easy technique to investigate genes and their functions. With the newborn RNA interference or for short RNAi (Figure 1-10), it is possible to target every known gene and to silence it.

In principle every cell has the RNAi machinery which marks a very important gene regulation mechanism. It is coded in different locations throughout the genome. Small RNAs, known as microRNAs (miRNA), are found in introns, exons and further regions, referred to as non-coding regions. The miRNAs are transcribed and then processed by a series of protein-complexes. Three main enzyme complexes are involved in miRNA processing: DROSHA, DICER and RNA induced silencing complex (RISC). The endonucleases DROSHA and DICER belong to RNaseIII

enzyme family. The exact function of DROSHA and DICER is still under investigation. DROSHA processes the primary-miRNA (pri-miRNA) in the nucleus by transforming the endogenous miRNA transcript into a hairpin structure, called precursor-miRNA (pre-miRNA). For exact cleavage DROSHA needs another protein called DGCR8 or PASHA in invertebrates. This protein determines the base of the dsRNA hairpins and DROSHA can produce the pre-miRNA. After the pre-miRNA has been exported to the cytosol, DICER further processes the miRNA, forming a double stranded RNA strand with a sense and antisense strand of about 21-25 nucleotides of length. DICER then cleaves the hairpin and thereby creates the double stranded RNA (Figure 1-9).

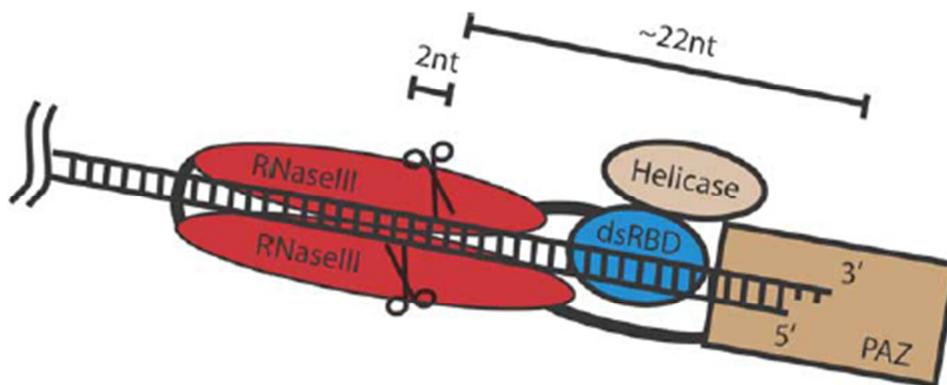


Figure 1-9 A model for dicer

The DICER domain PAZ binds to the 2nt overhang of the 3' end of the dsRNA. The RNaseIII domains form a pseudodimer and hydrolyse each strand of the dsRNA. (Hammond, 2005)

DICER consists of several domains but their functions still need to be fully investigated. An important domain is the PAZ domain which specifically binds to the 3' end of the dsRNA. The distance between the PAZ domain and the active cleavage site of the RNaseIII enzyme covers the distance of 25 nucleotides which is the final length of the dsRNA. It seems the domain structure of DICER is a molecular measuring tool that defines the length of the dsRNA by physical rather than biochemical aspects. In the next step the processed miRNA is loaded onto the RISC. For that two other enzymes are recruited, ARGONAUTE and a dsRNA-binding protein (TRBP) forming the RISC loading complex. The miRNA is transferred to the ARGONAUTE protein. The sense strand is removed and the antisense miRNA is the

guide for the RISC. Interestingly animal miRNAs are only partially complimentary to the target mRNA mainly in the 3' untranslated region. As a consequence, the target mRNA is only silenced by blocking translation. In plants the mRNA is cleaved by RISC. Plant miRNA is also a 100 % match to the target sequence (Hammond, 2005, Jinek and Doudna, 2009, Meister and Tuschl, 2004, Novina and Sharp, 2004).

siRNA is an artificially created double stranded RNA molecule. siRNAs are designed to be fully complimentary to the target sequence to guarantee the silencing of the target mRNA and are normally transfected into the desired cells. For this purpose mainly two methods are in use. The electroporation on the one hand uses an electric field that punches holes into the cell membrane and thus the siRNA can enter the target cell. On the other hand, there are several transfection reagents available to guide the artificial dsRNAs through the cell membrane into the cytoplasm where they then can act, e.g. Lipofectamine.

Currently in development are new forms of siRNA transfection methods such as nanotechnology or lipids. Thereby siRNAs are attached to nanoparticles or encapsulated into liposomes in order to change their charge and enhance the delivery. The uptake of these siRNA vehicles occurs then by the natural process of endocytosis.

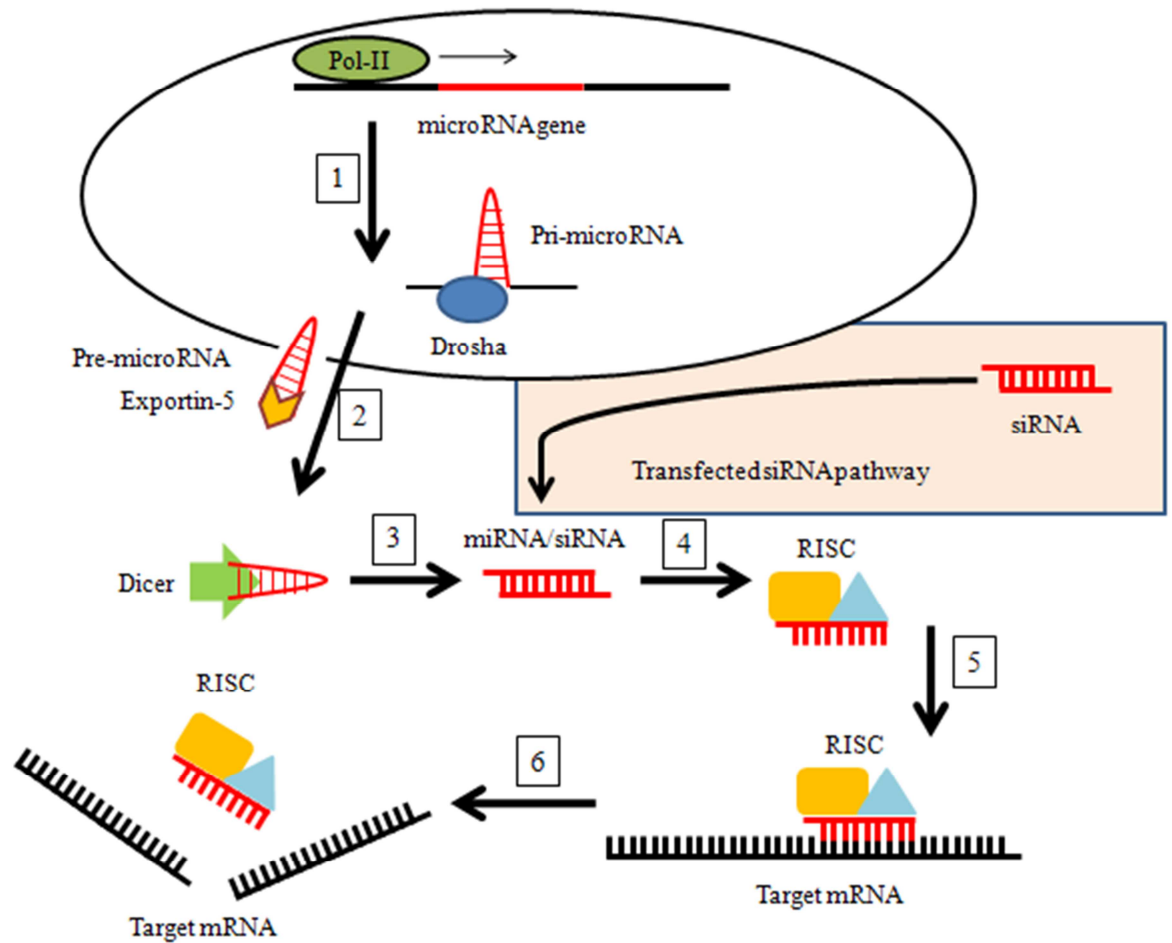


Figure 1-10 The miRNA and siRNA pathway – the principle of RNAi

Scheme of the siRNA and miRNA pathway. (1) The microRNAs gene is transcribed by RNA polymerase II and processed by DROSHA. After EXPORTIN-5 bound the pre-miRNA (2) it is exported to the cytosol where it is further processed by DICER (3). This is the status where transfected siRNA enters the RNAi machinery (red underlay). The interfering RNA (iRNA) antisense strand will be incorporated (4) by RISC and guides RISC to the target mRNA sequence (5). RISC hydrolyses the target mRNA (6) which is then further degraded. RISC will be recycled. (Hammond, 2005)

1.13 Preliminary work to this project

The subject of this thesis is based on results, which have been published in November 2005 in Blood (Thomas et al., 2005). In this publication the t(4;11) positive cell line SEM expressing the *MLL/AF4* fusion transcript was used. Treatment of the t(4;11) positive SEM cells with siRNA called “siMA6”, which is directed against the fusion gene transcript of der(11) *MLL/AF4*, led to a specific knockdown of the fusion gene transcript and protein expression (Figure 1-11, Figure 1-12).

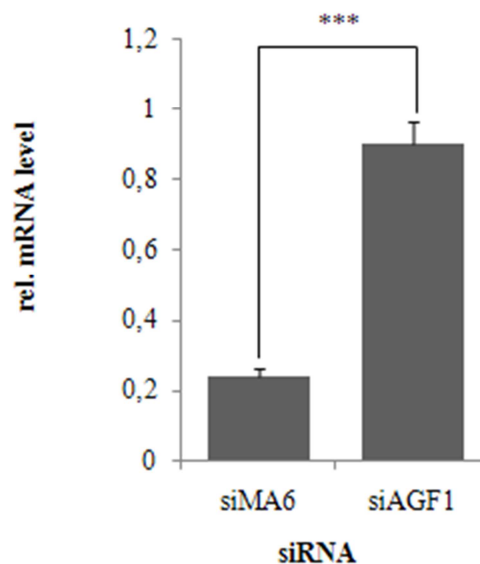


Figure 1-11 The siRNA siMA6 causes depletion of *MLL/AF4* mRNA.

The diagram on the left side shows a significant *MLL/AF4* mRNA reduction after 96hours of sustained siRNA treatment. siMA6 is the active siRNA against the fusion gene transcript, siAGF1 the siRNA control. Significance was tested with an unpaired student t test ($p < 0.005$).

In qRT-PCR experiments *MLL/AF4* mRNA was significantly reduced ($p < 0.005$) upon application of active siRNA siMA6 to SEM cells compared to treatment with siRNA siAGF1, an active siRNA against the fusion gene transcript *AML1/MTG8*, not present in SEM cells. The 96 hours of sustained treatment with siMA6 caused a 70% knockdown out of 3 independent experiments at the mRNA-level of expression of *MLL/AF4*. Western Blot experiments showed diminished *MLL/AF4* protein expression to confirm the successful inhibition of the *MLL/AF4* protein biosynthesis.

In addition MLL/AF4 depleted SEM cells showed a strong inhibition of proliferation compared to mock and control siRNA treated cells (Figure 1-13).

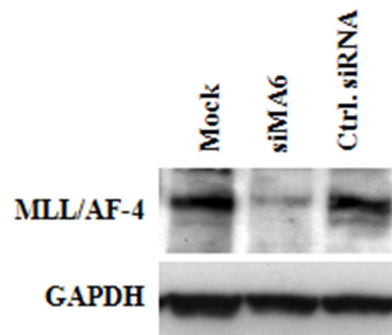


Figure 1-12 Western Blot against MLL/AF4

The picture shows the MLL/AF4 protein. siMA6 treatment results in depletion of the protein level, compared to the controls Mock and siRNA control siMM. GAPDH is used as loading control.

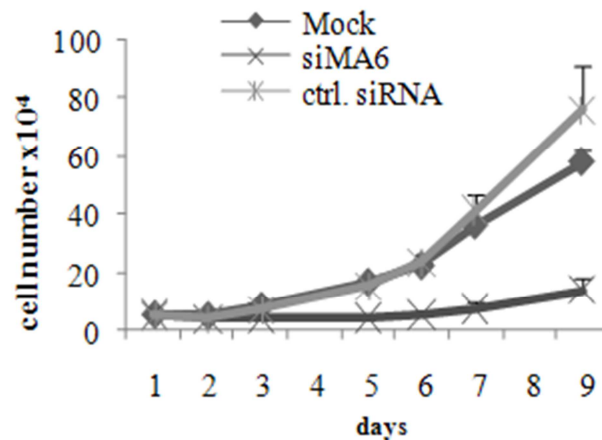


Figure 1-13 Proliferation curve of SEM cells after sustained siRNA treatment

The graph shows the proliferation of SEM cells (y-axis) after sustained treatment in days (x-axis) with active siRNA siMA6 (X) compared to Mock (♦) and control siRNA (*).

Furthermore, induction of apoptosis was associated with MLL/AF4 depletion. The SEM cells were harvested after 96 hours of continuous siRNA treatment and were analysed for apoptosis by annexin-V staining. A three-fold increase of apoptosis was observed compared to mock and a control siRNA. This finding was validated by

immunoblotting against the anti-apoptotic protein BCL-XL and cleavage of caspase 3 (CASP3), a clear induction of apoptosis pathway activation (Figure 1-14).

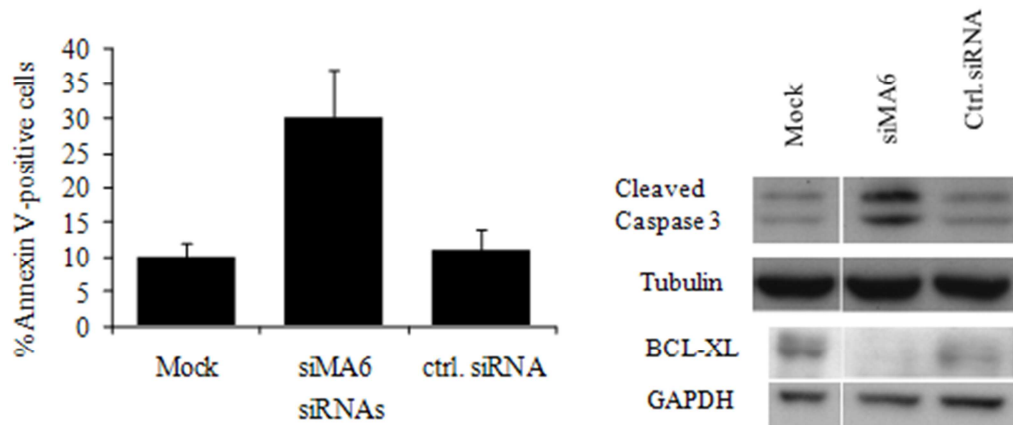


Figure 1-14 Induction of apoptosis upon MLL-/AF4 depletion

SEM cells were treated 96 hours with siRNA. The graph on the left side represents the result of an annexin-5 staining and shows a three-fold increase of apoptosis as consequence of MLL/AF4 depletion compared to mock and control siRNA treatment. The induction of apoptosis was further observed by western blot for cleaved CASP 3 and the anti-apoptotic protein BCL-XL.

1.13.1 Aim of this study

The aim of the research presented in this thesis was to identify additional novel target genes for MLL/AF4. Preliminary studies and publications (Milne et al., 2002, Schraets et al., 2003, Thomas et al., 2005) suggested *HOXA7*, *HOXA9* and *HOXA10*. The study was to examine other genes of the HOXA cluster. These genes are known to be regulators in early haematopoiesis so we hypothesised MLL/AF4 together with the HOXA genes play an important role in the stem cell character of the SEM cells.

A secondary objective was to investigate the inhibition of telomerase activity as consequence of siRNA mediated MLL/AF4 knockdown. Preliminary results strongly suggested an effect on telomerase activity upon MLL/AF4 knockdown. Further to telomerase activity, the major subunits of the telomerase, TERT and TERC, should be investigated.

2 Results

2.1 Genes of the HOXA cluster as possible MLL/AF4 targets

2.1.1 Introduction

Genes of the HoxA cluster are important genes as they play a major role in controlling early haematopoiesis in both differentiation and maturation. Whether those genes play a role in leukaemia has also been investigated, although so far, their contribution towards the malignant transformation of haematopoietic cells is unclear. These genes have been especially investigated in leukaemic cells positive for a MLL rearrangement. The link here is that *mll* has been shown to regulate *hox* gene expression in mice and their homologues do the same in *drosophila*. In ALL and AML cell line models and patients the posterior genes of the HoxA cluster, HoxA7 to HoxA10 in particular, have been shown to be alternatively regulated in the diseased cells. The other HoxA genes may have roles in early haematopoiesis but have not been investigated in a MLL rearranged leukaemia model.

Initially the effect of MLL/AF4 knockdown on all genes of the HoxA cluster was first examined. For this purpose the t(4;11) positive cell line SEM was treated with an siRNA specifically designed against the fusion transcript as published (Thomas et al., 2005). The cells were treated twice with siRNA in the beginning and 48 hours after the first treatment. Cells were then harvested 48 hours after the second siRNA treatment and analysed by qRT-PCR for HoxA gene expression. In addition significantly altered expressed genes were examined for their protein levels by western blot. This first series of experiments should reveal possible new target genes of MLL/AF4 within the HOXA cluster.

2.1.2 Real time PCR – important notes

Real Time PCR data is acquired by measuring the fluorescent signal strength in each reaction cycle of the PCR with a total of forty cycles. According to the literature real time PCR reaction analysis is divided into four phases (Figure 2-1). The first one is the linear ground phase, where no PCR product is measured. In this phase there is not enough PCR product to produce a measurable fluorescent signal. Following the ground phase comes the early exponential phase, where the first PCR products can be detected. This phase switches over to the log-linear phase were, if optimal primer

binding occurs, the PCR product is doubled during every cycle. In this phase the so called cycle threshold (CT) is determined. This value is most important for the calculation of the amount of PCR product. The CT is the cycle number at which the fluorescent signal, generated during the PCR reaction, exceeds a predefined threshold. This threshold is arbitrary defined by the machine's manufacturer and can be adjusted by the user. The optimum for setting the CT is the transition point between the early exponential phase and the log-linear phase. The starting point of the early exponential and log-linear phase very much depends on the gene of interest. In general it seems that highly expressed genes like GAPDH are measured during cycle 10 – 12. The log-linear phase is normally measured up to cycle 30. The final phase is the plateau phase. In this phase the PCR product is no longer doubled in every cycle. This might have various reasons, such as decreasing activity of the polymerase or exhausting reaction components. CT values determined within this phase must be considered with care as they might not reflect the real situation. CT values from cycle 30 onwards should only be considered as an approximate value.

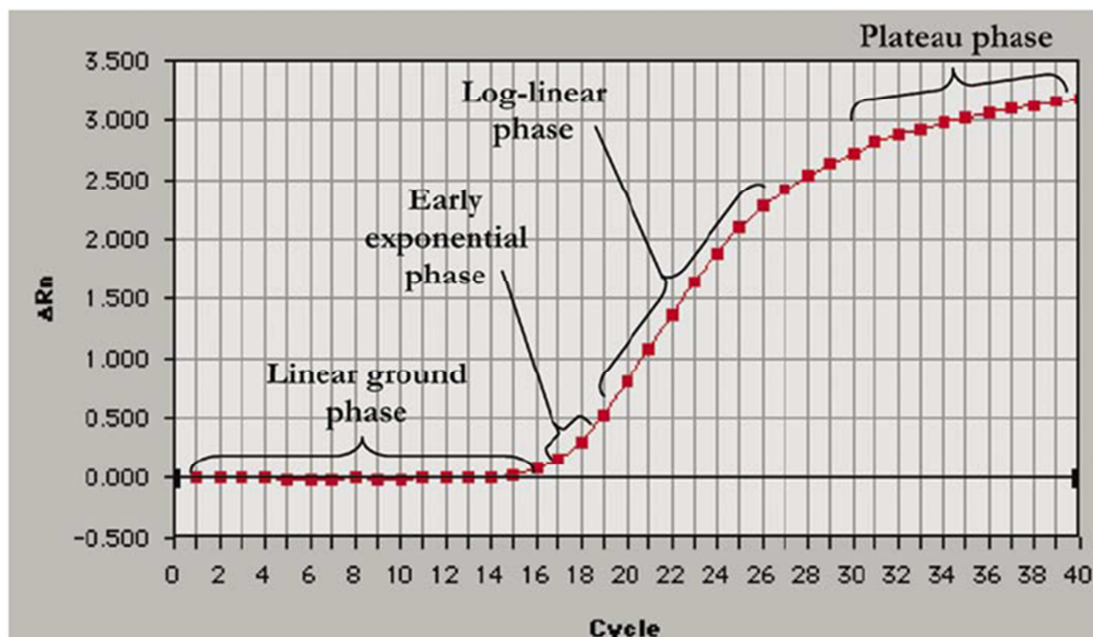


Figure 2-1 Phases of the PCR amplification

This graph demonstrates the four phases of the PCR amplification curve. On the y-axis, ΔRN describes the fluorescent signal, calculated from the signal of the fluorescent dye and a reference dye in the reaction. The x-axis shows the cycle numbers of the PCR reaction. The red line shows a typical amplification curve. The four phases of the amplification are shown. Taken from <http://www.dorak.info>

2.1.3 General HOXA gene expression in SEM cells

The first step was to determine basal mRNA expression levels of the HOXA genes in SEM cells, so relative gene expression of each individual HOXA genes was compared to internal GAPDH transcript control.

SEM cells were taken, RNA was extracted and mRNA levels were analysed by qRT-PCR. Results were analysed with SDS version 2.2 (Applied Biosystems) and Excel (Microsoft). Average ct cycle values were visualised as a bar chart (Figure 2-2) and relative HOXA gene expression levels were compared to the internal housekeeping gene GAPDH (Figure 2-3).

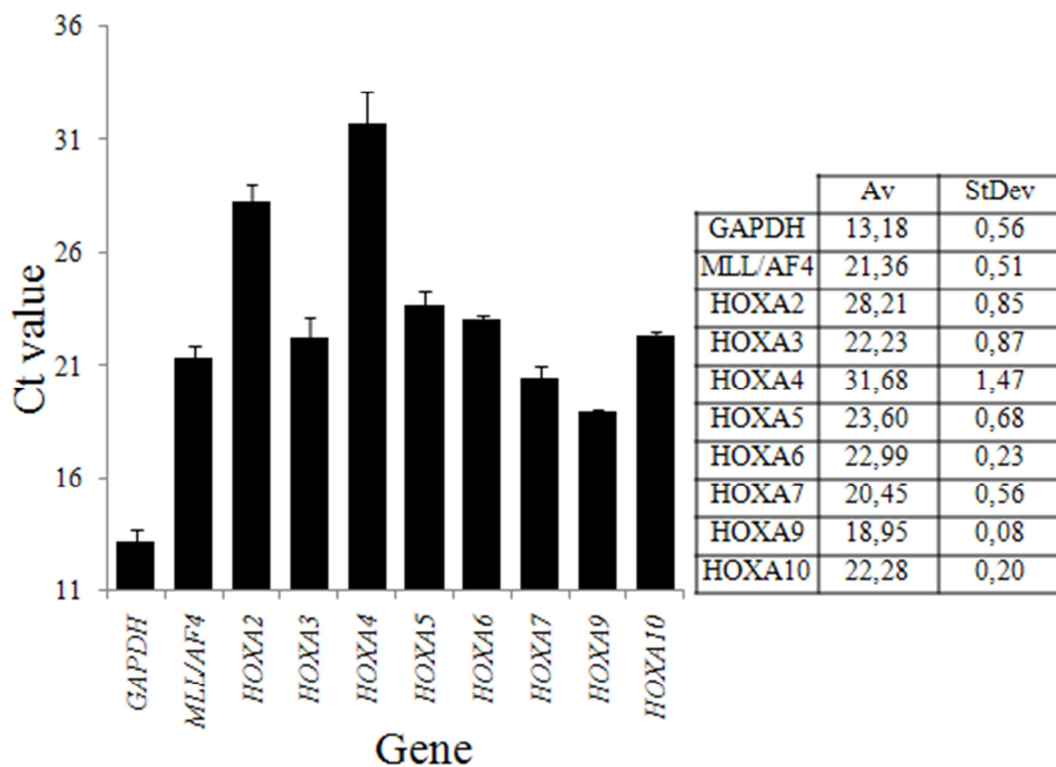


Figure 2-2 HOX and GAPDH gene expression in SEM cells

This bar chart shows the average CT cycle values of the HOXA genes. The error bars indicate average standard deviations of two (HOXA2 and 3) and four (HOXA4 – 10, GAPDH) independent experiments, respectively. The table shows the average CT cycle and standard deviation values of the according gene.

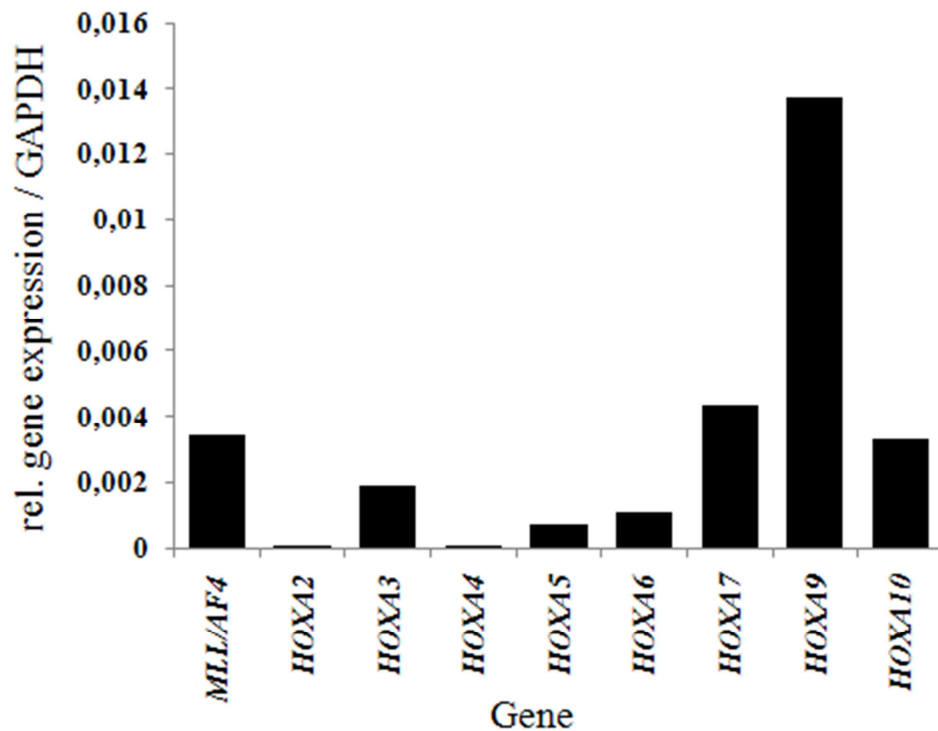


Figure 2-3 Relative expression of HOXA genes in SEM cells

Relative mRNA expression levels of the *HOXA* genes compared to *GAPDH* internal control.

The results show that *HOXA1*, *HOXA11* or *HOXA13* were not expressed. In contrast, *HOXA7*, *HOXA9* and *HOXA10* show the most abundant gene expression followed by the remaining *HOXA* genes A2-A6. *MLL/AF4* is also expressed at a higher level comparable to the posterior *HOXA* genes. Remarkable is the high expression of *HOXA9* which is about 4-fold compared to the other investigated *HOXA* genes and *MLL/AF4*.

The present results reflect the expression level of the *HOXA* genes in a cell line. This might be completely different in primary human leukaemic cells. To achieve this goal, primary leukaemic cells from patients with ALL should be compared where the *HOXA* genes should be compared to a suitable internal control, for example *GAPDH*. This data should be acquired from each patient and subsequently compared to the data from the other patients. This would result in a good overview of the *HOXA* gene activity in a cohort of ALL patients.

2.1.4 siRNA mediated knockdown of MLL/AF4 affects HOXA gene expression

Results of previous published work showed that the expression of at least two members of the HOXA gene cluster was influenced by *MLL/AF4* knockdown, namely the genes *HOXA7* and *HOXA9* (Thomas et al., 2005). Further to these results, primers were designed to investigate the other members of the HOXA cluster. In qRT-PCR experiments the effect of *MLL/AF4* knockdown on the HOXA genes. *HOXA2*, *HOXA3*, *HOXA4*, *HOXA5*, *HOXA6* and *HOXA10* were analysed. To support earlier results *HOXA7* and *HOXA9* were included in the experiments.

The main goal of the HOXA cluster analysis was to determine the effect of siRNA mediated *MLL/AF4* knockdown on the genes of the HOXA cluster. The siRNAs used were siMA6, which is targeted against the *MLL/AF4* mRNA, and siAGF6 which is a siRNA designed to target the fusion gene transcript *AML1/ETO*, a transcript that is present in AML cells with the t(8;21) translocation (e.g. SKNO cells or Kasumi-1 cells). This control was preferred over usual scrambled siRNA, as it successfully targets another fusion gene transcript (Heidenreich et al., 2003), thereby further demonstrating the specificity of the siRNA.

SEM cells were cultured and electroporated twice with 500nM siRNA on day zero and day two of the experiment. Cells were harvested two days after the second electroporation, mRNA was isolated and quantified by qRT-PCR. Results were analysed using Excel. Relative fold change of mRNA expression of *MLL/AF4* depleted and siAGF1 transfected non-target control cells was compared to untreated mock control. As *HOXA1*, *HOXA11* and *HOXA13* did not show any mRNA expression, those genes were not included in the experiment. The mRNAs of *HOXA2* and *HOXA3* were analysed in two independent experiments, but no knockdown was detected. *HOXA4* showed a knockdown of about 70% upon *MLL/AF4* depletion in two independent experiments, but due to the general very low expression levels (average CT value of 32) of *HOXA4* it was decided to concentrate on the main candidates. *HOXA5* was analysed in four independent experiment. No significant (student t test: $p < 0.9$) differences in gene expression were observed with *MLL/AF4* depletion (Figure 2-4).

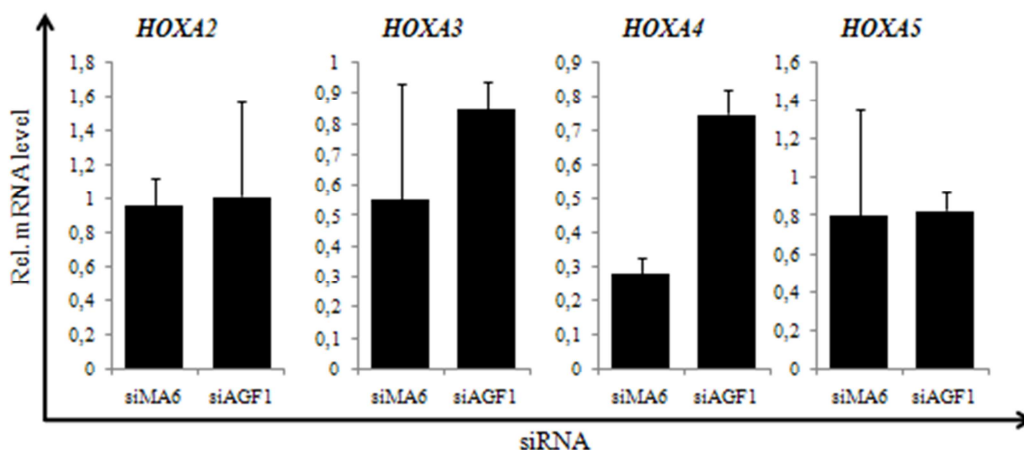


Figure 2-4 HOX gene expression after MLL/AF4 knockdown – part 1

This picture shows the qRT-PCR analysis of relative HOXA mRNA levels (y-axis) after 96 hours of sustained MLL/AF4 suppression by active siRNA siMA6 and siAGF6 control (x-axis) in SEM cells. *HOXA2*, *HOXA3* and *HOXA4* were analysed in two independent experiments. The error bars represent standard deviation of two experiments. *HOXA5* was analysed in four independent experiments. The error bars represent the standard deviation of four independent experiments. The result is not significant ($p < 0.9$), tested with student t test.

