Studying the role of the fusion protein MLL-AF4 in leukemogenesis with the help of siRNAs

der Fakultät für Biologie
der Eberhard Karls Universität Tübingen

zur Erlangung des Grades eines Doktors
der Naturwissenschaften

von

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D i s s e r t a t i o n

2007

Tag der mündlichen Prüfung:	11.10.2006
Dekan:	Professor Dr. F. Schöffl
1. Berichterstatter:	Professor Dr. A. Nordheim
2. Berichterstatter:	PD Dr. O. Heidenreich
3. Berichterstatter:	Professor Dr. R. Marschalek

To my deeply beloved parents,

my first teachers and scientific encourages,

my strictest judges and closest friends

PUBLICATIONS

During the course of this work the following articles have been published or submitted for publication:

Thomas M, Gessner A, Vornlocher HP, Hadwiger P, Greil J, Heidenreich O. (2005). Targeting MLL-AF4 with short interfering RNAs inhibits clonogenicity and engraftment of t(4;11)-positive human leukemic cells. Blood. **106**, 3559-66.

Thomas M, Greil J, Heidenreich O. (2006). Targeting leukemic fusion proteins with small interfering RNAs: recent advances and therapeutic potentials. Acta Pharmacol Sinica. **27**, 273-81.

Thomas M, Martinez Soria N, Heidenreich O. (2006). RNA Interference in Hematopoietic and Leukemic Cells. EDS. Systems of Biology, pp. 29-58, Springer Publishing House.

SUMMARY

Chromosomal aberrations giving rise to fusion genes are observed for many different leukemias. The chromosomal translocation t(4;11) marks an infant acute lymphoblastic leukemia associated with a particularly poor prognosis. To define the role of one of the corresponding fusion transcripts, *MLL-AF4,* in leukemogenesis more precisely, an RNAi approach was applied to inhibit the expression of this oncogene in leukemic cells.

RNAi is a cellular process leading to the enzymatic cleavage and breakdown of mRNA. Exogenously added synthetic siRNAs were shown to act as very potent and sequence-specific agents to silence gene expression, demonstrating their great potential not only for the analysis of gene function but also for gene-specific therapeutic approaches.

In this work I show that depletion of MLL-AF4 inhibits clonogenicity and proliferation, induces apoptosis in t(4;11)-positive leukemic cells and compromises their engraftment in a severe combined immunodeficiency (SCID) mouse xenotransplantation model. Furthermore, suppression of MLL-AF4 is associated with the decrease of telomerase activity in t(4;11) cells, which, in turn, is caused by the downregulation of the *hTERT* expression. Studies of the methylation status of *hTERT* promoter revealed that MLL-AF4 interferes with the genomic DNA methylation process. Thus, along with the significant phenotypical changes mediated by MLL-AF4 siRNA, direct targets and executors of MLL-AF4-mediated leukemogenesis were identified.

Targeted inhibition of *MLL-AF4* fusion gene expression may lead to an effective and highly specific treatment of this therapy-resistant leukemia.

ZUSAMMENFASSUNG

Chromosomale Veränderungen, die für die Entstehung von Fusionsgenen verantwortlich sind, sind häufig beobachtete Phänomene bei Leukämien. Die chromosomale Translokation t(4;11) ist ein Merkmal für eine akute lymphoblastische Leukämie bei Säuglingen mit sehr schlechter Prognose. Für die Definierung der Rolle des entsprechenden Fusionsgens *MLL-AF4* wurde die Technik der RNA-Interferenz benutzt, um die Expression dieses Gens in den leukämischen Zellen inhibieren.

Im Rahmen dieser Arbeit konnte gezeigt werden, dass eine Reduktion des MLL-AF4 Spiegels die leukämische Klonogenizität und Proliferation vermindert und die Apoptoserate von t(4;11)-positiven Zellen erhöht. Zusätzlich verhindert die siRNA-Behandlung die Etablierung einer Leukämie durch MLL-AF4-positive Zellen in einem SCID-Mausmodell. Ausserdem führt die Unterdrückung von MLL-AF4 zu einer Abnahme der Telomeraseaktivität, was wiederum auf einer Inhibierung der *TERT*-Expression beruht. Die Untersuchung des Methylierungsstatuses des *TERT* Promoters hat gezeigt, dass MLL-AF4 bei der Methylierung genomischer DNA eine wichtige Rolle spielt. In diesem Zusammenhang, wurden auch mit *HOXA7*, *HOXA9* und *MEIS1* Zielgene und Exekutoren von MLL-AF4 identifiziert.

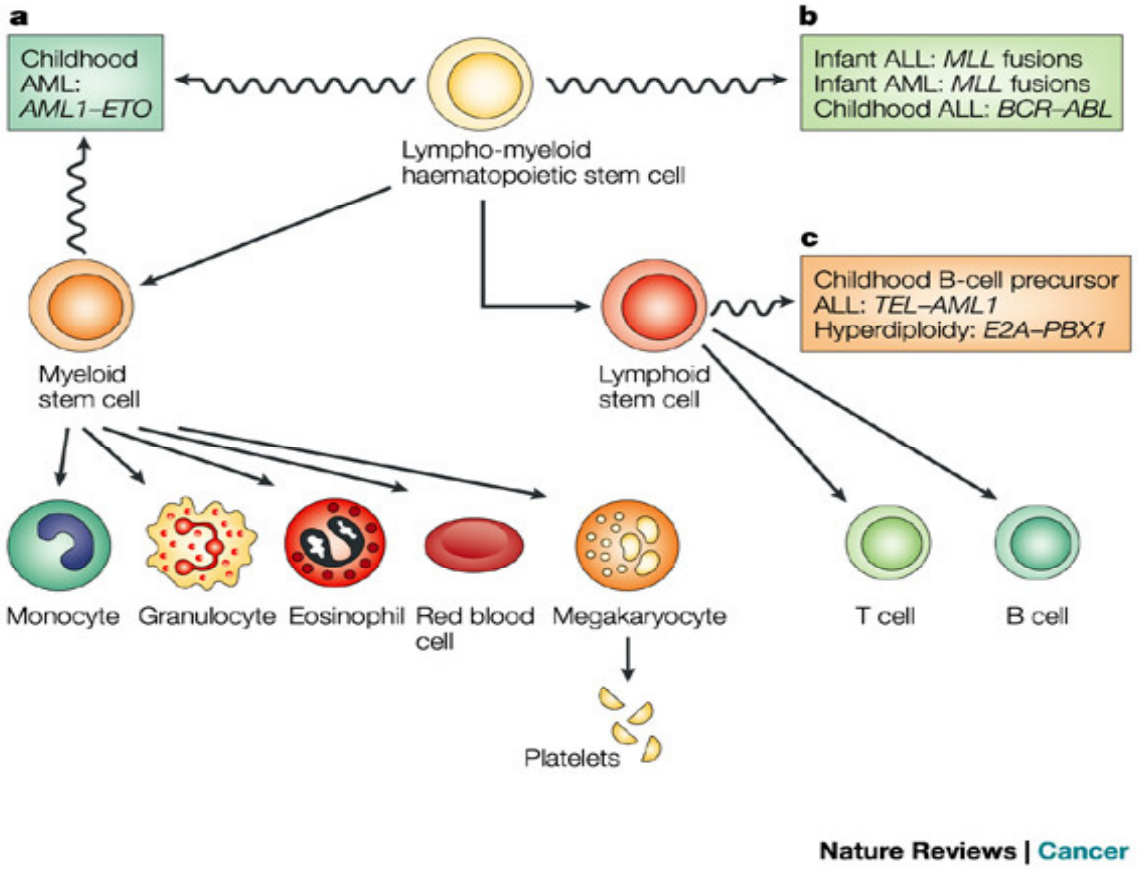
Die gezielte Hemmung der *MLL-AF4* Expression kann zur effektiven und hoch-spezifischen Behandlung dieser bislang therapieresistenten Leukämie führen. Die spezifische RNAi-vermittelte Abschaltung der Gene, die nur in den Leukämiezellen vorkommen und nicht in normalen Körperzellen, könnte eine attraktive Ergänzung der Chemotherapie werden.

I. INTRODUCTION

Specifications of blood cells (e.g. erythrocytes, granulocytes, macrophages, etc.), as well as the development of leukemia are paradigms of normal and pathologic combinatorial gene regulation, respectively. During hematopoietic lineage commitment, distinct sets of transcription factors coordinate cell division and cell-type specification. It appears that a set of transcription factor determines hematopoietic cell fate and proliferation. Mutations in critical transcription factors may interfere with their normal function in a dominant fashion, blocking differentiation and causing leukemia. In a large number of hematopoietic tumours consistent chromosomal rearrangements are found. In many cases, these rearrangements disrupt genes whose normal function is required for the proper development of blood cells. A malignant transformation happens at the initial stages of blood cell development on the levels of hematopoietic stem cells or precursors. The generation of chimeric fusion proteins by chromosomal translocations is a common pathogenic mechanism in human leukemias.

The disease itself is a genetically and phenotypically heterogeneous disorder. However, significant progress has been made in identifying mutant genes that are implicated in disease pathogenesis. There are common themes emerging in the signal transduction pathways and transcriptional programs, which are associated with malignant transformation. Mainly, the capacity of leukemic progenitors to self-renew appears to be a shared motif among all leukemias and result into therapies, which target these self-renewal capacities. In addition, chromosomal translocations and point mutations target pathways that confer proliferative and survival advantage to hematopoietic progenitors. Other mutations target hematopoietic transcription factors and phenotypically result in impaired hematopoietic differentiation. The attention of many hematologists is primarily attracted to therapies that focus on interrupting the proliferative and/or survival pathways, as well as developing agents that can override the block of

hematopoietic differentiation.

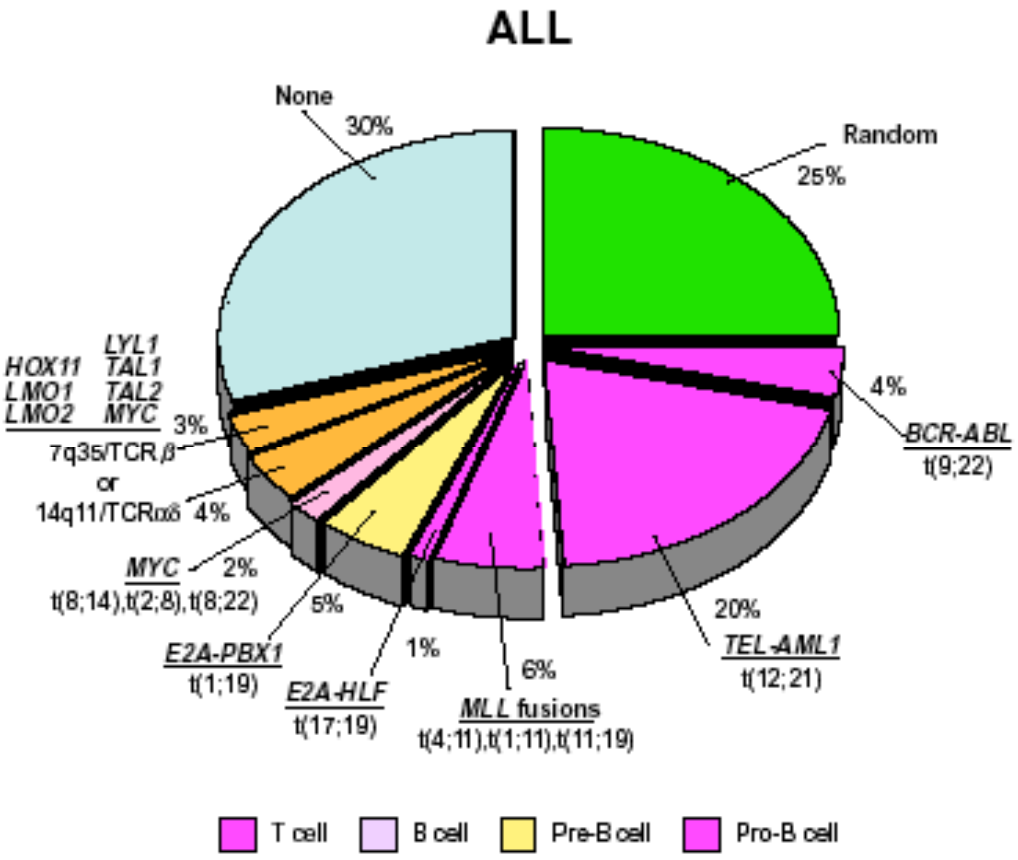


Scheme 1. General scheme of hematopoiesis and chromosomal translocations, which occur on different stages of hematopoietic differentiation and lead to the development of a various types of leukemia (Greaves et al. 2003).

I.1Hallmarks of ALL

Recurrent chromosomal abnormalities are a hallmark of lymphoblastic leukemias and provide insight into the molecular mechanisms of leukemogenesis. The most frequent targets of chromosomal translocations in the acute leukemias are genes that encode transcription factors, emphasizing the critical role of these "master" regulatory proteins in the control of blood cell development. The modular structure of transcription factors--including discrete DNA-binding, dimerization, and trans-effector domains--allows normally

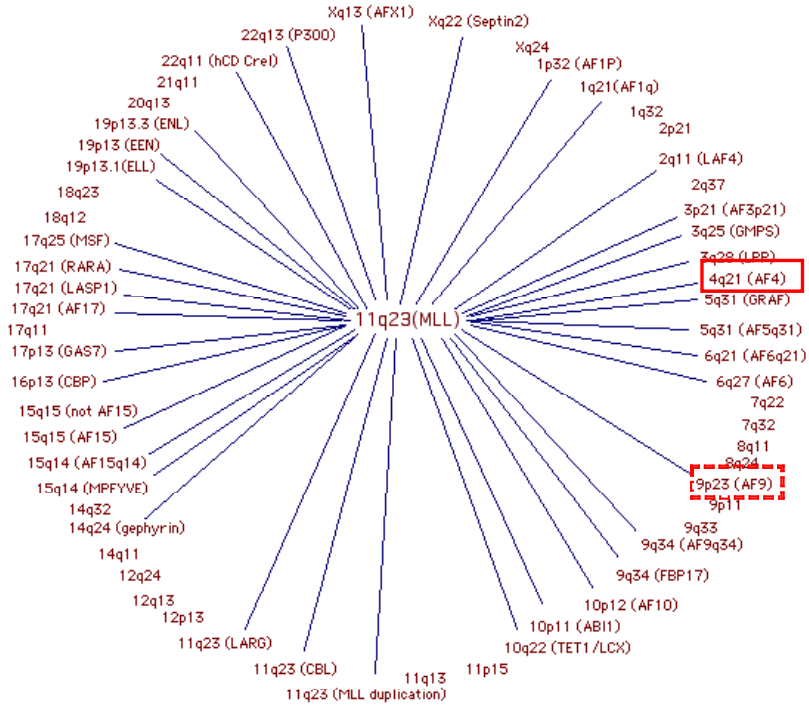
unrelated sequences from different chromosomes to be recombined into hybrid genes that encode fusion products with altered function. Commonly, the coding exons of genes, disrupted by a reciprocal translocation, are incorporated into a single "fusion" gene, which generates a chimeric protein with unique properties. The products of these aberrant genes are most often nuclear proteins active in transcription. Exceptions conclude several cytoplasmic proteins containing activated tyrosine kinase domain such as BCR-ABL, TEL-PDGFRβ, NPM-ALK and some others. In ALL, gene fusions tend to be specifically associated with one of the commonly recognized immunologic subtypes of the disease, as indicated by the color coding on the chart.



Scheme 2. Distribution of translocation-generated oncogenes among the acute lymphoblastic leukemias of children and young adults (Look 1997).

I.2 General aspects and molecular characteristics of infant acute lymphoblastic leukemia

Leukemias bearing translocations involving chromosome 11q23 are found in leukemic blasts from > 70% of the patients younger than 1 year old with the designated immunophenotype of AML or ALL (Biondi et al. 2000). Some infant leukemias express antigens characteristic for both lymphoblasts and monoblasts, and are sometimes designated as acute biphenotypic leukemias. Infants diagnosed with ALL harboring an 11q23 rearrangement have a particularly poor prognosis as compared to other children with ALL (Chen et al. 1993). The association of 11q23 rearrangements with either ALL or AML is unique in that most other translocations tend to be associated with leukemias of a particular hematopoietic lineage. These observations prompted the name mixed-lineage leukemia (*MLL*) for the gene on 11q23, which is involved in these types of aberrations.