The following results on *HOXA6*, *HOXA7*, *HOXA9* and *HOXA10* were obtained after SEM cells were treated twice with the *MLL/AF4* fusion transcript specific siRNA siMA6 and control siRNA siAGF1. All experiments were performed at least three times each in triplicates (Figure 2-5). Upon depletion of *MLL/AF4* mRNA levels of *HOXA6* were significantly ($p < 0.005$) reduced to about 70%. Western Blot analysis for the *HOXA6* protein confirmed the mRNA results (Figure 2-3). *HOXA7* and *HOXA9* have been analysed in previous experiments and results have been published (Thomas et al., 2005). To confirm the *HOXA7* knockdown, qRT-PCR analysis were repeated three times and western blot experiments were performed to confirm *HOXA7* protein depletion. *MLL/AF4* knockdown via siRNA caused a significant ($p < 0.02$) depletion of *HOXA7* mRNA of about 80% and a reduction of *HOXA7* protein expression (Figure 2-3). *HOXA9* mRNA reduction after *MLL/AF4* knockdown was around 65% and protein analysis by immuno blotting reflects this substantial change ($p < 0.003$) (Figure 2-3). The depletion of *MLL/AF4* with the siRNA treatment led to a significant reduction ($p < 0.03$) of *HOXA10* mRNA around 80%. However, western Blot on *HOXA10* was not successful.

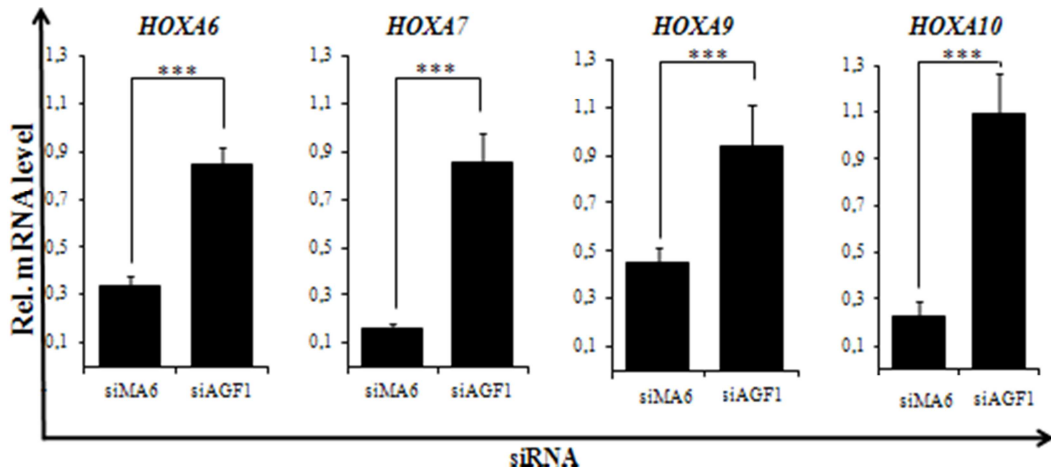


Figure 2-5 HOX gene expression after MLL/AF4 knockdown - part 2

The qRT-PCR analysis of relative HOXA mRNA levels (y-axis) after 96 hours of sustained MLL/AF4 suppression by siRNA (x-axis) in SEM cells. The error bars represent the standard error of the mean (SEM) of three independent experiments. All experiments were performed in triplicate. All results are significant (*HOXA6*: $p < 0.005$; *HOXA7* $p < 0.02$; *HOXA9* $p < 0.003$; *HOXA10* $p < 0.03$). Test of significance was performed by an unpaired student t test.



Figure 2-6 Protein analysis of several HOX genes after MLL/AF4 depletion

Protein analysis shows reduction of HOXA6 (~37kDa), HOXA7 (~25kDa) and HOXA9 (~36kDa) after MLL/AF4 depletion. Protein was yielded after 96 hours of sustained siRNA treatment. GAPDH (~37kDa) served as a loading control.

2.1.5 Summary and discussion

The first achievement during this thesis was the analysis of the HOXA cluster upon depletion of the MLL/AF4 fusion protein using a siRNA approach. The examination of the basal expression of the HOXA genes in the cell line SEM showed a low expression of the anterior HOX genes *HOXA2* – *HOXA5*. Anterior HOXA genes are expressed in very early stages of the haematopoiesis and since may have already passed the stage where those genes are active. Expression of the posterior HOXA genes *HOXA6*, *A7*, *A9* and *A10* was stronger, especially *HOXA7* and *HOXA9*. While *HOXA9* has been described in the literature to be highly upregulated in haematopoietic malignancies involving the MLL gene, there is sparse literature available on *HOXA7*, *HOXA6* and *HOXA10*. *HOXA1*, *HOXA11* and *HOXA13* were not detectable in the experiments. It is difficult to explain this finding. Since no positive control was used when testing the primers it is possible that the PCR did not work properly. It is also possible that expression of these HOXA genes may be silenced or deleted in SEM cells for unknown reasons.

The knockdown experiments show a comparable result. The majority of the anterior HOXA genes did not show a change in gene expression upon MLL/AF4 knockdown. An exception is *HOXA4* which did show a decrease in expression after siRNA treatment. Although this was a substantial knockdown of about 70% it was decided not to pursue those findings further, as the expression level of *HOXA4* is very low with a CT cycle value of about 32. As a rule of thumb values above 30 should not be considered as significant result but more as approximate values.

A new possible target of *MLL/AF4* was found in *HOXA6*. A significant reduction at the mRNA level of about 70% was found and confirmed in western blot experiments which clearly demonstrated *HOXA6* being affected by *MLL/AF4* regulation.

Knockdown of MLL/AF4 did also deplete mRNA levels of *HOXA7* and *HOXA9* significantly. Furthermore to those results western blot experiments for *HOXA7* and *HOXA9* support the findings. Those results confirm already published results (Thomas et al., 2005).

HOXA10 was also examined in three independent experiments. A significant knockdown of *HOXA10* upon MLL/AF4 depletion could be detected at the mRNA

level however all attempts to detect HOXA10 on protein level were unsuccessful. Further establishment of the HOXA10 western blot is necessary to show the effect of MLL/AF4 knockdown on this member of the HOXA cluster.

2.2 MLL/AF4 knockdown reduces telomerase activity

2.2.1 Introduction

A major hallmark of cancer cell lines is a high activity of the telomerase, a ribonuclear protein complex which is responsible for maintaining the telomeres. The telomerase is a complex consisting of two major sub units, the reverse transcriptase of the telomerase (TERT) and the RNA component (TERC).

After knockdown of MLL/AF4 proliferation and self renewal of the affected cells were disrupted. The hypothesis was that depletion of MLL/AF4 also may have an effect on telomerase activity. To check telomerase activity after MLL/AF4 depletion, siRNA treated SEM cells were analysed with a qPCR based telomeric repeat amplification protocol (TRAP) assay (Wege et al., 2003). The advantage of this method in comparison to the classic TRAP assay is, it is easy and quick in handling. Briefly SEM cells were treated twice with the relevant siRNAs (siMA6 and siAGF1 control). The SEM cells were lysed in a non-denaturing buffer to maintain protein activity after cell lysis. A protease inhibitor mix was added additionally. According to the literature, primers were designed, that mimic typical telomeric ends. Active telomerase would detect those “fake” telomeres and start to synthesise new telomeric repeats. With a second set of primers it is possible to perform real time PCR to detect those newly made telomeres. To compare telomerase activity of treated and untreated SEM cells a standard curve was created using a serial dilution of SEM cells. Using this standard curve, telomerase activity was determined after siRNA treatment.

2.2.2 Depletion of MLL/AF4 results in diminished telomerase activity

For the standard curve SEM cells were harvested and lysed in non-denaturing, proteinase inhibitor supplied buffer. Telomerase activity was determined from 50 000, 25 000, 10 000, 5 000 and 1 000 cells. Lysis buffer only was taken as zero control. From that data the correlation coefficient was calculated. It was 0.97 which is acceptable for the standard curve. For determination of telomerase activity in MLL/AF4 depleted cells, SEM cells were treated twice with siRNA siMA6 and siRNA control siAGF6, respectively. Cells were harvested and telomerase activity was determined from 25 000 cells. Based on the standard curve, telomerase activity in active siMA6 siRNA treated SEM cells was lowered to 30% due to MLL/AF4 depletion compared to the control siRNA experiment. The experiment was performed once in triplicate (Figure 2-7) and confirms results previously done by Maria Thomas.

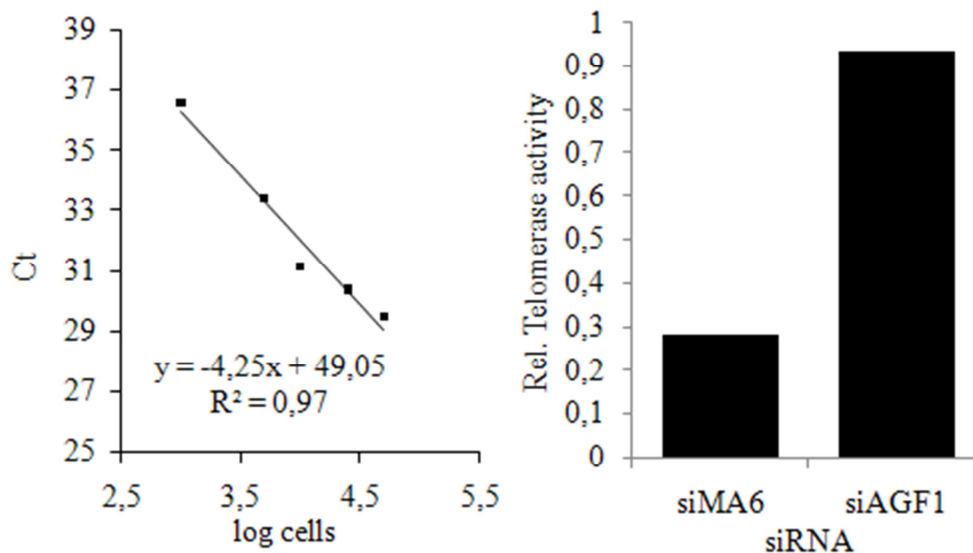


Figure 2-7 Telomerase activity of MLL/AF4 depleted SEM cells

The graph on the left side shows a standard curve created from a serial dilution of untreated SEM cells. On the right side a qRT-PCR analysis was performed on treated SEM cells. The amplicon cycle values (Ct) were compared with the standard curve Ct-values.

2.2.3 Effect of MLL/AF4 depletion on TERT and TERC

Initial experiments showed a decreased telomerase activity upon MLL/AF4 depletion in SEM cells. To investigate the effect of MLL/AF4 knockdown on the telomerase in detail, experiments were designed to examine the two major components of the telomerase TERT and TERC. To investigate their involvement in the telomerase activity reduction upon MLL/AF4 depletion SEM cells were treated twice with active siRNA siMA6, mismatch control siRNA (siAGF1) and mock. Two days after the second electroporation the cells were harvested, RNA and protein were isolated and analysed for TERT and TERC expression by qRT-PCR and Western Blotting.

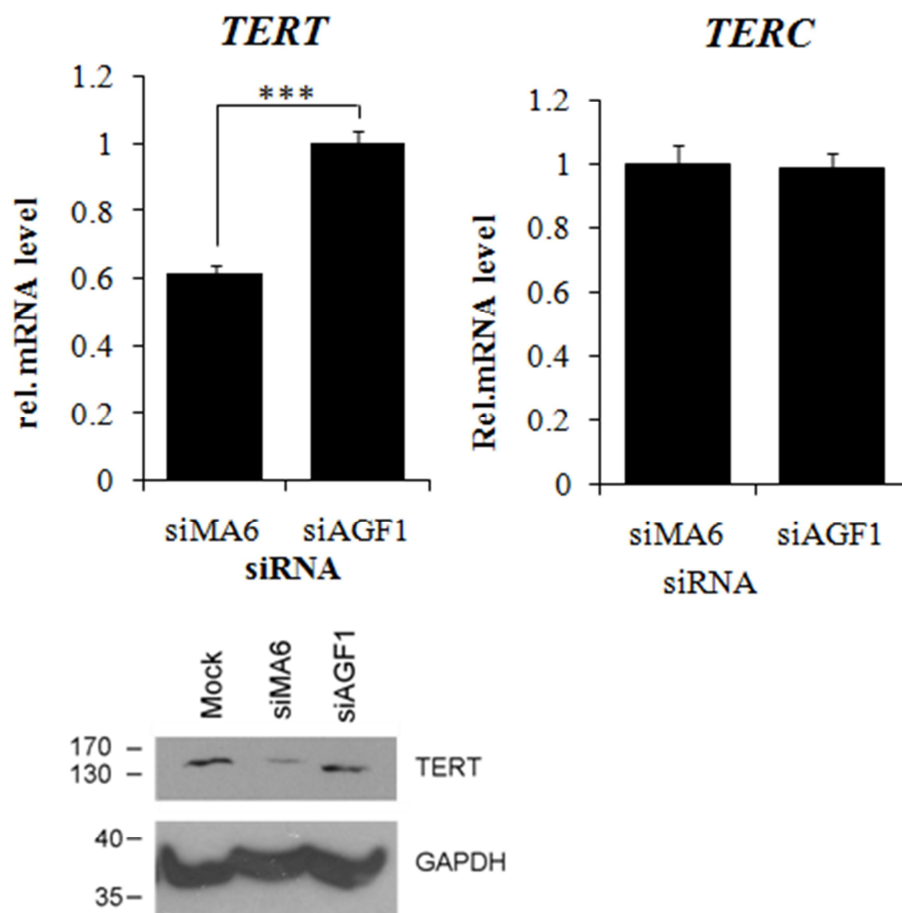


Figure 2-8 MLL/AF4 depletion affects TERT but not TERC expression

The upper row shows on the left side the result of three independent qRT-PCR experiments, performed in triplicate. A significant reduction ($p < 0.002$) of *TERT* mRNA upon siMA6 treatment of SEM cells was observed. Control siRNA siAGF1 and mock control show no effect. On the right side, *TERC* mRNA levels were investigated. No significant differences were detected ($p < 0.8$). The error bars represent the standard deviation of two independent experiments, performed in triplicate. The lower picture represents a western blot analysis of TERT. TERT protein is clearly reduced upon MLL/AF4 depletion. GAPDH serves as loading control. Test of significance was done by using an unpaired student t test.

A moderate but statistically significant ($p < 0.002$) mRNA reduction of 40% was detected for *TERT* after *MLL/AF4* knockdown by active siRNA siMA6 compared to siAGF1 and mock control. The experiment was performed three times in triplicate. This result was further analysed and confirmed in western blot experiments where a reduction of TERT protein was clearly detected upon depletion of *MLL/AF4* (Figure 2-8). *TERC* mRNA levels were investigated in two independent experiments also in triplicates. No significant differences were detected ($p < 0.8$).

2.2.4 Discussion

The results in this section are an important finding for the whole thesis. Here it is shown, that the *MLL/AF4* knockdown has also a negative effect on a mechanism which is of utmost importance for the survival of cells which are highly proliferative such as malignant haematopoietic cells. The importance of the telomerase has been discussed for nearly two decades. Since its description in the late 80's numerous publications have shown, that maintenance of the telomeres by telomerase is one of the most important mechanisms for cell survival. Especially for fast proliferating cells like haematopoietic cells and stem cells, active telomerase and telomere maintaining is vital. This seems also to be the case for most malignant cells, where a high telomerase activity is found. Exact mechanisms of telomerase regulation in cancerous cells are still under investigation.

The results of this chapter show a clear reduction of telomerase activity of roughly 70% after knockdown of *MLL/AF4* caused by siRNA treatment. This has been shown in the t(4;11) positive cell line SEM. To further investigate the effect of *MLL/AF4* on telomerase, the subunits TERT and TERC were analysed after *MLL/AF4* depletion. While TERC, the RNA component of the telomerase did not show any alterations in expression after *MLL/AF4* knockdown, the reverse transcriptase of the telomerase, TERT, showed a significant knockdown of 40% at the RNA level which was in addition confirmed by protein analysis in western blot experiments. As described by others (Thomas et al., 2005) knockdown of *MLL/AF4* has a fatal effect on leukaemic cells.

It has also been shown (unpublished data, PhD thesis Maria Thomas), that siRNA mediated knockdown of *HOXA7* in *MLL/AF4* positive cells also leads to reduction

of telomerase activity and downregulation of TERT. In the following experiments the aim was to investigate the effect of HOXA7 on TERT in detail.

2.3 TERT is a downstream target of HOXA7

2.3.1 Results

To further investigate whether there is a direct connection between HOXA7 and TERT, siRNAs against the *HOXA7* transcript (*siHOXA7*) were designed. SEM cells were electroporated twice with *siHOXA7*, *siMA6* and *siAGF1*. siRNA mediated knockdown of *MLL/AF4*, *HOXA7* and *TERT* were investigated by qRT-PCR. To confirm previous data by others (PhD thesis Maria Thomas) the experiment was performed once in triplicate (Figure 2-9). siRNA mediated knockdown of *MLL/AF4* showed diminished levels of transcript for *HOXA7* and *TERT*. Knockdown of *HOXA7* mRNA using *siHOXA7* had no effect on *MLL/AF4* mRNA but reduced the *TERT* transcript to 60 %, comparable to results using siRNA against *MLL/AF4*. This finding confirmed previous results in the group done by Maria Thomas.

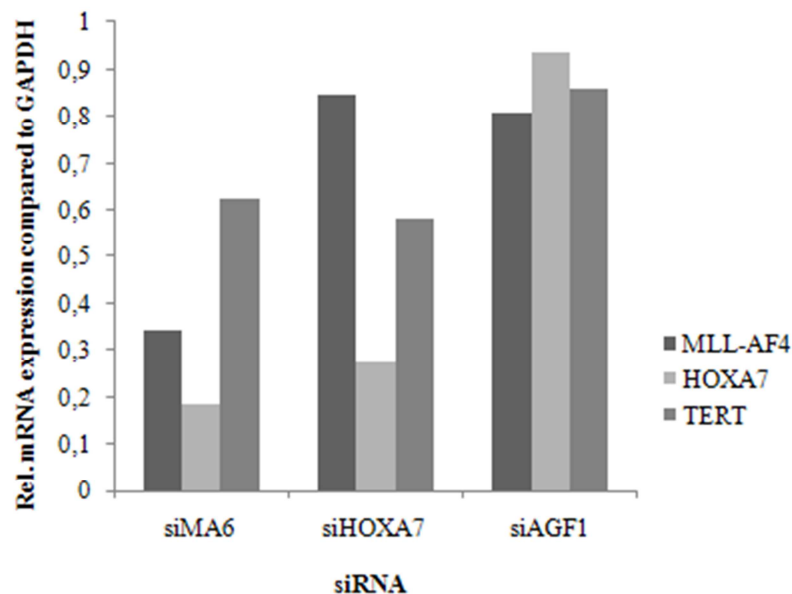


Figure 2-9 HOXA7 knockdown reduces TERT but not MLL/AF4 transcript levels

MLL/AF4 siRNA *siMA6* reduces *MLL/AF4* (dark gray bar), *HOXA7* (light grey bar) and *TERT* (medium grey bar) transcript levels. *siHOXA7* reduced *TERT* mRNA by 40 % but had no effect on *MLL/AF4*. Active non targeting siRNA *siAGF1* did not have any effect. GAPDH was used as a housekeeping gene.

2.3.2 Discussion

The effect of siRNA mediated MLL/AF4 knockdown on genes like HOXA7 and TERT give a valuable insight into the signalling mechanism of MLL/AF4. Both genes show a significantly decreased expression level at both the mRNA and protein level upon *MLL/AF4* knockdown. Knockdown of *HOXA7* does not influence *MLL/AF4* expression but *TERT* mRNA expression was reduced by 40%, a comparable value to MLL/AF4 knockdown. Confirmation at the protein level was not done due to time constraints, but would be important to confirm those results.

Those results suggest that MLL/AF4 has a direct effect on HOXA7 which in turn seems to have a regulatory effect on TERT. The loss of TERT is very likely the reason for the diminished telomerase activity. Publications show that telomerase activity is mostly dependent on the reverse transcriptase rather than on the RNA component (Wong et al., 2000) though this mechanism might be also active here. This could be further investigated by using siRNA against the *TERT* transcript. The aim here would be to investigate whether the loss of TERT does have a similar effect on MLL/AF4 positive cells as loss of MLL/AF4 or HOXA7.

2.4 **HOXA7 binds to the promoter of TERT**

2.4.1 Introduction

The results obtained so far strongly suggest that HOXA7 regulates TERT activity. Whether this regulation is direct or indirect was the research question for the next chapter. To investigate this matter, chromatin immuno precipitation (ChIP) was used. With this method it is possible to investigate direct binding of a certain transcription factor to the chromatin, for example transcription factor binding to a region within the promoter region of a gene of interest.

The critical point of this method is the choice of the right antibody. To receive good results the antibody must be very specific. As antibodies for HOXA7 are relatively rare and probably not specific enough for the ChIP, we used a plasmid that encoded for a murine *hoxa7*-flag protein. We then used antibodies which are specific for the flag sequence to perform the ChIP. Another more uncommon approach during the experiment was the use of inactivated bacteria particles for the pull down. Normally

beads covered with protein A are used for the pull down. Bacteria also have lots of protein A on their surface and gives a better pull down material with a larger surface thus produces a tighter pellet compared to the beads.

Our hypothesis was that HOXA7 binds somewhere within the *TERT* promoter. By ChIP the aim was to identify this region.

Briefly, SEM cells were transformed with a *hoxa7*-flag containing plasmid and incubated overnight. Cells were fixed with formaldehyde, lysed and chromatin was sheared by ultra-sonication. Flag-specific antibodies precipitated the *hoxa7*-flag construct together with bound chromatin. After removing the proteins by reverse crosslinking remaining DNA fragments were investigated by standard PCR.

Before performing the ChIP experiments, primers had to be designed to cover most of the *TERT* promoter. The described core promoter of *TERT* was selected for the ChIP experiment (Takakura et al., 1999). To further get a first idea on possible binding sites for HOXA7 in the *TERT* promoter region, a protein database and a browser based program called MatInspector® was used. This program investigates a customised sequence for possible transcription factor binding sites. All transcription factors have specific binding sequences and MatInspector uses this information for estimating the possibility of transcription factor binding. Thus it is possible to predict possible binding sites for HOXA7 within the *TERT* promoter.

2.4.2 Searching for HOXA7 binding sites in the TERT promoter region

The observed *TERT* depletion caused by siRNA mediated *HOXA7* knockdown led to the assumption that *TERT* is a direct target of HOXA. To investigate the possible connection between HOXA7 and *TERT* an *in-silico* experiment was performed. The browser based program MatInspector from Genomatix was used to investigate the promoter region of *TERT* for putative binding sites for HOXA7. After the sequence of the *TERT* promoter was analysed MatInspector identified several possible binding sites for HOXA7 (Figure 2-10).

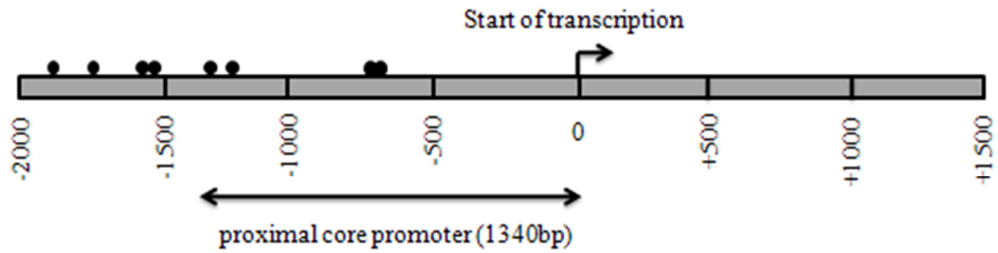


Figure 2-10 Putative binding sites for HOXA7 in the promoter of *TERT*

The schematic of *TERT* shows the promoter region from -2000bp upstream to +1500bp downstream of the start of transcription. The core promoter region has been defined by Takakura et al. (Takakura et al., 1999) and is illustrated by a black arrow. The black dots represent putative binding sites suggested by the MatInspector program from Genomatix.

It is important to realise that this is only a prediction of binding sites as the program only identifies fragments and calculates a possibility for transcription factor binding. As the putative binding sites were spread over a region from roughly -750 to -2000 base pairs (bp) primers were designed to cover the region from the start of transcription to -2000 bp. The program also revealed putative binding sites of other HOX proteins, mostly HOXA9 and HOX co-factors like MEIS and PBX proteins downstream of the transcription start site. Thus it was decided to include a region up to +1500 bp downstream of the transcription start into the analysis.

2.4.3 Chromatin immune precipitation reveals HOXA7 binding in the TERT promoter

Based on the suggestion of the MatInspector results for possible binding sites of HOXA7 in the *TERT* promoter (Takakura et al., 1999), several PCR primers were designed to cover a region from -2000bp to +1500bp for the start of transcription of *TERT* (Figure 2-11). For the experiment the plasmid *pMSCVpuro-fhoxa7* (gift from Dr. R. Slany, University of Erlangen), expressing FLAG-tagged murine *hoxa7* protein, was transiently introduced into SEM cells. As a specificity control, *pMSCVpuro-fhl2* (gift from Dr. S. Raimundo, University of Tuebingen), expressing FLAG-tagged *fhl2*, was transfected into control SEM cells.

16 hours after transcription the cells were harvested and the Chromatin-IP was performed (Figure 2-12). It showed that the FLAG-tagged *hoxa7* transcription factor binds to a region between 1.0kb and 1.5kb upstream of the transcription start of

TERT, but not FLAG-tagged *fhl2* which served as a specificity control. 6 % of total chromatin was used as input control.

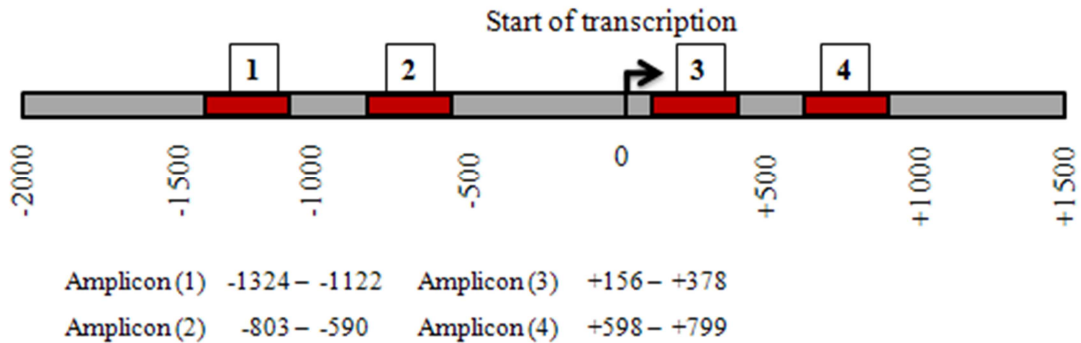


Figure 2-11 Scheme of the TERT promoter

The gray bar represents a scheme of the TERT promoter and coding region. The red bars represent the approximate position of the PCR amplicons, while the table shows their exact position. Numbers represent the bp from the start of transcription.

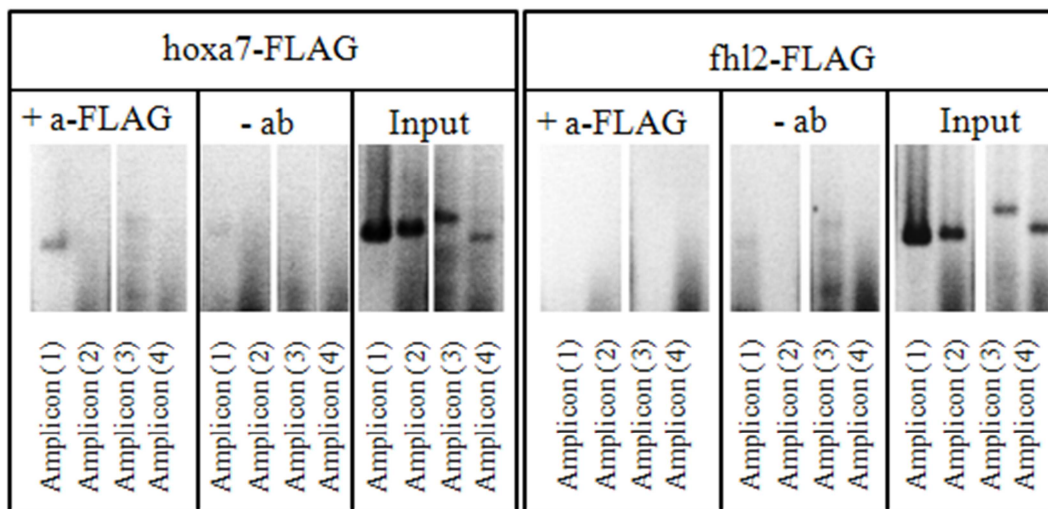


Figure 2-12 Chromatin IP reveals HOXA7 binding in the TERT promoter

SEM cells were transiently transfected with either a plasmid coding a FLAG-tagged *Hoxa7* or a plasmid coding a FLAG-tagged FHL2 as a specificity control. The left box shows the results of the amplicons from the SEM cells transfected with the plasmid coding the FLAG-tagged HOXA7 and the right box shows the results achieved with the specificity control. It can be clearly seen that the expressed fHoxa7 binds to the TERT promoter but not fFhl2. In both cases the negative controls without antibodies (-ab) did not show any binding and the input control of material in the Chromatin-IP was positive. 6 % of total chromatin was used as the input control.

2.4.4 Discussion

In this section the aim was to investigate a possible direct regulation of the reverse transcriptase of the telomerase (*TERT*) by the *HOXA7* transcription factor. Previous results have shown that knockdown of *HOXA7* affects *TERT* but not *MLL/AF4* expression. This implies *TERT* being a downstream target of *HOXA7*. The investigation was performed in two steps. Firstly the core promoter sequence of *TERT* was analysed with a browser-based program called MatInspector. This program calculates putative transcription factor binding sites within a specified sequence by aligning the given sequence with a transcription factor database. MatInspector predicted multiple binding sites for *HOXA7*, *HOXA9* and their cofactors *MEIS1* and *PBX* within a region spanning over 3500bp starting from -2000 bp up to 1500 bp from the transcription start of *TERT*. Possible *HOXA7* binding sites were only identified in the promoter region of *TERT* (Figure 2-10). Based on this prediction, primers were designed for the following chromatin immunoprecipitation (ChIP) (Figure 2-11). The ChIP was performed by immunoprecipitation of a murine *hoxa7*-FLAG construct which was transiently transfected into SEM cells. The ChIP revealed direct binding of *HOXA7* in the promoter region of *TERT*. This result was also confirmed by other group members investigating the same promoter region.

To improve the performed experiments, it is necessary to establish a ChIP protocol which uses a human anti-*HOXA7* antibody. Of course such a project relies either on the quality of commercially available antibodies or an antibody must be created by oneself. Further experiments should investigate also other *HOX* genes and their cofactors. *HOXA9* would be a strong candidate, as it is also downregulated after *MLL/AF4* knockdown. For the same reasons *MEIS1* represents another important factor that needs to be investigated.

2.5 MLL/AF4 knockdown and mRNA expression of transcriptional target during caspase inhibition

2.5.1 Introduction

Results in the previous sections demonstrated that MLL/AF4 knockdown induced apoptosis. It was hypothesised that the induction of apoptosis by MLL/AF4 knockdown might obscure molecular processes, thus knockdown was investigated whilst inhibiting apoptosis using zVAD, a cell permeable pan caspase inhibitor. The abbreviation zVAD stands for the chemical compound Z-Val-Ala-Asp(OCH₃)-Fluoromethylketone which acts as an irreversible, cell-permeable, broad-spectrum caspase inhibitor. This inhibitor binds irreversibly to the catalytic site of caspase proteases and inhibits activation of apoptosis.

To investigate the effect of apoptotic inhibition in the context of siRNA mediated knockdown of MLL/AF4 a combined experiment was performed where SEM cells were treated with the active siRNA siMA6, control siRNA siAGF1 and mock together with the pan caspase inhibitor zVAD and compared untreated controls. The cells were harvested two days after two or three successive siRNA treatments, combined with or without zVAD treatment or untreated. As zVAD was prepared in DMSO which can be cytotoxic, a DMSO control was employed which followed the same protocol as the zVAD experiment but without the inhibitor itself.

2.5.2 Results

Using qRT-PCR, the mRNA levels of *MLL/AF4*, *HOXA7* and *TERT* were investigated (Figure 2-13). While the addition of zVAD had no impact on the mRNA level changes of *MLL/AF4* and *HOXA7* after two and three electroporations of combined siRNA/zVAD treatment, *TERT* showed a prominent difference in the levels of reduction. Compared with the DMSO control, MLL/AF4 depletion and addition of zVAD reduced the mRNA level of TERT by 80%-90%. The decrease of TERT expression in the DMSO control was about 40%-50%. This effect was monitored in cells harvested after two and three electroporations.

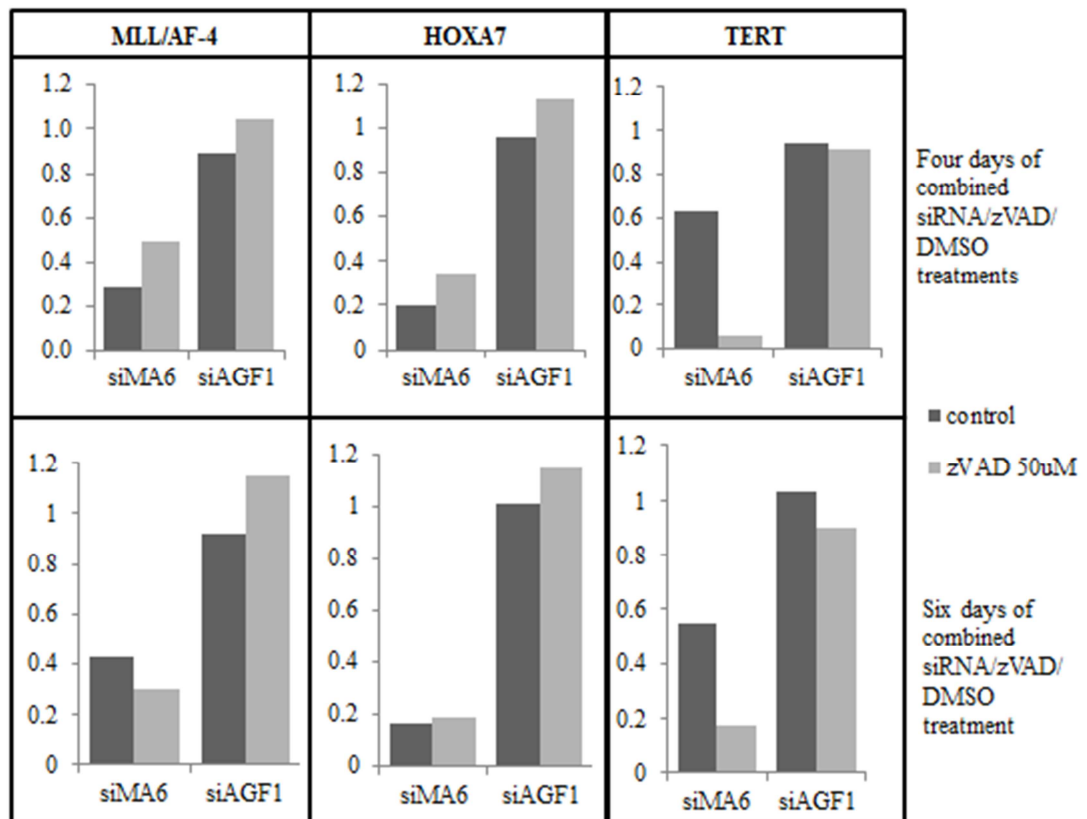


Figure 2-13 Inhibition of apoptosis by the pan caspase inhibitor zVAD

Analysis of the mRNA level performed after 4 and 6 days of combined siRNA/zVAD treatment. Y-axis shows relative fold change compared to mock control. GAPDH was used as housekeeping gene. Analysis with qRT-PCR revealed stronger knockdown of *TERT* in zVAD treated cells upon treatment with siMA6 compared to control cells treated with siAGF1. *MLL/AF4* and *HOXA7* levels remained unaffected.

To accompany these analyses the distribution of cells within the cell cycle was then determined using fluorescent activating cell sorting (FACS). During the cell cycle cells are distributed into four different stages which can be monitored by FACS. All living cells can be found in the G1/G0 phase, which is directly after cell division, the S phase and the subsequent G2/M phase. Dead cells appear in a population which is called the sub G1/G0 phase (Figure 4-8). FACS analysis showed that after addition of zVAD no alteration of the cell cycle distribution after two, four or six days with combined siRNA/zVAD treatment was observed. To monitor the effect of zVAD the sub G1/G0 population, which represents the amount of dead cells in the cell cycle, was analysed. After two days control cells showed about 3.5 % of the cells in the sub G1/G0 phase. zVAD treated cells contained a population of 1 – 1.5 % in the sub G1/G0 phase. After 4 days, DMSO treated cells showed a typical sub G1/G0 population for dead cells which is about 3%. Due to the siRNA treatment, the mock

and control siRNA treated cells also showed normal amounts of cells in the sub G1/G0 phase (~ 3-4%) while siMA6 treated cells showed 12% of dead cells. FACS analysis of the combined siRNA/zVAD treated cells showed a very low percentage of cells in the sub G1/G0 phase. Control cells had about 1% dead while the cells treated with active siRNA siMA6 showed only 2% dead cells. Interestingly, after 6 days of zVAD treatment a high amount of dead cells was detected in the sub G1/G0 population comparable with the DMSO control. In both samples MLL/AF4 depletion showed a subG1/G0 population of about 35% as. It was expected that zVAD would prevent the cells from dying by inhibiting the caspases (Figure 2-14). Experiments done in the group by Patricia Garrido Castro investigating this sub G1/G0 peak revealed that this population was not apoptotic as judged by Annexin-V staining or western blots against CASP3, CASP7 or PARP.

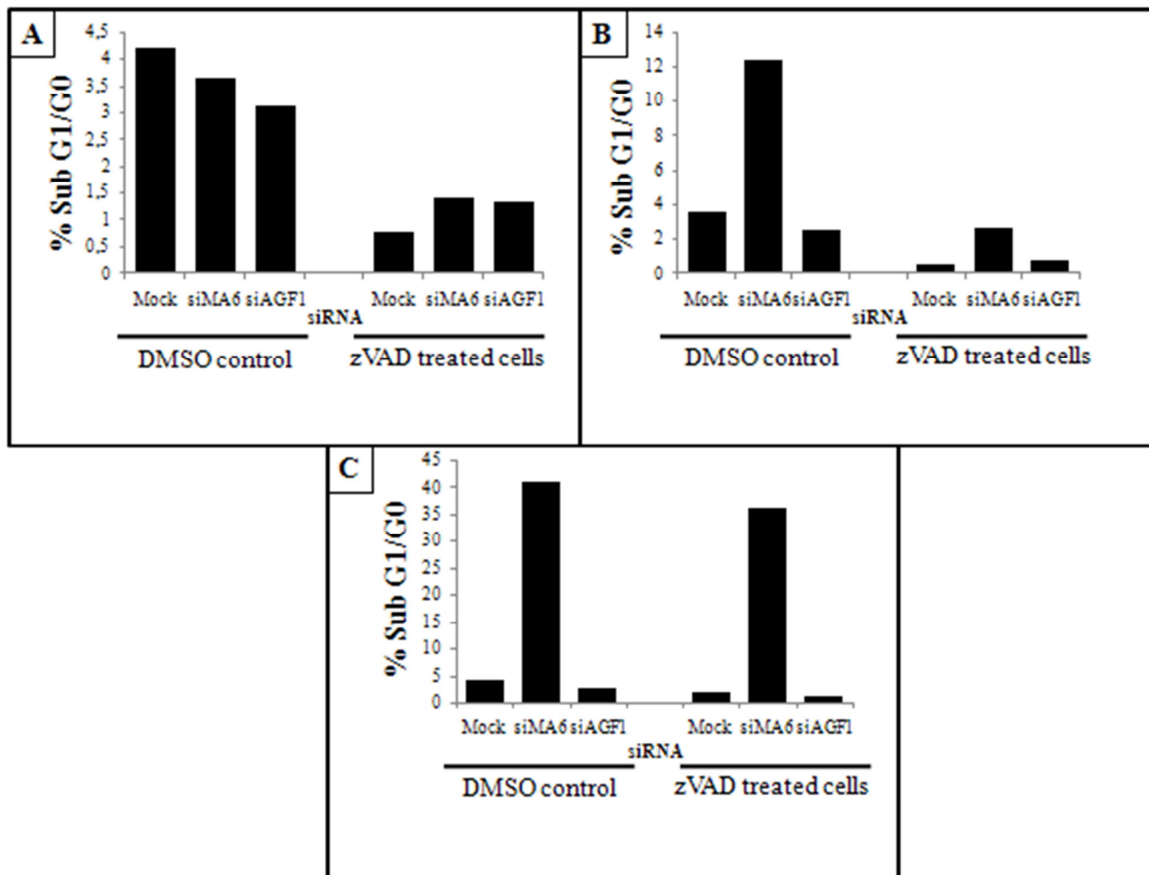


Figure 2-14 FACS analysis of subG1/G0 cell population after combined siRNA/zVAD treatment

SEM cells were treated with siRNA or mock together with either zVAD or DMSO. Cells were harvested after two (A), four (B) or six days (C) and analysed with FACS. The percentage of the cell population in subG1/G0 phase is represented on the y-axis, siRNA treatment on the x-axis.

2.5.3 Discussion

MLL/AF4 depletion leads to an increase in apoptosis and to down regulation of certain genes like HOXA7 and TERT. In this section of the thesis the effect of MLL/AF4 knockdown on HOXA7 and TERT were analysed while suppressing apoptosis by applying an anti-apoptotic agent called zVAD. zVAD is a pan-caspase inhibitor that blocks caspases by irreversible binding to their catalytic centre. As caspases are the main mediators of apoptosis their blocking results in apoptosis inhibition.

The results showed an increase in TERT reduction upon siRNA mediated MLL/AF4 knockdown with simultaneous apoptosis inhibition. The knockdown effect on HOXA7 was not altered which suggests an effect specific for TERT rather than a general one. It is also unlikely that it is a siRNA dependent effect as this should also be reflected in MLL/AF4 or HOXA7 knockdowns. One explanation could be that biologic processes during apoptosis might alter TERT expression. TERT has been reported to be anti-apoptotic so maybe during apoptosis cells try to survive by upregulating TERT. As apoptosis is not activated in zVAD treated cells, this hypothetical mechanism is also not active. As zVAD is targeting caspases, they could provide a target to further analyse this finding.

2.6 Rescue experiments for HOXA7 and TERT

2.6.1 Introduction

As shown in the previous sections *MLL/AF4* knockdown has dramatic effects on cell survival such as increased apoptosis, diminished self-renewal capability and inhibition of proliferation. Possible key players are HOXA7 and TERT. To investigate this, an artificial over-expression of HOXA7 or TERT should be realised in t(4;11) positive SEM cells. The hypothesis was that HOXA7 or TERT overexpression in the leukaemic cells would lead to a rescue of the cells after siRNA mediated knockdown of MLL/AF4. A lentiviral system was used to create a modified SEM cell line which over expresses either HOXA7 or TERT. To create HOXA7 overexpressing cells, a lentiviral vector was already available. The vector expresses a murine form of *hoxa7* together with a FLAG and eGFP construct. The TERT containing lentiviral vector had to be created.

2.6.2 *Generation of a lentiviral vector containing cDNA of human TERT*

In the basic lentiviral vector, pHR-SINcPPT-SIEW (pSIEW) (Figure 2-15) the sequence of the cDNA of *TERT*, the so called TERT fragment had to be integrated. The elements which are important for the lentivirus production are inserted between the 5' long terminal repeat (LTR) and the 3' LTR. The LTRs harbour several viral promoters and enhancers. During the integration of the wild type viral genome into the host genome, rearrangements of the LTRs take place which form a new element enabling viral reproduction, however for genetic engineering mutations within the LTR's sequence prevent the formation of the self-reproducing signal (SIN = Self Inactivation). The pSIEW vector contains a SFFV promoter which should drive the expression of the integrated gene. The SFFV promoter is derived from the spleen focus-forming virus. An *internal ribosomal entry site* (IRES) follows the integrated gene. Usually, in eukaryotes the translation can only be initiated at the 5' end of the mRNA molecule. The integration of the IRES allows for initiation of translation from a second open reading frame (ORF) of the mRNA molecule. Downstream of the IRES is a sequence coding for an *enhanced green fluorescent protein* (eGFP). The eGFP is a mutated form of the original GFP protein with brighter fluorescence. The function of the eGFP is to act as a reporter gene in the lentiviral background. Other integrated elements in the pSIEW vector are a *woodchuck posttranscriptional regulatory element* (WPRE) which enhances lentivirus-mediated transgenic expression. The WPRE element can form a RNA-protein complex which protects the freshly synthesised RNA from being degraded (Zufferey et al., 1999). Further to the WPRE, a *rev responsive element* (RRE) has been integrated into the pSIEW vector. Rev and RRE are necessary for the expression of different helper proteins and in addition rev is needed for transportation of full-length vector RNA. For security reasons, RRE and rev are found on different plasmids and can only work if transfected together in a packaging cell. Another element in the pSIEW vector is the *central polypurine tract* (cPPT) which improves the transduction efficiency by facilitating nuclear import of the vector's pre-integration complex in the transduced cells. The gag element encodes for a protein which is important for the encapsidation of the viral nucleic acid. The SL4 fragment is an element of the packaging sequence.

The TERT fragment was cut out of the plasmid pKS-TERT (Figure 2-16) and was then to be integrated into the *Bam*HI site of pHR-SINcPPT-SIEW via blunt end ligation. To achieve this both the *Eco*RI/*Sal*I-flanked TERT fragment and the *Bam*HI linearised plasmid pHR-SINcPPT-SIEW were separated in an agarose gel and subsequently purified (QIAGEN, Qiaquick Gel Extraction kit). Afterwards, both sequences underwent a fill-in reaction with Klenow enzyme.

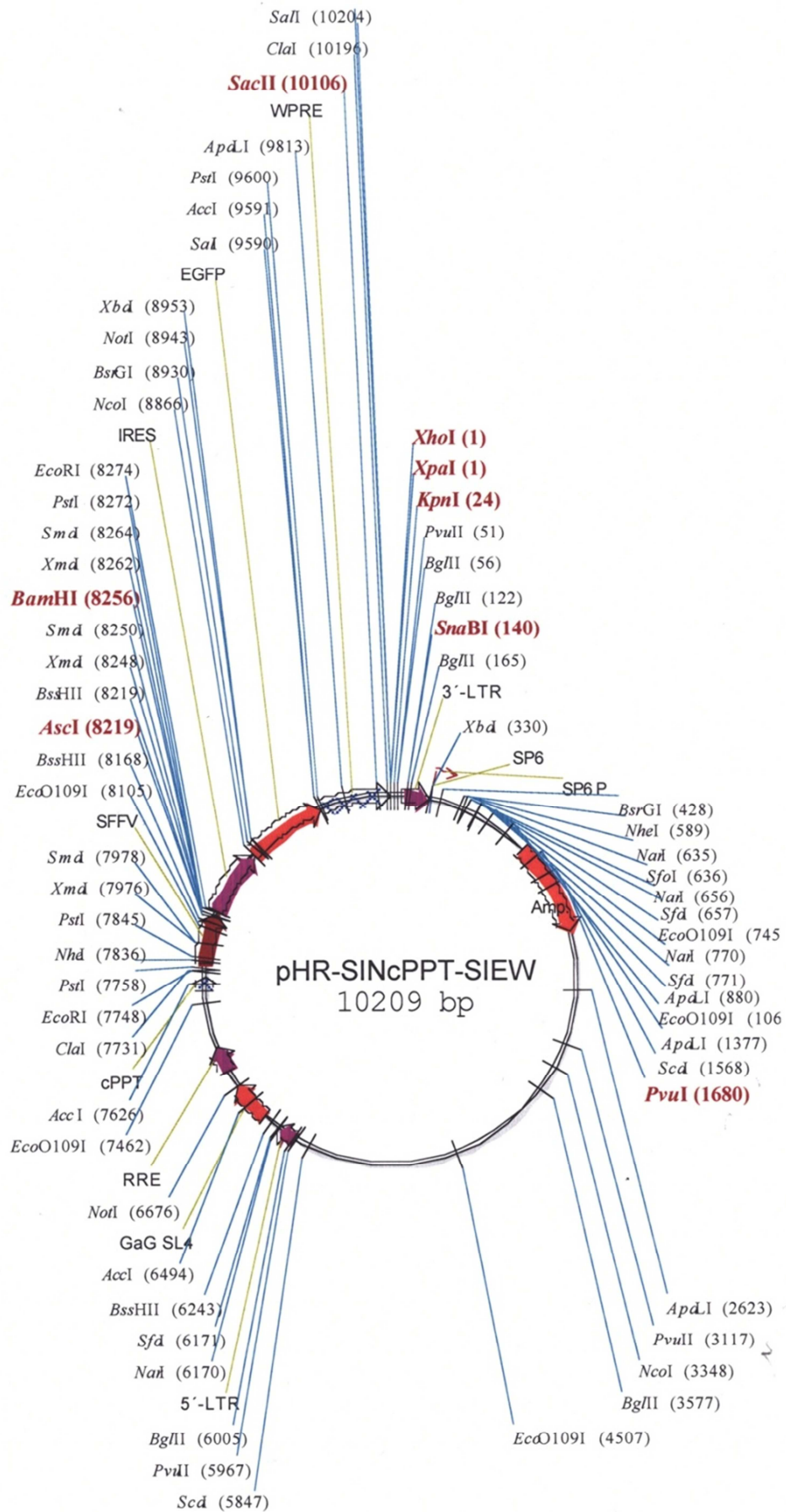


Figure 2-15 The basic lentiviral vector pHR-SINcPPT-SIEW

The lentiviral target vector has been dephosphorylated with Calf Intestinal Alkaline Phosphatase (CIAP) to prevent self-ligation. After successful ligation, the final lentiviral vector was transformed via heat shock into DH5 α *E.coli* bacteria. Unless otherwise stated, the bacteria strain DH5 α of *E.coli* was used.

The MiniPrep was cultivated in a shaking incubator at 37°C. After 24 to 48 hours the *E.coli* cells were harvested and the plasmids isolated. These plasmids were then checked by restriction analysis for successful integration of the TERT fragment. After several cloning attempts no clones were isolated and the strategy was modified. Because the restriction enzymes *Bam*HI and *Bg*III create compatible sticky ends it is possible to insert artificial *Bg*III sites into the pKS-TERT vector to flank the TERT fragment with *Bg*III. Later, the TERT fragment could then be integrated in the unique restriction site of *Bam*HI in the vector pHR-SINcPPT-SIEW. Therefore small oligonucleotides containing the *Bg*III site were designed. The first step was to design two small *Bg*III site containing oligo-linkers. They should insert the *Bg*III restriction site between the flanking restriction enzymes *Xba*I/*Eco*RI at the beginning of the TERT fragment and *Sal*I/*Acc*65I (*Kpn*I) at the end (Figure 2-16). For each side flanking the TERT fragment in the pKS vector, two single oligo strands were designed which after being hybridised form the full *Bg*III restriction site. The end of each double stranded oligo formed the sticky end of the relevant restriction site and so each double stranded oligo fragment was cloned into the pKS-TERT successively. Restriction analysis after integrating the first fragment *Xba*I-*Bg*III-*Eco*RI resulted in nine positive clones out of nine picked. Restriction analysis was done with *Bg*III and *Sal*I. After integrating the second fragment *Sal*I-*Bg*III-*Acc*65I two positive clones were identified out of nine picked. Restriction analysis was performed by using *Bg*III which should cut at each end of the TERT fragment and separate TERT. The new vector was named pKS-TERT-*Bg*III. Hence, the TERT fragment was obtained with the help of the artificially integrated *Bg*III restriction sites. So the transfer of the TERT fragment into the *Bam*HI site of the plasmid pHR-SINcPPT-SIEW could then be performed as described before although the fill-in reaction was not necessary in this case. Several attempts were made but also in the case of this modified strategy, all clones were negative. Since it was not possible to obtain the needed vector for the insertion of the TERT fragment into the SEM cells, another strategy was attempted to induce overexpression.

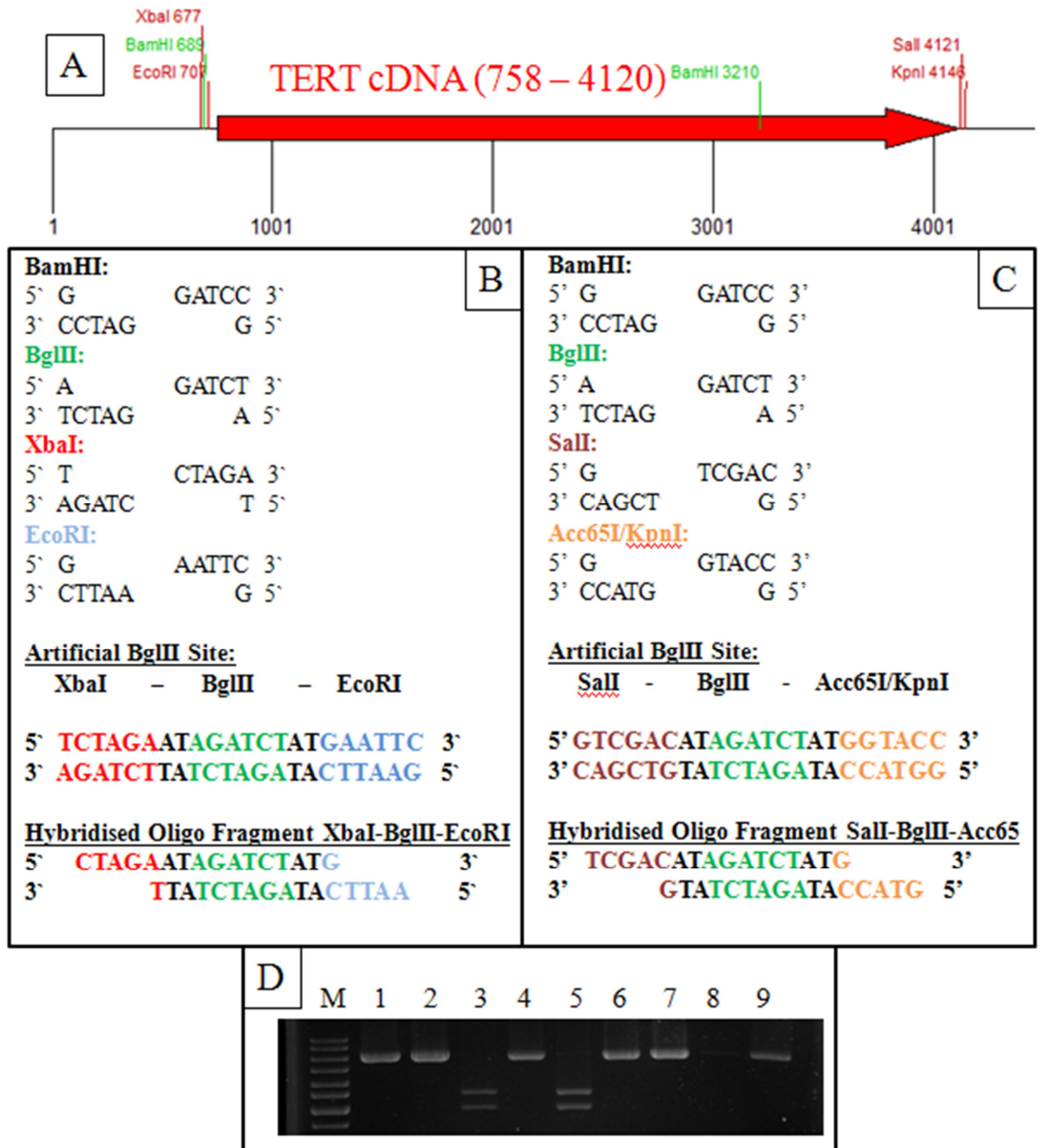


Figure 2-16: The pKS-TERT vector and insertion of two *BglIII* sites

(A) The picture shows the TERT fragment in the pKS-bluescript vector (red arrow) where the artificial restriction site of *BglIII* had to be integrated between the flanking restriction sites of *XbaI* and *EcoRI* at the beginning of the sequence of the cDNA coding for TERT and *SalI* and *Acc65I/KpnI* at the end of the sequence. The green restriction enzyme *BamHI* cuts two times in the vector, the enzymes in red cut the vector once. (B+C) Schemes to integrate the *BglIII* site into the vector pKS-TERT. The oligo fragments were designed as shown above and after hybridisation inserted into the desired site. Red represents the *XbaI* sequence, green *BglIII*, blue *EcoRI*, dark red *SalI* and yellow the *Acc65I/KpnI* site. (D) Restriction control shows insertion of both *BglIII* restriction sites; clone 3 and clone 5 were positive and used for further experiments. Restriction was done by using *BglIII*.

The first step of the new strategy involved the restriction of lentiviral vector pHR-SINcPPT-SIEW with the enzyme *NotI* at positions 8943 and 6676 to get a fragment which contains the *BamHI* restriction site. Restriction of the lentiviral vector revealed that the pHR-SINcPPT-SIEW vector did not contain an efficient *NotI* restriction site at position 8934bp despite the description in the plasmid, shown in Figure 2-15.

This fragment was then supposed to self-ligate into a small intermediate vector. This first intermediate vector should be linearised using the unique restriction site *BamHI*. Secondly the modified pKS-TERT-*BglII* vector was meant to be digested with the enzyme *BglII* flanking the TERT fragment. As *BglII* and *BamHI* produce compatible ends the TERT fragment should be integrated into the *BamHI* site of the intermediate vector to form the new transfer vector. The transfer vector could then be linearised again with *NotI* and used for insertion into the available plasmid, pHR-SINcPPT-SIEW.

At this point with pressing time constraints it was decided to concentrate on the overexpression of HOXA7.

2.6.3 Design of rescue experiments with HOXA7

The lentiviral vector which contains the sequence coding for the cDNA of *HOXA7* combined to the FLAG-tag was already available. As a reporter gene, the *hoxa7-FLAG* construct was connected to an *EGFP* construct via the *IRES* fragment. The control vector did not contain the *hoxa7-FLAG* construct. The experiment below describes the *hoxa7-FLAG* containing lentivirus. The control was treated the same way but will not be described separately.

To get a lentivirus containing the *HOXA7* over-expression cassette, the lentiviral vector with the integrated sequence encoding for the *HOXA7* cDNA was transfected together with the packaging vector *pCMVdeltaR8.91* and the enveloping vector *pMD2.G* into 293T cells. This was done by calcium precipitation. Three days after infection the 293Ts were examined with the fluorescence microscope for the expression of the enhanced green fluorescent protein (EGFP) which was used as reporter (Figure 2-17). EGFP expression was estimated by eye and showed an efficiency of almost 100% in both control and actual transfection experiments.

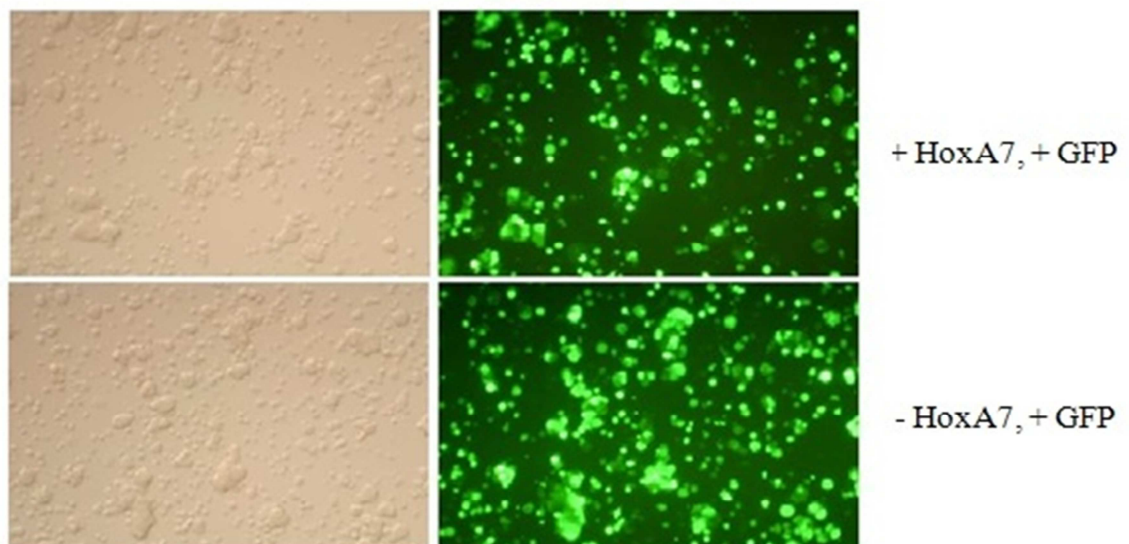


Figure 2-17 Virus production in 293T cells

293T cells three days after transfection of the lentiviral vector (+HoxA7, +GFP). The control vector (-HoxA7, +GFP) lacks the *HOXA7* insert.

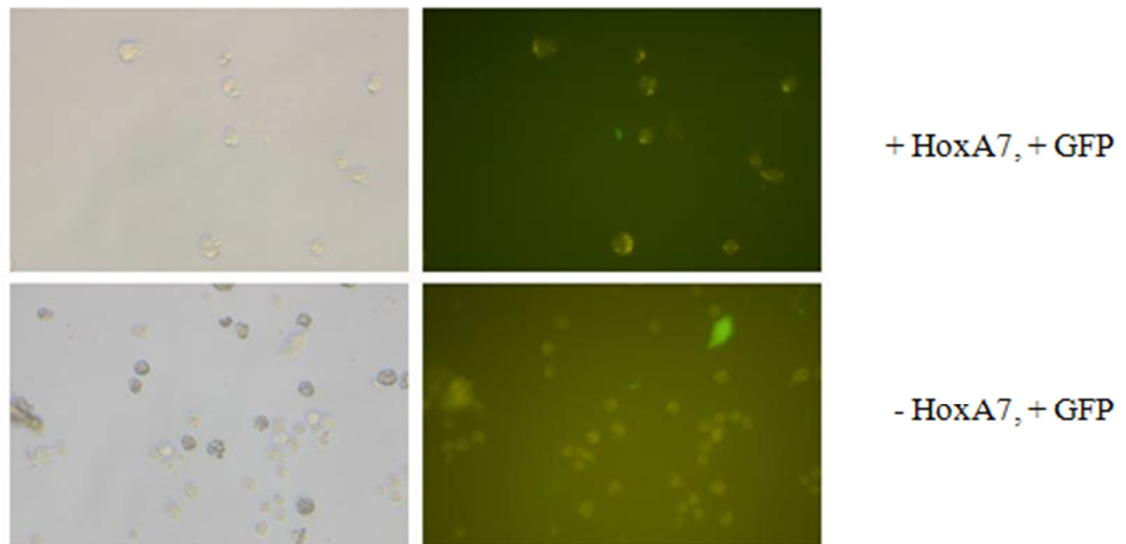


Figure 2-18 SEM cells after transduction with lentiviral particles
SEM cells three days after transduction with lentiviral particles.

On the fourth day after infection the lentiviruses were harvested and subsequently used for transduction of the SEM cells. Prior to the transduction the lentiviruses were concentrated by ultracentrifugation. Three days after the transduction the SEM cells were checked also for EGFP expression with a fluorescence microscope (Figure 2-18). To measure the transduction efficiency several FACS analysis to detect EGFP were done (Figure 2-19). Since the *hoxa7-FLAG* construct was connected to an *eGFP* construct via *IRES* overexpression it was assumed *eGFP* expression correlates directly with *hoxa7-FLAG* expression.