Scheme 3. Major translocation partners of MLL (picture is taken from the website www.genatlas.info.fr)

Red frames designate two partner genes –AF4 and AF9 – which are predominantly

found in infants.

As a result of chromosomal translocations, this gene is fused with more than 80 different partner genes, which may be of either nuclear or cytoplasmic origin. There are no known unifying properties shared by this vast array of MLL fusion proteins in human leukemia (scheme 3). All of them produce a fusion protein possessing the NH_2-terminus of MLL fused to COOH-terminus of the fusion partner (Ayton et al. 2001). Although MLL fusions can be found in either ALL or AML, particular translocations, such as t(4;11)(q21;q23), found most often in ALL and the t(9;11)(p21;q23) in AML. But this specificity is not absolute in that the t(9;11) is sometimes identified in blasts designated as ALL. The association of a particular translocation with the specific immunophenotype suggests that the fusion partner probably defines a phenotype but not the incidence of leukemia; but the molecular details of this association are unclear.

I.2.1 Diversity of translocation partners - common themes

In contrast to the majority of other fusion oncoproteins, MLL forms chimeras with a puzzling number of non-related proteins. MLL partners can be divided into two classes. The first group is mainly cytoplasmic and frequently associated with cytoskeleton-dependent signal transduction. The second group represents the majority of all 11q23 translocations. There, MLL is fused to nuclear proteins that resemble transcription factors. Three translocations within this group, the t(4;11), t(9;11) and the t(11;19) joining MLL with AF4, AF9 and ENL, respectively, make up for more than 75% of all observed cases.

There are evidences that the acquisition of novel properties by the combination of MLL with the fusion partners, rather than the loss of wild-type function, leads to the generation of active oncoproteins. A series of knock-in studies demonstrated that MLL needs a fusion partner to become leukemogenic, whereas a simple truncation did not cause leukemia in mice (Corral et al. 1996;

Dobson et al. 1999; Dobson et al. 2000). Moreover, several structure-function studies testing the transformation capacity of MLL fusions in a bone marrow transformation assay found a critical contribution of the fusion partner (Slany et al. 1998; Lavau et al. 2000; Luo et al. 2001). In several leukemia patients, MLL protein was found to acquire an internal partial tandem duplication (Whitman et al. 2005).

Nevertheless, all studies agreed on the necessity of two critical DNA binding motifs in the MLL portion of the fusion, namely the AT hooks and methyltransferase homology (MT) domain. Interestingly, in a knock-in experiment a fusion of MLL with the bacterial LacZ protein appeared to be sufficient to elicit leukemia in mice (Dobson et al. 2000). The authors of this report were the first to speculate that an intrinsic multimerization domain within LacZ supplies an essential function and suggested that a dimerization of the truncated MLL is the basic oncogenic trigger. In other studies, it has been postulated that transcriptional transactivation might be the common denominator of several fusion partners, and transactivator domains are indeed found in three most frequent fusion partners AF4, AF9, ENL (Prasad et al. 1995).

In this respect, there are currently two mechanisms of the oncogenic activation by MLL fusions in leukemia, recently summarized by So and Cleary (So et al. 2004). One subset of fusion partners already displays the required transcriptional activation potential required for leukemogenesis. The other subset acts via their homodimerization or oligomerization domains and therefore can lead in a dimerization-dependent pathway to deregulated transcription (So et al. 2004). Moreover, the necessity of the two DNA-binding domains of MLL supports the hypothesis that the presence of the MLL part is the crucial feature in the onset of leukemia, which could be also responsible for the target recognition.

Therefore, studying the hallmarks associated with MLL-AF4 leukemia, one of the most prevalent recurrent chromosomal translocation, may serve as a

model to get general clues in understanding of the MLL-associated leukemogenesis.

I.2.2 MLL fusions and *HOX* gene expression

The questions arise: what accounts for the leukemogenicity of MLL? Might there be some common functional thread tying together many of the fusion genes? At least one strong clue has emerged from the recognition that a major function of MLL, like its drosophila homologue Trithorax, is to serve as a maintenance factor for the expression of many members of the HOX family of transcription factors. *HOX* genes are now recognized as major components of the regulatory machinery of primitive hematopoietic cells. Strikingly, multiple lines of evidence link *HOX* genes directly to leukemic transformation (Buske et al. 2000; Owens et al. 2002). This evidence includes induction of leukemia in mice following engineered overexpression of certain *HOX* genes (*HOXA9*, *HOXA10*) and the observed overexpression of multiple *HOX* genes in MLL-associated leukemias. Analysis of MLL knockout mice suggests that this protein plays an important role in development and hematopoiesis through maintenance of appropriate *HOX* gene expression (Yu et al. 1995; Ernst et al. 2004; Ernst et al. 2004). The ability of wild-type MLL to regulate transcription of *HOX* genes suggests that its role in both hematopoiesis and leukemogenesis may be mediated by altering patterns of *HOX* gene expression. Given the apparent importance of *HOX* genes in leukemogenesis, it seems likely that translocations involving MLL alters expression of *HOX* genes that are critical for leukemogenesis. A satisfying model for at least some MLL-induced leukemias would be through induced deregulation of key *HOX* target genes.

I.2.3 Other characteristic features of MLL-associated leukemia

Armstrong and colleagues recently found the receptor tyrosine kinase FLT3 to be highly expressed in MLL-rearranged ALL as compared with other acute

leukemias (Armstrong et al. 2002). This prompted further assessment of FLT3 in this disease, in which they found approximately 20% of MLL-rearranged ALL samples to possess activating mutations in the activation loop region of a FLT3 molecule. These data support the idea that leukemogenic fusion proteins such as MLL fusions cooperate with activated kinases to promote leukemogenesis. Furthermore, FLT3 inhibitors appear to have activity against MLL-rearranged ALL in vitro and in murine models (Gilliland et al. 2002). Clinical trials to test the efficacy of FLT3 inhibitors in MLL-rearranged ALL are in development.

In general, the presence of MLL translocations in ALL often results in an early relapse after chemotherapy and is associated with an extremely poor prognosis (Secker-Walker, 1998, Leukemia). The reason for the dismal prognosis of the MLL-associated leukemias is the lack of a satisfying therapy approach. MLL translocations are typically found in infants in up to 80% of the cases (Heerema NA, Blood 1994), but also in a secondary chemotherapy-induced leukemia.

An unfortunate consequence of the successful treatment of the patients with hematological disorders, particularly those receiving radiation and/or various types of chemotherapy, is that some of them develop secondary acute leukemia. The majority of these patients had been treated with drugs such as alkylating agents that damage DNA. In the late 1980s, new drugs targeting topoisomerase II were developed. Topoisomerase II is an enzyme that cleaves DNA, making a double-strand break and unwinding the DNA. These drugs did not interfere with the cleavage process, but prevented religation of the double-strand breaks. This resulted in an increased number of chromosomes with double-strand breaks, which assumed to be related to their cytotoxic effects. Despite being very effective therapeutic agents, these drugs unexpectedly led subsequently to an increase of translocations involving MLL, almost certainly as a consequence of the treatment (Pedersen-Bjergaard J, 1994 Blood).

Selective induction of differentiation and apoptosis is a new strategy in the

treatment of other forms of leukemia. For example, All-trans retinoic acid (ATRA) and Arsenic (mixture of monomethylarsonic acid (MMA) and dimethylarsinic acid (DMA)) have been effective in the treatment of acute promyelocytic leukemia (APL) (Wang et al. 2000), which creates the possibility that other types of acute leukemia can be conquered by selectively inducing differentiation and/or apoptosis.

Such an approach could be considered in the treatment of MLL-associated leukemias. However, there is another hurdle in this aspect. Many studies have demonstrated that cells carrying *MLL* rearrangements are highly resistant to any death signals in vivo when compared to other leukemias (Dorrie et al. 1999; De Braekeleer et al. 2005). In particular, for MLL-AF4 leukemias was shown that these cells are growth factor independent and survive upon serum deprivation (Kersey et al. 1998).

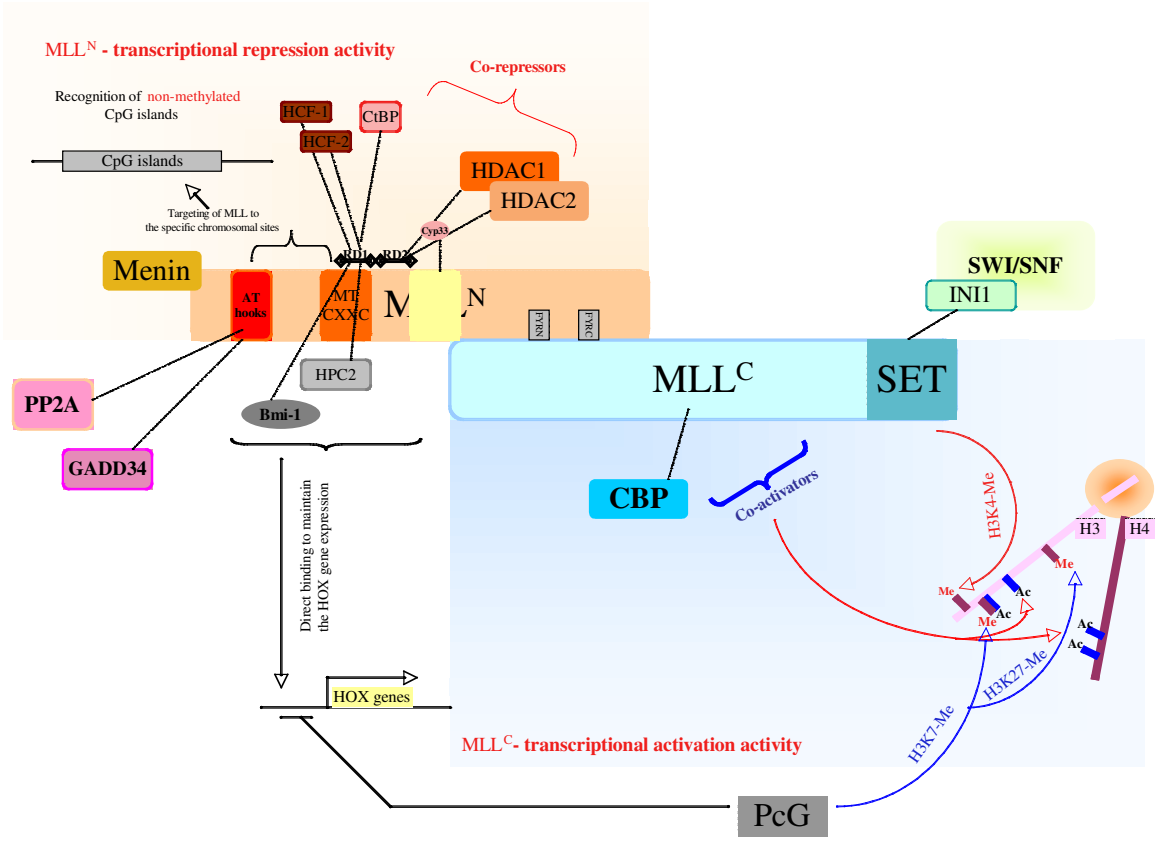
Abrogation of p53 functional activity is also a common feature of MLL fusions-mediated leukemogenesis. As it was described by Wiederschain (Wiederschain et al. 2005), several frequently detected MLL fusion proteins substantially downregulate p53-mediated induction of p21, MDM2 and BAX in response to DNA damage. Furthermore, the major mechanism of the inhibitory effect of MLL fusion proteins is the reduction of p53 acetylation by p300. MLL chimeras, inhibiting the stress-induced p53 acetylation by p300, interfere with the cellular DNA damage defense mechanism, providing an advantage towards malignant transformation.

I.3 MLL protein

The gene *MLL* is the mammalian counterpart of the Trithorax (*trx*) gene of Drosophila. In fruit flies, trx proteins have a positive role in the maintenance of cell type-specific patterns of *HOM-C* gene expression, apparently through epigenetic mechanisms that establish and sustain a receptive chromatin

configuration. An analogous role for MLL is supported by biochemical studies and phenotypic analysis of mice completely lacking or possessing single allele of a functional *MLL* gene.

The *MLL* gene encodes a 3969 amino acid DNA-binding protein that possesses multiple protein motifs including an NH_2-terminal DNA binding domain, transcriptional activation and repression domains, and a COOH-terminal SET domain that contains histone methyltransferase activity (Milne et al. 2002; Nakamura et al. 2002). Of interest, MLL protein is cleaved into two subunits by a recently identified novel protease (Yokoyama et al. 2002; Hsieh et al. 2003). The MLL^N protein, which displays transcriptional-repression activity, possesses 3 AT hooks and a transcriptional-repression domain (RD) (scheme 4). AT hooks have a homology to the high mobility group I (HMG-I) protein and possess DNA-binding activity. The RD consists of 2 functionally different domains, RD1 and RD2, and recruits 1 or more repressor complexes, including polycomb group protein such as HPC2, Bmi1 and the co-repressor C-terminal-binding-protein CtBP (via RD1) and histone deacetylases HDAC1 and HDAC2 (via RD2) (Xia et al. 2003). RD1 contains the DNA methyltransferase homology (MT) domain, also known as the CXXC domain, which has a homology to the methyl-CpG DNA-binding proteins, such as MBD1, MeCP1 and the DNA methyltransferase DNMT1 (Cross et al. 1997; Fuks et al. 2000). This MT domain also has a DNA-binding activity that is specific for unmethylated CpG DNA sequences (Birke et al. 2002) which has recently been shown to be essential for the transformation activity of MLL fusion proteins (Ayton et al. 2004). Also included in MLL^N are PHD (plant homeodomain) zinc fingers, which are thought to be involved in the transcriptional regulation and chromatin-remodeling activity of MLL, possibly through interaction with Cyp33, a known suppressor of HOXA9 and HOXC8 transcription (Fair et al. 2001).



Scheme 4. 2 subunits of MLL protein with recruited partners.

Abbreviations used on the scheme (alphabetical order): Bmi1-lymphoid oncogene; CBP-CREB-binding protein; CtBP-C-terminal binding protein; FYRC-FYRC domain; GADD34-protein phosphatase 1; H3/H4-histones H3/H4; H3K4/H3K7/H3K27-Me-methylation of lysine 4/7/27 of histone 3; HCF1/2-host cell factor 1/2; HDAC1/2-histone deacetylase 1/2; HPC2-chromobox homolog 2; INI1-SWI/SNF related, matrix associated, actin dependent regulator of chromatin; PcG-polycomb group proteins; PP2A-protein phosphatase 2A; SWI/SNF-protein complex encoded by "SWItched" and "Sucrose Non Fermentation" genes.

MLL^C possesses a transcriptional-activation domain that binds to CBP (CREB-binding protein) (Ernst et al. 2001), a histone acetylase that promotes transcriptional activation by acetylating histone H3 and H4 at target gene loci. The C-terminal-located SET (su[var]3-9, enhancer of zeste, trithorax) domain, a highly evolutionary conserved region, has a lysine-directed histone methyltransferase activity, that methylates lysine 4 of histone H3, thereby maintaining active chromatin (Milne et al. 2002).

I.3.1 MLL and its fusions have an epigenetic function

MLL fusions are responsible for chromatin alterations on target genes,

whose expression is critical to stem cell development or lineage specification in hematopoiesis. Alterations in the 'histone code' or in the DNA methylation content occur as consequence of aberrant targeting of the corresponding enzymatic activities.

The MLL protein was shown to possess HMTase activity toward H3-K4 via its SET domain, leading to transcriptional activation at *HOX* gene promoters, the expression of which is high in progenitor cells and downregulated during differentiation and maturation. Additionally, MLL functions in a large super-complex of at least 29 proteins (scheme 4), which is involved in the remodeling, acetylation, deacetylation and methylation of nucleosomes and histones through association with SWI/SNF, TFIID, NuRD, MBDs, HDACs and HAT CBP.

MLL fusion proteins, which are neither cleaved nor reassociate with MLL^N or MLL^C are still capable of activating the 5´-located and later-expressed *HOX* genes (Milne et al. 2002). Therefore, MLL fusion proteins, which lack C-terminal MLL amino acid residues and SET domain, are also capable of activating some *HOX* genes, suggesting a distinct mechanism for gene regulation by MLL and MLL fusion proteins.

H3-K4 methylation is usually associated with transcriptional activation, presumably in part through the disruption of the binding of the nucleosome remodeling and deacetylase (NuRD) repressor complex to H3 tails and the creation of the docking site for a transcriptional co-activator such as hSNF2H. Methylation of H3-K9 or H3-K27 by some of the PcG proteins, in contrast, is correlated with transcriptional repression, presumably in part through preferential binding of PcG proteins to H3 tails (Cao et al. 2002). Because few histone demethylases have been identified, these findings suggest an attractive model in which lysine methylation serves as a stable epigenetic mark and explain the long-standing functional antagonism between Trithorax and PcG proteins (Milne et al. 2005).

Taken together, these data indicate that MLL and its fusions assemble into

a chromatin-modifying transcription-regulatory supercomplex to coordinate gene expression through such epigenetic pathways as DNA methylation as well as methylation and acetylation, deacetylation and other modes of remodeling of nucleosome-attached histones.

I.4 t(4;11) translocation

One of the most prevalent MLL translocations found in 40% of all MLL rearrangements is the translocation t(4;11)(q21;q23). This reciprocal chromosomal rearrangement results in two fusion products: *MLL-AF4* and *AF4-MLL*. The translocation t(4;11) is associated with a very aggressive and therapy-resistant acute lymphoblastic leukemia in infants and with therapy-related secondary leukemias (Heerema et al. 1994).

I.4.1 MLL-AF4 and AF4-MLL

The *MLL-AF4* gene generated by the t(4;11)(q21;q23) was cloned in the early 1990s. *MLL-AF4* encodes a protein of 2304 amino acids, with the NH_2-terminal 1439 amino acids derived from MLL on chromosome 11, and COOH-terminal 865 amino acids from the AF4 gene on chromosome 4 (Ziemin-van der Poel et al. 1991; Gu et al. 1992; Tkachuk et al. 1992; Domer et al. 1993).

As it was already mentioned, MLL and at least some of its leukemic derivatives are involved in mechanisms controlling *HOX* gene transcription. Moreover, the *HOX* genes *HOXA7* and *HOXA9* in combination with the homeotic gene *MEIS1*, which is a transcriptional regulator of *FLT3*, are necessary for the transformation induced by several different MLL fusion genes. Such a crucial role has not been yet reported for MLL-AF4. Nevertheless, expression levels of several *HOX* genes and of *MEIS1* are raised in both primary t(4;11) ALL and t(4;11) leukemic cell lines.

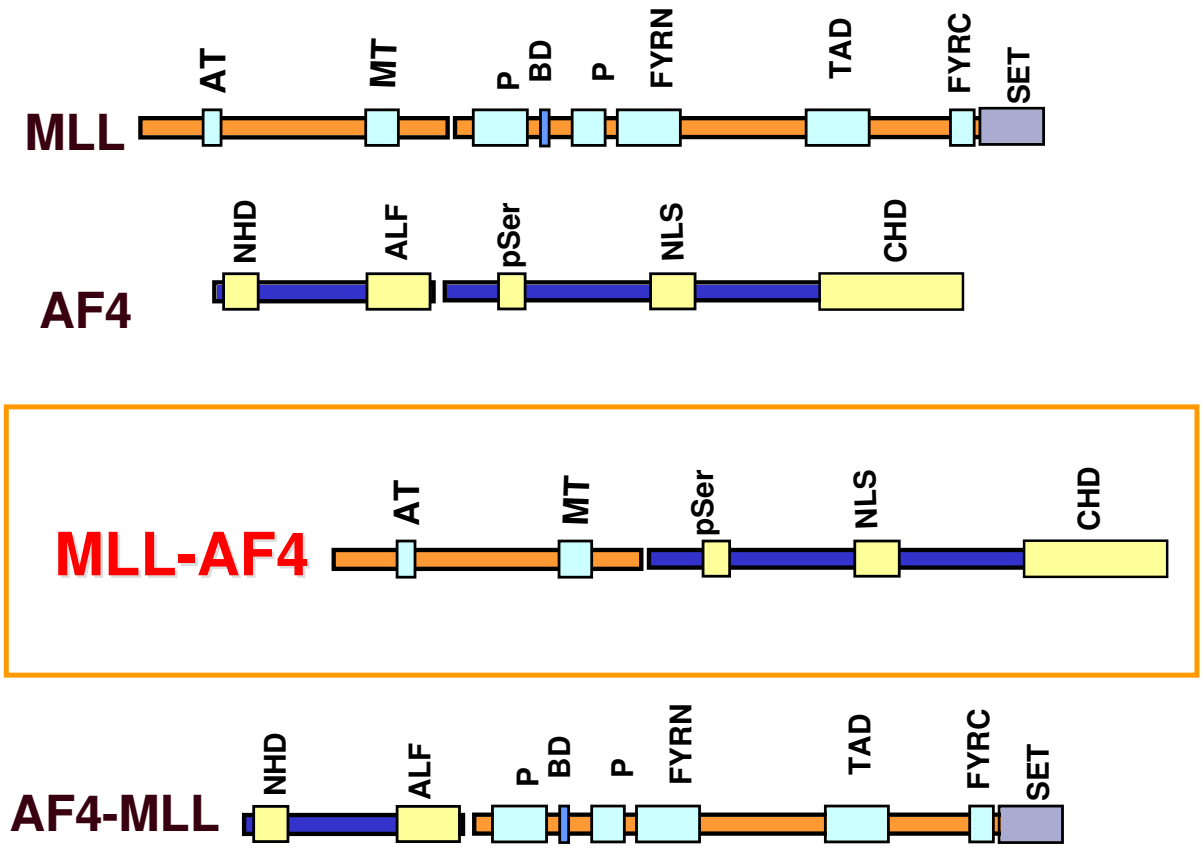
The ALL-1 fused gene from chromosome 4 (*AF4*) encodes a serine/proline-

rich protein containing a nuclear localization signal and a guanosine triphosphate (GTP)-binding domain. It localizes to the nucleus (Li et al. 1998) and is probably involved in the control of gene transcription. Whereas homozygous inactivation of MLL is embryonally lethal, AF4-deficient mice exhibit imperfect T-cell development and modest alterations in B-cell development (Isnard et al. 2000).

The significance of either fusion gene for leukemogenesis is currently not completely understood. *AF4-MLL* has recently been shown to interfere with ubiquitin-mediated AF4 degradation and to transform murine embryonic fibroblasts (Bursen et al. 2004). The authors of this report show that AF4-MLL directly binds and traps SIAH1 proteins disabling the ubiquitin-mediated degradation in t(4;11)-positive cells. These proteins belong to the group of RING Finger E3 ubiquitin ligases that specifically target proteins for their degradation at the 26S proteasome. The major function of E3 ubiquitin ligases is the control of steady-state level of several key proteins. Binding of SIAH1 to these key proteins may be compromised due to a reduction of a steady state of SIAH1 protein levels, which are trapped in the complex with AF4-MLL. This mimics a functional inactivation of SIAH1 protein that is correlated with the inability to promote tumor suppression, execute apoptosis, to cause cell cycle arrest and in general is associated with the aberrant regulation of the ubiquitin-mediated degradation in t(4;11)-positive cells.

Several studies suggest that MLL-AF4 supports cell survival in the t(4;11) context. Cells with t(4;11) translocation survive extended serum starvation (Kersey et al. 1998) and are resistant to CD95-mediated apoptosis (Dorrie et al. 1999).

The aim of this thesis was to address the role and significance of MLL-AF4 in the maintenance of the leukemic phenotype. For that, an RNA interference approach to suppress *MLL-AF4* expression in the SEM and RS4;11 cell lines was developed.



Scheme 5. Domain structure of wild-type MLL and AF4 proteins and of the resultant fusion proteins MLL-AF4 and AF4-MLL.

I.5 RNA interference

RNA interference (RNAi) is induced by exogenously introduced double-stranded RNAs (dsRNAs), RNA viruses, transposons and endogenous short dsRNAs, and may be an ancient defense system against viral infections and other genetic invaders (Huppi et al. 2005). The term RNA interference was first introduced in 1998, after the discovery by Andrew Fire and collaborators that injection of long dsRNA into Caenorhabditis elegans led to efficient and specific gene silencing (Fire et al. 1998). However, in contrast to the situation in invertebrates, introduction of dsRNAs longer than 30 base pairs into mammalian somatic cells activates interferon responses, finally leading to a general inhibition of gene expression. However, the identification of small interfering RNAs (siRNAs), double-stranded RNAs of 21 to 28 nucleotides in length, as

intermediates of the RNAi process paved the way for applying RNAi in mammalian cells (Tuschl et al. 1999).

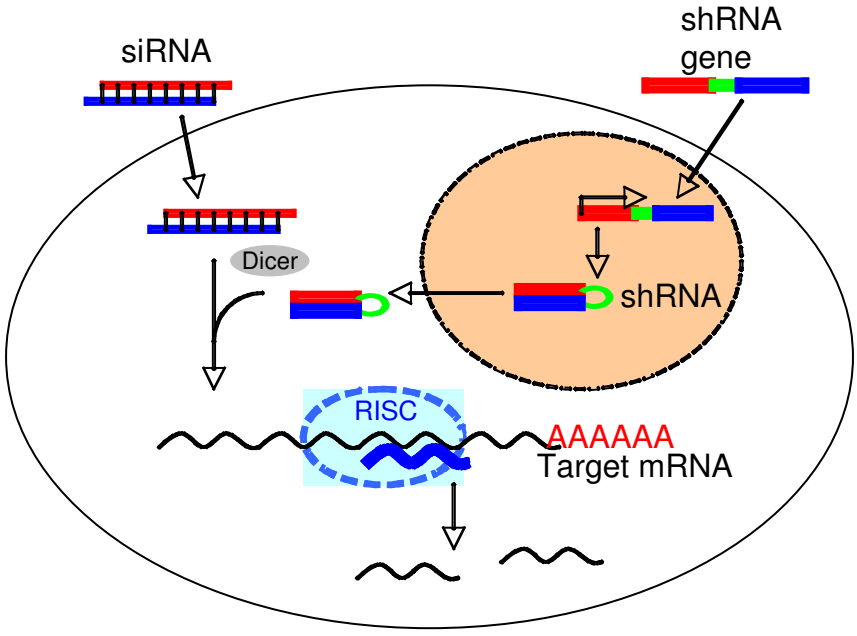
Because of their short lengths, siRNAs only rarely induce interferon responses, but cause an efficient and sequence-specific inhibition of gene expression. An increasing number of vector systems for the ectopic expression of small hairpin RNAs (shRNAs), the intracellularly expressed cousins of siRNAs, complement improved chemical synthesis of siRNAs for transient applications. All these advancements have allowed RNA interference to become a standard tool in molecular and cellular biology within the last 3 years. Moreover, both siRNAs and shRNAs have been successfully applied in vivo in animal models. Because of its high reproducibility, specificity and efficacy, in combination with rapid advances in the delivery of RNAi-inducing molecules, RNAi holds great promise for the development of a new therapeutic strategies (Cheng et al. 2003; Caplen 2004).

I.5.1 Short overview of the siRNA mechanism

Naturally occurring siRNAs are generated from long double-stranded RNAs by the RNase III-type enzyme Dicer. The cleavage creates 5'-phosphate and 3'-hydroxyl termini and yields 21–28 long double stranded RNAs with 2 nucleotide-long 3'-overhangs (Elbashir et al. 2001). The siRNAs associate then with a multiprotein complex to form the RNA-induced silencing complex (RISC; scheme 6). RISC is activated by unwinding the siRNA duplex and by discarding one of the strands. Recent data suggest that a Dicer-containing protein complex may also facilitate these 2 steps (Chendrimada et al. 2005). The remaining strand guides RISC to complementary RNA sequences. RISC does not seem to freely diffuse through the cytoplasm, but has been shown to be part of the cytoplasmic P-bodies (Liu et al. 2005; Sen et al. 2005). The RISC component AGO2 cleaves the target sequence 11 nucleotides away from the 5'-end of the siRNA, leading to the rapid degradation and, thus, inactivation of the

target transcript (Meister et al. 2004; Okamura et al. 2004). Alternatively, particularly in the case of imperfect homology between target sequence and guide strand, RISC may not cleave the target transcript, but may instead inhibit its translation.

Translational interference without degradation of the transcript is also a feature of endogenously expressed micro RNAs (miRNAs). The eminent roles of miRNAs in the regulation of gene expression and the consequences for cellular processes such as differentiation or proliferation have only just recently come to the fore. In contrast to siRNAs, animal miRNAs have an imperfect homology to their binding sequence, which seems to be responsible for the inability of miRNAs to trigger RNA cleavage. Nevertheless, despite differences in the mechanism (RNA degradation versus inhibition of translation), siRNAs and miRNAs share protein components to form the corresponding nucleoprotein complexes (Hutvagner et al. 2002). Both siRNA-mediated RNA degradation and miRNA-mediated inhibition of translation take place in the cytoplasm (Zeng et al. 2002).



Scheme 6. Delivery and processing of siRNA and shRNA.

Inside the cytoplasm, a Dicer-containing complex promotes the dissociation of the double-stranded siRNA to 2 single strands and the formation of catalytically competent RISC. In the case of shRNAs, a vector containing an shRNA expression cassette enters the nucleus via transfection

or viral infection. Inside the nucleus, the transcribed shRNAs are exported into the cytoplasm. Picture is taken from (Thomas et al. 2006).

Thus, post-transcriptional gene silencing takes place in defined structures, like many other essential cellular processes.

I.5.2 siRNA design and delivery ex vivo

The efficiency and specificity of an siRNA is dependent on both siRNA and target site properties. One important point to consider is the choice of the guide strand by RISC. Strand selection is controlled by the thermodynamic stability of the siRNA termini. The strand with the lower 5'-terminal thermodynamic stability is more likely to stay with RISC. Furthermore, an adenosine residue at position 11 of the antisense strand, the complementary position of the cleavage site, can be advantageous for cleavage efficiency, as RISC prefers to cleave RNA to the 3' side of a uridine. However, these rules are not absolute, and siRNAs, which do not adhere to these rules, do not necessarily have inferior activity.

Targeting the fusion site of a leukemic fusion transcript may, depending on the sequences flanking this site, not allow to strictly obey these rules. Instead, a compromise sequence must be found, or, if this is not possible, “non-consensus” siRNAs can be tried. For instance, in the present work active and specific siRNAs were identified by scanning a leukemic fusion site with several siRNAs, which did not follow any of the rules mentioned here (Thomas et al. 2005).

Transient transfections of siRNA duplexes into mammalian cells can be performed with various commercially available cationic lipid formulations, or with electroporation (Ui-Tei et al. 2004; Fedorov et al. 2005). The advantage of such a transient approach is the lack of cell adaptation to reduced target protein levels. For that reason, genes essential for cell proliferation can be targeted by transient siRNA approaches. However, there is no single transfection method that can be successfully applied to all cell types under all experimental

conditions.

It is therefore important to optimize transfection conditions so that maximum gene silencing is achieved. The following transfection parameters have been shown to affect transfection and gene silencing efficacy: cell culture conditions, including cell density and medium composition; the type and amount of transfection agent; the quality and quantity of siRNA; and the length of time that the cells are exposed to the siRNA. Differences have been reported in the ability to transfect and silence gene expression between adherent and nonadherent cells. Post mitotic cells such as neurons and muscle cells tend to be more difficult to transfect using liposomes compared with mitotic cells such as stem cells, fibroblasts and tumor cells.

A major disadvantage of transfections using preformed siRNA lies directly in their transient nature: a long-lived protein may not be affected by such an approach. To overcome this limitation, several siRNA expression systems based on plasmid and viral vectors have been developed to achieve a sustained "knockdown" of the gene of interest (Donze et al. 2002). In most cases, the expression cassettes contain an RNA polymerase III-dependent promoter such as the U6 or the H1 promoters. The siRNAs are expressed as hairpin structures known as short hairpin RNAs (shRNAs), which are processed by Dicer to the mature siRNAs (Brummelkamp et al. 2002).

Because the application of plasmid vectors is frequently hampered by inefficient stable transfection rates and by silencing of the shRNA genes, viral vector systems for the expression of shRNAs have been developed. In particular, lentiviral and oncoretroviral systems have been demonstrated to be effective in most cell lines and primary cell types. Because lentiviruses are able to infect non-cycling and post-mitotic cells, they are now widely used to establish a stable RNAi in, for example, neuronal cells, stem cells and transgenic mouse models (Abbas-Terki et al. 2002).