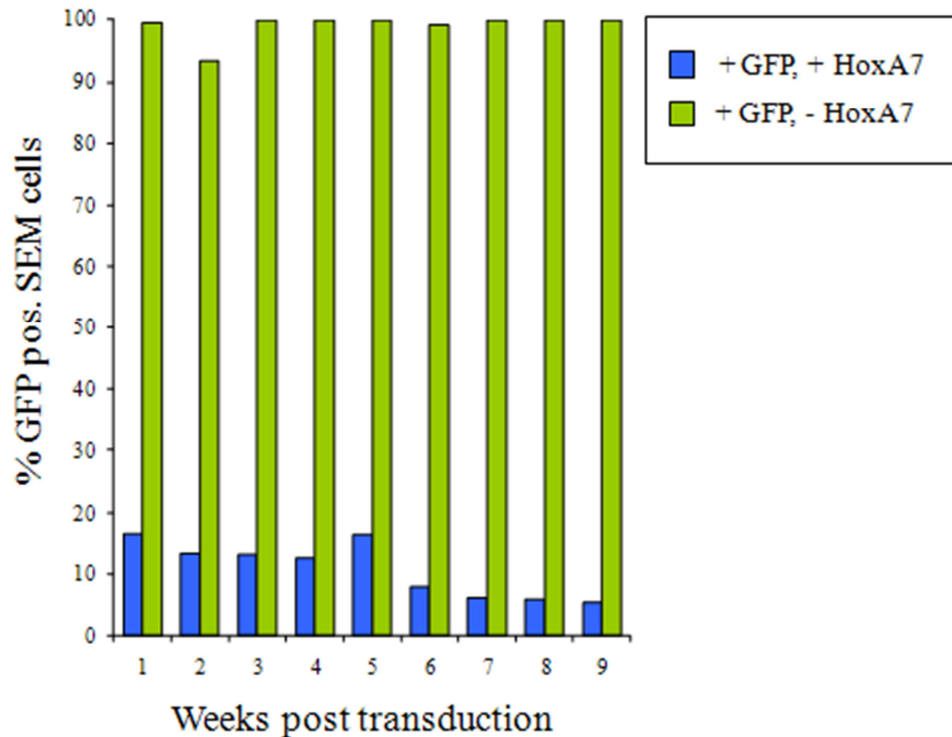


Figure 2-19 FACS analysis of GFP positive SEM cells after lentiviral transduction

Analysis of SEM cells after lentiviral transduction shows GFP positive cells in both cases. Blue bars represent transduced cells with HoxA7-FLAG containing lentiviruses, green bars represent HoxA7 lacking lentiviruses. The number of weeks after transduction is shown on the x-axis, percentage of GFP positive cells is shown on the y-axis.

One week after transduction approximately 16% of the cell population transduced with *HOXA7* positive lentiviruses showed an expression of the reporter protein EGFP measured with a FACS machine (FACSCalibur, Beckton Dickinson, UK). The control cells were almost 100% positive. This *HOXA7* positive population remained stable at 16% until week five, when a sorting attempt was made. The attempt to culture sorted GFP positive cells had to be aborted, because after sorting the separated cells died while being cultured in standard conditions. The remaining mixed cell population was kept under optimum conditions (0.5×10^6 cells/ml culture medium) and was monitored over several weeks in incubation flasks using FACS analysis (Figure 2-19). It was observed that the amount of GFP positive cells decreased slowly over the cultivation period. As shown in Figure 2-19 the GFP positive control cells did not decrease at all. At this point of study it was decided to halt the experiment and to archive the remaining cells in cryo stocks. After thawing and re-culturing the *HOXA7* positive cells FACS analysis showed no EGFP

expression. Control cells remained fine. As two of three cryopreserved stocks were negative the experiment was aborted.

In another attempt, three different cell lines SEM, RS4;11 and MV4;11 were used as target cells for a similar lentiviral production and transduction using the *pHR-SINcPPT-SIEW* lentiviral vector containing the *hoxa7-FLAG* cDNA. Shortly after the lentiviral transduction the cell numbers decreased rapidly. Interestingly also the control cells decreased rapidly but started growing after a few days. Finally the experiment had to be aborted as almost none of the pSIEW-HOXA7 transduced cells survived. The numbers of viable cells were determined by counting with the Neubauer cell counting chamber and the use of trypan blue. The number of trypan blue positive cells was not determined but clearly visible and rising during the time of the experiment. Figure 2-20 shows cell numbers at three different days.

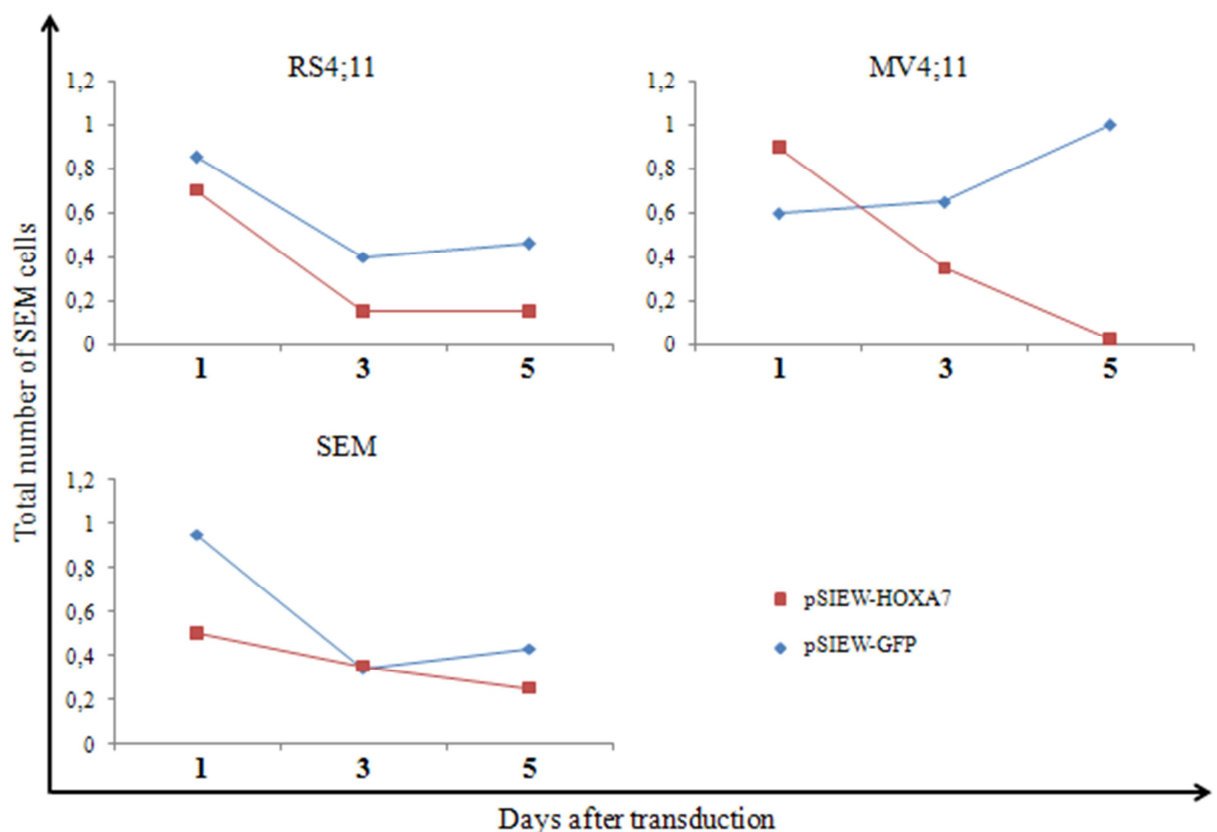


Figure 2-20 Different cell lines transduced with lentiviral particles

Cell numbers on the y-axis and days after transduction on the x-axis show that in all cell lines HOXA7 transduced cells (red) decreased rapidly, while control cells (blue) start to grow after the second measurement. Shortly after the third measuring on day 5 the experiment had to be aborted as no more HOXA7 positive cells were available.

2.6.4 Discussion

In this section the aim was to create a SEM cell line that overexpresses either TERT or HOXA7.

The creation of a lentiviral vector that overexpresses TERT failed. Multiple cloning attempts were made but the final step to integrate the TERT containing cassette into the lentiviral vector failed. The likely problem is the size of vector and insert. The vector itself is about 10kb in size, and the TERT insert has 3kb. This size is too big for most bacteria as bacterial polymerases have difficulties to replicate vectors that are more than 10kb in size. Another problem was the inaccuracy of the only available map of the base vector pSIEW. Many cloning strategies were unsuccessful as predicted restriction sites detailed on the map were not present. For successful creation of a TERT containing vector, another lentiviral basal vector is needed.

The creation of SEM cells that overexpress the murine *hoxa7* was successful as judged by GFP expression. Unfortunately those cells were unstable and died. The reasons remain unclear. *Hoxa7* overexpression seems to have a fatal effect on the cells.

The method for creating these cells could not be fully established by the end of this thesis. Virus production must be monitored more closely by virus titer determination. Furthermore exact virus doses for transduction of target cells must be evaluated. This would also give information about the efficiency of the virus production itself and might help to improve it. Titration with known virus amounts could help to improve transduction efficiency and may lead to the creation of a cell line that overexpresses the target gene of interest.

2.7 General expression of HOXA genes and TERT

2.7.1 Introduction

For this thesis, leukaemic cell lines were used to investigate changes in gene expression after siRNA mediated depletion of the fusion oncogene, *MLL/AF4*. To put this into context, the general expression level of the investigated genes within different leukaemic cell lines was investigated. The overall expression of certain genes of the *HOXA* cluster and *TERT* in different leukaemic cell lines was therefore investigated to reveal possible correlations. The following genes were chosen from the *HOXA* cluster: *HOXA6*, *HOXA7*, *HOXA9* and *HOXA10*. Different leukaemic cell

lines were used to investigate the expression levels of the above mentioned genes by qRT-PCR. 293T cells were used as reference cell line, as it is a non leukaemic cell line. Table 5 gives a short overview of the cell lines used in this experiment. The experiment was performed once in triplicates.

Table 5 Cell lines for expression study

SEM	ALL cell line carrying the t(4;11) translocation with breakpoint at e9-e4
RS4;11	ALL cell line carrying the t(4;11) translocation with breakpoint at e10-e4
MV4;11	FAB M5 categorised AML cell line carrying the t(4;11) translocation with breakpoint at e9-e5
REH	ALL cell line carrying the t(12;21) translocation for TEL-AML1 fusion gene
697	ALL cell line carrying the t(1;19) translocation for E2A-PBX fusion gene
TK6	Cell line carrying the BCR-ABL fusion gene t(9;22); no leukaemic phenotype
NALM6	ALL cell line carrying the t(5;12) translocation
K562	CML cell line carrying the t(9;22) translocation for BCR-ABL fusion gene
Kasumi-1	FAB M2 categorised AML cell line carrying the t(8;21) translocation for AML1-MTG8 fusion gene.

2.7.2 Results

As shown in Figure 2-7 *HOXA6* and *HOXA7* were only expressed in the t(4;11) positive ALL cell lines SEM and RS4;11. *HOXA9* and *HOXA10* were expressed in all investigated t(4;11) positive cell lines. Interestingly compared to the t(4;11) negative cell lines used in this experiment, HOX expression was relatively high in the t(4;11) positive cell lines but not in the other cell lines.

The expression level of *TERT* was also analysed in this experiment. *TERT* mRNA levels were high in all cell lines but MV4;11 (Figure 2-21). The t(4;11) positive cell lines SEM, RS4;11 and MV4;11 revealed an interesting correlation between *HOXA6*, *HOXA7* and *TERT* expression. In the ALL cell lines, a high expression of *HOXA6* and *HOXA7*, together with a high expression of *TERT* was detected. In the AML cell line MV4;11, *HOXA6* and *HOXA7* mRNA levels were very low, as was the mRNA level of *TERT* (Figure 2-22). Interestingly compared to the other cell line, the t(4;11) positive cell lines did not show the highest level of *TERT* expression. REH and 697 cells showed a much higher *TERT* expression. All other cell lines had comparable expression levels to the t(4;11) positive cells.

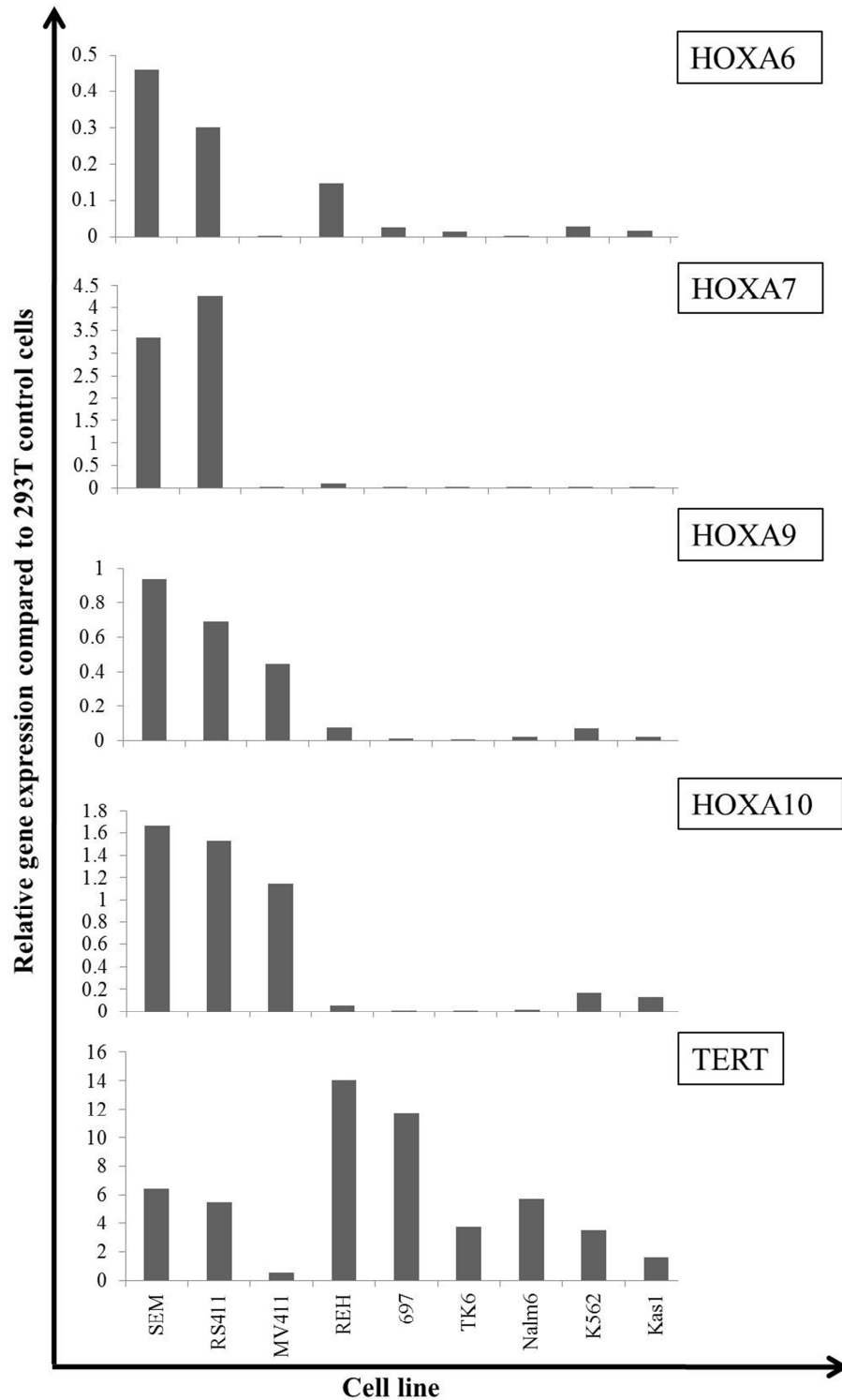


Figure 2-21 Comparison of gene expression of the HOXA cluster and TERT

Several leukaemic cell lines were used to determine the gene expression of *HOXA6*, *HOXA7*, *HOXA9*, *HOXA10* and *TERT* relative to the gene expression in 293T cells. Genes were normalised against the housekeeping gene *GAPDH*.

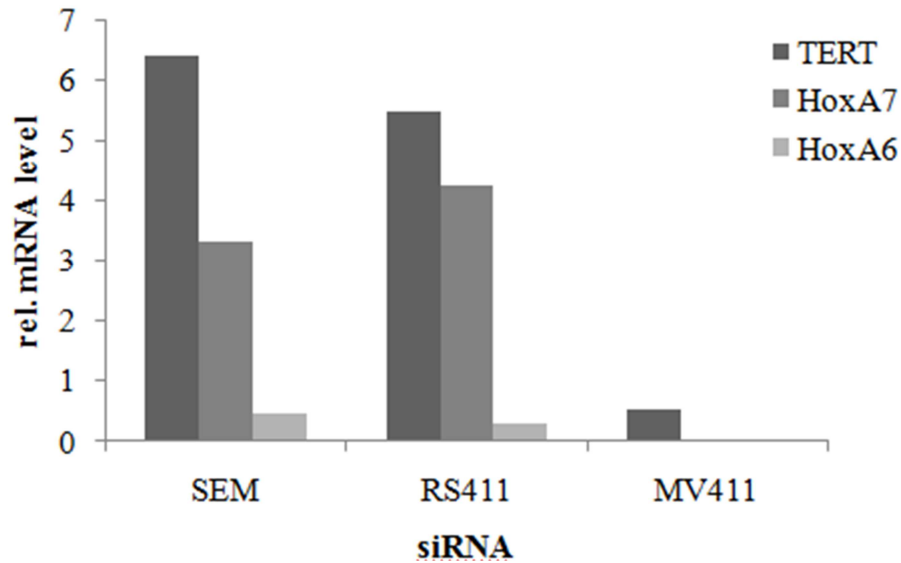


Figure 2-22 Possible correlation between *HOX* and *TERT* expression levels

A direct comparison of mRNA expression levels of *HOXA6*, *HOXA7* and *TERT* showed an interesting correlation. While *TERT* is expressed highly in SEM and RS4;11 cell lines, it is very low in MV4;11 cells. The expression pattern of *HOXA6* and *HOXA7* is comparable to *TERT*.

2.7.3 Discussion

The aim of this section was to determine the expression levels of several *HOX* genes (A6, A7, A9 and A10) and *TERT* within a range of leukaemic cell lines. It was of interest whether t(4;11) positive cell lines show a different gene expression compared to other leukaemic cell lines. 293T cells were used as a control cell line.

Interestingly all t(4;11) positive cell lines showed *HOXA9* and *HOXA10* expression. *HOXA6* and *HOXA7* were only expressed in the ALL cell lines SEM and RS4;11. All the tested t(4;11) negative cell lines showed no or much lower expression of the investigated *HOX* genes. This strengthens the findings of others that *HOX* gene overexpression is a hallmark of MLL rearranged cells.

It is interesting that MV4;11 cells do not have the *HOXA6* and *HOXA7* overexpression. One reason could be that MV4;11 is an AML cell line and *HOX* gene expression is differently regulated in myeloid compared to lymphoid cells.

TERT expression was also evaluated in all cell lines. It was present in all tested cell lines. The lowest expression was measured in MV4;11 cells, while the highest values were found in REH cells. All other cell lines had equal levels. There is an interesting correlation between *TERT* and *HOX* gene expression in the t(4;11) positive cell lines. In SEM and RS4;11 cells, *HOXA6* and *HOXA7* expression was found, together with

TERT. Interestingly in MV4;11 cells which show no HOXA6 and A7 expression, also TERT expression was lowest. This further strengthens the hypothesis that TERT may be regulated by HOXA7. Whether HOXA6 also plays a role in TERT expression needs to be further analysed. It would be of interest to see whether other MLL rearranged cells have a HOX-TERT pattern. This pattern could be evaluated in patient material and may lead to a biomarker for MLL rearranged leukaemia and better risk assessment of patients.

3 Discussion

3.1 MLL/AF4 has an effect on HOX gene expression

Acute lymphoblastic leukaemia (ALL) is the most common leukaemia diagnosis in children, between 3 and 10 years. In general, ALL therapy results in an overall survival of nearly 80 % of all cases. Nevertheless, in some cases the prognosis is exceptionally poor. One of these high-risk ALL subgroups are infants of one year or younger. Although initial therapy achieves a remission rate of nearly 95 %, most of those infants relapse within the first year. The overall survival rate of those patients is 44 % (Stam et al., 2006).

A hallmark of infant ALL is the occurrence of a translocation involving the *MLL* gene (11q23) found in 80 % of cases (Pieters et al., 2007). So far, over one hundred partner genes have been identified for this gene (Meyer et al., 2009). The most common partner is the *AF4* gene, detected in about 50 % of the *MLL* rearranged patients (Pieters et al., 2007). This reciprocal translocation t(4;11) (q21;q23) leads to both the fusion *MLL/AF4* as well as *AF4/MLL* (Domer et al., 1993). In our group we especially have addressed the role of *MLL/AF4* by applying siRNA against the fusion site of this transcript (Thomas et al., 2005). Effects of the knockdown of the expression of *MLL/AF4* were inhibition of proliferation, diminished self-renewal potential and a strongly increased rate of apoptosis for example in the cell line SEM. Furthermore, we showed that *MLL/AF4* depletion also reduces the rate of engraftment of leukaemic cells in a severe combined immuno-deficient mice (SCID-Mice) model.

The overall aim of the study was to investigate the role of *MLL/AF4* in t(4;11) positive B-ALL cells with regards to the interaction between *MLL/AF4* and the genes of the *HOXA* cluster. As published data showed lethal consequences for t(4;11) positive cells upon siRNA mediated knockdown of *MLL/AF4* together with a substantial decrease of *HOXA7*, *HOXA9* and *MEIS1* expression (Thomas et al., 2005) it was hypothesised that the *MLL/AF4* – *HOXA* combination plays a key role for stemness and self-renewal potential.

The first aim of this study was to investigate the effect of siRNA mediated *MLL/AF4* knockdown on all genes of the *HOXA* cluster. This cluster located on chromosome 7 consists of 11 sequentially expressed genes, namely *HOXA1*, 2, 3, 4, 5, 6, 7, 9, 10, 11

and 13. These genes have been reported to be key transcriptional regulators for haematopoiesis (Sauvageau et al., 1994). Thus, during haematopoiesis *HOXA* genes play a role in differentiation. During early differentiation the so called 3' or anterior *HOXA* genes (*HOXA1* to *A6*) are active, while the ones located more at the 5'-end of the *HOXA* cluster, termed posterior *HOXA* genes (*HOXA7* to *A13*) are active in more differentiated haematopoietic cells (Pineault et al., 2002). Therefore we investigated the effect of *MLL/AF4* knockdown on these target genes. As some HOX gene primers had no positive controls, absent expression must be viewed with caution as this may be due to inefficient primer design.

The results showed a distinctive pattern between the group of anterior and posterior *HOX* genes. With two exceptions, *HOXA4* and *HOXA6*, the anterior HOX genes showed very similar behaviour upon *MLL/AF4* depletion in that they did not show a substantial alteration in their expression.

In two recent publications, the anterior *HOXA* genes were studied. In a study by Bach and colleagues murine bone marrow cells (MBMC) were transduced with *MLL/ENL* and murine *Hox* gene expression was analysed. They found that with the exception of *Hoxa2* all other anterior Hox genes were substantially upregulated. Transducing the MBMCs with each individual anterior *HOXA* gene resulted in transformation of the cells, with the exception of *HOXA2* and *HOXA5*. If each individual *HOXA* gene was cotransfected with the HOX cofactor *MEIS1*, with the exception of *HOXA5*, all other anterior HOX genes showed transforming ability. When trying to establish permanent cell lines from those experiments, only *HOXA1*, *HOXA4* and *HOXA6* transformed cells, with or without *MEIS1*, survived and expanded permanently in culture. The three permanent *HOXA+MEIS1* cells were subsequently transplanted into mice and all three transplanted mice groups developed an AML phenotype where the combination *HOXA4+MEIS* was most and *HOXA6+MEIS1* the least aggressive combination (Bach et al., 2010).

In the study by Guenther and colleagues, t(4;11) positive SEM cells were investigated for the binding of *MLL/AF4*, wildtype *MLL*, wildtype *AF4*, H3K4 and H3K79 to the locus of the anterior *HOXA* genes with global ChIP-on-chip microarrays. There was no direct binding of *MLL*, *AF4* or the fusion protein to any of the anterior *HOXA* genes detected. In the case of *HOXA1* H3K4 binding was

detected and *HOXA5* and *HOXA6* were found to be bound by H3K79 (Guenther et al., 2008). This signature suggests transcriptional active genes (Jones et al., 2008).

In this thesis *HOXA1*, *HOXA2*, *HOXA3* and *HOXA4* were investigated in two, *HOXA6* in three and *HOXA5* in four independent experiments. While there was no *HOXA1* expression detected, *HOXA2*, *A3* and *A5* were analysed but showed no substantial change in the level of expression upon *MLL/AF4* depletion. *HOXA4* and *HOXA6* show a substantial decrease of 70 % in two and three experiments respectively. For *HOXA4* only the transcript level was investigated and more experiments are necessary on both mRNA and protein level to confirm these preliminary data. However, *HOXA6* was analysed on mRNA as well as on protein level. Both methods demonstrated a substantial knockdown which in the case of the transcript level statistically significant.

HOXA1 and *HOXA2* have been described to be active during myeloid differentiation (Zhang et al., 2009). In *MLL* knockout mice (*MLL*^{-/-}) an increase of *HOXA1* has been reported (Schraets et al., 2003). However, this was investigated in murine fibroblasts and does not necessarily represent the situation in human haematopoietic cells. Nevertheless H3K4 methylation was found within the *HOXA1* locus (Guenther et al., 2008) which implies transcriptional activity, although this was not detectable during this project. The results of the Bach study indicate a role of a *MLL* fusion protein, *MLL/ENL*, in *HOXA1* regulation as well as the potential of *HOXA1* to induce an AML phenotype in mice (Bach et al., 2010). Assessment of *HOXA2* in the lymphoid t(4;11) SEM cell line showed expression, hence it would be of interest to investigate whether the AML t(4;11)-positive cell line MV4;11 has a higher *HOXA2* expression, and whether there might be an effect caused by *MLL/AF4* knockdown in this cell line. However, the MV4;11 cell line was established from AML cells which were classified as FAB M5, meaning that the differentiation process is more advanced; but expressions of *HOXA1* and *HOXA2* are suspected in earlier differentiation phases. Despite this, it would be worthwhile to investigate MV4;11 cells for *HOXA2* and also *HOXA1* expression as *MLL/AF4* can influence those genes. Although no direct link between *MLL* and *HOXA2* can be found, this demonstrates the oncogenic potential and thereby the potential contribution to leukaemogenesis of *HOXA2*.

HOXA3 showed similar results like *HOXA2* in the Bach study as well as in the Guenther study (Bach et al., 2010, Guenther et al., 2008).

The role of *HOXA5* in leukaemia is still widely unknown and controversial results have been published. On the one hand it has been reported that overexpression of MLL/AF9 or CALM/AF10 in bone marrow cells results in upregulation of *HOXA5* (Argiropoulos and Humphries, 2007, Okada et al., 2006). ALL patients with a MLL rearrangement were analysed for *HOXA* gene expression and amongst others *HOXA5* was clearly upregulated compared to ALL patient without MLL rearrangements (Armstrong et al., 2002). On the other hand, in studies by Strathdee and colleagues methylation assays revealed strong hypermethylation, a sign of inhibition of transcription, of CG-rich regions, so called CpG islands, in the promoter regions of *HOXA4* and *HOXA5* (Strathdee et al., 2007a, Strathdee et al., 2007b) in childhood acute leukaemia. The data presented here shows expression of *HOXA5* but as it did not show substantial gene expression alteration upon MLL/AF4 knockdown, we conclude that *HOXA5* together with *HOXA3*, *HOXA2* and *HOXA1* do not play a substantial role in our study.

Two independent MLL/AF4 knockdown experiments were done and *HOXA4* expression analysed. A reduction of *HOXA4* upon MLL/AF4 knockdown could be observed on mRNA level. Although the *HOXA4* signal was detected quite late during the qRT-PCR (around cycle 33), hence it was assumed that *HOXA4* is expressed but at very low level. The literature supports these findings. It has been reported that there is an indicative correlation between *HOXA4* and other members of the four HOX clusters which may play a role in the overall HSC development (Bijl et al., 2006). The decrease of *HOXA4* expression as a consequence of MLL/AF4 depletion suggests that *HOXA4* is a target for MLL/AF4 but possibly indirectly as in the Guenther study *HOXA4* was not detected to in the CHIP-on chip study in SEM cells. The results of Bach (Bach et al., 2010) plus the data here suggest that MLL fusions have an effect on *HOXA4* expression which itself seem to be a potent contributor to acute haematopoietic malignancies. This is supported by the findings of Armstrong and colleagues who detected *HOXA4* upregulation in an ALL patient cohort with MLL rearrangement (Armstrong et al., 2002). They compared this result to ALL

patients without MLL rearrangement and found that *HOXA4* expression to be exclusive to MLL rearranged patients. More studies regarding the role of *HOXA4* in t(4;11) positive cells are necessary to explore *HOXA4* contribution to this kind of disease. Confirmation of *HOXA4* knockdown upon MLL/AF4 depletion would be the first step in this direction. It would be interesting to clarify whether *HOXA4* plays a role in ALL onset or is more important for myeloid malignancies.

In this study a new downstream target of *MLL/AF4*, *HOXA6*, was identified. A statistically significant reduction of expression on mRNA level of about 70 % after siRNA mediated *MLL/AF4* knockdown experiments in SEM could clearly be demonstrated. This together with confirmatory western blot results showed that *HOXA6* was affected by MLL/AF4. In the literature, *HOXA6* has been shown to be important in the early phase of haematopoietic differentiation (Dickson et al., 2009). *HOXA6* was also demonstrated to have an influence on colony forming and self-renewal. Therefore the interaction between *MLL/AF4* and *HOXA6* is another indication for *HOXA* genes playing a central role in the stem cell behaviour of malignant haematopoietic progenitor cells. Whether *HOXA6* plays an important role in the development and maintenance of ALL still needs to be investigated. Direct interaction of MLL, AF4 or MLL/AF4 was not reported in the Guenther study (Guenther et al., 2008). However H3K79 binding to the *HOXA6* locus was reported which indicates involvement of the DOT1L methyltransferase complex. In this study, *HOXA6* showed similar expression levels as *HOXA3* or *HOXA5* in SEM cells. In contrast, *HOXA6* expression was clearly affected by MLL/AF4 knockdown. Examination of *HOXA6* expression in the cell lines SEM, RS4;11 MV4;11 and the Kasumi-1 showed that AML cell lines MV4;11 and Kasumi-1 did not have a substantial *HOXA6* expression while it was detectable in all in the ALL cell lines RS4;11 and SEM. In the ALL cell lines REH, 697 and NALM6 *HOXA6* expression was comparably low to the aforementioned AML cell lines. It is also worth noting, that *HOXA6* showed a similar expression level and MLL/AF4 knockdown pattern as *HOXA7*. This has to be investigated further. Knockdown experiments with siRNA specially against *HOXA6* should be performed to clarify whether *HOXA6* plays a functional role in t(4;11) positive leukaemia. By using AML and ALL cell lines it should be possible to see if *HOXA6* has an influence on lineage determination.

As already briefly mentioned above the knockdown of MLL/AF4 on mRNA level resulted in depletion of *HOXA7* (Thomas et al., 2005) which was confirmed on protein level by western blot in this study. Furthermore it was shown that siRNA treatment against *HOXA7* of SEM cells had a similar effect as MLL/AF4 knockdown. These data were produced by qRT-PCR which showed an 85 % knockdown of *HOXA7*. In the literature, *HOXA7* has been described as acute lymphoblastic leukaemia associated although it is not clear so far if *HOXA7* expression is vital for leukaemic development involving rearranged *MLL* (Armstrong et al., 2002, Rozovskaia et al., 2001). The Bach study demonstrated induction of *HOXA7* expression in murine haematopoietic cells by transfection with a *MLL/ENL* construct. Moreover they showed a transforming ability of *HOXA7* but which was less potent than *HOXA6*. The coexpression of *MEIS1* increased *HOXA7* transforming potential. From those cells it was possible to establish a permanent cell line (Bach et al., 2010). In global ChIP-on-chip studies *HOXA7* was clearly detected as a target of MLL/AF4 and showed epigenetic involvement of H3K4 as well as H3K79 at the *HOXA7* locus (Guenther et al., 2008). The data presented here therefore proves that in the SEM cell line model, *HOXA7* is clearly necessary for the upkeep of the leukaemic cells as *HOXA7* depletion leads to rapid onset of apoptosis. When comparing *HOXA7* expression levels in several leukaemic cell lines, a high expression of *HOXA7* in the t(4;11) positive ALL cell lines SEM and RS4;11 was detected but no *HOXA7* expression was measurable in MV4;11, the t(4;11) positive AML cell line. Also in three other ALL cell lines e.g. REH, 697 and NALM6 no *HOXA7* expression was detected. This suggests a role of *HOXA7* in the two t(4;11) positive ALL cell lines. Interestingly those cell lines were all obtained from patients at relapse (Greil et al., 1994, Stong and Kersey, 1985), whereas MV4;11 was derived from a patient with *de novo* t(4;11) AML (Lange et al., 1987). In a publication from Bertrand and colleagues another t(4;11) positive cell line BLIN-3 was reported to be *HOXA7* negative. BLIN-3 was established from an infant with *de novo* ALL (Bertrand et al., 2003, Bertrand et al., 2001). These results, although only observed in cell lines so far, indicate a role of *HOXA7*, and maybe also of *HOXA6*, in the onset and also the severity of relapse resulting in t(4;11) ALL, because the expression of these *HOXA* genes was only detectable in cell lines established from relapsed patients. In contrast to this, are the findings of two research group who investigated ALL patients carrying t(4;11) translocations (Stam et al., 2009, Trentin et al., 2009). They found

that not all t(4;11) positive ALL patients showed upregulation of *HOXA7* as well as *HOXA3*, *HOXA5*, *HOXA9* and *HOXA10*. Those lacking *HOXA* overexpression had a very high risk of relapse compared to the patient cohort with up regulated *HOXA* genes (Stam et al., 2009). This still needs to be clarified whether the genetic phenotype of *de novo* ALL's matches the relapse genotype and if *HOXA* gene expression plays an important role in relapse. Nevertheless, as in this study, *HOXA7* knockdown had lethal consequences for the cell lines, thus *HOXA7* and *HOXA6* could be potential targets for ALL relapse therapy.

To further investigate the role of *HOXA7* the t(4;11) positive cell lines SEM, RS4;11 and MV4;11 were transduced with a lentiviral construct expressing a murine FLAG tagged *Hoxa7* combined with *eGFP* as an expression marker. The cell line SEM was transduced twice and RS4;11 as well as MV4;11 once, but did not result in a stable *HOXA7* over expressing cell line. The transduced cells from RS4;11 and MV4;11 died within five days after transduction while SEM cells in the first experiment survived for several weeks but the eGFP expression decreased constantly. An attempt to sort and cultivate eGFP positive cells failed in the first instance. In the second transduction experiment the eGFP positive SEM cells also died rapidly within five days post transduction. The reasons for these results are not clear. One reason could be that *HOXA7* overexpression would need the often discussed additional *MEIS1* coexpression although this does not explain the high apoptosis rate of cells with overexpressed *HOXA7*. Control cells, transduced with a construct which only lacks the *Hoxa7* FLAG tag showed no signs of apoptosis or any other negative effects. This rules out the possibility of toxicity of the lentiviral construct integration or the *eGFP*. During the first transduction experiment with SEM a decreasing eGFP fluorescence could be monitored over time by fluorescence microscopy. This indicates that cells with high *Hoxa7* overexpression died earlier than cells with moderate or low *Hoxa7* overexpression. It is also possible that the virus concentration plays a major role in the successful transduction of a cell line. A weakness of this study was not to determine a virus titer. One suggestion at this point is to always determine the virus titer which would help to standardise these transduction experiments. For future work it is important to determine the virus titer to use a median virus concentration for each transduction which was not done in these experiments. To avoid time consuming titer determinations it is possible to use

at least a dilution series of the concentrated virus to determine cell viability and eGFP expression. As virus production is not equal every time the dilution series cannot be a standard procedure but would be helpful as a starting experiment to collect the first data. Figure 3-1 illustrates the hypothesis of the correlation between cell viability and HOXA7-eGFP expression in SEM cells. This model can also be used for other cell lines as well.

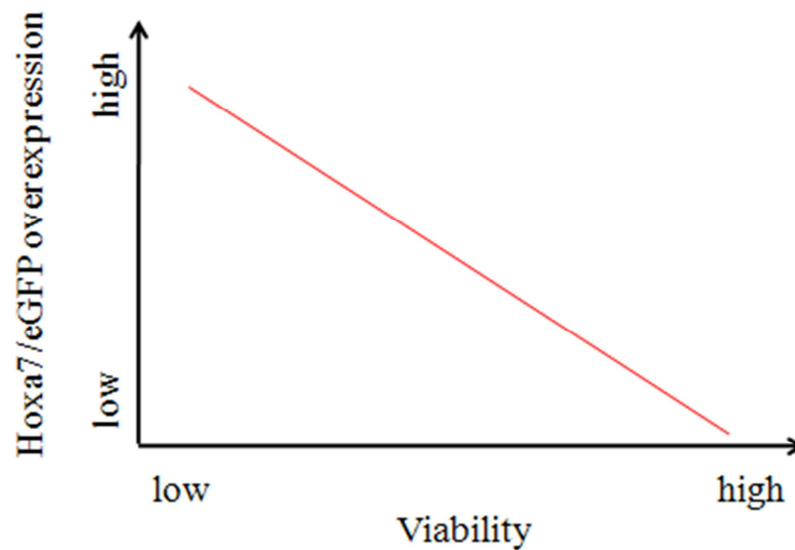


Figure 3-1 Viability model of transduced SEM cells

This diagram illustrates the model for the survival of cells transduced with a *Hoxa7-eGFP* expressing cassette. The eGFP expression is a marker for *Hoxa7* expression as both genes are driven by the same promoter. Cells which show the highest expression of *Hoxa7/eGFP* have the lowest viability. With decreasing *Hoxa7/eGFP* expression, depending on the number of integration sites, viability of the cells increases. Further experiments should reveal the optimal concentration of virus particles to create a permanent *Hoxa7/eGFP* overexpressing cell line.

Discussions about the HOXA gene cluster and their interactions in ALL, especially MLL/AF4, *HOXA9* will be described in the next chapter. Our own results show that *HOXA9* mRNA expression is decreased after MLL/AF4 knockdown. This result was confirmed by western blot. Comparison of several leukaemic cell lines (SEM, RS4;11, MV4;11, REH, 697, TK6, NALM6, K562 and Kasumi1) showed *HOXA9* expression only in t(4;11) positive cells but low or abundant expression in others. This would support the argument that *HOXA9* contributes to MLL rearranged

leukaemias. There exist a large number of studies describing the role of *HOXA9* in leukaemogenesis. *HOXA9* upregulation is detected in most t(4;11) related leukaemias (Armstrong and Look, 2005, Armstrong et al., 2002). In animal models it was demonstrated, that *HOXA9* overexpression leads to increased proliferation and to a leukaemic phenotype (Thorsteinsdottir et al., 2002). More recent work shows that *HOXA9* is necessary for MLL rearranged leukaemia. It was shown, that *HOXA9* depletion in MLL rearranged cells led to inhibition of proliferation and caused apoptosis (Faber et al., 2009). In contrast to this, it was reported that transformation of haematopoietic *HOXA9* negative precursor cells with *MLL/GAS7* led to colony forming blasts but which were less potent than after restoring *HOXA9* expression. This indicates the importance of *HOXA9* contribution to the rapid development of the leukaemic phenotype (So et al., 2004). Bertrand and colleagues also reported a lack of *HOXA9* expression in the already mentioned t(4;11) positive cell line BLIN-3 (Bertrand et al., 2003). The studies of the Bach group demonstrated an induction of *HOXA9* expression by transfection of murine haematopoietic cells with *MLL/ENL* as well as the transforming potential of *HOXA9*. The transfection of murine primary bone marrow cells with *HOXA9* led to a differentiation block and onset of proliferation which was further increased by coexpression of the HOXA cofactor *MEIS1*. Those cells were taken into permanent cell culture and cell lines were established. Transplantation of those cells then led to rapid onset of an acute myeloid leukaemia phenotype in recipient mice and a quick death of the animals (Bach et al., 2010). Global ChIP analyses revealed the binding of MLL/AF4 to *HOXA9* and of H3K4 and H3K79 to the *HOXA9* locus (Guenther et al., 2008).

The *MLL/AF4* knockdown effect on *HOXA10* was investigated in three independent experiments on mRNA and protein level. The detection of *HOXA10* in several western blot experiments failed. *MLL/AF4* depletion led to a significant 80 % reduction of the transcript level in SEM cells. Similar to *HOXA9*, *HOXA10* was expressed in three t(4;11) positive cell lines (SEM, RS4;11 and MV4;11) but was less expressed in other leukaemic cell lines (REH, 697, TK6, NALM6, K562 and Kasumi1). *HOXA10* has been described to play an important role in transforming myeloid progenitors. The overexpression of *HOXA10* leads to onset of acute myeloid leukaemia in murine haematopoietic cells (Thorsteinsdottir et al., 1997). *HOXA10* was also reported to impair haematopoiesis (Buske et al., 2001) and is a critical

regulator for development of haematopoietic stem cells, and erythroid and megakaryocyte development (Magnusson et al., 2007). There is sparse literature available on the role of *HOXA10* in acute lymphoblastic leukaemia. By transfecting murine bone marrow cells with *HOXA10* a differentiation block and induction of proliferation could be observed. Further experiments showed colony forming ability which was pronounced by coexpression of *HOXA10* with *MEIS1*. From both types of transfected cells, *HOXA10* with or without *MEIS1*, it was possible to generate permanent cell lines (Bach et al., 2010). This result underlines the potency of *HOXA10* in leukaemic transformation. *HOXA10* was also found to be upregulated in a subset of T-cell acute leukaemia bearing an inversion within chromosome 7 (Speleman et al., 2005). In global ChIP-on-chip analysis MLL/AF4 was found to bind to *HOXA10* and also epigenetic involvement at the *HOXA10* locus was found (Guenther et al., 2008). Together with the presented results here, this suggests that *HOXA10* has an important role in leukaemogenesis.

It was not possible to detect expression of *HOXA11* and *HOXA13* in two independent *MLL/AF4* knockdown experiments. As for *HOXA1*, primers were not tested for specificity or with a positive control. *HOXA11* like *HOXA10* has been reported to be upregulated in inv(7) positive T-cell acute lymphoblastic leukaemia (Speleman et al., 2005). However, *HOXA13* has been reported to be essential for the onset of T-Cell leukaemia (Su et al., 2006). Bach and colleagues showed upregulation of *HOXA11* as well as *HOXA13* in *MLL/ENL* transfected murine haematopoietic cells. *HOXA11* and *HOXA13* transformed murine bone marrow cells were able to form colonies and enhanced colony formation was detected with coexpression of *MEIS1*. Nevertheless it was not possible to form a permanent cell line from those cells (Bach et al., 2010). ChIP-on-chip studies revealed that H3K4 trimethylation was detected within the *HOXA11* and *HOXA13* locus which suggests involvement of the MLL methylation complex (Guenther et al., 2008). Unlike genes like *HOXA7* or *HOXA9*, *HOXA11* and *HOXA13* did not show any noticeable dysregulation in microarray studies which could mean that *HOXA11* and *HOXA13* do not play an important role in infant acute leukaemia.

In summary, the whole *HOXA* cluster was investigated for changes in *HOXA* gene expression as a consequence of *MLL/AF4* depletion. *HOX* genes have been reported to play important roles during haematopoiesis and development of leukaemia (Argiropoulos and Humphries, 2007) but also at least partly in cell cycle control (Mishra et al., 2009). A significant reduction of *HOXA* gene expression for *HOXA6*, *HOXA7* and *HOXA9* on both mRNA and protein level was found which could indicate possible target genes in MLL rearranged ALL cases. *HOXA10* only showed a significant reduction at the mRNA level but it was not detected at the protein level. Another possible target gene, *HOXA4*, was found although more experiments need to be done to confirm this result. *HOXA7*, *HOXA9* and *HOXA10* were also downregulated and are genes which are strongly correlated with acute leukaemia, thus the findings are not very surprising. These genes have been shown to be induced by MLL fusion genes like *MLL/ELL*, *MLL/AF6*, *MLL/AF9*, *MLL/AF10* and *MLL/ENL* (Ayton and Cleary, 2003, Bach et al., 2010). *HOXA6* activation by a MLL fusion was recently reported by Bach and colleagues (Bach et al., 2010). Furthermore *HOXA7* and *HOXA9* have been reported to be essential for the maintenance of leukaemia (Ayton and Cleary, 2003, Faber et al., 2009). Key cofactors for *HOXA* genes have been found in *MEIS1* and *PBX1* (Bach et al., 2010, Mamo et al., 2006, Wang et al., 2006, Zeisig et al., 2004). *MEIS1* and *PBX1* can alter HOX-DNA binding specificity, increase HOX-DNA binding affinity and enhance gene transcription activity (Argiropoulos et al., 2007). *MEIS1* has been found to form trimeric complexes with *HOXA9* and *PBX1* (Shen et al., 1997, Shen et al., 1999). *MEIS1* has been found to play a major role in MLL leukaemia stem cell potential by regulating self-renewal, the differentiation arrest and also the *in vivo* generation of LSCs from myeloid progenitors (Wong et al., 2007) Also *MEIS1* has been reported to be downregulated as a consequence of *MLL/AF4* knockdown (Thomas et al., 2005). The Guenther study showed that *MEIS1* was bound by *MLL/AF4*, H3K4 and H3K79 in SEM cells. All these data indicate the importance of *MEIS1* and *PBX1* in leukaemogenesis. Another common signature of *HOXA7*, *HOXA9* and *HOXA10* has been found during global ChIP-on-chip studies. All three genes show binding to *MLL/AF4* as well as binding of H3K4 and H3K79 to the locus of the genes (Guenther et al., 2008). Thus, in summary, all direct binding of *MLL/AF4* to the *HOXA* genes seems to be a key mechanism in MLL rearranged leukaemia. The question remains as to how MLL exactly effects their gene expression. Evidence in

the literature suggests that binding of MLL to the CpG cluster within the *HOXA9* promoter protects it from methylation by DNA methyl transferases (DNMT). DNA methylation would inhibit the transcription of the gene (Erfurth et al., 2008). Thus one assumes that an uncontrolled permanent binding of MLL/AF4 via the DNA methyltransferase domain to the promoter leads to permanent transcription of the *HOXA9* gene and presumably the same mechanism contributes to *HOXA7* and *HOXA10* gene activation. Knockdown of MLL/AF4 could then allow DNMT binding, DNA methylation and thereby gene transcription inhibition. As it has been shown that at least *HOXA7* and *HOXA9* are vital for survival this could explain the fatal effect of the MLL/AF4 knockdown. siRNA directed against *HOXA7* confirmed the critical role of this gene. The cells show a similar phenotype on lack of *HOXA7* expression as they do when *MLL/AF4* is depleted. Targeting *HOXA6*, *HOXA9* and *HOXA10* with siRNA could provide further insights into this matter and thus may clarify if the other *HOXA* genes also have critical roles in the survival of *MLL/AF4* positive cells. An interesting correlation was found when comparing expression levels of *HOXA7* of four t(4;11) positive cell lines SEM, RS4;11 and MV4;11 and BLIN-3 (Bertrand et al., 2001). *HOXA7* expression was found in the t(4;11) positive ALL cell lines SEM and RS4;11 but not in the AML cell line MV4;11. This may suggest that *HOXA7* is exclusively expressed in ALL. However, gene expression studies of the BLIN-3 cells, a cell line established from an infant diagnosed with ALL, also did not show *HOXA7* expression. This is supported by further findings that not all t(4;11) positive infant leukaemias show overexpression of *HOXA7* (Stam et al., 2009, Trentin et al., 2009). Interestingly HOXA-negative patients had a worse prognosis than HOXA-positive ones. Interestingly SEM and RS4;11 are cell lines established from patients in relapse. MV4;11 and BLIN-3 had their origins in patients with *de novo* development of t(4;11) positive leukaemia. Whether *HOXA7* overexpression is important for relapse has yet to be revealed. If *HOXA7* can be identified as a key player in the development of aggressive relapse leukaemia, it may provide a possible target for treatment.

Another point of discussion about the expression pattern of *HOXA6*, *HOXA7*, *HOXA9* and *HOXA10* at the mRNA level in t(4;11) positive cell lines (SEM, RS4;11 and MV4;11) is that these cell lines had high expression of *HOXA* genes compared to other cell lines which showed very low gene expression. Whereas the ALL cell lines,

SEM and RS4;11, showed expression of all four mentioned *HOXA* genes *HOXA6* and *HOXA7* were exclusively expressed in the ALL cell lines. Atkinson and colleagues investigated epigenetic marking of the HOX genes during differentiation of pluripotent cells (Atkinson et al., 2008). They showed that during differentiation of pluripotent cells the HOXA genes (*HOXA4* to *HOXA11*) are epigenetically marked by di- and trimethylation and these methylations suggest gene activation. As MLL fusion genes are thought to be constantly bound at the *HOXA7* to the *HOXA10* sites in t(4;11) positive ALL cells, this would support the hypothesis that differentiation is blocked due to the constant activity of *HOXA* genes in malignant haematologic diseases, driven by *MLL* fusion gene expression (Slany, 2009). In the B-cell precursor cells from SEM and RS4;11 it seems that *HOXA6* and *HOXA7* drove the cells towards the lymphoid lineage. This may indicate that these genes are important for the lineage determination in the process of differentiation. Both *HOXA6* and *HOXA7* activation could drive the cell towards the lymphoid lineage, while the inactivation of those genes may lead to the myeloid lineage. Because *HOXA* genes are expressed in transcriptional cascades it is difficult to argue with this theory. The activation of a certain *HOXA* gene activates the transcription of the next *HOXA* gene in a 5' direction and represses transcription of the 3' *HOXA* gene, although this sequential gene activation cascade is discussed controversially in the literature.

In the lymphoid development there is also activation of *HOXA9* expression but this is transient and a specific stage of development. The following picture visualizes the expression of the HOXA genes in a cascade and the involvement of MLL/AF4 expression as an interrupter of differentiation in the affected cell (Figure 3-2).

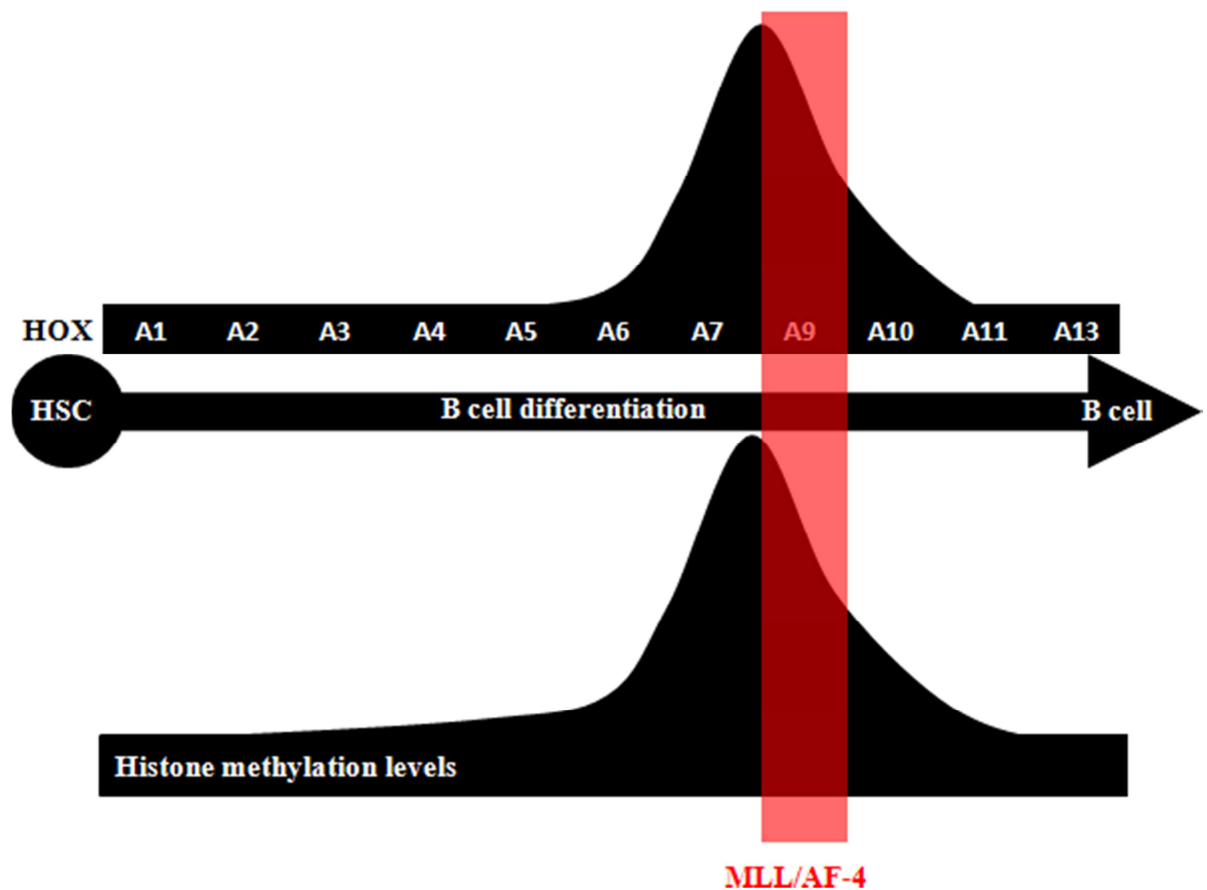


Figure 3-2 Halt of differentiation by MLL/AF4

Illustration of how MLL/AF4 expression could affect progression of the HOXA gene transcription cascade. Atkinson and colleagues (Atkinson et al., 2008) showed a correlation between epigenetic marking and activation of HOXA genes in pluripotent human stem cells. Here this model is translated to haematopoietic stem cells (HSCs). Differentiation of HSCs can progress until the appearance of MLL/AF4 (RED) which blocks further differentiation by constant stimulation of HOXA7/HOXA9 expression via mechanisms yet not fully understood.

Differentiation is a tightly controlled process and the *HOXA* genes play an important role. However, the way a haematologic stem cell differentiates is not known in detail. Often there are agonists and antagonists for certain groups of genes. For example, the trithorax group (trx-G), of which MLL is a member, are agonists for HOXA gene expression and antagonists of genes of the polycomb group (PcG). PcG genes have been shown to be crucial in the development of mice and are implicated in leukaemogenesis. Both trx-G and PcG are groups of histone methyltransferases. While the genes of the trx group activate gene expression by methylation of H3K4, PcG genes silence gene expression by methylation of H3K27 (Schuettengruber et al., 2007). A role in leukaemia has been reported for PcG proteins which are deregulated

in AML. Interestingly, this is associated with deregulation of the PcG target genes *HOXA4*, *HOXA9* and *MEIS1* (Grubach et al., 2008). The PcG gene *BMI1* for example has been found to be crucial for the development of human leukaemia CD34 positive cells as *BMI1* knockdown impaired self-renewal and induced apoptosis of the cells (Rizo et al., 2009). Their particular role in the development in *MLL* rearranged forms of leukaemia has not been fully investigated yet.

3.2 A correlation between *MLL/AF4*, *HOXA7* and *TERT*

Over the past years a considerable amount of literature has been published on the involvement of the enzyme complex telomerase and its subunits in cancer biology. These subunits are *TERT*, the reverse transcriptase of the telomerase, and *TERC*, the RNA component of the telomerase. In these publications an elevated telomerase activity has been demonstrated in a substantial number of cancer types (Hahn, 2005, Shay and Bacchetti, 1997). In the present study telomerase activity was investigated after depletion of *MLL/AF4* by siRNA mediated knockdown in the t(4;11) positive cell line SEM. TRAP assays showed that depletion of *MLL/AF4* was associated with a 70 % reduced telomerase activity in these cells. A high activity of telomerase and a therefore assumed permanent occurring prolongation of the telomeres in the cell override the activation of apoptosis and can lead to a malignant cell. The data presented here suggest *MLL/AF4* is involved in processes which are very important for the stem-like behaviour of the malignant cells. More detailed investigations on the subunits of the telomerase namely *TERT* and *TERC* revealed a knockdown effect on *TERT* but not on *TERC* upon depletion of the fusion transcript *MLL/AF4*. Treatment of SEM cells with siRNA against *MLL/AF4* or *HOXA7* showed in both cases moderate but significant *TERT* depletion of about 40 % ($p < 0.002$). As a consequence of *MLL/AF4* knockdown, not only the transcripts of *HOXA7* but also *TERT* were affected. Applying siRNA against *HOXA7* revealed that only *TERT* but not *MLL/AF4* was affected. This shows that *HOXA7* is downstream of *MLL/AF4* and so is *TERT*. Furthermore it was demonstrated that *TERT* is a downstream target of *HOXA7*. As knockdown of *HOXA7* diminished *TERT* expression, a direct regulation was hypothesised. The simplified model to this hypothesis is shown in the picture for the *MLL/AF4* signalling pathway Figure 3-3. *HOXA7* has been reported to

transform haematopoietic pluripotent cells into colony forming blasts thereby suggesting contribution to self-renewal potential (Bach et al., 2010). TERT also has been reported to immortalise cells by bypassing the p53 and Rb apoptosis pathway (Chapman et al., 2006). The interaction between MLL/AF4, HOXA7 and TERT could be a key mechanism in the self-renewal potential of the SEM cells, and thus for HOXA positive t(4;11) ALL. These experiments suggest the possible signalling order shown in figure 3-3 but direct gene regulations have not been shown so far.

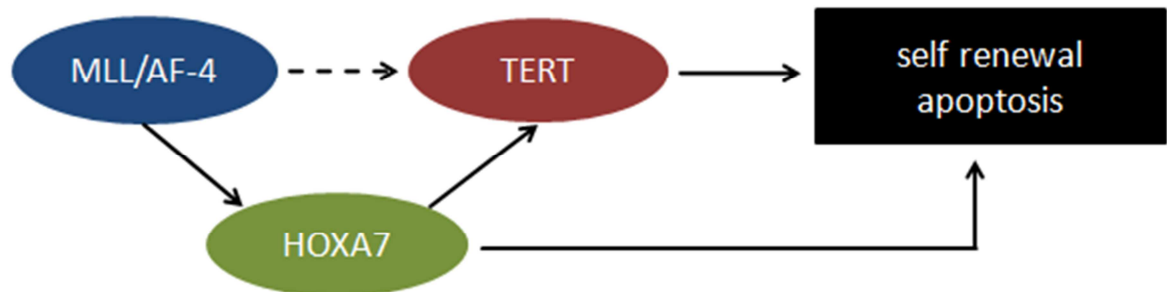


Figure 3-3 Model of MLL/AF4 target genes

Knockdown experiments showed HOXA7 being influenced by MLL/AF4. In knockdown experiments it also showed HOXA7 is a downstream target of MLL/AF4 and TERT being a downstream target of HOXA7. The dashed line illustrates that there is no clear evidence whether MLL/AF4 directly influences TERT expression. Both HOXA7 and TERT are correlated with the potential of self-renewal and regulating cell death.

A direct effect of *MLL* or *MLL/AF4* on *TERT* also has not been detected in the global ChIP analysis from the group of Guenther. Epigenetic modifications such as histone methylation of the TERT promoter by *MLL* or *MLL/AF4* is possible as H3K4 and H3K79 have been found to bind within the TERT locus(Guenther et al., 2008). This indicates that gene activation involves the MLL methyltransferase complex. In addition to MLL there are also other histone methyltransferases like MLL2, MLL3,

MLL5, SET1a, SET1b and ASH1L (Slany, 2009) which could all be involved in the H3K4 methylation.

To investigate the promoter of *TERT* for possible binding sites of HOXA7 an *in silico* approach was used as a first step.

In this study an internet browser based program called MatInspector[®] (Genomatix Software GmbH, Germany) was used to investigate the promoter of TERT for potential HOXA7 binding sites. After providing the sequence of the core TERT promoter the program suggested several possible binding sites for *HOXA* genes or their cofactors such as *PBX1* and *MEIS1*.

After selecting the most suitable sites primers were designed for the designated promoter sequences. Using these specifically designed primers, a chromatin immunoprecipitation assay (ChIP) was carried out. The ChIP assays revealed direct binding of *Hoxa7* to the promoter of *TERT*. The binding position is about 1kb upstream of the transcription start of *TERT*. While the software MatInspector predicted this, it is the first time that direct binding of a HOXA transcription factor to *TERT* has been demonstrated. Further investigations should point out whether this binding is crucial or to what extent it contributes to the leukaemic phenotype of MLL/AF4 positive cells. As the MatInspector program also suggested general HOX binding to the *TERT* promoter it is likely that also other HOXA transcription factors can bind such as HOXA6 or HOXA9. In addition MEIS1 or PBX1 binding was also suggested. As the experiments were performed with FLAG-tagged *Hoxa7*, endogenous HOXA7 binding to the TERT promoter has not been demonstrated. The data presented here show direct binding of murine *hoxa7* to the promoter of *TERT*. TERT overexpression leads to a constantly active telomerase which maintains the telomeres, thus leading to a high proliferation rate without telomere shortening and cells are immortalised.

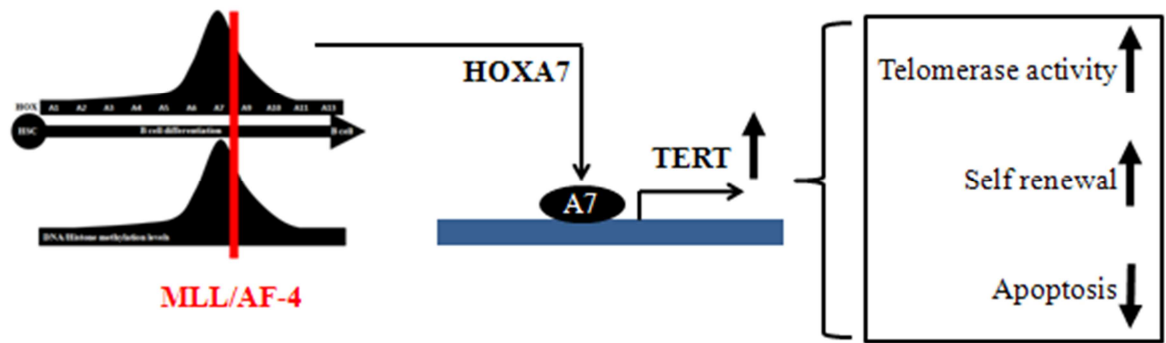


Figure 3-4 Mechanism for TERT activation

TERT over-expression is regulated by *HOXA7*, which is constantly overexpressed by *MLL/AF4* binding. *TERT* maintains a constant telomerase activity which contributes to the immortality of the cells.

To what extent this mechanism contributes to the stem cell potential of the leukaemic cells is not clear. We have shown so far, that knockdown of *MLL/AF4* or *HOXA7* leads to the onset of apoptosis and diminished colony forming rate. As *MLL/AF4* and *HOXA7* knockdown also result in *TERT* reduction it is likely that *TERT* contributes to the stem cell potential of the investigated leukaemic cells. In the literature there are reports of *TERT* overexpression in normal cells which leads to immortalisation of these cells (Chapman et al., 2006). Also, the high telomerase activity in a high variety of cancer, including haematopoietic malignancies, indicates that *TERT* is playing a central role in tumour maintenance (Shay et al., 1996, Hiyama et al., 1995b, Kim et al., 1994).

On the one hand, the protein *MLL* alone is known to generally methylate lysine 4 of the protein histone 3 (*H3K4*) in the promoter regions of genes and thereby activates their transcription. On the other hand, *AF4* is known to form a complex with the elongation factor *DOT1L* and is involved in modifying methylation of histone 3 lysine 79 (*H3K79*) which also modulates gene transcription activity. In addition the literature describes *MLL/AF4* fusion proteins having a striking impact on gene activity via abnormal *H3K79* methylation (Krivtsov et al., 2008). Global ChIP-on-chip analysis revealed gene activating *H3K4* and *H3K79* signature on the promoter of *TERT* in *t(4;11)* positive cells but also in *t(4;11)* negative REH cells suggesting that the involvement of *MLL/AF4* in the epigenetic marking of *TERT* is unlikely (Guenther et al., 2008).