Such an approach of creating a cell line with the stable expression of

shRNA would not be accessible for studying the functions of MLL-AF4. As it is demonstrated in this work, the reason is that a sustained downregulation of MLL-AF4 leads to the inhibition of proliferation and massive induction of apoptosis of t(4;11)-positive cells, so that 10 days of a continuous MLL-AF4 suppression results in more than eight-fold reduction of a cell population. Therefore, a transient inhibition of MLL-AF4 was used for examining its functions.

I.6 Aim of the project

The aim of the thesis was to analyze the role of the fusion oncogene MLL-AF4 in leukemogenesis and, in particular, its role in proliferation, clonogenicity and apoptosis. In parallel, the molecular mechanisms of MLL-AF4 – mediated gene expression were investigated. As a tool for these studies, siRNAs targeting MLL-AF4 were used to inhibit expression of the fusion transcript in leukemic cells. First of all, the most active and specific siRNA had to be found by scanning the breakpoint site of the fusion gene. The role of MLL-AF4 in clonogenicity, proliferation, apoptosis, differentiation, telomerase activity and engraftment in mouse models was studied by specific MLL-AF4 inhibition with siRNAs, selected by this approach.

To date, the presence of *MLL* rearrangements are indicative of a poor clinical outcome, partially due to the lack of specific pharmacological inhibitors. Therefore, a long-term goal is to establish siRNA approach as an alternative option for developing new strategies for the treatment of these aggressive types of leukemia. The results presented in this work support the role of siRNAs as a specific, but still flexible, and thus promising therapeutic tool for the treatment of t(4;11) ALL.

II. MATERIALS

II.1 Devices

ABI-PRISM-Taqman 7000	Applied Biosystems Detection System, Darmstadt
ABI-PRISM-Sequence-Electroporation-Impulse generator	EPI 2500, Heidelberg
FACSCalibur	Beckton Dickinson, Heidelberg

II.2 Buffers and solutions

Standard chemicals were purchased from AppliChem (Darmstadt), Fluka (Munich), Merck (Darmstadt), Sigma (Munich), Roth (Darmstadt).

Cell culture

RPMI-Medium (Invitrogen, Karlsruhe)	RPMI 1640 with Glutamax-I and 25 mM Hepes 10% FCS
Trypan Blue (Sigma, Munich)	0.5% Trypan Blue 0.9% NaCl in H_2O sterilized with 0.45 µM filter
1x PBS (AppliChem, Darmstadt)	137 mM NaCl 2.7 mM KCl

4.3 mM Na_2HPO_4
1.4 mM KH_2PO_4
pH 7.4

Protein extracts and western blotting

Urea Buffer	9 M Urea 4% CHAPS 1% DTT pH 5.0 stored at –20C
Complete Mini, EDTA-free	Roche, Mannheim
Bradford Reagent Concentrate (BIO-RAD Laboratories, Munich)	Bio-Rad Protein Assay
2x SDS loading buffer	100 mM Tris, pH 6.8 2.5 mM EDTA, pH 8.0 25% Glycerin 0.05% Bromphenolblue 4% SDS 100 mM DTT
Separating Gel	0.5 M Tris, pH 8.8 (5%-15%) 5-15% Acrylamid/Bisacrylamid (37.5:1) 0.1% SDS 0.07% APS

	0.13% TEMED
Stacking Gel	125 µM Tris, pH 6.8 4.8% Acrylamid/Bisacrylamid (37,5:1) 0.1% SDS 0.04% APS 0.4% TEMED
Precast gradient gels for western blotting with MLL-AF4	345-0027 Criterion Tris-HCl Gel, 4–15%, (12+2 well, 45 µl, 13.3 x 8.7 cm) Bio-Rad, Munich
Electrophoresis Buffer	0.3% Tris-Base 1.44% Glycine 0.1% SDS
Coomassie-Blue-staining solution	0.25% Coomassie Brilliant Blue R250 40% Methanol 10% acetic acid filter with Whatman No.1 Filter
Transfer buffer	0.3% Tris-Base 1.44% Glycine 20% 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 Detection Reagents	Amersham Biosciences, Freiburg
Stripping Buffer	100 mM β-Mercaptoethanol
	2% SDS
	62.5 mM Tris, pH 6.7

E.coli transformation

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

Hybridization of siRNAs

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

Reverse Transcription and real-time PCR

Recombinant RNasin Ribonuclease Inhibitor	Promega, Mannheim
5x MMLV-RT-Buffer	250 mM Tris/HCl 375 mM KCl 15 mM $MgCl_2$ 50 mM DTT pH 8.3 Promega, Mannheim
dNTPs	10 mM dATP 10 mM dGTP 10 mM dCTP 10 mM dTTP Promega, Mannheim
SYBR Green PCR Master Mix	Applied Biosystems, Darmstadt

Colony formation assay

Medium for CFA	5 ml PBS 0.56 g Methylcellulose autoclave +75 ml RPMI1640 +20 ml FCS mix for 16-40 h at 4C

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

Complete™ Protease Inhibitor Cocktail Tablets	Chymotrypsin, 1.5 µg/ml Thermolysin, 0.8 µg/ml Papain, 1mg/ml Pronase, 1.5 µg/ml Pancreatic extract, 1.5 µg/ml Trypsin, 0.002 µg/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

ChIP assay

Cell lysis buffer	5 mM PIPES, pH 8
	85 mM KCl
	0.5% NP40
	+Complete, 1 tablet
Nuclei lysis buffer	50 mM Tris-Cl, pH 8.1
	10 mM EDTA
	1% SDS
	+Complete, 1 tablet
IP Dilution Buffer	0.01% SDS
	1.1% Triton X100
	1.2 mM EDTA
	16.7 mM Tris-Cl, pH 8.1
	167 mM NaCl
1x Dialysis buffer	2 mM EDTA
	50 mM Tris/HCl. pH 8
IP Wash buffer	100 mM Tris/HCl. pH 9
	500 mM LiCl
	1% NP40
	1% deoxycholic acid

IP elution buffer	50 mM $NaHCO_3$ 1% SDS
5x PK buffer	50 mM Tris/HCl, pH 7.5 25 mM EDTA 1.25% SDS

II.3 Kits

TRAPeze Telomerase Detection Kit	Integrin Applied Biosystems, Oban Argyll, Scotland
RNeasy Total RNA Isolation Kit	Qiagen, Hilden
DNeasy Tissue Kit	Qiagen, Hilden
QIAquick PCR Purification Kit	Qiagen, Hilden
Qiaquick Gel Extraction Kit	Qiagen, Hilden
EZ DNA Methylation Kit	Zymo Research, Freiburg
TA cloning Kit	Invitrogen, Karlsruhe
AMAXA Nucleophection Kit	AMAXA, Cologne
Annexin V/FITC Kit	Bender MedSystems, Austria
Miniprep Palsmid Isolation Kit	Qiagen, Hilden

II.4 Enzymes

M-MLV Reverse Transcriptase, RNase H Minus, Point Mutant	200 U/µl Promega, Mannheim
RNase A	70 U/mg, 100 mg/ml Qiagen, Hilden
TaqPolymerase	1 U/µl

	Promega, Mannheim
Benzonase	Merck KGaA, Darmstadt
T4 DNA Ligase	Invitrogen, Karlsruhe

II.5 Antibodies

MLLT2, polyclonal, Rabbit	0.2 mg/ml 1:1000 in 10% Milk-blocking solution No. 10852 Orbigen, San Diego, CA
GAPDH Mouse anti-rabbit	4.2 mg/ml 1:20000 in 10% Milk-blocking solution No. 5G4 HyTest, Turku, Finnland
Tubulin Ab-4	1 mg/ml 1:1000 in 10% Milk-blocking solution No. MS-719-PO NeoMarkers, Fermont, CA
Bcl-xL/S Mouse	0.2 mg/ml 1 µg/ml No. 556499

	BD Pharmingen, Heidelberg, Germany
c-Myc	0.7 mg/ml 1:1000 in 5% Milk blocking solution clone 9E10 Biolabs, Hamburg, Germany
Phospho-c-Myc	0.2 mg/ml 1:1000 in 5% BSA and 0.1% NaAzid Cell Signaling Technology, Beverly, MA
Caspase 9	1 mg/ml 1:1000 in TST No. AAM-139 Stressgen, Hamburg, Germany
Cleaved caspase 3 (Asp 175)	0.2 mg/ml 1:1000 in TST No. 9661 Cell Signaling Technology, Beverly, MA
ECL anti-mouse IgG, HRP-conjugated	1:10000 in TST Amersham Biosciences, Freiburg, Germany

ECL anti-rabbit IgG, HRP-conjugated	1:10000 in TST Amersham Biosciences, Freiburg, Germany

II.6 Fluorescent dyes

Propidiumiodid	1 mg/ml in PBS Sigma-Aldrich, Munich

II.7 Synthetic oligonucleotides

siRNAs

Alnylam (Kulmbach)

MLL-AF4 siRNA sequences.

	MLL AF4
	ACAAAAACCAAAAGAAAAG / CAGACCUACUCCAAUGAAG
siMA1	5’ - AAG / CAGACCUACUCCAAUGAA -3’
	3’ - UUUUC / GUCUGGAUGAGGUUACUU -5
siMA2	5’ - AAAG / CAGACCUACUCCAAUGA - 3’
	3’ - CUUUUC / GUCUGGAUGAGGUUACU - 5’
siMA3	5’ - AAAAG / CAGACCUACUCCAAUG - 3’
	3’ - UCUUUUC / GUCUGGAUGAGGUUAC - 5’
siMA4	5’ - GAAAAG / CAGACCUACUCCAAU - 3’
	3’ - UUCUUUUC / GUCUGGAUGAGGUUA - 5’
siMA5	5’ - AGAAAAG / CAGACCUACUCCAA - 3’
	3’ - UUUCUUUUC / GUCUGGAUGAGGUU - 5’

siMA6 5’ - AAGAAAAG / CAGACCUACUCCA - 3’

3’ - UUUUCUUUUC / GUCUGGAUGAGGU - 5’

siMA7 5’ - AAAGAAAAG / CAGACCUACUCC - 3’

3’ - GUUUUCUUUUC / GUCUGGAUGAGG - 5’

siMA8 5’ - AAAAGAAAAG / CAGACCUACUC - 3’

3’ - GGUUUUCUUUUC / GUCUGGAUGAG - 5’

siMA9 5’ - CAAAAGAAAAG / CAGACCUACU - 3’

3’ - UGGUUUUCUUUUC / GUCUGGAUGA-5’

siMA10 5’ - CCAAAAGAAAAG / CAGACCUAC - 3’

3’ - UUGGUUUUCUUUUC / GUCUGGAUG - 5’

siMA11 5’ - ACCAAAAGAAAAG / CAGACCUA - 3’

3’ - UUUGGUUUUCUUUUC / GUCUGGAU - 5’

siMA12 5’ - AACCAAAAGAAAAG / CAGACCU - 3’

3’ - UUUUGGUUUUCUUUUC / GUCUGGA - 5’

siMA13 5’ - AAACCAAAAGAAAAG / CAGACC - 3’

3’ - UUUUUGGUUUUCUUUUC / GUCUGG - 5’

siMA14 5’ - AAAACCAAAAGAAAAG / CAGAC - 3’

3’ - GUUUUUGGUUUUCUUUUC / GUCUG - 5’

siMM 5’ - AAAAG / C**U**GACCU**U**CUCCAAUG - 3’

3' - UCUUUUC / G**A**CUGGA**A**GAGGUUAC - 3'

Bold letters in siMM indicate mismatches with MLL-AF4 fusion site.

siMARS

siMARSs 5’ - ACUUUAAGCAGACCUACUCCA - 3’
siMARSas 3’ - CCUGAAAUUCGUCUGGAUGAGGU - 5’

siHOXA7

siHOXA7s 5’ - CCGUUCCGGGCUUAUACAAUG - 3’
siHOXA7as 3’ - CUGGCAAGGCCCGAAUAUGUU – 5’

siHOXA9

siHOXA9s 5’ - GAUGCCAUUUGGGCUUAUUUA- 3’
siHOXA9as 3’ - CACUACGGUAAACCCGAAUAA– 5’

siMeis1

siMeis1s 5’ - GCAAAGGUACGACGAUCUACC- 3’
siMeis1as 3’ - CGCGUUUCCAUGCUGCUAGAU– 5’

siMenin

siMenin s 5’ - GUCGCAAGUGCAGAUGAAGUU- 3’
siMenin as 3’ -UUCAGCGUUCACGUCUACUUC– 5’

Primers for conventional PCR

Purimex (Grebenstein)

hTERT_ChIP	forward	5´-CAG GCC GGG CTC CCA GTG GAT-3´
hTERT_ChIP	reverse	5´-TGC GCA GCA GGG AGC GCA CG-3´
hTERT_ChIP_2	forward	5´-AGC ACT TCC TCT ACT CCT CAG-3´
hTERT_ChIP_2	reverse	5´-TCC TCG GGG GCC GCC ACA GAG C-3´

Primers for methylation-specific PCR

TERT.F1	forward	5´-GTT TTT AGG GTT TTT ATA TTA TGG-3´
TERT.R1	reverse	5´-AAC TAA AAA ATA AAA AAA CAA AAC-3
TERT.F2	forward	5´-GGG TTA TTT TAT AGT TTA GGT-3
TERT.F4	forward	5´-GGA GGG ATT GGG GAT T-3´
TERT.F5	forward	5´-TTT GGG GTT TTA GGG TTG G-3´
TERT.F6	forward	5´-TAG GTG TTT TGT TTG AAG GAG T-3´
TERT.R6	reverse	5´-CAA CTA AAA ACC ACC AAC-3´
TERT.R2	reverse	5´-AAT CCC CAA TCC CTC C-3´
TERT.F3	forward	5´-GGT TTT TTT AGT TTT TTT TTT-3´
Apaf_AS4	reverse	5´-ATC TTC CCA ACC TAT AAC ACC CTT CCC-3´
Apaf_S4	forward	5´-TAA AAG GGA TAG AAT TAG AGG TGG-3´

Primers for quantitative real-time RT-PCR

APAF1-all	forward	5´-ACC CCT AAT TCC CGG TGG A-3
APAF1-all	reverse	5´-TTT GCG CCT AGG TCT TAG TGG-3´
APAF1-2;3	forward	5´-GGA ATA CAG ACT CAC GTT CA-3´
APAF1-2;3	reverse	5´-GTT CTT CCA TTA ATG AGT T-3
SEPT-all	forward	5´-CTA CCG GGC ACA GTG CAT C-3´
SEPT4-all	reverse	5´-TCA TAC TTG CGA TTC CGT TCC-3´
SEPT_4.4	forward	5´-CAA AGG CCT GAA AGG GAG AGA-3´
SEPT_4.4	reverse	5´-ATG CAT CAC GGT GAG GAG C-3´
SEPT_4.2	forward	5´-ACA GGG TGC ACT GCT GCC-3´
SEPT_4.2	reverse	5´-CAG GAG CCT CAG GCT TGG-3´
SEPT_4.1;3	forward	5´-TGT ACT TCA TCT CAC CCT TC-3´
SEPT_4.1;3	reverse	5´-TCC ACT TCG GGA GGT GTC AG-3
BCL-xL	forward	5´-TGT GGA ACT CTA TGG GAA CAA-3´
BCL-xL	reverse	5´-GTG AGC CCA GAA CCA CGC CG-3´
BCL-x	forward	5´-GCG TAG ACA AGG AGA TGC AGG-3´
BCL-x	reverse	5´-GGT CAT TCA GGT AAG TGG CCA-3
CD133	forward	5´-ATG GCA ACA GCG ATC AAG G-3´
CD133	reverse	5´-GTA CTT TGT TGG TGC AAG CTC T-3´
Meis1.2	forward	5´-GTT GAC AAC CTC GCC TGT GAT-3´
Meis1.2	reverse	5´-CCC CTC AGA CCC AAC TAC CA-3´
GAPDH	forward	5´-GAA GGT GAA GGT CGG AGT C-3´
GAPDH	reverse	5´-GAA GAT GGT GAT GGG ATT TC-3
HOXA10.2	forward	5´-CAG GCC ACC TCG TGC TCT T-3
HOXA10.2	reverse	5´-TTT GTC CGC CGA GTC GTA G-3´
Menin_2	forward	5´-CGG ACC TGG TGC TCC TTT C-3´
Menin_2	reverse	5´-TGG TAG GGA TGA CGC GGT T-3´
MLL-AF4	forward	5´-ACA GAA AAA AGT GGC TCC CCC-3´
MLL-AF4	reverse	5´-TAT TGC TGT CAA AGG AGG CGG-3´

AF4-MLL	forward	5´-CAG AAG CCC ACG GCT TAT GT-3´
AF4-MLL	reverse	5´-GCA AAC CAC CCT GGG TGT TA-3´
AF4-MLL(-11)	reverse	5´-CAA CAC CAA TTT TCC AGC TGG-3´
HOXA7	forward	5´-CGC CAG ACC TAC ACG CG-3´
HOXA7	reverse	5´-CAG GTA GCG GTT GAA GTG GAA-3´
HOXA9	forward	5´-CCA CCA TCC CCG CAC A-3´
HOXA9	reverse	5´-AAC AGG GTT TGC CTT GGA AA-3´
OAS1	forward	5´-TCC AAG GTG GTA AAG GGT GG-3´
OAS1	reverse	5´-AGG TCA GCG TCA GAT CGG C-3´
STAT1	forward	5´-CAT CAC ATT CAC ATG GGT GGA-3´
STAT1	reverse	5´-GGT TCA ACC GCA TGG AAG TC-3´
hTERT	forward	5´-CCA GGG CCT CCA CAT CAT-3´
hTERT	reverse	5´-CAG CGC TGC CTG AAA CTC-3´

II.8 Plasmids

pMSCVpuro-flag-HoxA7 Flag-tagged murine HoxA7 cDNA cloned into the retroviral vector pMSCVpuro (kind gift from R. Slany)

II.9 Cell lines

SEM - human B cell precursor leukemia (Greil et al. 1994), t(4;11)-positive cells, DSMZ No. ACC546

RS4;11 - human B cell precursor leukemia (Stong et al. 1985), t(4;11)-positive cells, DSMZ No. ACC508

MV4;11 - human acute monocytic leukemia (Lange et al. 1987), t(4;11)-positive cells, DSMZ No. ACC102

HL60 - human acute myeloid leukemia (Collins et al. 1977), DSMZ No. ACC3

K562 - human chronic myeloid leukemia in blast crisis (Lozzio et al.