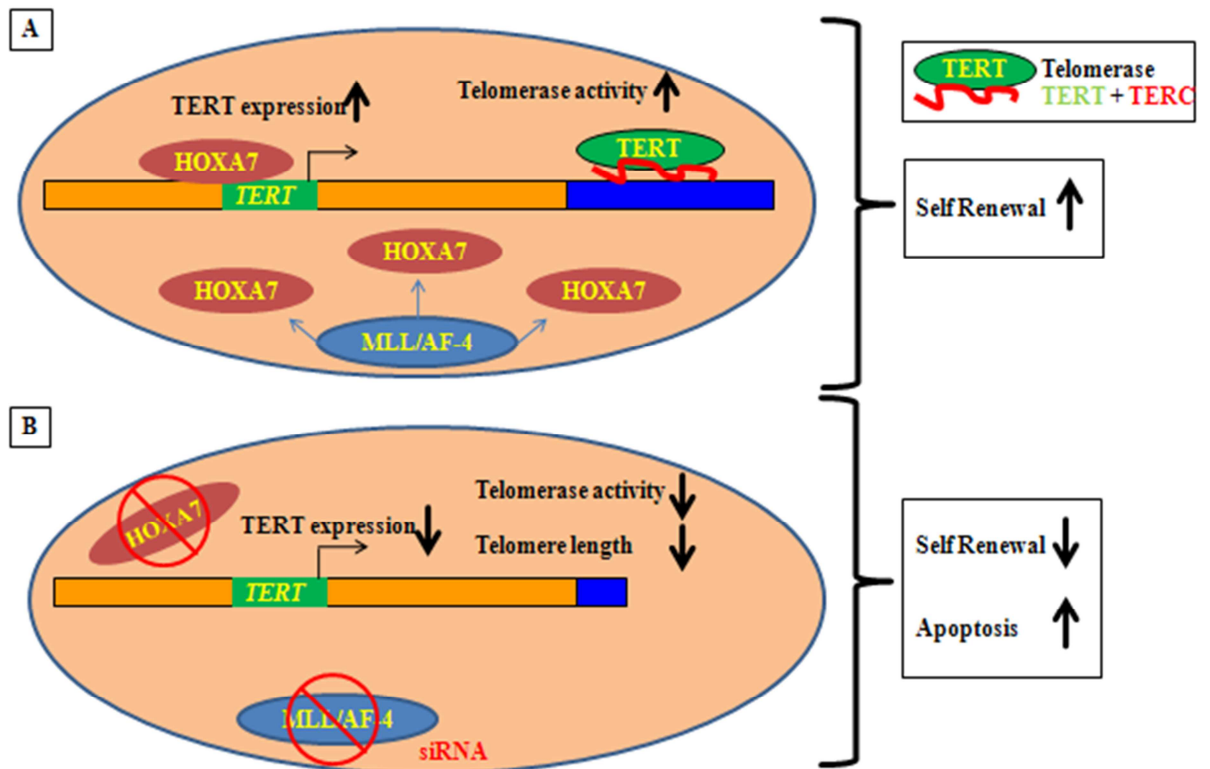


Figure 3-5 MLL/AF4 knockdown consequences

MLL/AF4 regulates *TERT* expression via *HOXA7*. (A) Direct binding of *HOXA7* in the promoter region of *TERT* was identified suggesting an indirect link between MLL/AF4 and *TERT* via *HOXA7*. (B) siRNA mediated knockdown (red) of MLL/AF4 causes a reduction of *HOXA7* thereby a diminished *TERT* expression and consequently a lowered telomerase activity. The loss of telomerase activity thus causes shortening of the telomere length and consequently damage of the coding chromosomal region which may trigger apoptosis.

The consequence of MLL/AF4 knockdown (Figure 3-5) is the direct depletion of *HOXA7* in the affected cells. Direct *HOXA7* binding to the *TERT* promoter explains the diminished *TERT* expression which results in reduction of telomerase activity. Thus the telomeres are not maintained anymore and will be reduced to a critical point where damage of coding regions of the DNA occurs. This DNA damage may then trigger the DNA-damage p53 response and finally induction of apoptosis.

Using siRNA against MLL/AF the knockdown of *TERT* at the mRNA level was twofold and a considerable knockdown of the *TERT* protein 4 was observed (Figure

2-6). During analyses of these effects in SEM cells, an increasing number of apoptotic cells was observed when the time duration of siRNA treatment increased. By inhibiting apoptosis with the pancaspase inhibitor zVAD the aim was to rescue the cells and investigate them. The TERT knockdown was much stronger upon MLL/AF4 depletion after combined zVAD treatment compared to a control experiment without inhibitor. The level of mRNA of *TERT* was reduced to about 10% in siRNA/zVAD treated cells compared to only 50 % in siRNA/control cells. FACS analyses showed the effectiveness of apoptosis inhibition by zVAD (Figure 2-14). Interestingly after 144 hours of constant combined siRNA/zVAD treatment a subG1\G0 population of dead cells was observed. Closer investigations done by Patricia Garrido Castro in the group showed that this population did not consist of apoptotic cells, as key apoptotic markers were not seen however, there was evidence of necroptosis. This would lead to the assumption that the knockdown of MLL\AF4 leads to inevitable cell death. Ongoing experiments further investigate this matter.

Reports in the literature show that TERT has alongside its role in telomerase maintenance also an anti-apoptotic effect (Lee et al., 2008, Zhang et al., 2003). One reason for the resistance of the disease regarding many forms of treatment may be the high TERT expression in the surviving cells. As most anti-cancer agents block or damage crucial functions for cells like inhibition of DNA structure modification which normally induces apoptosis a high TERT expression might be sufficient to survive those treatments. The remaining problem in cancer therapy is not the blast-crisis itself but the complete removal of malignant cells from the haematopoietic system of the individual. Thus a small residual population of TERT-induced apoptosis resistant cells may be enough to induce a relapse.

When apoptosis is not suppressed by pancaspase inhibitors most cells die upon MLL/AF4 depletion. So far, there is no obvious reason that some cells do survive. Whether TERT plays a role in this resistance to apoptosis needs to be further investigated.

Since *MLL/AF4* knockdown results in *HOXA7* depletion which then has a negative effect on *TERT* expression, it is perplexing that qRT-PCR results from zVAD treated cells do not correlate with the *HOXA7* and *MLL/AF4* results. One would expect

MLL/AF4 and *HOXA7* to show an increased knockdown effect in apoptosis inhibited cells similar to TERT, assuming that there is a direct regulation of *TERT* by *HOXA7*. It may be that there is a mechanism which restores TERT expression during the knockdown and a small subpopulation of cells recovers from the siRNA mediated knockdown. *HOXA7* may not be the only regulator of TERT expression (Wu et al., 1999, Toh et al., 2005) and rescue mechanisms may be activated during the knockdown experiments. Those rescue mechanisms could depend on pro-apoptotic caspase activation as this is the only major change in the experimental setup (Figure 3-6). With inhibition of the caspases by zVAD, this rescue mechanism does not take place and TERT expression remains low. As TERT has been reported to act in an anti-apoptotic way (Zhang et al., 2003, Lee et al., 2008) this rescue mechanism would clearly contribute to the survival and immortality of the cancer cells. This rescue mechanism may be present only in therapy resistant t(4;11) positive relapse acute lymphoblastic leukaemias.

With regard to the *HOXA* genes, this mechanism would be *HOXA* independent. With the reports on *HOXA* negative and *HOXA* positive ALLs (Stam et al., 2009, Trentin et al., 2009) together with the poor outcome of *HOXA* negative patients, investigations regarding the TERT expression might show existence of a mechanism of TERT expression independent of the *HOXA* genes and more resistant to apoptosis.

In our cell line comparison of *HOXA7* and *TERT* expression, it was shown that *TERT* expression in MV4;11 cells is much lower compared to *HOXA7*-positive SEM or RS4;11 cells. However, this *HOXA7* independent TERT expression might have a higher impact on chemotherapy resistance than the *HOXA7* dependent TERT expression. Investigations into this matter might provide new insight into the mechanisms of t(4;11) positive leukaemias.

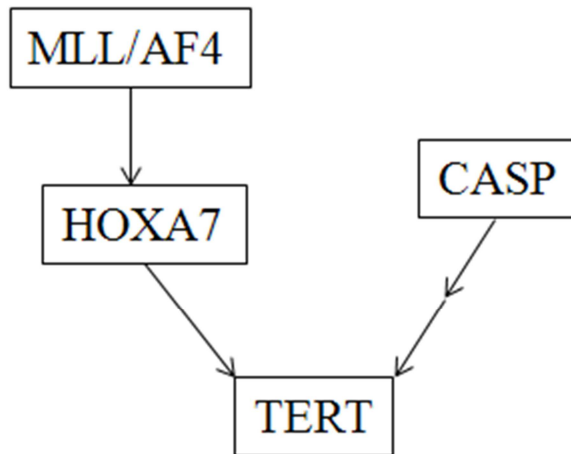


Figure 3-6 TERT is modulated by two independent mechanisms

TERT regulation by MLL/AF4 is mediated via HOXA7. An alternative caspase dependent regulation may also exist.

3.3 Summary

The overall aim of the study was to investigate the role of MLL/AF4 in t(4;11) positive B-ALL cells with regard to the interaction between MLL/AF4 and the genes of the HOXA cluster. The hypothesis was that MLL/AF4 – HOXA played a key role in both stemness and self-renewal potential.

Acute lymphoblastic leukaemia (ALL) is the most common form of leukaemia in children of five years and under. In infants with ALL up to the age of 12 months rearrangement of the MLL gene is detected in more than 80 % (Pieters et al., 2007). More than 100 partner genes have been identified so far for MLL (Meyer et al., 2009). The most common is AF4, detected in nearly 50 % of MLL rearranged infant leukaemias (van der Linden et al., 2009). This translocation t(4;11) marks a very aggressive form of leukaemia with a poor prognosis. The role of *MLL/AF4* was investigated by applying siRNA against the fusion gene transcript. Knockdown of MLL/AF4 in the t(4;11) positive cell line SEM led to diminished proliferation, increased rate of apoptosis. Two homeobox genes, *HOXA7* and *HOXA9* along with HOX cofactor *MEIS1* were also reported to be depleted upon *MLL/AF4* knockdown (Thomas et al., 2005).

The first goal was to investigate the whole HOXA cluster for altered expression upon MLL/AF4 knockdown. During the study it became clear that *HOXA7* is vital to the survival of MLL/AF4 positive malignant cells. In knockdown experiments with *HOXA7* its central role in maintaining the viability of MLL/AF4 positive SEM cells could clearly be shown. Beyond that, *HOXA7* binding to the promoter of *TERT*, the reverse transcriptase that maintains the telomeres, was found and thereby contributed to the immortality of the malignant cells. Interestingly over expression of *HOXA7* in t(4;11) positive cells turned out to be lethal. The reason for this remains unclear. There is still the question as to whether *HOXA7* by itself could transform HSCs into their malignant form.

While *HOXA7*, *HOXA9* and *HOXA10* knockdown was concomitant with previous data, a new target gene was identified in *HOXA6* and another possible target gene in *HOXA4*. The *HOXA* genes have been recently described as bearing transforming potential in haematopoietic pluripotent cells (Bach et al., 2010) and to be directly

associated to the MLL complex (Guenther et al., 2008), especially HOXA7 and HOXA9. In addition, the important HOX co factor MEIS1 was demonstrated to be essential for leukaemic survival and greatly improves the transforming potential of malignant haematopoietic cells (Ayton and Cleary, 2003, Faber et al., 2009, Mamo et al., 2006, So et al., 2004). The exact role of the HOX genes in leukaemogenesis is still unclear. Their contributing characteristics are widely discussed in the literature. Comparing cell lines from *de novo* patients (MV4;11 and BLIN-3) and relapse patients (SEM and RS4;11) it appears that HOXA7 may play a role in the relapse patients which suffer from a more aggressive and therapy resistant form of leukaemia. One must be careful as this is data based on cell lines and may not reflect the data from primary patient material. Here the question rises as to why t(4;11) positive ALL patients with positive HOXA status have a better prognosis than HOXA negative patients (Stam et al., 2009, Trentin et al., 2009). More investigations in the genomic expression pattern of *de novo* patients compared with relapse patients would clarify this situation.

A common finding in most forms of cancer is a high activity of the telomerase, an enzyme complex that maintains the telomeres, thereby contributing to the immortality of the malignant cells (Davison, 2007). The question was addressed as to whether the telomerase activity is affected by *MLL/AF4* knockdown. A decrease in telomerase activity of about 70 % was found upon *MLL/AF4* depletion. Further analyses were performed to see whether the protein subunit *TERT* or the RNA component *TERC* was affected by *MLL/AF4* knockdown. We identified *TERT*, the reverse transcriptase of the telomerase, as being affected by *MLL/AF4* knockdown. Further experiments showed that *TERT* was also depleted by siRNA mediated knockdown of *HOXA7* which suggested *TERT* being a target of *HOXA7*. *TERC* did not show any changes upon *MLL/AF4* depletion. Subsequently the question was asked as to whether *TERT* is a direct target of *HOXA7*. In first instance, *in silico* experiments were performed and several putative binding sites for *HOXA7* in the promoter region of *TERT* were found. Primers were designed for several regions in the *TERT* promoter and ChIP experiments were performed to investigate the matter. Binding of *HOXA7* directly to the promoter of *TERT* was detected. Epigenetic

marking of TERT has been previously reported by others (Guenther et al., 2008). In our scenario it looks like the binding of *HOXA7* is crucial for *TERT* expression. Comparing t(4;11) cell lines SEM, RS4;11 and MV4;11, a correlation was shown between *HOXA6*, *HOXA7* and *TERT* expression. Both ALL cell lines SEM and RS4;11 showed *HOXA6*, *HOXA7* and *TERT* expression while the AML cell line MV4;11 did not. Whether this difference is dependent on the lineage AML vs. ALL or relapse vs. *de novo* disease has to be confirmed in further experiments. Application of siRNA against *TERT* could also lead to results which show the importance of *TERT*. As TERT is a key component in the telomerase and thereby for telomere maintenance, the loss of *TERT* would have result in telomere shortening and induction of apoptosis as a consequence. The t(4;11) positive cell line SEM showed an increased apoptosis rate upon *MLL/AF4* depletion whereupon *TERT* depletion is a consequence. Whether *TERT* depletion is decisive for apoptosis induction needs to be investigated.

The function of TERT being an inhibitor for apoptosis is currently discussed in the literature (Lee et al., 2008, Zhang et al., 2003). SEM cells were treated with siRNA against *MLL/AF4* and additionally with or without zVAD, a pancaspase inhibitor which inhibits apoptosis induction. zVAD treated cells showed lower *TERT* expression after *MLL/AF4* depletion than cells without zVAD. This leads to the assumption that cells with low *TERT* expression die faster than cells with high *TERT* expression. Here it is possible that TERT inhibits apoptosis by a mechanism so far not fully understood. This mechanism involves a family of proteins called 14-3-3 which may shuttle TERT to the mitochondrion and prevents cytochrome c release thus inhibiting apoptosis (Seimiya et al., 2000). 14-3-3 proteins or even their genes are also possible targets to be investigated for treatment in leukaemia. These proteins are involved in many processes like signalling, cell cycle and apoptosis (van Hemert et al., 2001).

3.4 Future work

Knockdown of *MLL/AF4* causes depletion of *HOXA6*, *HOXA7*, *HOXA9* and *HOXA10*. Furthermore *HOXA4* seems to be affected but needs to be validated in more *MLL/AF4* knockdown experiments and at the RNA as well as at the protein level. To investigate the function of *HOXA4*, *HOXA6*, *HOXA9*, *HOXA10* and also *TERT*, siRNAs must be designed and knockdown experiments in t(4;11) cell lines performed. The inclusion of the cell line BLIN-3 to the already available cell lines SEM, RS4;11 and MV4;11 would allow the influence of *HOXA* genes on the malignant cells to be further investigated. Initial comparison experiments should be performed to investigate overall expression of the *HOXA* genes and *TERT* in all cell lines, including other AML and ALL cell lines. One could also think of adding primary patient material and carefully distinguish between *de novo* and relapsed patients.

Another important step would be the generation of leukaemic cell lines that stably over express one of the candidate *HOXA* genes or *TERT* or both, using the existing cell lines such as SEM, RS4;11 and MV4;11. *MLL/AF4* knockdown experiments could give then further insight into the importance of *HOXA* genes. Overexpression of *TERT* and knockdown experiments targeting the *HOXA* genes would clarify the importance of *TERT*. Attempts done in this thesis did not lead to the establishment of such cell lines.

To investigate *HOXA7* binding to the *TERT* promoter, luciferase assays could be established. As a starting point, the already published sequence of the *TERT* core promoter could be used (Takakura et al., 1999). Attempts could be made to clone this promoter into a luciferase gene harbouring vector and to express the protein under control of the *TERT* promoter to measure the binding of recombinant *HOXA7* in an overexpressing cell line. Investigating truncated versions of the *TERT* promoter could then finally lead to the exact position of the *HOXA7* binding site.

Another subject for further studies could be the 14-3-3 proteins and their role in the *TERT* localisation in the malignant cell. It is possible that investigation of the role of these proteins in the regulation of cellular processes in leukaemia may give rise to further studies or even lead to targets for treatment. Firstly, it should be investigated

if they are aberrantly expressed in leukaemic cells. Following this, the effects of MLL/AF4 downregulation on 14-3-3 proteins could be investigated. Several forms of the *14-3-3* genes have been identified to be affected by *MLL* and *AF4*. Epigenetic modification of H3K4 and H3K79 at the locus of those forms has also been reported (Guenther et al., 2008). One of these forms is 14-3-3zeta which is already associated with TERT nuclear localisation (Seimiya et al., 2000, Zhang et al., 2003). This form would be the primary target for further analyses.

4 Materials and Methods

4.1 Materials

All details and specifications are standard unless stated otherwise.

General lab equipment is from Scientific Laboratory Supplies (Nottingham, UK), VWR (Lutterworth, Leicestershire, UK) or Fisher Scientific (Loughborough, Leicestershire, UK).

4.1.1 *Devices*

ABI 7000 Real Time detection system Germany	Applied Biosystems, Darmstadt, Germany
ABI 7500 Fast Real-Time PCR system	Applied Biosystems, USA
ABI 7000 SDS program	Applied Biosystems, USA
ABI 7900HT Sequence detection system	Applied Biosystems, USA
ABI SDS 2.2	Applied Biosystems, USA
Agarose gel electrophoresis unit Herts., UK	BIO-Rad, Hemel Hempstead, Herts., UK
Electroporation-Impulsegenerator EPI 2500	Heidelberg, Germany
FACSCalibur Germany	Beckton Dickinson, Heidelberg, Germany
FACScan	Beckton Dickinson, Oxford, UK
FACSCanto	Beckton Dickinson, Oxford, UK
FujiFilm Luminescent Image Analyser LAS-300 Japan	FujiFilm Microdevices Co., Ltd., Japan
Fuji X-Ray Film processor RG11 Japan	Fuji Photo Film Co. Ltd., Tokyo, Japan

MINI-PROTEAN II electrophoresis cell Herts., UK	BIO-Rad, Hemel Hempstead,
MINI Trans-Blot Electrophoretic transfer cell Herts., UK	BIO-Rad, Hemel Hempstead,
MSE Soniprep 150 plus sonicator	MSE (UK) limited, London, UK
ND-1000 spectrophotometer USA	Nanodrop technologies Ltd.,
Spectramax 250 multiwell plate reader	Molecular devices, Crawley, UK

Centrifuges

Centrifuge 5415C	Eppendorf, Hamburg, Germany
Centrifuge 5415R	Eppendorf, Hamburg, Germany
L870M Ultracentrifuge	Beckman, High Wycombe, UK
Allegra® X-12 benchtop centrifuge Wycombe, UK	Beckman Coulter, High

4.1.2 Buffers and solutions

All standard chemicals were purchased either from SIGMA (Poole, Dorset, UK), Fisher Scientific (Leicestershire, UK) or BDH (Dorset, UK).

Protein extracts and western blotting

Urea Buffer

9 M Urea

4 % CHAPS

1 % DTT

pH 5.0

stored at -20°C

Bradford Reagent Concentrate Bio-Rad Protein Assay BIO-Rad, Hemel
Hempstead, UK

2x SDS Loading buffer

100 mM Tris, pH 6.8

2.5 mM EDTA, pH 8.0

25 % Glycerin

0.05 % Bromphenolblue

4 % sodium dodecyl sulphate (SDS)

100 mM DTT

Separating Gel:

	7 %	8 %	10 %	12 %	15 %
H ₂ O	5.75 ml	5.5 ml	5 ml	4.5 ml	3.7 ml
4x separation gel-buffer	2.5 ml				
40 % PAA	1.75 ml	2 ml	2.5 ml	3 ml	3.8 ml
Mix well					
10 % APS	100 µl				
TEMED	10 µl				

Stacking Gel:

3.9 ml H₂O

5 ml 2x stacking gel buffer

1 ml 40 % PAA (Sigma, 19:1)

Mix well

100 µl 10 % APS

10 µl TEMED

10x Tris – Glycine buffer 14.4 % Glycine

3 % Tris

1x electrophoresis buffer 1/10 vol. 10x Tris-Glycine buffer

0.1 % SDS

1x blotting buffer	1/10 vol. 10x Tris-Glycine buffer
	10 % Methanol
TST	10 mM Tris, pH 7.5
	100 mM NaCl
	0.1 % Tween20
	1 mM EDTA, pH 8.0
Blocking solution	TST buffer + 10% skim milk
ECL Plus Western Blotting	Amersham Biosciences, Freiburg, Germany
Detection Reagents	
Immobilon Western	Millipore, Watford, Hertfordshire, UK
Chemoluminescent HRP solution	
Immobilon-P Membrane, PVDF	Millipore, Watford, Hertfordshire, UK

Stripping Buffer	100 mM β -Mercaptoethanol
	2% SDS
	62.5 mM Tris, pH 6.7

E.coli transformation

LB-Medium	10 g/l Bacto-Trypton (Applichem, Darmstadt)
	5 g/l Yeast Extract
	10 g/l NaCl
	pH 7.4
	sterilized by autoclaving

Modifying Enzymes

Klenow Fragment

Calf intestinal alkaline phosphatase, CIAP

Chromatin Immunoprecipitation (ChIP) Assay with Staphylococcus aureus

Cell lysis buffer	5mM	PIPES, pH8
	85mM	KCl
	0.5%	NP40
Nuclei lysis buffer	50mM	Tris-Cl, pH 8.1
	10mM	EDTA
	1%	SDS
	+ Complete, 1 tablet (right before use)	
IP dilution buffer	0.01%	SDS
	1.1%	Triton X100
	1.2mM	EDTA
	16.7mM	Tris-Cl, pH 8.1
	167mM	NaCl
	+ Complete, 1 tablet (right before use)	
1x Dialysis buffer	2mM	EDTA
	50mM	Tris/HCl, pH 8

IP Wash buffer	100mM	Tris/HCl, pH 9
	500mM	LiCl
	1%	NP40
	1%	deoxycholic acid
IP elution buffer	50mM	NaHCO ₃
	1%	SDS
5x PK buffer	50mM	Tris/HCl, pH 7.5
	25mM	EDTA
	1.25%	SDS

- 37% Formaldehyde
- 1.25M Glycine
- Complete™ Mini, EDTA-free, REF 11 836 170 001, 25 tablets, Protease Inhibitor cocktail tablets, Roche, Basel, CH
- PBS (+ Complete™)
- RNaseA (10mg/ml)
- 5M NaCl
- 100% EtOH
- TE Buffer
- 1:1 mixture of Phenol and Chloroform/Isoamyl alcohol (24:1)
- Chloroform/Isoamyl alcohol (24:1)
- 3M NaAc

Hybridization of siRNAs

SiRNA Hybridization buffer	100 mM NaCl
	25 mM Tris-HCl, pH7.5

Cell cycle analysis

Citrate buffer	0.25 M Saccharose
	40 mM Natriumcitrate, pH 7.6

DNA-staining and lysis buffer	PBS
	20 µg/ml PI
	0.5% NP-40
	0.5 mM EDTA

Cell cycle wash buffer	PBS
	0.5% BSA

FACS-buffer	PBS
	2 mM EDTA
	0.1% BSA

Telomerase activity assay

Roche Complete™ Protease Inhibitor Cocktail Tablets	Chymotrypsin, 1.5µg/ml
	Thermolysin, 0.8µg/ml
	Papain, 1mg/ml
	Pronase, 1.5µg/ml
	Pancreatic extract, 1.5µg/ml

	Trypsin, 0.002µg/ml
Telomerase lysis buffer	10 mM Tris/HCl, pH 7.5
	1 mM MgCl ₂
	1 mM EGTA
	5 mM β-Mercaptoethanol
	0.5% CHAPS
	10% Glycerol
	+Complete™, 1 tablet

Real Time RT-PCR

SYBR Green QCR Supermix w/ROX	Invitrogen, Paisley, UK
RevertAid™H Minus First Strand cDNA Synthesis Kit	Fermentas, York, UK

Electroporation

Cuvettes 4mm par 50 sans pipet Southampton, UK	Eurogentec,
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4.1.3 Kits

RNeasy Total RNA Isolation Kit UK	Qiagen, Crawley, West Sussex,
QIAquick PCR Purification Kit UK	Qiagen, Crawley, West Sussex,
QIAquick Gel Extraction Kit UK	Qiagen, Crawley, West Sussex,

Miniprep Plasmid Isolation Kit UK	Qiagen, Crawley, West Sussex,
Maxiprep Endofree Plasmid Isolation Kit UK	Qiagen, Crawley, West Sussex,
AMAXA Nucleofection Kit	Lonza
Nucleofector solution R	

4.1.4 Antibodies

Primary

Anti HOXA6 Antibody #H00003203-M01	Abnova Cooperation, UK
Anti HOXA7 Antibody #ab-70027-100	Abcam, Cambridge, UK
Anti HOXA9 Antibody #07-178	Millipore/Upstate, Watford, UK
Anti hTERT Antibody #1531-1 Germany	Epitomics/BIOMOL, Hamburg,
Anti glyceraldehyde-3-phosphate dehydrogenase (GAPDH) Antibody #5G4	HyTest Ltd. Turku, Finland

Secondary HRP conjugated

Anti rabbit IgG #P0217	Dako, Ely, Cambridgeshire, UK
ECL™ Anti mouse IgG #NA931V	GE Healthcare, Bucks, UK
Anti goat IgG #SC-2020	Santa Cruz, USA

4.1.5 Synthetic Oligonucleotides

Oligonucleotides were purchased from Purimex (Grebenstein, Germany), Alnylam Europe (Kulmbach, Germany), VHBio (Gateshead, UK), Eurofins-MWG-Operon (London, UK) or Qiagen (Crawley, UK)

siRNA

MLL/AF4 siRNA “siMA6”

sense; 5'-AAG AAA AGC AGA CCU ACU CCA-3'

antisense; 5'-UGG AGU AGG UCU GCU UUU CUU UU-3'

siRNA control siMM

sense; 5'-AAA AGC UGA CCU UCU CCA AUG-3'

antisense; 5'-CAU UGG AGA AGG UCA GCU UUU CU-3'

siRNA control siAGF1

sense; 5'-CCUCGAAAUCGUACUGAGAAG-3'

antisense; 5'- UCUCAGUACGAUUUCGAGGUU -3'

HOXA7 siRNA “siHOXA7”

sense; 5' - CCGUUCCGGGCUUAUACAAUG - 3'

antisense; 5' - UUGUAUAAGCCCGGAACGGUC – 3'

hTERT siRNA “siRNA-5 TERT”

sense; 5' AAC ACG GUG ACC GAC GCA CUG 3'

antisense; 5' UUC AGU GCG UCG GUC ACC GUG 3'

hTERT siRNA "siRNA-41 TERT"

sense; 5' AAA AGA GGG CCG AGC GUC UCA 3'

antisense; 5' UUU GAG ACG CUC GGC CCU CUU 3'

Primers for RealTime-PCR

HoxA1	forward	5'-ACC CCG CCA GGA AAC G-3'
HoxA1	reverse	5'-GGA ACG CAG GGC GAA GA-3'
HoxA2	forward	5'-AGA ACT GTG GAG CTG GCC TAA AC-3'
HoxA2	reverse	5'-GAA AAC GCT AAA GTC CTG CAA AGA-3'
HoxA3	forward	5'-CCA ATC TGC TGA ACC TCA CTG A-3'
HoxA3	reverse	5'-GCA TGC CCT TGC CCT TCT-3'
HoxA4	forward	5'-GGT GTA CCC CTG GAT GAA GAA G-3'
HoxA4	reverse	5'-CTT AGG CTC CCC TCC GTT ATA AC-3'
HoxA5	forward	5'-CGC CCA ACC CCA GAT CTA C-3'
HoxA5	reverse	5'-GGC CGC CTA TGT TGT CAT G-3'
HoxA6	forward	5'-CGG TTT ACC CTT GGA TGC A-3'
HoxA6	reverse	5'-GCC CAT GGC TCC CAT ACA C-3'
HoxA7	forward	5'-GAG GCC AAT TTC CGC ATC TA-3'
HoxA7	reverse	5'-GCG GTT GAA GTG GAA CTC CTT-3'
HoxA9	forward	5'-CCA CCA TCC CCG CAC A-3'
HoxA9	reverse	5'-AAC AGG GTT TGC CTT GGA AA-3'

HoxA10	forward	5'-CAG GCC ACC TCG TGC TCT T-3'
HoxA10	reverse	5'-TTT GTC CGC CGA GTC GTA G-3'
HoxA11	forward	5'-TCG TCT TCC GGC CAC ACT-3'
HoxA11	reverse	5'-TCT CGG ATC TGG TAC TTG GTA TAG G-3'
HoxA13	forward	5'-CCA AAT GTA CTG CCC CAA AGA-3'
HoxA13	reverse	5'-AGA CCA CGT CGG GCA GAG T-3'
TERC	forward	5'-GCT GTT TTT CTC GCT GAC TTT CA-3'
TERC	reverse	5'-GCA GCT GAC ATT TTT TGT TTG C-3'
TERT	forward	5'-GGA GAA CAA GCT GTT TGC GG-3'
TERT	reverse	5'-AGG TTT TCG CGT GGG TGA G-3'
GAPDH	forward	5'-TGG CAT GGC CTT CCG T-3'
GAPDH	reverse	5'-TCT CCA GGC GGC ACGT T-3'
HPRT	forward	5'-TGA CAC TGG CAA AAC AAT GCA-3'
HPRT	reverse	5'-AGC TTG CGA CCT TGA CCA TC-3'
RPLP01	forward	5'-TCT GAT TGG CTA CTT TGT TGC C-3'
RPLP01	reverse	5'-CCT TCC ACG AGG ACG CCT-3'
28S	forward	5'-AAG CAA GGC CTC TGG AAA AC-3'
28S	reverse	5'-ATG CAG TAT GTC TTA GCC TTA A-3'
RPLP0M	forward	5'-TGG GCA AGA ACA CCA TGA TG-3'
RPLP0M	reverse	5'-AGT TTC TCC AGA GCT GGG TTG T-3'

Plasmid Maps:

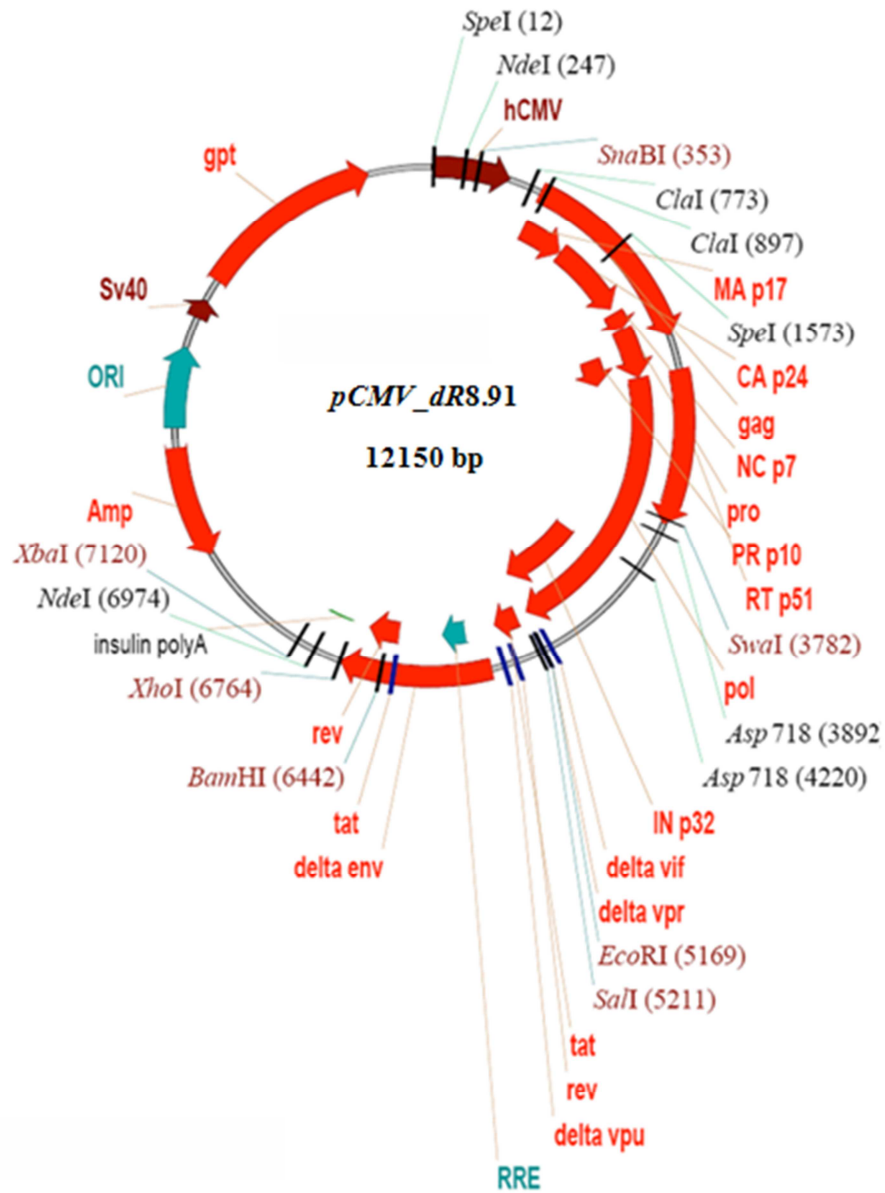


Figure 4-1pCMV_dR8.91
Plasmidmap of the pCMV_dR8.91 packaging vector

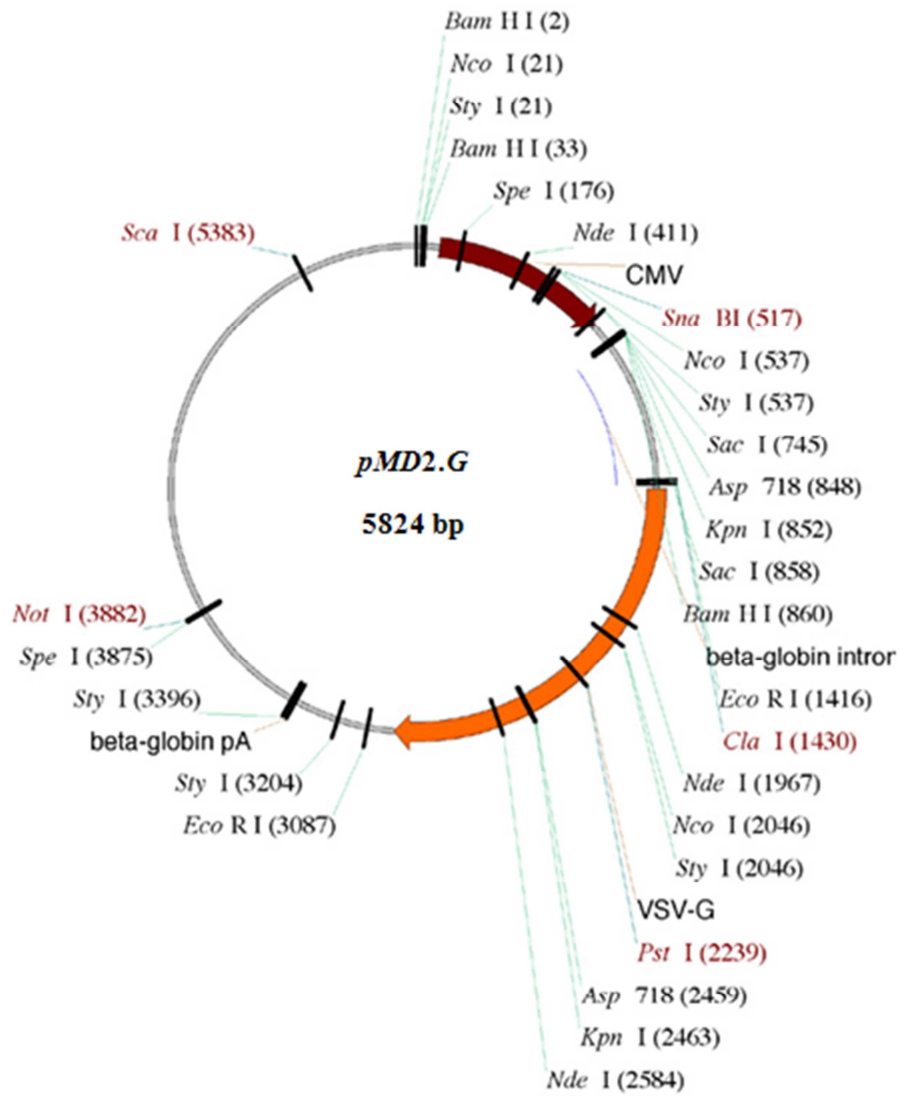


Figure 4-2 pMD2.G
Plasmidmap of the pMD2.G enveloping vector

4.2 Methods

4.2.1 Cell lines and Cell culture (Sources: DSMZ, Germany)

Leukaemic cell lines (immunology and cytogenetics from DSMZ, Germany):

MV4;11 (AML):

Original cells were obtained from a 10 year old boy with acute monocytic leukaemia (AML FAB M5). The MV4;11 cell line was established in 1987 by Lange and colleagues (Lange et al., 1987).