1978), t(9;22)-positive cells, DSMZ No. ACC10

Kasumi-1 - human acute myeloid leukemia (Asou et al. 1991), t(8;21)-positive cells, DSMZ No. ACC220

SKNO-1 - human acute myeloid leukemia (Matozaki et al. 1995), t(8;21)-positive cells

U937 - human histiocytic lymphoma (Sundstrom et al. 1976), t(10;11)-positive cells, DSMZ No. ACC5

III. METHODS

III.1 Abbreviations

3'UTR	3' untranslated region
APS	ammonium persulphate
bp	base pairs
BSA	bovine serum albumin
CIP	chloroform:isoamylalcohol:phenol (24:1:25)
DAPI	4',6'-diamidino-2-phenylindole hydrochloride
ddH2O	double-deionized water
DTT	dithiothreitol
EDTA	ethylenediaminetetraaceticacid
FACS	fluorescence-activated cell sorter
FCS	fetal calf serum
FITC	fluorescein isothiocyanate
GM-CSF	granulocyte-macrophage colony-stimulating factor
kD	kilodalton
PAGE	polyacrylamide gel electrophoresis
PCR	polymerase chain reaction
rpm	revolutions per minute
SDS	sodium dodecyl sulphate
TEMED	tetramethylethylenediamine
X-gal	5-bromo-4-chloro-3-indolyl-3-D-galactoside

III.1. Cell culture

Cell lines

The human leukemia cell lines SEM, RS4;11 (obtained from DSMZ, Braunschweig) and MV4;11 (obtained from J. Krauter, Medical School Hannover) carry the chromosomal translocation t(4;11)(q21;q23) but express different MLL-AF4 variants due to different break points. These cell lines were cultivated in RPMI 1640 Glutamax medium with 10%FCS (PAN Biotech, Aidenbach). Further leukemic cell lines used in this study HL60, K562, Kasumi-1 and U937 were cultivated under the same conditions. SKNO-1 cells were maintained in RPMI 1640 Glutamax medium supplemented with 20% FCS and 7 ng/ml GM-CSF (BD Pharmingen, Heidelberg). All cell lines were incubated at 37°C, 5% CO_2 and 95% air humidity. Highly purified CD34+-selected cells from healthy donors, which had been sampled for reasons of quality control and stored in liquid nitrogen for several years, were thawed and used according to instructional guidelines after confirmation that these cells were no longer useful for the recipient.

Freezing and thawing of the cell lines

The cells were concentrated to 1-2x10^6/ml and resuspended in chilled medium, containing 40 % FCS, 10 % DMSO and 50 % normal medium. The total volume of one frozen stock was 800 µl. For delayed freezing, the tubes were stored in special „freezing“-boxes, filled with isopropanol, for several days at –70°C and afterwards were transferred to the liquid nitrogen.

For recovering, frozen cells into culture medium, cells were quickly thawed, slowly transferred to the 10 ml of room-temperature medium and centrifuged at 1200 rpm for 5 min. After the second wash with the same amount of medium, the cells were resuspended in an appropriate medium volume. The

medium was changed after 24 h of cultivating of freshly thawed cells.

III.2 Transfections

Electroporation

Cells were electroporated in 100–800 µl culture medium at a density of 10^7/ml in 4 mm electroporation cuvettes. SiRNAs were added immediately before electroporation. If not otherwise indicated, the siRNA concentration during electroporation was 500 nM. Electroporations were performed using a rectangle pulse EPI 2500 electroporator. Parameters were 330 V and 10 ms (milliseconds) for Kasumi-1 cells, 370 V and 10 ms for RS4;11 cells and 350 V and 10 ms for all other cell lines. Fifteen minutes after electroporation, cells were diluted twenty fold in culture medium and incubated at 37°C, 5 % CO_2. Using this protocol, we routinely observe less than 10 % of dead cells after electroporation with siRNA transfection efficiency close to 100 %.

Nucleophection

The procedure was performed as described in the protocol to the AMAXA Nucleophection Kit. Cells were concentrated to $1x10^6$/100 µl and transferred into cuvettes, provided with a kit. 2 µg of plasmid DNA were subsequently added to the cells, mixed by pipetting and pulsed with the electroporator. The parameters were 250 V and 15 ms. after the pulsing; the cells were resuspended in fresh medium to the final concentration $5x10^5$/ml.

III.3 RNA isolation

Total RNA extraction was performed with the RNAeasy Kit according to

the protocol. The cells were centrifuged, washed with PBS and resuspended in 350 µl RLT Buffer with 1% β-Mercaptoethanol. The lysates could be either stored at –70°C or immediately used for further extraction. Next, the lysates are transferred to the DNA shredder and centrifuged for 3 minutes at maximum speed. After adding 350 µl of 70 % EtOH to the flow-through, the mixture is applied to an RNAeasy column and centrifuged for 15 seconds at 10.000 rpm. The flow-through after the column is used for subsequent protein lysate preparation (see the chapter below). The column with an immobilized RNA is further treated as described in the manufacturer's protocol.

III.4 Protein lysate preparation

To the flow-through from the RNAeasy column, 100 % acetone was added till the edge of the tube, closed with cups provided and stored at –20°C for at least 1 hour. Afterwards, the acetone mixtures were centrifuged at +4°C, for 20 minutes at 14.000 rpm and the pellets were air-dried. Meanwhile, the urea buffer was thawed at room temperature and the pellets were resuspended in 50 µl of urea buffer. The lysates were vortexed, spun down and the concentration was finally measured using Bradford analysis.

III.5 Real-time RT-PCR

cDNA synthesis

One µg of total RNA was taken for the cDNA synthesis. For the annealing of random hexamers, 5 µl of 100 µM random hexamers were added and the total volume of reaction was adjusted with water to 13 µl. The mixture was incubated for 10 minutes at 70°C and afterwards put immediately on ice. Additional components, such as 4 µl of 5xRT Buffer, 2 µl of 10 mM dNTPs, 0.5 µl of

MMLV-RT and 0.5 µl of RNase Inhibitor were added to the mixture. Following incubation for 10 min at room temperature, the tubes were kept at 42°C for 45 minutes and, finally, put at 99°C for 3 min for enzyme inactivation. To each probe were added 30 µl of water.

Taqman PCR

For the real-time RT-PCR Master Mix were added the following components:

375 nM forward Primer

375 nM reverse Primer

62,5% (v/v) Sybr-Green Mix (Applied Biosystems, Darmstadt)

water up to 12 µl.

For MLL-AF4 primers, a concentration of 62.5 nM was used.

The probes were pipetted in triplicates, 12 µl of master mix and 3 µl of cDNA. The standard Taqman PCR conditions were used:

95°C	10 min	1x
50°C	2 min	1x
95°C	15 sec	40x
60°C	1 min	

III.6 Colony formation assay

Cell lines

Twenty-four hours after cell electroporation with siRNAs, 10,000 cells were plated in 0.5 ml of RPMI 1640 medium containing 20 % FCS and 0.56 % methylcellulose, in 24-well plates. In the case of RS4;11, cell numbers were increased to 12,000 per well. Colonies containing more than 20 cells were counted 14 days after plating. Under these conditions, mock-transfected cells

(electroporated without siRNAs) yielded 50 to 100 colonies per well dependent on the cell line examined.

Primary bone marrow progenitors

Human Colony-Forming Cell Assays were performed using MethoCult® Methylcellulose-based media (CellSystems, St. Katharinen, Germany). After electroporation, 5000 human primary $CD34^+$ cells were plated in duplicate in 35-mm culture dishes with 1 ml of methylcellulose medium. The numbers of CFU-GEMMs and CFU-GMs were counted 10 days after plating.

III.7 MTT test

Cells were electroporated twice within 48 h and were plated on 96-well plates at the density of $0.5x10^5$ cells in 100 µl/well. Every 24 h later, 10 µl of MTT solution (Roche, Mannheim, Germany) was added. After incubation for 4 hours at 37°C, cells were lysed with the solubilization solution according to the manufacturer's instruction. The OD measurements were performed using ELISA Reader (Dynex, Frankfurt/M, Germany) at 560 nm, and 650 nm as a reference wavelength. Cell numbers were calculated by cell dilution series.

III.8 FACS analysis

Cell-cycle analysis

At indicated time points after electroporation, $0.5x10^6$ cells were suspended in 200 µl citrate buffer (250 mM sucrose, 40 mM sodium citrate pH 7.6) followed by the addition of 800 µl staining solution (phosphate-buffered saline containing 20 mg/l propidium iodide, 0.5 % (w/w) NonIdet P40, 500 µM

EDTA) and 10 µl boiled RNase A (10 g/l). Cells were kept for 30 minutes on ice and analyzed by flow cytometry (FACSCalibur, Becton Dickinson, Heidelberg, Germany). Data analysis was performed with ModFit (Verity Software House, Inc) or FCSPress 1.3 http://www.fcspress.com.

Apoptosis assay

Apoptosis was examined with human Annexin V/FITC Kit, according to the provider's instructions. Briefly, 2-5x10^5 cells were washed with PBS at the indicated time points after electroporation followed by incubation in the presence of Annexin-V-FITC solution for 10 min at room temperature. The cells were washed again with PBS and stained with propidium iodide. The samples were then immediately analyzed by flow cytometry using a FACSCalibur.

CD surface marker staining

Surface expression of CD133 was monitored by its staining with a phycoerythrin-conjugated CD133 antibody (Miltenyi Biotech, Berhisch Gladbach). The cells were centrifuged, resuspended in 50 µl of FACS buffer and then 10 µl of antibody was added. After incubation at room temperature in the darkness, the cells were washed with PBS and finally resuspended in 200 µl of PBS followed by flow cytometry analysis.

III.9 Western blotting

Total lysates (50 µg for MLL-AF4 detection, 10-20 µg for all other immunoblots) were analyzed as follows. The lysates were separated by 5 % sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred onto polyvinylidenefluoride membranes. After blocking with Tris-buffered saline/0.1 % Tween20 containing 10 % fat-free milk, blots were incubated overnight in Tris-buffered saline/0.1 % Tween20 containing 1 % fat-free milk and 2.5 mg/L

of an appropriate antibody. As secondary antibody, a corresponding IgG horseradish peroxidase conjugate (1:10000 dilution; Amersham, Freiburg, Germany) was used as recommended by the manufacturer. Detection was performed with enhanced chemiluminescence plus (Amersham) using Hyperfilm ECL (Amersham).

III.10 Xenotransplantation of SCID mice

Female 4-5 week old CB-17/lcrCrj-SCID/SCID mice were obtained from Charles River Germany. SEM cells ($2x10^7$) were electroporated on day one and day 3 either without (Mock) or with 500 nM of the indicated siRNA. On day 4, the cells were counted with Trypan blue and were injected intraperitoneally into mice. Animals were maintained and treated according to protocols approved by the Regional Board Tübingen.

III.11 Histology

Organs were removed and fixed in neutrally buffered 4% formalin at room temperature for 4-5 days followed by dehydration, embedding into paraffin and sectioning. The tissues were stained with hematoxylin (Meyer´s hemalum solution, Merck, Darmstadt, Germany) and eosin (Eosin Y, Merck, Darmstadt, Germany) for light microscopy. Light microscopy was performed with a Zeiss Axioplan microscope (Zeiss, Guttingen, Germany) using a 20 x Plan-Neofluar or 40 x Plan-Neofluar 1.3 oil lens. Images were captured using Axio Vision 4 Software provided with the microscope and Adobe Photoshop (Adobe Systems, San Jose, CA, USA).

III.12 Telomerase activity assay

III.12.1 Conventional TRAP assay

Telomerase repeats amplification protocol (TRAP) assay was performed using a TRAPeze Telomerase Detection Kit according to the manufacturer's protocol with some modifications. Briefly, a master mix containing all of the reagents outlined below was made for a final volume of 50 µl. The reagents included 5 µl of 10x TRAP buffer, 1 µl of TS primer, 1 µl of TRAP primer mix, 1 µl of 50x dNTPmix, 1 µl of Taq polymerase (5 units/µl) and RNase and DNase free distilled water to a final volume of 48 µl. 2 µl of cell extracts in CHAPS lysis buffer (2 µg/2 l) was added to each tube containing the reagent mix. 2 µl of CHAPS lysis buffer and 1 µl of TSR8 (supplied with TRAP kit) were used as negative and positive control respectively. All the tubes were incubated at room temperature for 30 minutes before the PCR was performed with the two steps PCR protocols (30 seconds at 94°C and 30 seconds at 59°C) for 30 cycles. The PCR products (one half) were electrophoresed on sequencing 10 % polyacrylamide gel and visualized with camera.

III.12.2 SYBR Green RQ-TRAP analysis

This assay was performed according to the original paper (Wege et al. 2003) with some modifications.

Preparation of telomerase samples

$5x10^3$ and $7x10^3$ cells (2 samples for each experimental condition) were pelleted and washed with 200 µl of PBS. After the centrifugation, the cells were resuspended in 60 µl of Telomerase Lysis Buffer and incubated for 30 minutes

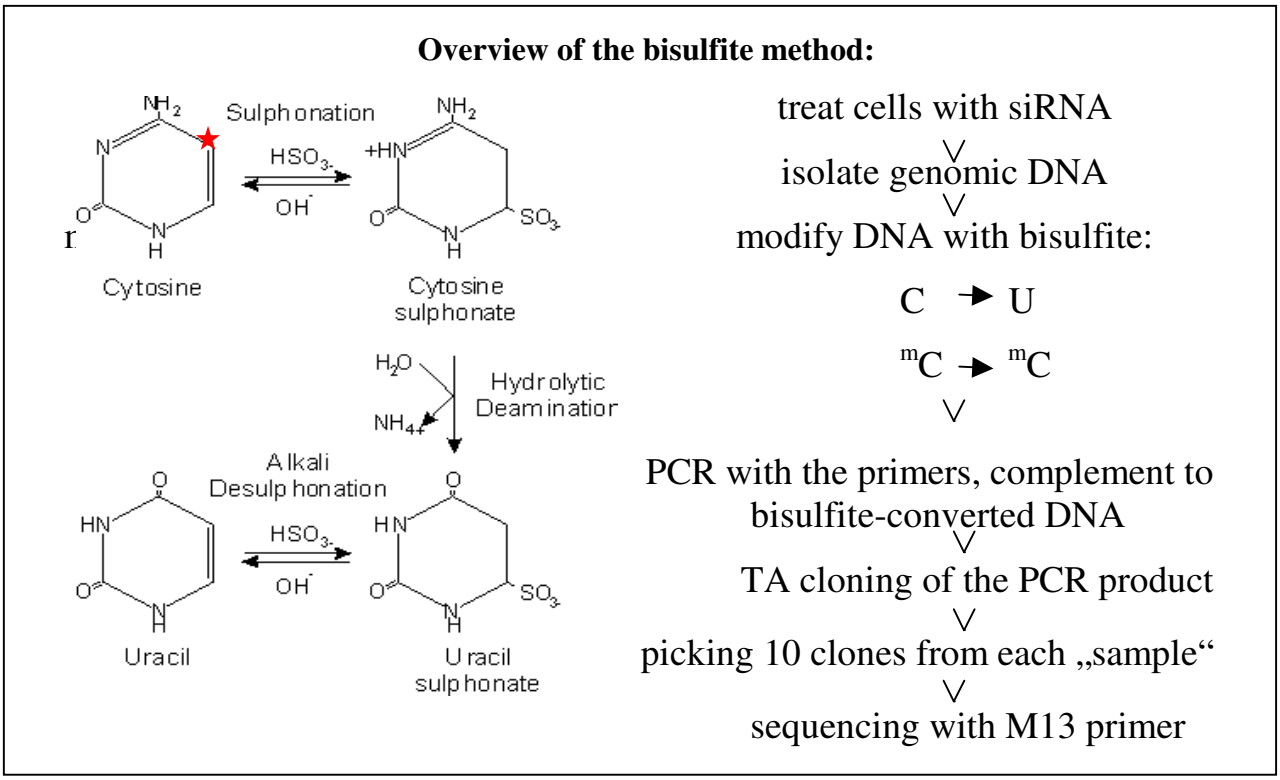
on ice. Afterwards, the cells were centrifuged for 30 minutes at 4°C at 14.000 rpm and 2 µl of the supernatant were subsequently used for Taqman-PCR. The final samples were stored at 4°C.

Taqman-PCR

For the Taqman PCR 2µl of the telomerase sample, 0.1 µg of telomerase primer TS, and 0.05 µg of anchored return primer ACX, in 25 µl with SYBR Green PCR Master Mix (Applied Biosystems, Darmstadt) were mixed. Using the ABI Prism 7700 thermal cycler (Applied Biosystems), samples were incubated for 20 min at 25°C and amplified in 40 PCR cycles with 30 s at 95°C and 90 s at 60°C (two-step PCR). The threshold cycle values (C_t) were determined from semi-log amplification plots (log increase in fluorescence versus cycle number) and compared with standard curves generated from serial dilutions of the same telomerase samples (1000, 100, 10, 1 cell). Standards, inactivated samples and lysis buffer were assayed anew on every plate. Each sample was analyzed at least in triplicates.

III.13 DNA methylation analysis

For the detection of DNA methylation, the method of direct sequencing of bisulfite treatment was used. The general overview of the method is outlined on the scheme 7. This technique consists of treating DNA with bisulfite, which causes unmethylated cytosines to be converted into uracil while methylated (shown with the star on the scheme) cytosines remain unchanged. Further, the treated DNA is amplified by PCR and the resulting PCR products are cloned in TA vectors. The colonies, which contain an insert, are picked and transferred into liquid medium for growing overnight. After the plasmid isolation, every plasmid was analyzed by sequencing with M13 primer. In total, 10 clones per each sample were analyzed.



Scheme 7. Overview of the bisulfite method of DNA methylation analysis (A. Gratchev "Analysis of methylation using bisulfite sequencing" http://www.methods.info/Methods/DNA_methylation/Bisulphite_sequencing.html).

Isolation of genomic DNA

Genomic DNA isolation was performed with DNeasy Kit from Qiagen according to the provided protocol. Briefly, 2-5x10^6 cells were pelleted by centrifugation with 1200 rpm (400 g in Sorvall ST-H750 rotor) for 5 minutes and resuspended in 200 µl of PBS. After addition of 20 µl of proteinase K and 200 µl of buffer AL, the sample was vortexed and incubated at 70°C for 10 minutes. Next, 200 µl of 100 % ethanol was added and the whole mixture was transferred onto the DNeasy mini spin column and centrifuged at 8000 rpm (3600 g in Sorvall 3328 rotor, table centrifuge) for 1 minute. After the centrifugation, the column was placed onto the fresh collection tube and 500 µl of AW1 buffer was added to the column. The sample was then centrifuged at 8000 rpm for 1 min and the washing step with 500 µl of buffer AW2 was repeated, but this time by centrifuging at 14000 rpm (11000 g in Sorvall 3328 rotor) for 3 minutes to dry the column. The DNA was eluted with 200 µl of AE

buffer by centrifugation for 1 minute at 8000 rpm. The DNA concentration was determined photometrically.

Treatment of genomic DNA with bisulfite

The treatment of genomic DNA with bisulfite was performed using EZ DNA Methylation Kit™ according to the provided protocol. Briefly, for one modification reaction 2 µg of the isolated genomic DNA were mixed with 5 µl of M-dilution buffer and the sample was filled with water up to 50 µl. The sample was then incubated for 15 minutes at 37°C and after this 100 µl of the prepared CT Conversion Reagent (i.e. resuspended in 750 µl of water and 210 µl of M-dilution buffer) and slightly vortexed. The sample was then incubated for 16 hours in the darkness at 50°C. After the incubation, the sample was placed on ice for 10 minutes and then 400 µl of a binding buffer was added and the sample was thoroughly mixed. The mixture was loaded onto Zymo-Spin I column and centrifuged for 30 seconds at 10000 rpm (5590 g in Sorvall 3328 rotor). For washing the column, 200 µl of M-wash buffer was added onto the column and centrifuged for 30 seconds at 14000 rpm (11000 g in Sorvall 3328 rotor). Next, 200 µl of M-desulphonation buffer were loaded onto the column for 15 minutes and after the incubation spun at 14000 rpm for 30 seconds. Two wash steps with 200 µl of M-wash buffer and centrifuging at 14000 rpm for 30 seconds are following. Finally, the modified DNA was eluted with 10 µl of M-elution buffer and centrifugation at 14000 rpm for 1 minute.

Amplification of the converted DNA

Total volume of PCR reaction was 40 µl, consisting of the following components:

2 µl of bisulfite-treated DNA

4 µl of 10x TaqPolymerase Buffer

1 µl of 10mM dNTPs

2 µl of DMSO

1 µl of 10µM forward primer

1 µl of 10µM reverse primer

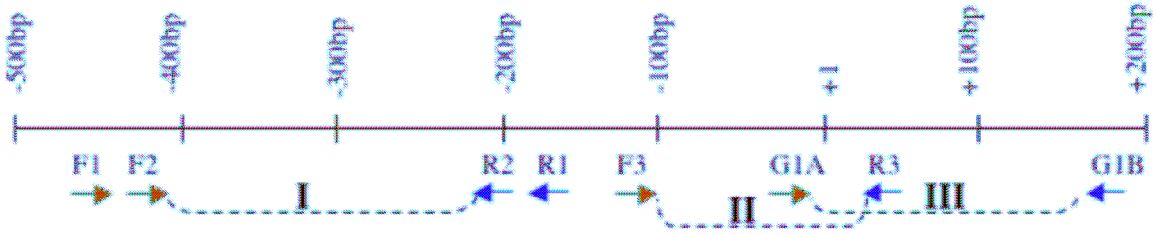
2 µl of Taq Polymerase (New England Biolabs, Frankfurt)

27 µl of water

The PCR conditions were following:

4 minutes at 95°C	x1
30 seconds at 95°C	x35
30 seconds at 56°C	
1 minute at 72°C	
7 minutes at 72°C	x1

For the amplification of the region I, a nested PCR was performed. For the first round the primer pair F1-R1 was taken and for the second round 2µl of the first-round reaction was amplified with the primer pair F2-R2 (scheme 8). The regions II and III were amplified with the primers F3-R3 and G1A-G1B, respectively.



Scheme 8. Schematic representation of the primer positions for the analysis of DNA methylation within hTERT promoter.

Red arrows designate forward primers, blue arrows-reverse primers. The roman numbers indicate regions, separately analyzed for DNA methylation. The base pair numbers are calculated in regard to the transcription start.

Extraction of the PCR product

After the PCR, the whole reaction was loaded on 2 % agarose gel and the corresponding PCR product was excised and cleaned up with the QIAquick Gel

Extraction Kit from Qiagen according to the protocol. Briefly, 3 volumes of buffer QG were added to the excised band and the sample was incubated at 50°C till complete diffusion of the agarose in the tube. The sample then was applied onto QIAquick spin column and centrifuged for 1 min at maximum speed. For washing, 750 µl of buffer PE were added and the column was centrifuged for 1 minute at maximum speed. For the final elution, the column was placed in a clean tube and 50 µl of water were added directly onto the column matrix. The DNA was eluted by centrifugation at maximum speed for 1 minute.

TA cloning

For the cloning into TA vector, the TA cloning® Kit was applied according to the provided protocol. The following ligation mixture was prepared:

5 µl of 2x Rapid Ligation Buffer

1 µl of pGEM-T Easy (50 mg/l)

3 µl of PCR product

1 µl of T4-DNA-Ligase (3 U/µl)

After mixing by pipetting up and down, the ligation mixture was incubated for 1 hour at room temperature.

Transformation of E.coli DH5α

100 µl of KCM solution were added to the ligation mixture and mixed by pipetting. The competent cells were thawed and the ligation mixture with KCM solution was given to the competent cells. After the incubation for 20 minutes on ice, the heat-shock at 42°C for 2 minutes was performed, followed by ice incubation for 5 minutes. Then, 400 µl of LB medium was added, and the cells were incubated for 1 hour at 37°C on the rotating platform. After the incubation, 200 µl of the bacteria were plated on each LB-Amp/X-Gal plates, followed by overnight incubation at 37°C.

Picking the colonies and mini-preparation of the plasmids

White colonies were picked and transferred into 1 ml of liquid LB medium, supplemented with ampicillin. The plasmid isolation was made with Miniprep Plasmid Isolation Kit according to the provided protocol with some modifications. After overnight incubation in the shaker by 37°C, the cells were pelleted and resuspended in 100 µl of the P1 buffer. Next, 100 µl of the buffer P2 were added, the tubes were 5 times inverted and incubated for 4 minutes at room temperature. After addition of 100 µl of buffer P3, the tubes were again inverted and stored for 5 minutes on ice. Further, the lysates were centrifuged at 14.000 rpm for 7 minutes and the supernatants were transferred in new tubes. 500 µl of EtOH was added, the whole mixture was vortexed and centrifuged for another 10 min at 14.000 rpm. The pellet was resuspended in 50 µl of water.

Sequencing

PCR reaction

For the sequencing PCR, the following mixture was set up:

0,5 µl of 10 µM M13 primer

0,5 µl of the plasmid

2 µl of Big DyeMix (Applied Biosystems, Darmstadt)

7 µl of water

The following PCR conditions were used:

1 minute at 96°C	x1
10 seconds at 96°C 5 seconds at 55°C 4 minutes at 60°C	x30

Precipitation and extraction of the PCR product

After the PCR reaction, 1 µl of 3 M NaAc (pH 5,5) and 25 µl of EtOH were added, and the samples were kept at –70°C for 1 hour or at –20°C overnight. The reactions were centrifuged for 20 minutes at 13.000 rpm (9500 g in Sorvall 3328 rotor) and the pellets were washed with cold 70 % EtOH. After the subsequent centrifugation, the pellets were air-dried and resuspended in 25 µl of TSR solution. The probes were then incubated for 2 minutes at 90°C and the tube lids were substituted with the gum covers for the sequencer.

III.14 Treatment with 5-aza-2´-deoxycytidine

Cells were treated with 20 µM 5-Aza-dC (Sigma-Aldrich, Munich) for 96 hours. After the plating on the first day, the medium was supplemented every day with the fresh 5-Aza-dC, since the substance is not stable in culture medium for longer than 24 hours. The medium was completely changed every second day and the genomic DNA or total RNA were isolated on day 4.

III.15 Chromatin Immunoprecipitation (ChIP) assay

The protocol for ChIP assay was taken from the lab of P. Farnham (Weinmann et al. 2002). Briefly, SEM cells were transfected with 2 µg of the HOXA7-flag or FHL2-flag (control) construct using an AMAXA Nucleophection protocol. After 16 hours, transfected cells were cross-linked with formaldehyde at a final concentration of 1 % for 15 min at 37°C. The fixation was stopped by the addition of glycine to a final concentration of 0.125 M. The cells were collected by centrifugation and once rinsed with cold PBS. The cell pellets were resuspended in cell lysis buffer, incubated on ice for 20 min and the nuclei were collected by microcentrifugation and then resuspended in nuclei lysis buffer. The samples were incubated on ice for 10 minutes and

subsequently sonicated. The parameters for sonication were: duty cycle 50, output 5, pulse for 10 seconds; each sample was pulsed 5 times with the break of 2 minutes in between. The chromatin solution was precleared with the addition of *Staphylococcus aureus (Staph A)* cells, which express protein A (Cowan 1 strain, kindly provided by the lab of Prof. A. Peschel) for 15 min at 4°C. Prior to use, the Staph A cells were blocked with 1 µg of sheared herring sperm DNA (Gibco, Karlsruhe) /µl and 1 µg of bovine serum albumin (Applichem, Darmstadt) /µl for at least 4 h at 4°C. Precleared chromatin from 10^6 cells was incubated with 1 µg of anti-flag antibody or no antibody and rotated at 4°C for approximately 12 to 16 h. The next day, 15 µl of blocked Staph A cells were added to the samples and rotated for 15 minutes at 4°C. After the pull-down, the probes were centrifuged at 14.000 rpm for 3 minutes at room temperature and the pellets were washed with dialysis buffer in a following way: 1.4 ml of dialysis buffer was added, thoroughly pipetted up and down, the sample was rotated for 3 minutes at room temperature and centrifuged for 3 minutes at 14.000 rpm (11000 g in Sorvall 3328 rotor). The same washing procedure was subsequently repeated five times with IP Wash Buffer and finally the immune complexes were eluted with 150 µl of freshly prepared IP Elution Buffer, vigorously vortexed for 15 minutes, pelleted by centrifugation for 3 minutes at 14.000 rpm and the supernatant was transferred into new tube. The elution procedure was repeated with another 150 µl of IP elution buffer and the final eluates were combined. Prior to the first wash, 10 % of the supernatant from the reaction with no primary antibody for each time point was saved as total input chromatin and was processed with the eluted immunoprecipitates beginning at the cross-link reversal step. Cross-links were reversed by the addition of NaCl to a final concentration of 200 mM, and RNA was removed by the addition of 10 µg of RNase A per sample followed by incubation at 65°C for 4 to 5 h. The samples were then precipitated at -20°C overnight by the addition of 2.5 volumes of ethanol and then pelleted by microcentrifugation. The samples were

resuspended in 100 µl of Tris-EDTA (pH 7.5), 25 µl of 5x proteinase K buffer and 1.5 µl of proteinase K (Roche Mannheim) and incubated at 45°C for 2 h. Samples were extracted with phenol-chloroform-isoamyl alcohol (25:24:1) followed by extraction with chloroform-isoamyl alcohol and then precipitated with 1/10 volume of 3 M NaOAc (pH 5.3) and 2.5 volumes of ethanol. The pellets were collected by microcentrifugation, resuspended in 30 µl of H_2O, and analyzed by PCR.