Immunology: CD3 -, CD4 +, CD5 -, CD8 -, CD10 -, CD13 +, CD14 -, CD15 +, CD19 -, CD21 -, CD25 -, CD33 +, CD37 -, CD68 +, CD138 -, HLA-DR +

Cytogenetics: human hyperdiploid karyotype - 48(46-48)<2n>XY, +8, +18, +19, -21, t(4;11)(q21;q23)

RS4;11 (ALL):

Original cells were obtained from a 32 year old woman with acute lymphoblastic leukaemia in first relapse. The cell line was established by Stong and colleagues in 1985 (Stong and Kersey, 1985)

Immunology: CD3 -, CD10 -, CD13 -, CD19 +, CD20 -, CD34 (+), CD37 -, cyCD79a +, CD80 -, CD138 -, HLA-DR +, sm/cyIgG -, sm/cyIgM -, sm/cykappa -, sm/cylambda -

Cytogenetics: human hyperdiploid karyotype - 47/48<2n>X/XX, +8, +18, t(4;11)(q21;q23), i(7q) - sideline without +8/18 - carries MLL-AF4 fusion gene - resembles published karyotype

SEM (ALL):

Original cells were obtained from the peripheral blood of a 5 year old girl at relapse of acute lymphoblastic leukaemia (ALL). The cell line was established by Greil and colleagues (Greil et al., 1994)

Immunology: CD3 -, CD4 -, CD13 +, CD14 -, CD15 +, CD19 +, CD33 +, CD34 -, HLA-DR +, sm/cyIgG -, sm/cyIgM -, sm/cykappa -, sm/cylambda –

Cytogenetics: human hypodiploid karyotype with 1.5% polyploidy - 45(40-46)<2n>XX, -13, t(4;11)(q21;q23), del(7)(p14) - carries t(4;11) with breakpoints at AF4 and MLL - matches published karyotype

Other cell lines:

293T

Human embryonic kidney cell line, modified with human adenovirus type 5 (Graham et al., 1977). The SV 40 large T-antigen was introduced into the cells which makes them a perfect host for virus production. The SV 40 large T antigen allows for replication of transfected episomes that contain the SV 40 origin of replication. An episome is a piece of plasmid DNA which is located in the nucleus but not integrated into the genome of the host cell.

Cytogenetics: human flat-moded near-triploid karyotype; 61-73<3n>XXX, add(1)(p32), add(1)(q44)x1-2, der(4;10)(q10;q10), del(8)(p12), t(9;9)(p24;q21), der(13)t(6;13)(q21;q32), der(14)t(13;14)(q32;q32), del(17)(p11), der(22)t(17;22)(q21;q22)

4.2.2 Cell culture

Cells from the cell lineages SEM, RS4;11 and MV4;11 were cultured in RPMI1640 Medium (SIGMA, HEPES Modification, #R5886) supplemented with 10% FCS (SIGMA, #F7524) and 2mM L-Glutamine (SIGMA, #G7513). The 293T cells were cultured in DMEM (SIGMA, #D6171) and also supplemented with FCS and 2mM L-Glutamine. All cells were incubated at 37°C, 5% CO₂ and 95% air humidity. SEM, RS4;11 and MV4;11 cells were split every two days to a final concentration of 5x10⁵cells/ml. 293T cells were split at a ratio of 1:10 when their confluence reached about 80%.

4.2.3 Freezing and thawing of cells

Cells were frozen in 800 µl of medium, containing 40% FCS and 10% DMSO. A typical total cell number for freezing was 5x10⁶ cells. The cells were put into cryovials (Nunc) and frozen at -80°C for 24 hours. After that time the cells were put into liquid nitrogen for long term storage.

For thawing, the cells were quickly thawed at 37°C and pre-warmed medium was added to the thawed solution. The cells were pelleted by centrifugation and subsequently resuspended in pre-warmed medium to dilute the cells to the desired concentration.

4.2.4 Determination of cell concentration

To determine the cell concentration in a culture flask a hemacytometer was used (Figure 4-3). The hemacytometer is a counting chamber called “Neubauer improved Counting Chamber”.

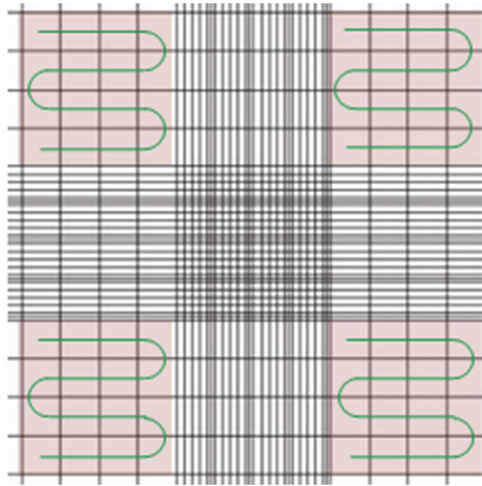


Figure 4-3 Cell counting using a Neubauer counting chamber

The picture shows the counting grid on a improved Neubauer counting chamber. Cells in the four red highlighted were counted as illustrated in the picture (green lines); taken from www.carl-roth.de .

After counting all squares, the following equation gave the concentration of cells per ml medium:

$$\frac{\text{counted cellnumber}}{4} * 10^4 = \text{cells/ml medium}$$

4.2.5 Treatment of SEM cells with zVAD

zVAD or zVAD-FMK (carbobenzoxy-valyl-alanyl-aspartyl-[O-methyl]-fluoromethylketone) is a pan caspase inhibitor (*pan* = Greek meaning “all”) which binds irreversibly to the catalytic centre of the major caspases and by this blocks apoptosis. zVAD inhibits all caspases except CASP2.

The substance was dissolved in DMSO following manufacturer’s protocol, resulting in a 20 mM stock solution. As DMSO is cytotoxic, the stock dilution was pre-diluted 10-fold in medium, yielding a second stock solution with concentration of 2 mM. Just before seeding the electroporated or otherwise treated cells, the 2mM stock solution was added to the cell culture medium to get the desired concentration. Cells

were cultured as described in section 4.2.2. The end concentration in medium was 50 μ M. 1mg of zVAD was diluted in 107 μ l DMSO to get 20 mM stock solution. Frozen at -20° C, the solution is stable for about a month. Before use, the DMSO stock solution was diluted 1:10 in medium (no additives) (2mM end concentration). The final dilution was 1:40 in cell culture medium.

4.2.6 Hybridisation of siRNA

siRNA sense and antisense strands were diluted in hybridization buffer to a final concentration of 20 μ M, placed into a thermal block and incubated at 95° C for 30 seconds. Subsequently the heat-block was cooled down to room temperature. When the thermal block reached this temperature, the siRNA solution was aliquoted (in order to avoid unnecessary handling of the sample) and frozen at -20° C.

4.2.7 Molecular Cloning

Restriction of plasmid DNA

The restriction of plasmid DNA was performed for three purposes: Either to cut a plasmid for a following cloning step, to check the size of a certain plasmid or to separate and isolate a specific fragment of the plasmid.

The plasmid DNA was digested according to the manufacturer's specifications. Normally 1 μ g to 2 μ g of plasmid DNA were used for digestion. The reaction mix contained 1x concentration of the enzyme specific reaction buffer, normally 5 units of enzyme and was filled to the desired volume with ddH₂O. The reaction was incubated for 1 hour at 37°C if not otherwise stated. For double digestion the reaction buffer was chosen after using the Fermentas Double digest program (<http://www.fermentas.com>). The restriction enzymes were inactivated by the addition of EDTA followed by incubation at 65°C for 20 minutes.

Isolation of plasmid DNA fragments

To isolate the desired DNA fragment, the digested plasmid DNA solution was separated on a 1 % agarose gel by electrophoresis. After addition of DNA loading dye the solution was pipette into the loading pockets of the gel and run with 120V for

one hour or until the bromophenol blue marking has reached the lower quarter of the gel.

After electrophoresis, the gel was stained with ethidium bromide (EtBr) for at least 30 minutes. The ethidium bromide bath contained 200ng EtBr diluted in one litre water. Subsequently the gel was placed onto a UV table and the desired band was cut out with a scalpel. The fragments were then isolated by using the QiaQuick Gel Extraction Kit. Briefly the gel slice was weighed and dissolved in buffer QG. After adding one gel volume of isopropanol, the solution was transferred to a QiaQuick column. After several washing steps the fragments were eluted with water or EB buffer.

Modification of plasmid DNA

For molecular cloning it is sometimes necessary to modify the linearised vector to avoid religation in following steps or to fill in sticky ends to perform a blunt end ligation.

For some cloning reactions a blunt end ligation seems to be the optimal way. If the plasmid DNA was cut with enzymes which produce sticky ends these have to be altered to blunt ends. To perform this fill-in reaction the Klenow fragment was used according to the manufacturer's protocol. The Klenow fragment is part of the polymerase I enzyme and has endonuclease activity, but no exonuclease activity.

For the fill-in reaction 20 µl of the digested plasmid solution was mixed with 1 µl EcoPol buffer (10 mM Tris-HCl at pH 7.5; 5 mM MgCl₂; 7.5 mM DTT), 1 µl 10mM dNTPs, 1 µl Klenow (5u/ µl) and filled with 7 µl of water. The reaction was incubated for 15 minutes at room temperature and subsequently deactivated by heating to 95°C for 3 minutes. The plasmid DNA was isolated by agarose gel electrophoresis and extracted by using the QiaQuick gel Extraction Kit.

To avoid religation of the linearised vector, the digested plasmid was treated with calf intestinal alkaline phosphatase (CIAP) according to the manufacturer's protocol. CIAP dephosphorylates the ends of an open vector. Without a free phosphate-group the ends of the opened vector cannot religate. For dephosphorylation the desired plasmid fragments were mixed with 10xCIAP buffer (50mM Tris-HCl (pH 9.3 at

25°C), 1mM MgCl₂, 0.1mM ZnCl₂ and 1mM spermidine), 2 µl CIAP (1u/ µl) and water. The dephosphorylation was performed for 30 minutes at 37°C. For inactivation the solution was incubated at 95°C for 3 minutes.

Ligation

For the ligation, the insert fragment and the vector were mixed in a 3:1 ratio based on their molecular weight. The ligation mix contained the insert-vector mix, 10% v/v ligase buffer (400 mM Tris-HCl, 100 mM MgCl₂, 100 mM DTT, 5 mM ATP (pH 7.8 at 25°C), 1 µl of T4 ligase (4u/ µl) and was filled to 20 µl with water. The reaction was incubated for 4-6h at room temperature. The reaction was inactivated by adding EDTA and incubation at 65°C for 20 min. The complete ligation was then used for the transformation.

Transformation of bacteria

For transformation of expression and cloning vectors the *E. coli* strain DH5α was used. All lentiviral vectors were replicated by transforming the *E. coli* strain Stbl3 from Invitrogen (One Shot ® Stbl3™ chemically competent *E. coli*; Genotype: F-*mcrB mrr hsdS20* (r_B⁻,m_B⁻) *recA13 supE44 ara-14 galK2 lacY1 proA2 rpsL20*(Str^R *xyl-5 λ-leu mtl-1*). 100 µl of the desired competent bacteria were mixed with the whole ligation solution and incubated on ice for 20 minutes. The mixture was then incubated for exactly 90 seconds in 42° C warm water and put back on ice for another 5 minutes. After addition of 400 µl of warm LB-buffer the mixture was incubated for 30 minutes at 37°C while constantly shaking. Thereafter the mixture was plated onto agar plates containing ampicillin as the selection marker. The plates were then incubated at 37°C overnight.

Isolation of plasmid DNA from bacteria

MiniPrep: For preparation of a MiniPrep, one single colony from was picked from a selective plate and inoculated in 3ml LB Medium overnight at 37°C on a shaking platform. The next day, the bacteria were harvested by centrifugation at 6800g for 3 minutes. The pellet was resuspended in buffer P1 (50 mM Tris-Cl, pH 8.0; 10 mM EDTA; 100 µg/ml RNaseA) and transferred to a micro centrifuge tube. Lysis buffer P2 (200 mM NaOH; 1% SDS w/v) was added subsequently and the tube was

inverted several times. After complete lysis of the bacteria, the neutralisation buffer P3 (3.0 M potassium acetate, pH 5.5) was added and the tube again was inverted. The tubes were then centrifuged for 10 minutes at 17 000 g to pellet the debris. The supernatants were transferred to a QiaPrep Spin Column and centrifuged at 17 000 g for 1 minute followed by two washing steps with buffer PB. The plasmids were eluted by adding 50 µl water to the column and centrifugation for one minute at 17 000 g.

MaxiPrep (optional endofree): For the MaxiPrep, 2ml of cultured plasmids (remaining culture of the MiniPrep pre-culture) were added to 100 ml – 150 ml LB medium and cultured overnight at 37° C on a shaking platform. The next day, the bacteria were harvested by centrifugation with 6000 g at 4° C for 20 minutes. The pellet was resuspended in buffer P1 and lysed by addition of buffer P2 followed by a 5 minute incubation at room temperature. The lysis was neutralised by addition of buffer P3 and incubation on ice for 20 minutes. The mixture was then applied to a QiaFilter cartridge and filtered into a new 50ml tube. Where required an endotoxin removal step was performed at this point by adding buffer ER to the filtered lysate. During the incubation, the QiaTip 500 columns were equilibrated by applying buffer QBT to them. The neutralised mixture was then centrifuged for 30 minutes with 20 000 g at 4° C. The supernatant was applied to the equilibrated Tip500 and washed twice with buffer QC. The plasmids were eluted with buffer QF.

4.2.8 Typical setup for transfection experiment

Day 0	Transfection of target cells with siRNA MOCK, active siRNA, control siRNA 1st electroporation
Day 1 24h	↓
Day 2 48h	<u>Optional</u> : harvest cells and analysis
	Transfection of target cells with siRNA 2nd electroporation
Day 3 72h	↓
Day 4 96h	<u>Optional</u> : harvest cells and analysis
	Transfection of target cells with siRNA 3rd electroporation
Day 5 120h	↓
Day 6 144h	Harvesting of treated cells. Analysis by various methods including protein analysis and qRT-PCR

Figure 4-4 Overview of a typical transfection experiment

The flowchart shows the typical setup for the transfection of a target cell line with siRNAs and procedures for subsequent analysis.

Figure 4-4 shows briefly how a typical transfection experiment was performed. On day 0 target cells were electroporated with according siRNAs and cultured for 48h at optimal cell line specific conditions. Depending on the experimental setup cells were harvested on day 2 and analysed or electroporated for a second time. This procedure was repeated on day 4. On day 6 all remaining cells were harvested and analysed. A range of analytical methods was used included fluorescent activating cell sorting (FACS), protein analysis in form of western blots and mRNA quantification by quantitative reverse transcriptase polymerase chain reaction.

4.2.9 Electroporations

The standard method for the transfection of siRNAs was the electroporation with the pulse generator EPI2500 (Dr. L. Fischer, Heidelberg), shown in figure 4-5. Cells were electroporated in 100 – 800 µl culture medium in 4mm cuvettes at a concentration of 1×10^7 cells/ml. If not indicated otherwise, the final siRNA concentration was 500 nM diluted from a 20 µM stock. The electroporation conditions varied between the different cell lines. SEM and MV4;11 cells were electroporated at 350V with a 10ms pulse. For RS4;11 cells, 370V and a 10ms pulse was used. After siRNA treatment, the cells were left in the cuvettes for 15 minutes at RT and subsequently diluted twenty-fold into culture flasks.

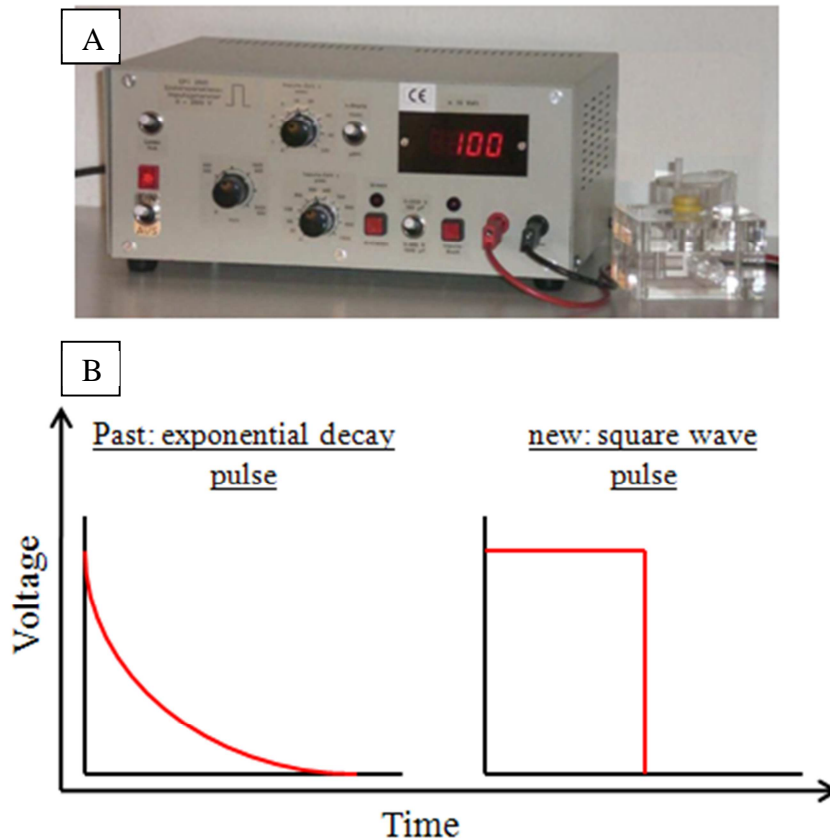


Figure 4-5 The Fischer EPI2500 electroporator

(A) Picture of the EPI2500 electroporator used during this study. (B) represents the advantage of the square pulse, generated by the EPI2500. The advantage compared to older methods is the constant upkeep of the voltage during the electroporation time (red line). Taken from www.electroporation.eu

For nucleofection, the transfection of plasmid DNA directly into the nucleus, the procedure was performed according to the protocol for the AMAXA Nucleofection kit. Cells were electroporated in cuvettes provided with the kit at a concentration of 1×10^6 cells/100 μ l. As suggested by the protocol, 2 μ g of plasmid DNA was added and immediately electroporated with a 15ms pulse at 250V. Subsequently the cells were cultured in the according medium at the optimum cell concentration.

4.2.10 Isolation of total RNA and protein

For the isolation of total RNA and protein, the Qiagen RNeasy Kit was used and the method was performed according to the protocol. Basically cells were harvested and washed once with PBS. After centrifugation, the cell pellet was lysed in RLT buffer supplemented with β -Mercaptoethanol (10 μ l/1ml RLT). The lysate was transferred

onto a Qiagen Shredder column and spun for 2min at full speed (~13000 rpm, depending on the centrifuge) in a table centrifuge at room temperature (RT). After adding 350 µl of 70% ethanol to the flow through, the lysate was transferred to a QIAGEN RNeasy column and spun for 1min at full speed. The flow through was collected for further protein isolation, the RNA was further isolated according to the manufacturers protocol. The total RNA yield was determined by spectrophotometrical measuring with a ND-1000 UV-Vis Spectrophotometer.

The flow through with the protein was transferred to a 2 ml Eppendorf tube and filled up with 100% acetone. The tubes were closed and kept on ice for 30 minutes. Subsequently the protein lysates were centrifuged at full speed in a table centrifuge for 15 min at RT. The supernatant was discarded, the pellet briefly air dried and then solubilised in urea buffer. The protein concentration was determined by Bradford protein assay.

4.2.11 cDNA synthesis – reverse transcriptase PCR (RT-PCR)

To analyse the mRNA it has to be converted to cDNA which is then used in the real time PCR. For the cDNA synthesis, the RevertAid™H Minus First Strand cDNA Synthesis Kit from Fermentas was used according to the manufacturers protocol. In a PCR tube, one microgram of RNA was mixed with random hexamers and the total volume was adjusted to 12 µl. The mixture was incubated at 70° C for 5min. After the incubation, 4 µl of reaction buffer, 2 µl of dNTP mix, 1 µl of RiboLock RNase inhibitor and 1 µl of MuMLV reverse transcriptase polymerase were added. The tube was incubated in a PCR machine with the following conditions:

10min 25°C

60min 42°C

10min 70°C

After the synthesis was done, 30 µl of RNase-free water was added to the mixture. The cDNA was then stored at -20°C.

4.2.12 Quantitative real time polymerase chain reaction (qPCR)

The real time PCR was performed in two different real time PCR machines. The qPCR analysis is made by calculating the cycle threshold (CT) values and plotting them to a graph. The earlier a signal is detected, the more substrate was present in the sample. This can then be correlated with the amount of mRNA extracted from the cells and so levels of expression can be determined.

The following procedure was performed when using the ABI PRISM 7000 Sequence Detection System from Applied Biosystems:

The end concentration for each standard primer except the MLL-AF4 primers is 375 nM. Standard primers were pre diluted to 100 μ M each.

Each MLL-AF4 primer was used at an end concentration of 62.5 nM. The MLL-AF4 primers were pre diluted to 5 μ M each.

For the real time PCR on this machine the following mix was prepared in a 96-well plate:

Master-mix using standard PCR primers:

SYBR [®] Green (2x)		7.5	μ l
Primer fw	(100 μ M \rightarrow 375nM)	0.05	μ l
Primer rev	(100 μ M \rightarrow 375nM)	0.05	μ l
<u>dH₂O</u>		<u>4.4</u>	<u>μl</u>
	$\Sigma =$	12	μ l

For MLL/AF4 primers the following mix has been prepared:

SYBR®Green (2x)		7.5	μl
Primer fw	(5μM→62.5nM)	0.15	μl
Primer rev	(5μM→62.5nM)	0.15	μl
<u>dH₂O</u>		<u>4.1</u>	<u>μl</u>
		Σ =	12 μl

Each sample was prepared in triplicate.

In a 96-well plate 12 μl qPCR-Mix + 3 μl RT-PCR cDNA were mixed.

After preparation, the plate was sealed, short-spun and put into the real time PCR machine (ABI PRISM 7000 Sequence Detection System, Applied Biosystems).

The following program was used:

50°C 2min 1 cycle

95°C 10min _____

95°C 15sec 40 cycles

60°C 1min

The data were analysed with the programs ABI 7000 SDS and Microsoft Excel.

For broader analysis of mRNA the ABI 7900HT Sequence detection system was used. This machine uses 384 well plates and therefore the units for each well changed. Primer concentrations remained the same as for the ABI PRISM 7000 system. Each sample was prepared in triplicate.

Master-mix for all standard primers (except MLL-AF4)

SYBR®Green (2x)	5	μl
Primer fw (100μM → 375nM)	0.03	μl
Primer rev (100μM → 375nM)	0.03	μl
<u>dH2O</u>	<u>2.94</u>	<u>μl</u>
	Σ = 8	μl

Master-mix for MLL-AF4 primers only:

SYBR®Green (2x)	5	μl
Primer fw (5μM → 62.5nM)	0.1	μl
Primer rev (5μM → 62.5nM)	0.1	μl
<u>dH2O</u>	<u>2.8</u>	<u>μl</u>
	Σ = 8	μl

In a 384 well multi-plate 8 μl master-mix together with 2 μl cDNA were prepared in triplets. Acquired data were analysed with the program SDS version 2.2 from Applied Biosystems. Additional to the data from the cDNA amplification, a dissociation curve was acquired. This curve shows at which temperature the amplicons dissociates and the curve shows specificity of the primer and detects unspecific products and primer dimers.

4.2.13 Bradford assay

Before analysing the protein, the protein concentration had to be determined. This was done by using the Bio-Rad protein assay. This assay uses colorimetry to quantify the amount of protein in a certain solution based on the Bradford method. The assay uses Coomassie Brilliant Blue G-250. During the assay, the red form of Coomassie interacts with the protein and denatures it. During the process the blue form of

coomassie becomes stable and the absorption shifts from 465 nm to 595 nm determined with a spectrometer. Briefly a standard curve was created using BSA (0 – 10 µg/ µl). All samples were measured in triplicates. 10 µl of diluted samples and standards were mixed with 190 µl Bradford reagent in a 96-well plate and measured in a Spectramax 250 multi-well plate reader using the SOFTmax PRO analysis software (Molecular Devices).

4.2.14 SDS Gel Electrophoresis and Western Blot

For protein separation a SDS-PAGE (sodium dodecyl sulphate – polyacrylamide gel electrophoresis) was performed. For western blotting 20 - 30 µg protein were diluted with SDS-loading dye and separated on a polyacrylamide (PAA) gel.

To get the right PAA solution, the two components acrylamide and bis-acrylamide had to be mixed in the ratio 19:1. While acrylamide forms long polymers, bis-acrylamide cross-links the long polymers. The addition of tetramethylethylenediamine (TEMED) and ammonium per-sulphate (APS) starts the polymerisation.

The loading dye contains SDS and DTT to denaturise the protein and to give it a negative charge. The protein was loaded into pockets in the gel and first run through the stacking gel to concentrate and even the protein distribution. After passing the stacking gel, the proteins were separated according to their size in the separation gel. Gels were run at 80V for 10 minutes and 120V for one hour or until the bromophenol blue band has reached the end of the gel.

During the western blot procedure the proteins were transferred from the PAA gel to a Millipore Immobilon PDVF membrane. For this, a mini trans-blot system from BIO-RAD was used. The proteins were blotted for one hour at 400mA. Subsequently the membrane was incubated in Ponceau red for two minutes to check the blotting procedure for loading and possible bubbles. Ponceau red stains reversibly proteins and the membrane can be destained with TST or water. The gel was stained in colloidal coomassie overnight to visualise remaining proteins as a loading control. For blocking, the membrane was incubated in a 5% or 10% milk-TST solution for

one hour, depending on the experimental setup. Blocking a membrane can lower the risk of unspecific antibody binding. After blocking the membrane was briefly rinsed with TST and subsequently incubated with the primary antibody at 4°C overnight on a shaking or rolling platform. The next day, the membrane was washed three times for five minutes in TST and subsequently incubated with the secondary antibody for one hour at room temperature. After a repeating washing procedure the membrane was incubated with the detection solution (Millipore Immobilon Chemoluminescent HRP substrate) for five minutes. Signal detection was done by using x-ray films (Kodak) and an automatic film developer.

4.2.15 Telomerase activity assay

The PCR based telomerase assay (TRAP assay) was described elsewhere (Wege et al., 2003) and is a modified version of the classical TRAP assay published by Kim and colleagues in 1997 (Kim and Wu, 1997). The sample cells were lysed with a non-denaturising buffer. A PCR mix was prepared containing SYBR[®]Green and two primers ACX and TS. Those two primers mimic the telomere end and active telomerase binds to the primers and elongate them. The amplicons can be analysed with real time PCR. The amplicon cycle values (Ct) were compared with standard curve Ct-values generated from untreated cells. Figure 4-6 illustrates the basics of the qPCR based TRAP assay.

For the standard curve 1×10^5 untreated cells were collected and pelleted by spinning at 250 g for 5 minutes at room temperature. Cells were washed once with 1 ml PBS. Cells were lysed in telomerase lysis buffer to an equivalent of 25 000 cells/ μ l lysis buffer. The cells were incubated on ice for 30 minutes. After successful lysis, the lysed cells were spun for 30 minutes at 4° C at full speed in a table top centrifuge (> 10 000 g).

Untreated cells were used for standard curve and the cell lysate was diluted corresponding to 50,000, 25,000, 10,000 and 5,000 cells.

25,000 treated cells were used for the analysis of the telomerase activity.