ChIP PCR

The following components were mixed for PCR reaction:

1 µl of dNTPs (10 mM)

1,5 µl of forward primer (10 µM)

1,5 µl of reverse primer (10 µM)

5 µl of 10x Taq Buffer

1 µl of the template

37,5 µl of water

2,5 µl of Taq Polymerase (1 U/µl)

The following PCR conditions were used:

2 minutes at 94°C	x1
30 seconds at 94°C 30 seconds at 58°C 30 seconds at 72°C	x35
7 minutes at 72°C	x1

Staph A cells

Staph A cells from 1 liter of overnight culture were pelleted and resuspended in 10 ml of 1x dialysis buffer. The cells were then centrifuged at 10.000 rpm (5600 g in Sorvall 3328 rotor) for 5 minutes at 4°C and washed

similarly one more time. Next, the pellets were resuspended in 3 ml of 1x PBS+3 % SDS+10 % β-Mercaptoethanol and boiled for 30 minutes. The samples were afterwards centrifuged at 10.000 rpm for 5 minutes, washed in 1x dialysis buffer and centrifuged again at 10.000 rpm for 5 minutes. The washing step was repeated. Finally, the probes were resuspended in 4 ml of 1x dialysis buffer, divided into 100 µl aliquots, snap frozen and stored at –70°C.

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IV. RESULTS

IV.1 Identification of specific and efficient siRNAs using an siRNA scan

In order to identify efficient MLL-AF4 siRNAs, an siRNA scan of the *MLL-AF4* fusion site of 36 nucleotides in length was performed. For that, 13 different siRNAs were synthesized with target sites moved by one single nucleotide each (siMA1 to siMA14, sequences in "Materials" section). In contrast to the common siRNA design with two 3'-overhangs, siRNAs consisting of a 21 nucleotides long sense and a 23 nucleotides long antisense strand were used. As a consequence, these siRNAs form only a single 3'-overhang by the antisense strand. The efficiencies of the different siRNAs to reduce the level of *MLL-AF4* mRNA were examined in the t(4,11)-positive SEM cell line. Electroporation of SEM cells with 500 nM of different MLL-AF4 siRNA reduced *MLL-AF4* mRNA levels by 5 % to 75 % (Fig. 1). The total RNA was isolated 24 h after electroporation and real-time quantitative RT-PCR analysis was performed in triplicates for each probe. Mismatch control siRNA, siMM, or the sequence-unrelated siRNAs siK4 and siGL2 targeting neomycin phosphotransferase and luciferase mRNA, respectively, hardly affected *MLL-AF4* levels. Two most active siRNAs, siMA3 and siMA6 were chosen for the further experiments. SiMA13 reduced *MLL-AF4* mRNA levels only by 10 % and was included in the experiments as a further control siRNA.

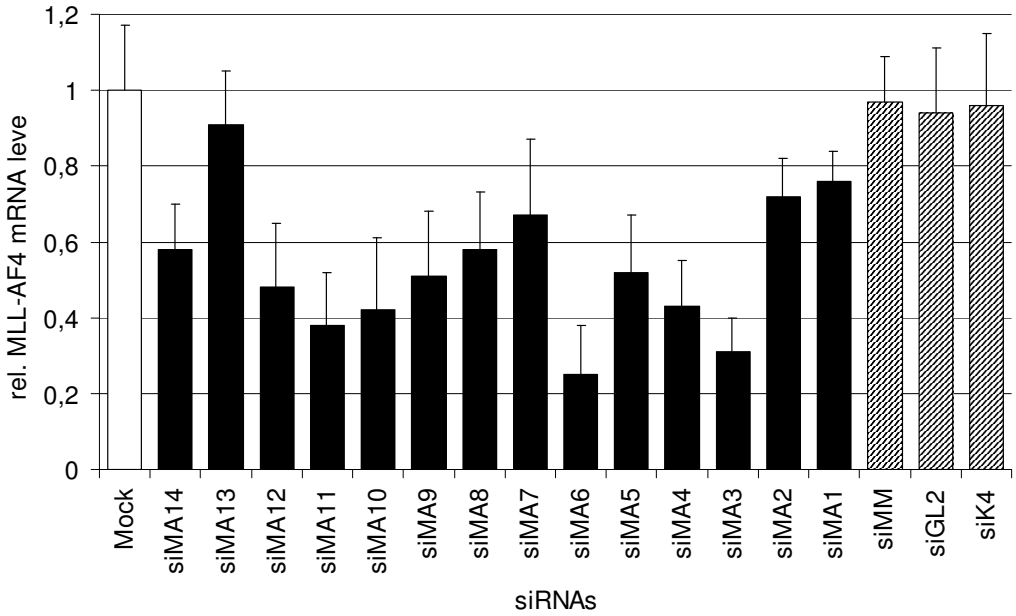


Fig. 1. siRNA scan of the *MLL-AF4* fusion site.

One of two experiments yielding very similar results is shown. Total RNA was isolated 24 h after electroporation with 500 nM of the indicated siRNAs and analyzed by real time RT-PCR. *MLL-AF4* mRNA levels normalized to the *GAPDH* mRNA levels are shown. White column, mock control; hatched columns, control siRNAs; black columns, MLL-AF4 siRNAs.

IV. 2 Effects of siRNAs on MLL-AF4 mRNA

IV.2.1 Decrease of MLL-AF4 protein level

The reduction of *MLL-AF4* mRNA levels by siMA6 lead to decreased MLL-AF4 protein levels (Fig. 2). MLL-AF4 was detected with an antibody targeting the C-terminus of AF4. The molecular weight of MLL-AF4 fusion protein is 240 kDa, whereas the predicted molecular weight of AF4 is 130 kDa. The cells were electroporated with 500 nM of siMA6 or siMM, total cell lysates were isolated using RNeasy column 48 h later and 50 µg of the protein were loaded on the gradient Tris-HCl PAA gel (4%-15%). SEM cells, electroporated with siMA6, contained substantially less MLL-AF4 protein than cells either electroporated without any siRNA ("Mock") or cells treated with the mismatch

control siMM. Unspecific bands, running at 70 kDa, 130 kDa and 180 kDa were unaffected. Amounts of GAPDH protein were detected for the loading control.

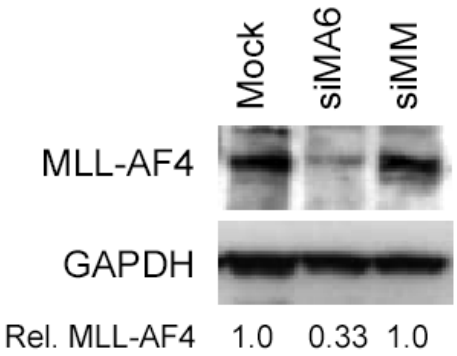


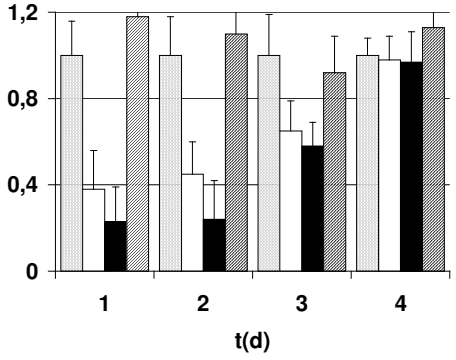
Fig. 2 Depletion of MLL-AF4 protein upon siMA6 transfection.

GAPDH served as a loading control and for normalization. Normalized MLL-AF4 protein levels, calculated using densitometric software Image Gauge V3.12 (Fuji Film Co.) are indicated at the bottom. The experiment was repeated three times.

IV.2.2 Time-course and dose-dependence of siRNA treatment

Next, the time course and dose dependency of the *MLL-AF4* suppression were examined. Electroporation of SEM cells with 500 nM of siMA3 or siMA6 resulted in more than a twofold reduction of *MLL-AF4* mRNA levels. The extent of reduction reached its maximum one day after electroporation and stayed unchanged till the second day. *MLL-AF4* transcript levels showed a complete recovery after four days (Fig. 3A). The depletion of *MLL-AF4* with siRNA was also dose-dependent. The siRNA concentration of 100 nM resulted in a two-fold reduction of *MLL-AF4* transcript levels. The working concentration for the further experiments was chosen to be 500 nM (Fig. 3B).

A



B

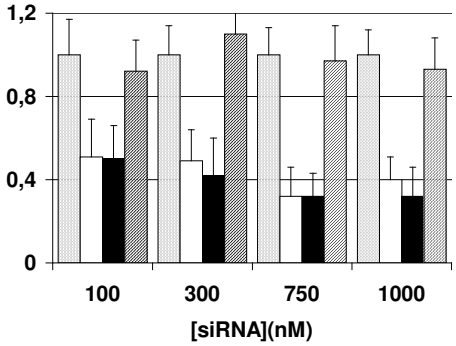


Fig. 3 siRNA-mediated inhibition of MLL-AF4 expression in SEM cells.

A. Time course of MLL-AF4 suppression. Total RNA was isolated at the indicated time points after transfection with 500 nM of siRNA and analyzed by real-time RT-PCR. The average of at least three independent experiments in triplicates is shown here. The error bars indicate standard deviations between three experiments. B. Dose-dependent suppression of fusion transcript by MLL-AF4 siRNAs. SEM cells were electroporated with the indicated concentrations of siRNAs. Twenty four hours later, total RNA was isolated and analyzed by real-time RT-PCR. One representative experiment is shown here, the experiment was repeated twice in triplicates. The error bars show standard deviations.

IV.2.3 Specificity of MLL-AF4 siRNAs

To test the specificity of chosen siRNAs, the levels of wild-type *AF4* and *MLL* mRNA levels were analyzed using real-time RT-PCR. Whereas siMA6 affected neither unfused *AF4* nor *MLL* mRNA levels, siMA3 substantially reduced *AF4* levels (Fig. 4). Next, the specificity of siRNAs was tested in several cell lines. These cell lines included t(8;21)-positive cell lines Kasumi-1 and SKNO-1, human histiocytic lymphoma cell line U937, acute promyelocytic leukemia cell line HL-60, human chronic myeloid leukemia cell line K562,

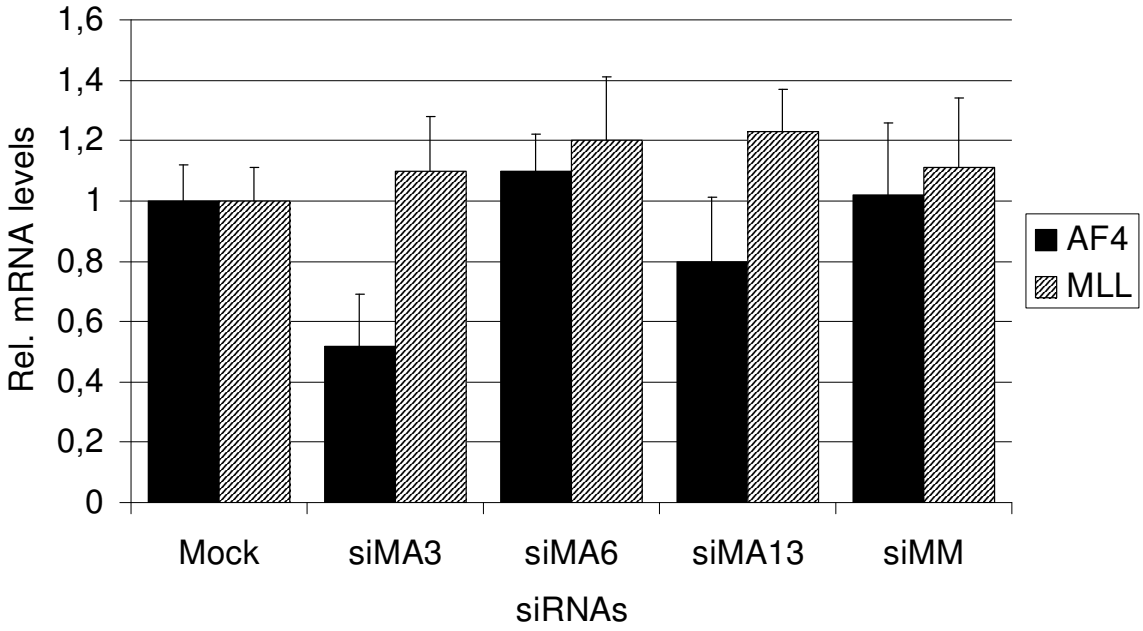


Fig. 4 Effects of MLL-AF4 siRNAs on wild-type *MLL* and *AF4* mRNA levels.

The cells were electroporated with the indicated siRNAs and the RNA was isolated 24 hours later. The average of at least three independent experiments in triplicates is shown here. The error bars indicate standard deviations.

t(4;11)-positive cell lines with different chromosomal breakpoints RS4;11 and MV4;11. The summarized outcome of the Taqman analysis of several cell lines after transfection with indicated siRNAs is shown in Table 1. It appeared that siMA3 affected the levels of *AF4* mRNA in all tested cell lines, except MV4;11. Therefore, siMA3 was excluded from further experiments, because of the lack of specificity towards reducing *MLL-AF4* mRNA levels.

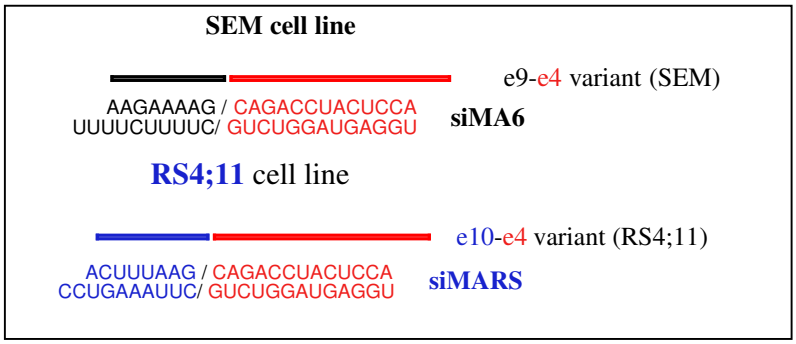
	siRNA			
Transcript	siMA3	siMA6	siMA13	siMM
MLL-AF4	+	+	-	-
alt. *MLL-AF4*	+[a]	-	-[c]	-
MLL	-	-	-	-
AF4	+[b]	-	-[c]	-

Table 1. Effects of MLL-AF4 siRNAs on fusion and wildtype mRNA levels.

+, decrease by 40% to 70%; -, less than 40% decrease. Alt. *MLL-AF4*, *MLL-AF4* variants expressed in MV4;11 and RS4;11 (see scheme 1). [a] Only in RS4;11, not in MV4;11; [b] No decrease in MV4;11; [c] Not tested in HL60 and K562. Each Taqman analysis was performed in triplicates at least twice.

IV.3 Characterization of a similar siRNAs in RS4;11 cell line

The *MLL-AF4* fusion site varies between different t(4;11)-positive cell lines. Whereas SEM cells express a transcript containing an e9-e4 fusion, RS4;11 cells (Domer et al. 1993) express an e10-e4 variant.



Scheme 9. Sequences and positions of siMARS, targeting e10-e4 variant in RS4;11 cells and siMA6, directed against e9-e4 variant in SEM cells.

An siRNA, positioned analogously to siMA6 towards e10-e4 variant was synthesized (Scheme 9) and its activity and specificity was tested in RS4;11 cells. This siRNA was given a name siMARS (*MLL-AF4* transcript in RS4;11 cells). The siMA6 and siMARS share more than 60% homology.

IV.4 Specificity and activity of siMARS and siMA6 in RS4;11 and SEM cell lines: „cross-over"-experiment

To test the specificity of siMA6 and siMARS in targeting corresponding *MLL-AF4* transcripts, a „cross-over"-experiment was performed. In these sets of experiments, the indicated siRNAs were transfected either into SEM or in RS4;11 cells. mRNA levels of *MLL-AF4*, wild-type *MLL* and *AF4* were tested 24 hours after electroporation using real-time RT-PCR. In spite of a homology of more than 60 %, siMA6 did not diminish levels of the e10-e4 isoform in RS4;11 cells, whereas a perfectly homologous siRNA, siMARS, reduced the *MLL-AF4* e10-e4 variant in RS4;11 by 60%, without affecting *AF4* or *MLL* expression (Fig. 5).