The following mix was prepared. The protocol shows the mixture for a single well.
Each sample was prepared in triplets:

Primer Sequences (Kim and Wu, 1997):

TS: 5'- AAT CCG TCG AGC AGA GTT -3'

ACX: 5'- GCG CGG [CTTACC]₃ CTA ACC -3'

0.4 µM telomerase primer TS

0.1 µM anchored return primer ACX

1x SYBR Green master mix

2 µl lysate

Ad 15 µl H₂O

RealTime PCR program:

1 cycle:

25°C 20min

95°C 10min

40 cycles:

95°C 30sec

60°C 90sec

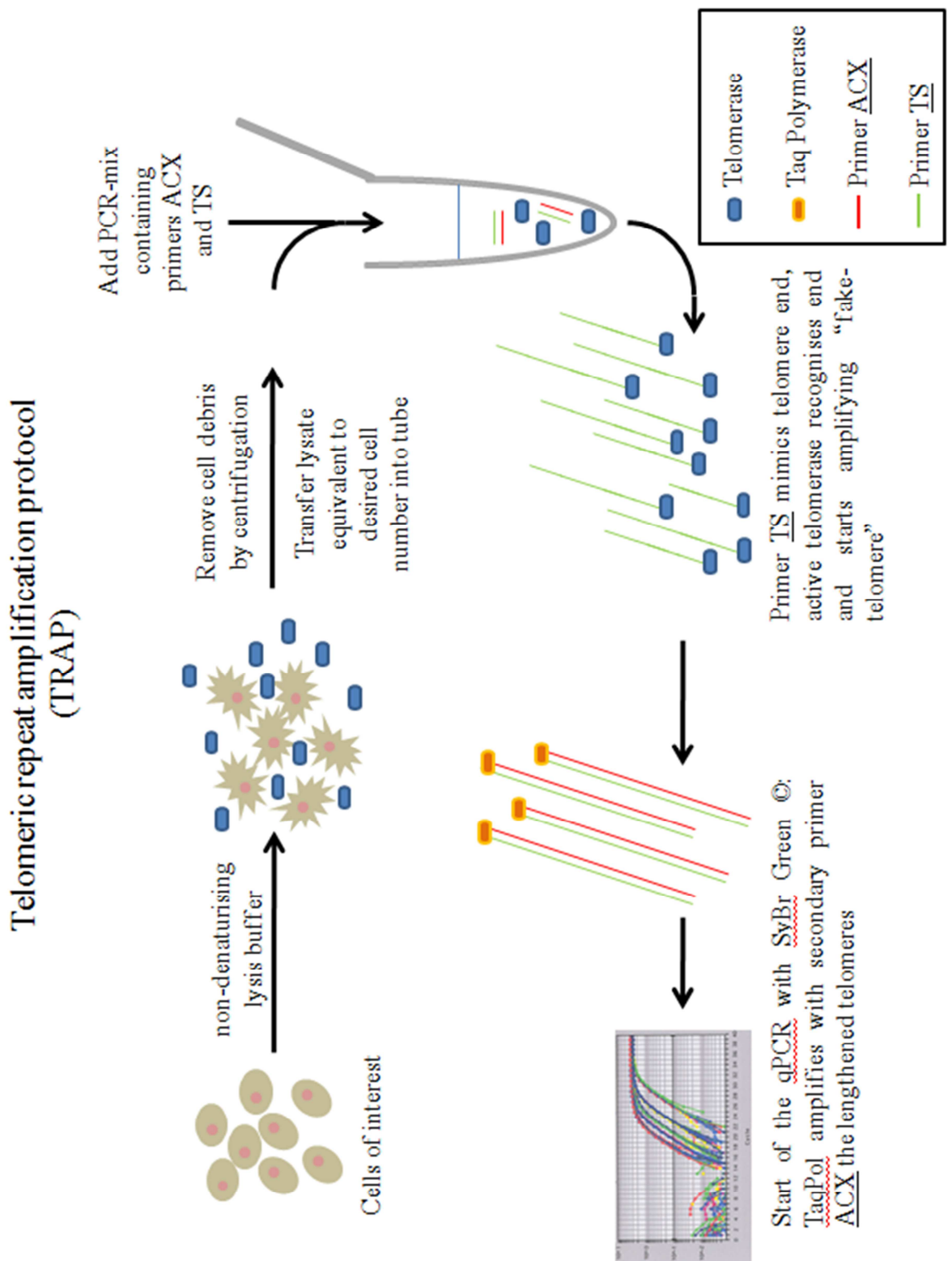


Figure 4-6 Scheme of the TRAP assay

This illustration shows the principle of the telomeric repeat amplification protocol (TRAP). Cells of interest are lysed in non-denaturing lysis buffer. After lysis the cell debris are removed and the lysate is transferred to a real time PCR 96-well plate. The transferred lysate volume is equivalent to the desired number of cells. Additionally to the lysate, a qPCR mix is added including primers TS and ACX. TS is designed so that the telomerase in the lysate recognises TS as telomere end and elongates it to "fake-telomeres". After an elongation time

the qPCR starts and TaqPol amplifies the “fake-telomeres” with ACX as secondary primer. Results are then analysed with qPCR program SDS2.2 (Wege et al., 2003).

4.2.16 Fluorescent activated cell sorting (FACS)

FACS analysis was mainly used for analysing the cell cycle and monitoring GFP expression.

During the cell cycle a cell divides into two. The cell cycle consists of four major phases which are the M phase, the G1 phase, the S phase and the G2 phase (Figure 4-7). During the M phase (M for mitosis) the mitosis takes place which is the segregation of the chromosomes and finishes with the cytokinesis, the division of the cytoplasm. Following the M phase is the G1 phase. During this phase, the cell grows and starts to express the needed proteins. Next comes the S phase (S for synthesis), where the DNA is replicated in preparation for the next M phase followed by the G2 phase which is like the G1 phase used to express proteins and cell growth.

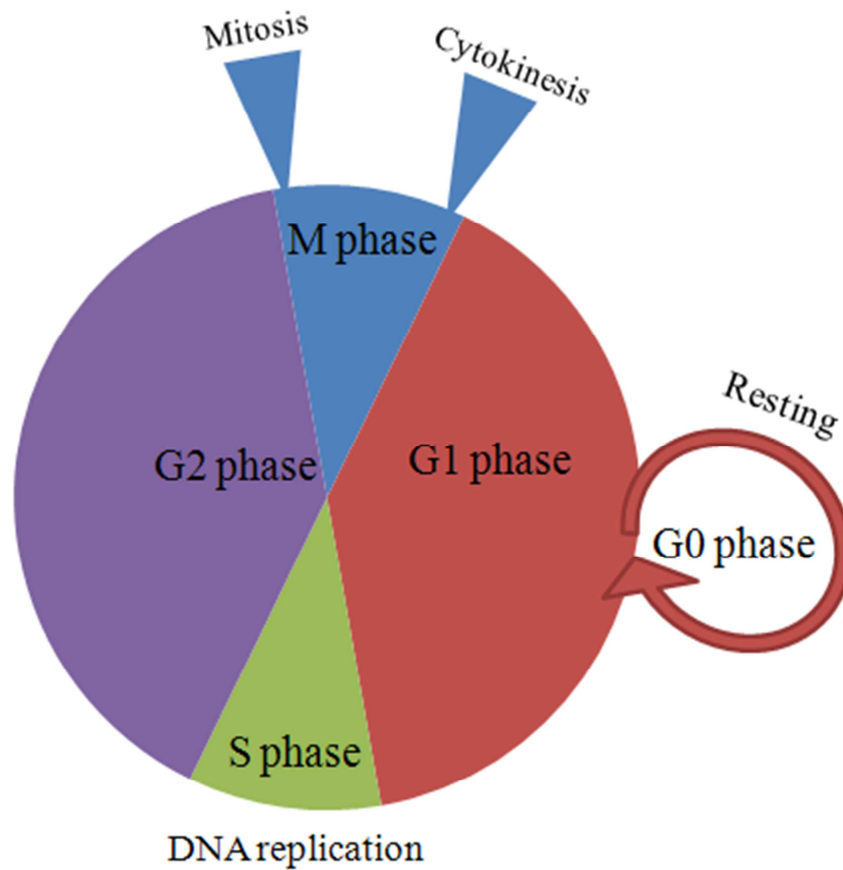


Figure 4-7 The cell cycle

The phases of the cell cycle. In the M phase mitosis and subsequently the cytokinesis take place. In the following phases continuous cell growth happens. During the S phase the DNA is being replicated. During G1 and G2 phase increasing cell growth and protein expression takes place. Some cells enter the G0 phase either temporarily or permanent. During this phase the cell cycle is on hold or completely switched off. Protein expression continues. During the G0 phase repair mechanisms also can also take place (Alberts, 2002).

With DNA staining it is possible to monitor the distribution of cells within the cell cycle. As DNA content of each cell is increasing during the cell cycle, the analysis gives a typical result (Figure 4-8).

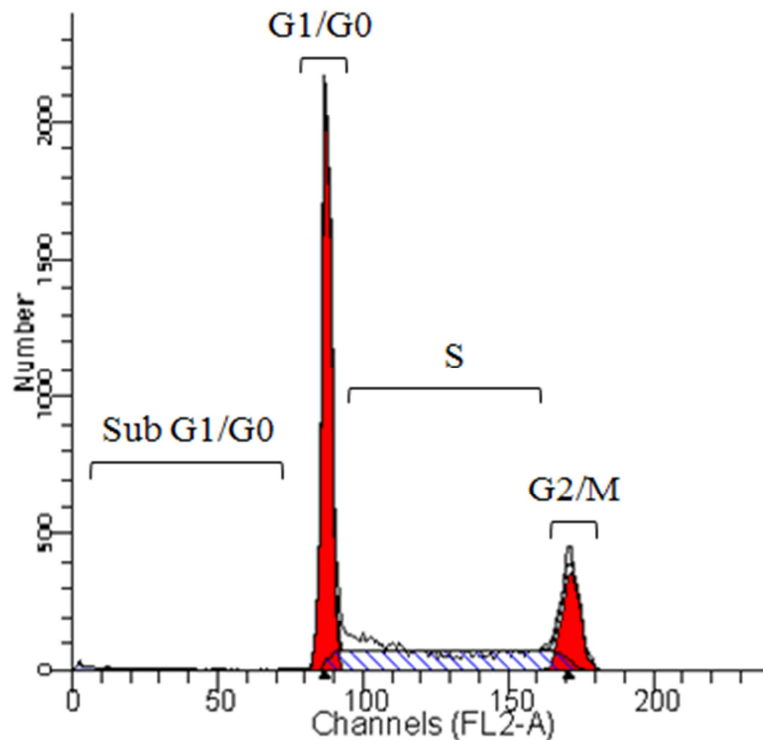


Figure 4-8 Typical distribution of cells within the cell cycle

The picture represents SEM cells after 96 hours of continuous mock treatment. The y-axis represents the cell count and the x-axis shows the DNA content. Cells in the G1/G0 phase have a haploid set of chromosomes and thereby represent the population of viable cells with lowest DNA content. During the S phase the genome is replicated therefore the DNA content increases. In the G2/M phase the cells bear a diploid set of chromosomes and this is visible on the diagram as the peak for G2/M has the highest amount of DNA. When cells die the DNA degrades into smaller fragments. Therefore dying cells are represented in the Sub G1/G0 phase which has the lowest DNA content of all cells analysed during a FACS/Cell cycle experiment. During this representative experiment the cells were distributed as followed: 48.75% in G1/G0 phase, 35.33% in S phase, 15.92% in G2/M phase and 3.95% in the sub G1/G0 phase which represents the dead cells. Analysis was done with Mod Fit LT™ (© Varity Software House)

For cell cycle analysis 50,000 – 100,000 cells were harvested, washed in PBS once and resuspended in 100 µl citrate buffer. In a FACS tube 400 µl propidium iodide solution together with 3 µl RNaseA were prepared. The cells in the citrate buffer were added and incubated for 5 minutes. The cells were then analysed in a FACS machine (ABI FacsCalibur) using the programs CellQuest, Mod Fit and WinMDI.

The green fluorescent protein (GFP) was first discovered in the jellyfish *Aequora victoria* and described by Osamu Shimomura and colleagues in 1962 (Shimomura et al., 1962). Over the years many mutants were biologically engineered which

increased the intensity of the emitted fluorescent light and also changed the excitation peak for better use in commonly used FACS machines. Today GFP is broadly used in cell biology as it is possible to combine the GFP gene to a gene of interest and so to receive an expressed fusion protein with an attached GFP signal. Thus it is possible to monitor protein expressing directly in a cell, for example the distribution during certain cell events.

For eGFP expression, 100000 cells were harvested, washed with PBS once and resuspended in 500 µl PBS. The expression of eGFP was analysed in FACS machine using the programs CellQuest and WinMDI.

4.2.17 ChIP – chromatin immuno precipitation

The ChIP protocol was established in the Lab of P. Farnham (Weinmann and Farnham, 2002).

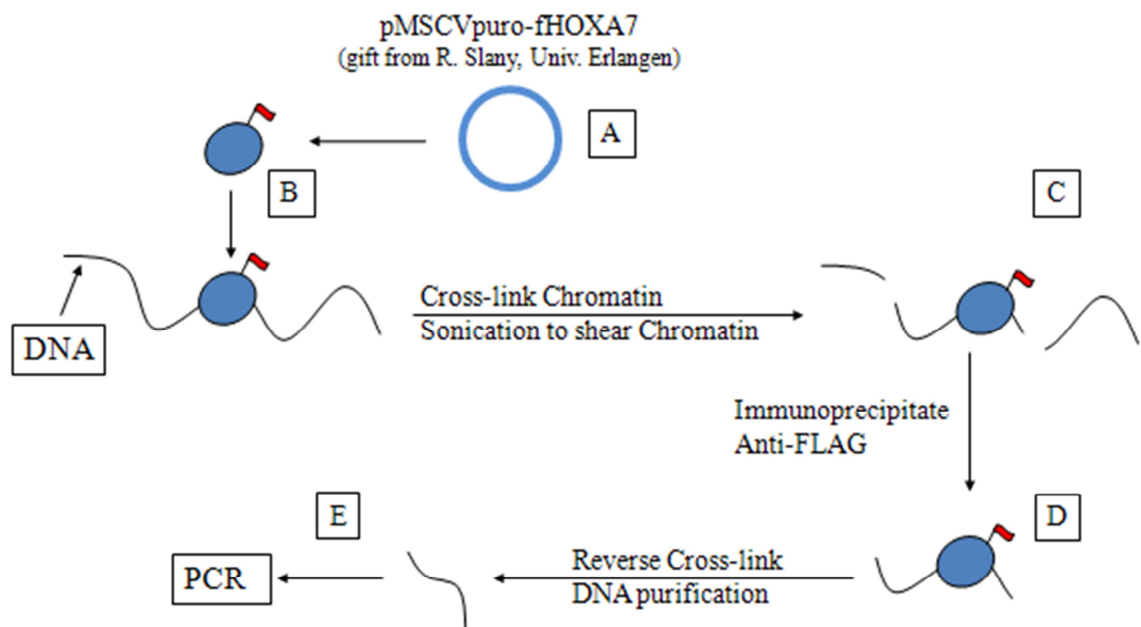


Figure 4-9 The chromatin immuno precipitation (ChIP)

The chromatin IP presented as an illustration. (A) A plasmid containing an expression cassette for FLAG tagged HOXA7 was transiently transfected into the nucleus of SEM target cells via nucleofection (Amaza™ Nucleofection Kit®). (B) The FLAG tagged HOXA7 protein binds to the DNA. (C) The protein-chromatin bindings are cross-linked and by

sonication the DNA is sheared into fragments between 500bp and 1000bp. (D) By incubation with an anti-FLAG antibody and subsequently performing an immuno precipitation reaction it is possible to isolate the cross-linked protein-chromatin complex. (E) After removing the protein by reverse cross-link reaction and purifying the DNA it is possible to analyse the DNA fragments with PCR. As a specificity control a plasmid expressing a FLAG tagged FHL2 protein was used.

SEM cells were taken and transfected with 2 μ g plasmid of pMSCVpuro-fHOXA7 or pFHL2-FLAG per 10^6 cells. The plasmids were transfected by electroporation with the Nucleofection Kit from AMAXA.

One litre of *Staphylococcus aureus* (StaphA) bacteria (Cowan 1 strain, kindly provided by Prof. Andreas Peschl from the University of Tuebingen) were cultured overnight. When they reached an optical density of about 0.6 the bacteria were spun down and the pellet was resuspended in 10ml dialysis buffer followed by a centrifugation step with 5600g for 5min at 4°C. This step was done twice. The bacteria were then resuspended in 3ml of 1xPBS with 3%SDS and 10% β -Mercaptoethanol and boiled for 30min. The bacteria were then again twice centrifuged and resuspended in 10ml dialysis buffer. In a final step the bacteria were resuspended in 4ml dialysis buffer and divided into 100 μ l aliquots, quickly frozen in liquid nitrogen and stored at -80°C.

For one ChIP assay a single vial of StaphA cells was thawed. 10 μ l of herring sperm DNA (10mg/ml) and 10 μ l of BSA (10mg/ml) were added to preclear the bacteria overnight at 4°C on a rotating platform. The next day the precleared bacteria cells were centrifuges for 3min at full speed (> 10000g) and the supernatant was discarded. The pellet was washed twice with 1x dialysis buffer and was then resuspended in 100 μ l 1x dialysis buffer.

Cross linking the chromatin was done by incubating the transfected SEM cells with formaldehyde (1% end concentration) for 10min at 37°C. The reaction was stopped by adding 1/10 vol. of 1.25M glycine. The cells were spun down, washed once with PBS and resuspended in the cell lysis buffer. After 10min incubation on ice, the nuclei were collected by spinning the cell lysate at 5000rpm for 5min at 4°C. The supernatant was discarded; the pellet was resuspended in nuclei lysis buffer and incubated on ice for 10min. Each sample was subsequently sonicated 5 times alternately with a two minute break in between for 10 seconds (= 10 pulsed) with the following settings: Duty cycle 50, output 5. After sonication the samples were

centrifuged at 10000g for 10min at 4°C. The lysates were then incubated with 15 µl pre blocked StaphA cells for 15min at 4°C. The lysates were spun down and the supernatants collected. To the precleared lysates 1 µl of anti-FLAG antibody or no antibody (control) was added and incubated on the rotating platform at 4°C overnight. On the following day to each lysate 15 µl of precleared bacteria cells were added and rotated for 15min at 4°C. Subsequently all samples were centrifuged at 10 000g for 3min. The supernatant of the antibody control was collected as chromatin input control, the other supernatants were discarded. For washing the pellets, 1.4ml dialysis buffer was added to the samples, rotated for 3min and subsequently centrifuged at 10000g. The supernatants were discarded. This washing step was performed 5 times. The whole washing process was then performed using IP wash buffer. After the last wash, the pellets were resuspended in 150 µl IP elution buffer and vortexed strongly. The samples were centrifuged at 10,000g for 3min at room temperature and the supernatants collected in new tubes. The elution step was then performed a second time with the bacteria pellets and the supernatants were merged. The reverse cross linking was performed by adding NaCl (200 mM end concentration) and 10 µg RNaseA followed by 5 hour incubation at 67°C. The samples were then precipitated overnight by adding 2.5 volume of absolute ethanol at -20°C. On the next day, the samples were centrifuged at 10 000g at 4°C for 20min and resuspended in 100 µl TE buffer. Subsequently 25 µl of 5x proteinase K buffer and 5 µl proteinase K was added and incubated for 1 hour at 45°C. The chromatin was then isolated with phenol-chlorophorm-isoamyl alcohol extraction (25:24:1) followed by chloroform-isoamyl alcohol extraction (24:1). After precipitation with 1/10 vol. of 3M NaAc and 2.5 vol. absolute ethanol the chromatin pellets were air dried, resuspended in 30 µl of water and analysed by PCR.

4.2.18 Production of recombinant pseudotyped lentivirus

The process of the lentiviral production is based on a protocol from D. Trono's Laboratory of Virology and Genetics (EPFL, Lausanne, Switzerland). The protocol was modified by Vasily V. Grinev from the University of Minsk (Department of Genetics, Biology Faculty, Belarusian State University, Minsk, Belarus).

The cell line 293T was used as lentivirus producer cell line. One day before transfection, $2-3 \times 10^6$ viable 293T cells were seeded in a 100mm Tissue Culture dish in 10ml DMEM medium, completed with 10%FCS and 2mM L-Glutamine and cultured overnight in an incubator (5% CO₂, 37°C, 95% air humidity).

On the day of transfection the cells had a confluence of about 30%. The method for transfection was by calcium precipitation. For each lentiviral plasmid the following mix was prepared. In an Eppendorf tube, 5 µg of the pMD2.G envelope plasmid, 15 µg of the pCMVdeltaR8.91 packaging plasmid and 20 µg of the third generation lentivirus transfer plasmid were mixed with special water (125µl of 1M pH 7.3 HEPES solution dissolved in 50 ml water) and 0.5M CaCl₂ solution to an end volume of 500 µl. This solution was then mixed very slowly with 500 µl 2xHeBS. The prepared mix was then incubated for 30min at RT so that the precipitates can form. Subsequently the mix was added slowly; drop wise on the 293T cells. The plate was gently stirred and placed back in the incubator and cultivated overnight. On the next day, a fine, sandy precipitate was visible all over the plates but not in the surroundings of the cells. A very careful medium change was done to minimize any damaging to the cell monolayer. The cells were then incubated under standard conditions for 3 more days. On the third day the medium was collected and spun down (500g, Beckman Coulter Allegra® X-12 benchtop centrifuge) to get rid of any cells. The supernatant was again collected and filtered through a 0.45µm filter to remove any remaining cell debris. To concentrate the virus, the solutions were transferred into *Beckman Instruments Inc* thickwall-style polyallomer conical tubes (ref. 358126) with Derlin PKGED'1 adapters (ref. 358156). The tubes were filled up to 30ml with medium and then put into a SW 28 swinging bucket rotor and spun at 26,000 rpm (120,000 g) for 120 min at +4°C in a Beckman ultracentrifuge. The supernatant was discarded and the virus pellet was resuspended in medium according to the experimental design. Figure 4-10 gives an overview about the process.

4.2.19 Lentiviral transduction of suspension cells

One day prior to the transduction, 2×10^6 cells per lentiviral transduction were seeded to a well of a 6-well plate in 2 ml complete medium. On the day of transduction, 1ml containing the recombinant virus particles was added to each well, according to the experimental setup. The virus titer was not determined. The plates were sealed with parafilm, wrapped in cling film and centrifuged at 1500g for 2h at 32°C. The cells were then put into an incubator at standard conditions overnight. The next day the cells were collected, washed with PBS once and resuspended in 3ml of fresh medium to remove any unbound lentiviral particles. Then the cells were treated under standard conditions.

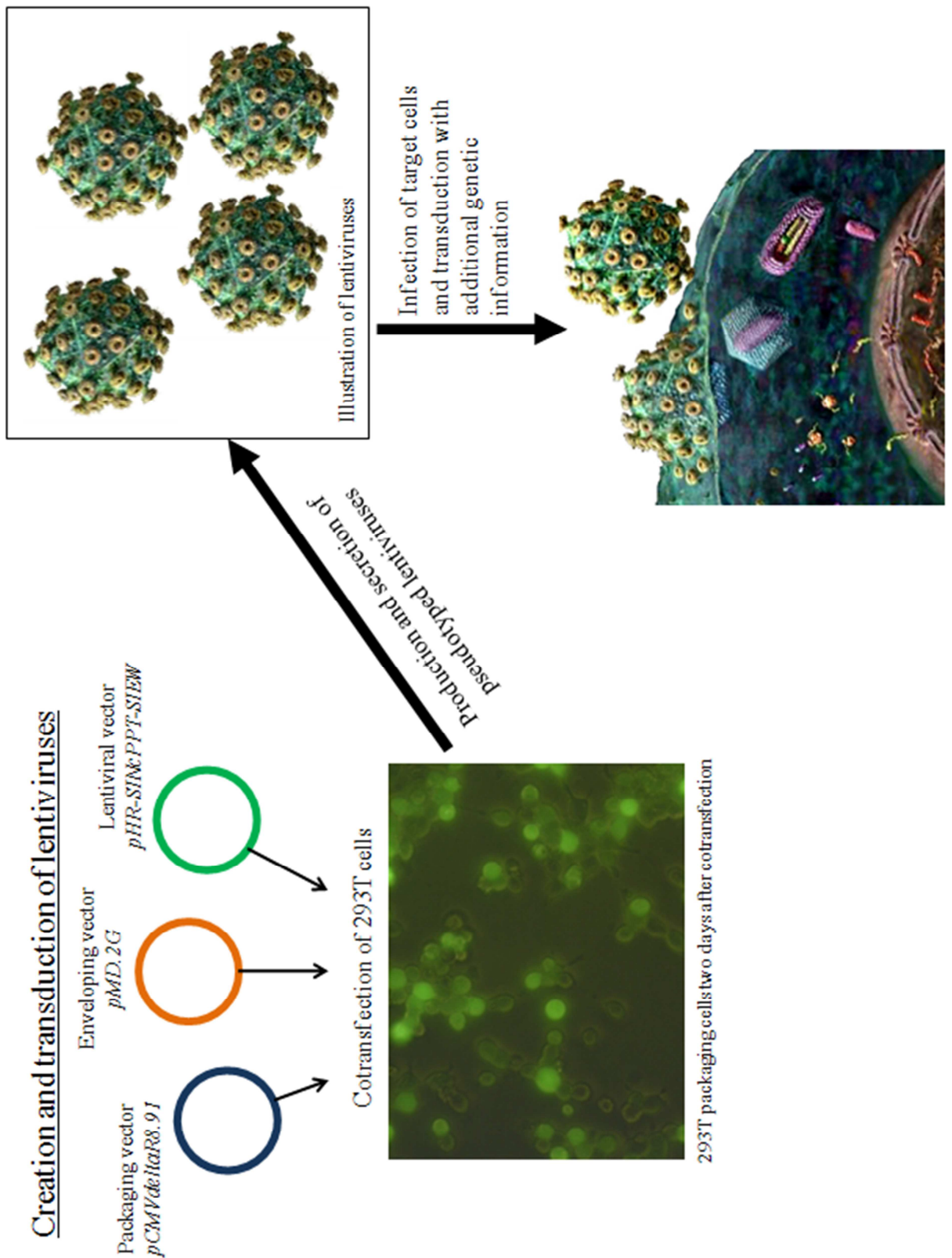


Figure 4-10 Illustration of lentivirus creation and transduction of target cells

In this scheme the production of the lentiviruses followed by the transduction of target cells is demonstrated. 293T cells are being transfected with three plasmids containing all genetic information for creating a virus with the gene of interest. In those 293T cells, the viruses is produced and secreted into the medium. The lentiviruses are harvested and concentrated for

the transduction which is the last step for the successful transduction of genetic material into a target cell.

5 Appendix

5.1 References

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5.2 List of abbreviations

AD	transcriptional activation domain
AF4	ALL-1 fused gene on chromosome 4
ALL	acute lymphoblastic leukaemia
ALT	alternative lengthening of telomeres
AML	acute myeloblastic leukaemia
APS	ammonium persulphate
AT-H	AT Hook
ATP	adenosine triphosphate
BCR	breakpoint cluster region
bp	Base pair
BSA	bovine serum albumin
CD	cluster of differentiation
cDNA	complementary desoxyribonucleic acid
CHAPS	3[(3-Cholamidopropyl)dimethylammonio]-propanesulfonic acid
CHD	C-terminal homology domain
ChIP	chromatin immunoprecipitation
CIAP	calf intestinal alkaline phosphatase
CLL	chronic lymphoblastic leukaemia
CML	chronic myeloblastic leukaemia
cPPT	central polypurine tract
CR	complete remission
DMSO	demethylsulphoxide
DNA	desoxyribonucleic acid
dsRNA	double stranded ribonucleic acid
DTT	Dithiotreitol
EDTA	Ethylene diamine tetraacetic acid
EFS	event free survival
eGFP	enhance green fluorescent protein
EGTA	ethylene glycol tetraacetic acid
FACS	fluorescent activated cell sorting
Fw	forward

GAPDH	glycerine aldehyde 3 phosphate
GFP	green fluorescent protein
H3K4	histone 3 lysin 4
H3K79	histone 3 lysin 79
HDAC	histone deacetylase
HOX	homeobox
HRP	Horse radish peroxidase
HSC	hematopoietic stem cell
IRES	internal ribosomal entry site
LTR	long terminal repeat
MEF	murine embryonic fibroblast
miRNA	micro ribonucleic acid
MLL	mixed lineage leukaemia
MT	methyltransferase domain
NHD	N-terminal homology domain
NLS	nuclear localisation sequence
NOD/scid	nonobese diabetic severe combined immunodeficiency
NP40	nonyl phenoxy polyethoxy ethanol
ORF	open reading frame
PAA	poly acryl amide
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate buffered saline
pcG	polycomb group
PCR	polymerase chain reaction
PHD	plant homeodomain
PI	propidium iodide
PIPES	piperazine-N,N'-bis(2-ethanesulfonic acid)
Pol II	RNA polymerase II
pSer	proline-serine rich region
PVDF	Polyvinyliden flouride
qPCR	quantitative polymerase chain reaction
qRT-PCR	quantitative reverse transcription polymerase chain reaction
RB	retinoblastoma
Rev	reverse

RISC	ribonucleic acid induced silencing complex
RNA	Ribonucleic acid
RRE	rev responsive element
RT	room temperature
RT-PCR	reverse transcription polymerase chain reaction
SDS	sodium dodecyl sulphate
SET domain	Su(var)9-3, Enhancer of Zeste and trithorax histone methyltransferase domain
SFFV	spleen focus forming virus
SL-IC	scid leukaemia – initiating cell
SIN	self inactivation
siRNA	small interfering ribonucleic acid
t(4;11)	reciprocal translocation between chromosome 4 and chromosome 11
TEMED	tetramethylethylenediamine
TERC	telomerase ribonucleic acid component
TERT	reverse transcriptase of the telomerase
TRAP	telomeric repeat amplification protocol
trxG	trithorax group
WPRE	woodchuck posttranscriptional regulatory element
zVAD-FMK	carbobenzoxy-valyl-alanyl-aspartyl-[O-methyl]- fluoromethylketone

5.3 Abstract

The t(4;11) fusion protein MLL/AF4 regulates TERT expression

About 50% of all infants suffering from acute lymphoblastic leukaemia (ALL) show the translocation t(4;11)(q21;q23) which creates the fusion genes *MLL/AF4* and *AF4/MLL*. This reciprocal translocation identifies a therapy resistant form of leukaemia with a poor prognosis. In order to gain a better insight into the molecular mechanisms of t(4;11) leukaemias we used the two ALL cell lines SEM and RS4;11, both harbouring the t(4;11) translocation, however with different fusion sites. Specific small interfering RNAs (siRNAs) against the two fusion site variants of the *MLL/AF4* fusion transcript were designed and transfected into the respective cells via electroporation, using very mild conditions. Serial electroporations at two-day intervals resulted in sustained depletion of the *MLL/AF4* transcript up to 70-80%, and depending on the experimental setup, cells were analysed after two or three electroporations. Knock-down of *MLL/AF4* resulted in strong inhibition of proliferation and clonogenicity in the two t(4;11)-positive cell lines SEM and RS4;11, along with induction of apoptosis. *MLL/AF4* depletion resulted in a 65% decrease in telomerase activity in both SEM and RS4;11 cells, with telomerase reverse transcriptase (*TERT*) expression being reduced twofold on both transcript and protein levels. Notably, *TERT* reduction was even stronger (90% depletion) when apoptosis caused by *MLL/AF4* knock-down was suppressed with the caspase inhibitor zVAD. In contrast, levels of the RNA component of the telomerase, *TERC*, were not affected by *MLL/AF4* knock-down. Additionally, *MLL/AF4* knock-down was associated with reduced expression of several members of the HOXA gene cluster, *HOXA6* (65% reduction), *HOXA7* (85% reduction), *HOXA9* (60% reduction) and *HOXA10* (75% reduction). Interestingly, siRNA-mediated knock-down of the *MLL/AF4* target gene *HOXA7* also induced apoptosis and resulted in a 70% decrease of *TERT* levels in two t(4;11) positive cell lines without affecting *MLL/AF4*. Chromatin immunoprecipitation assays revealed *HOXA7* binding to the promoter of *TERT*. Therefore, *MLL/AF4* regulates *TERT* expression, at least in part, via *HOXA7*. These data suggest that t(4;11) positive cells with substantially lower *TERT* expression undergo apoptosis, and that *TERT* may play an antiapoptotic role in t(4;11) positive ALL. Furthermore, these studies identify *TERT* as a putative new therapeutic target in this therapy-resistant infant leukaemia.

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