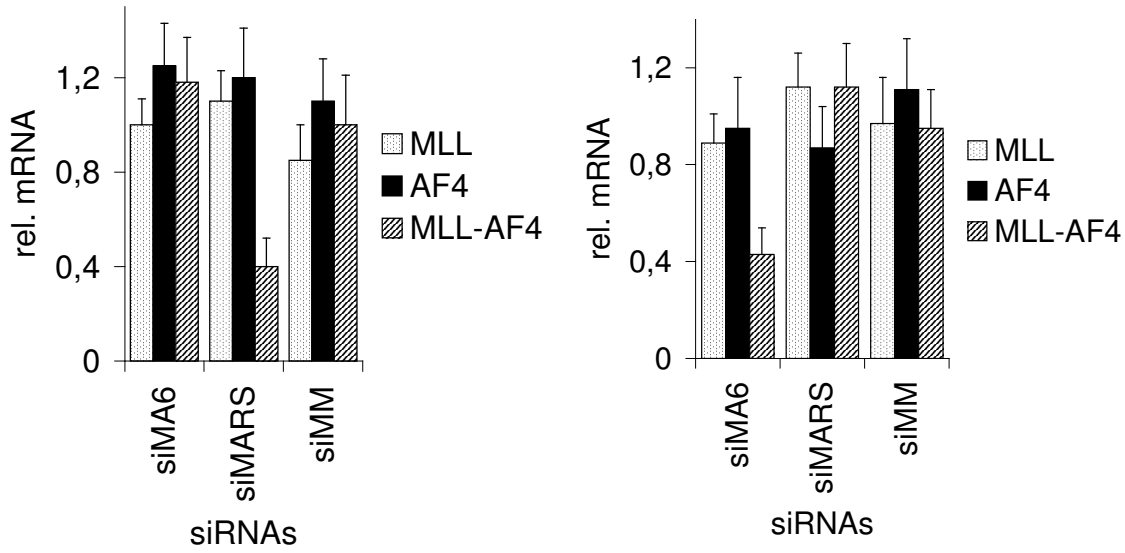


Fig. 5 Exclusive specificity of siMARS and siMA6 towards targeting the corresponding *MLL-AF4* transcripts without affecting wild-type *MLL* and *AF4* mRNA levels.

Real-time RT-PCR analysis 24h after electroporation of RS4;11 cells (left) with indicated siRNAs and SEM cell line (right). On each diagram, the average of at least three independent experiments in triplicates is shown. The error bars indicate standard deviations between three experiments. The reduction of *MLL-AF4* transcripts is statistically significant ($p < 0.005$).

IV.5 Analysis of interferon response induction by siMA6 and siMM

It was shown (Bridge et al. 2003) that both chemically synthesized siRNAs as well as intracellularly expressed shRNAs may induce a limited interferon response both in cell culture and *in vivo*. This induction proceeds via Toll-like receptors and/or protein kinase R, but the responsible parameters are just currently becoming clear (Kim et al. 2004; Scacheri et al. 2004; Hornung et al. 2005). One possible method of controlling siRNA- triggered interferon response is to examine the induction of classical interferon response genes such as *STAT1* and 2´-5´-oligoadenylate synthase 1 (*OAS1*). To investigate whether the used siRNAs siMA6 and siMM induce an interferon response, the mRNA levels of *STAT1* and *OAS1* were analyzed after the electroporation of SEM cells with these siRNAs (Fig. 6). Transfection with polyIC, a strong inducer of interferon

response, increased *OAS1* transcript levels more than 50-fold and *STAT1* mRNA levels more than 10-fold, demonstrating that interferon response pathways can be induced in these leukemic cells. Neither siMA6 nor siMM induced *STAT1* and *OAS1* expression, demonstrating a lack of an interferon response induction by these siRNAs.

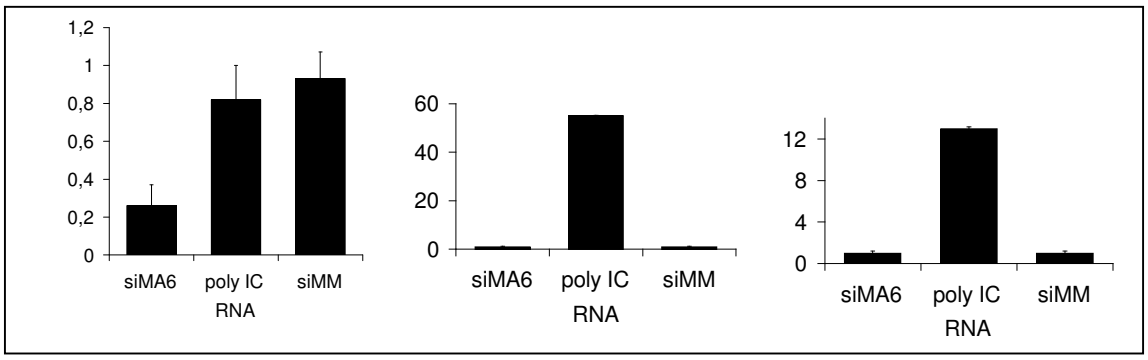


Fig. 6 Analysis of the interferon response induction by siMA6 and siMM in SEM cells.

Real-time RT-PCR analysis of *OAS1* and *STAT1* mRNA after treatment of SEM cells with siMA6 and siMM; polyIC was used as a positive control of interferon response induction. Two independent experiments yielding very similar results were performed, one representative experiment is shown. The error bars show standard deviations.

IV.6 Phenotypic effects of MLL-AF4 suppression

IV.6.1 MLL-AF4 affects leukemic clonogenicity

To study the relevance of MLL-AF4 for leukemic clonogenicity, t(4;11)-positive SEM cells were transfected with siRNAs followed by incubation in semisolid medium. SiMA6-mediated depletion of *MLL-AF4* reduced the number of colonies fivefold (Fig. 7). This effect was specific, since colony formation of the t(8;21)-positive leukemic cell line Kasumi-1 was not affected by siMA6. *Vice versa*, transfection with the AML1/MTG8-specific siRNA siAGF1 compromised Kasumi-1 colony formation without interfering with SEM colony formation (Martinez et al. 2004). None of the mismatch controls (siMM and siAGF6) affected leukemic clonogenicity.

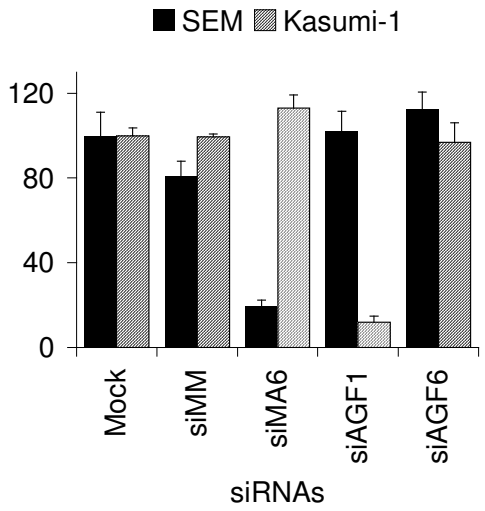


Fig. 7 Specificity of MLL-AF4 and AML1/MTG8 siRNAs.

SEM cells express *MLL-AF4*, whereas Kasumi-1 cells express *AML1/MTG8*. Colony numbers of siMA6-treated SEM cells are significantly lower than SEM controls ($p < 0.001$). The average of at least three independent experiments in quadruplicates is shown here. The error bars reflect standard deviations.

Colony formation assay was performed in another 6 cell lines: K562, Kasumi (CD34-), HL-60, SKNO-1, U937 and MV4;11. Neither of the t(4;11)-negative leukemic cell lines HL60, K562, SKNO-1 and U937 nor the t(4;11)-positive cell lines RS4;11 and MV4;11 expressing *MLL-AF4* variants not affected by siMA6 showed impaired colony formation upon siRNA transfection (Fig. 8 and Table 2).

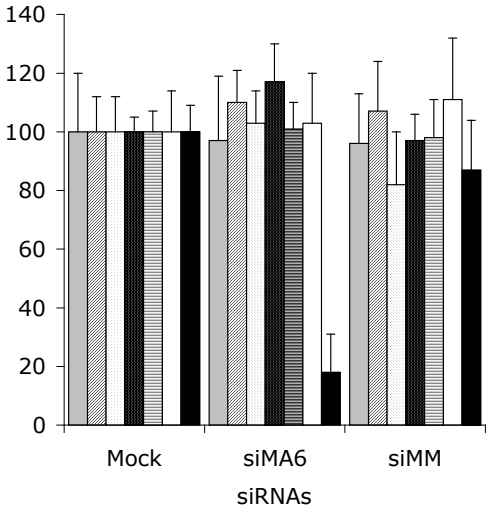


Fig. 8 Lack of unspecific effect of siMA6 in the other tested cell lines.

siMA6 reduces clonogenicity exclusively in SEM cells, expressing perfectly homologous *MLL-AF4* transcript variant. Neither t(4;11)-negative leukemic cell lines (HL-60, K562, Kasumi-

1, SKNO, U937), nor t(4;11)-positive cell lines (MV4;11) showed impaired colony formation upon siRNA transfection.

Interestingly, the colony formation analysis using siMA3 showed a reduction of colony numbers in RS4;11 cells. Whereas MV4;11 clonogenicity was hardly affected upon siRNA treatment, RS4;11 cells electroporated with siMA3 or siMA13 had threefold or almost twofold lower colony numbers, respectively. In contrast to siMA3 and siMA13, siMA6 reduced exclusively the clonogenicity of SEM cells, and the mismatch control siMM had no effects in any t(4;11)-positive cell line (Table 2).

	siRNA			
Cell line	siMA3	siMA6	siMA13	siMM
SEM	++‡	++‡	-	-
Kasumi-1	+†	-	+†	-
SKNO-1	+†	-	+†	-
U937	+†	-	+†	-
HL60	+†	-	-	-
K562	+†	-	-	-
MV4;11	-	-	-	-
RS4;11	+‡	-	+‡	-

Table 2: Effects of MLL-AF4 siRNAs on colony formation of leukemic cell lines.

++, decrease by more than 70%; +, decrease by 40% to 70%; -, less than 40% decrease; ‡ significantly lower compared to mock or siMM treated cells ($p < 0.001$ according to unpaired, two-tailed student's t-test); † significantly lower compared to mock or siMM treated cells ($p < 0.01$ according to unpaired, two-tailed student's t-test)

RS4;11 clonogenicity was more than twofold reduced upon siMARS-mediated suppression of the *MLL-AF4* e10-e4 variant thereby demonstrating for another t(4;11) cell line the dependence of clonogenic efficacy on MLL-AF4 (Fig. 9). Here, a similar „cross-over"-experiment was performed. Both siMARS

and siMA6 were transfected in SEM and RS4;11 cell lines and 24 h later the cells were plated in a semisolid medium, containing 20 % FCS. Two weeks later the colonies containing at least 20 cells were counted. Only the siRNA with perfect homology toward fusion site of *MLL-AF4* transcript, i.e. siMA6 in SEM cells and siMARS in RS4;11 cells, significantly reduced the colony formation of the corresponding cell line.

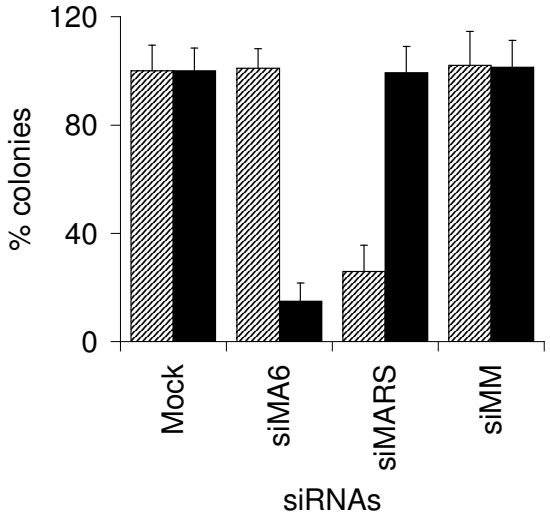


Fig. 9 Inhibition of SEM and RS4;11 clonogenicity is dependent on the perfect homology of siRNA to the MLL-AF4 fusion site.

Colony numbers of siMA6-treated SEM cells and siMARS-treated RS4;11 cells are significantly lower than the corresponding controls ($p<0.001$). The average of at least three independent experiments in quadruplicates is shown here. The error bars indicate standard deviations.

MLL-AF4 siRNA electroporation of primary human hematopoietic $CD34^+$ cells did neither affect the numbers of GEMM nor those of GM colonies (Fig. 10). This lack of effect cannot be attributed to inefficient siRNA transfections, since human hematopoietic $CD34^+$ cells the can be efficiently transfected with functional siRNAs (Dunne et al. 2003; Heidenreich et al. 2003; Scherr et al. 2003).

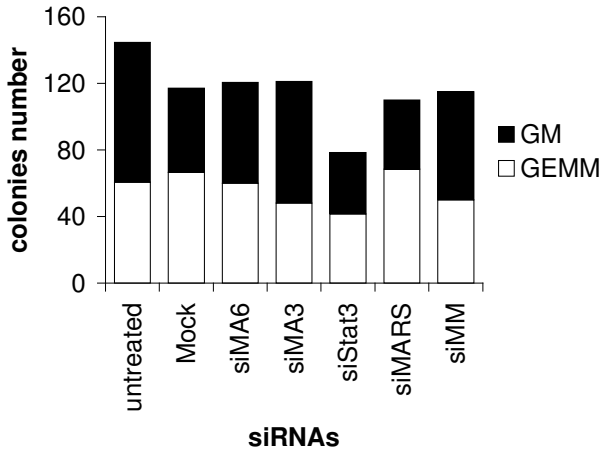


Fig. 10 MLL-AF4 siRNAs do not affect colony formation of primary human CD34+ hematopoietic cells.

siMA3, which was shown to affect the level of *AF4* mRNA was also included in this experiment, as well as siStat3 which was taken as a positive control. Two independent experiments in quadruplicates yielding very similar results were performed, one representative experiment is shown.

IV.6.2 Suppression of *MLL-AF4* inhibits leukemic proliferation

Next, the effects of different MLL-AF4 siRNAs on the proliferation of t(4;11)-positive SEM cells in suspension culture were examined. A single electroporation with 750 nM of an indicated siRNAs did not significantly affect the doubling time of these cells (bold lines on Fig. 11). However, a second electroporation 48 h later resulted in the proliferation decrease of cells electroporated with siMA6 in comparison with siK4 (dashed lines on Fig. 11).

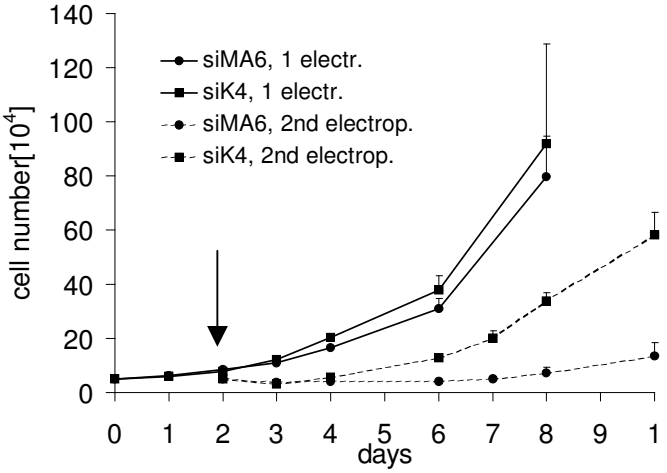


Fig. 11 MTT cell proliferation test following cells after single and double

electroporations of SEM cells with active siRNA, siMA6, and control siRNA, siK4.

Arrow indicates the day, when the cell population was splitted: one part was left in culture without any further treatment, whereas the other part was again electroporated with indicated siRNAs. Two independent experiments yielding very similar results were performed, one representative experiment is shown. Error bars represent the standard deviation between triplicates in 96-well plate. A titration curve with cell dilutions was used for the conversion of the raw OD values to the corresponding cell numbers.

Whereas a single electroporation with siMA6 did not affect the proliferation of t(4;11)-positive SEM cells (Fig. 11), repeating siRNA electroporation for every second day resulted in a sustained inhibition of proliferation of SEM cells by siMA6, and of RS4;11 cells by siMARS (Fig. 12). Thus, proliferation was only inhibited by the siRNA homologous to the corresponding *MLL-AF4* fusion site demonstrating the specificity of these MLL-AF4 siRNAs. Mock or control siRNAs electroporated SEM or RS4;11 cells had doubling times of 1.4 days demonstrating that the repeated electroporation did not seriously affect their proliferation (Fig. 12). Since MLL-AF4 protein levels decreased within 48 h after a single siRNA treatment (see Fig. 2), the necessity of repeated siRNA electroporations is unlikely to be caused by a long MLL-AF4 half-life. Instead, an extended MLL-AF4 knockdown might be required to down-modulate proliferation-supportive signals provided by, for instance, cell-cell contacts or secreted growth factors.

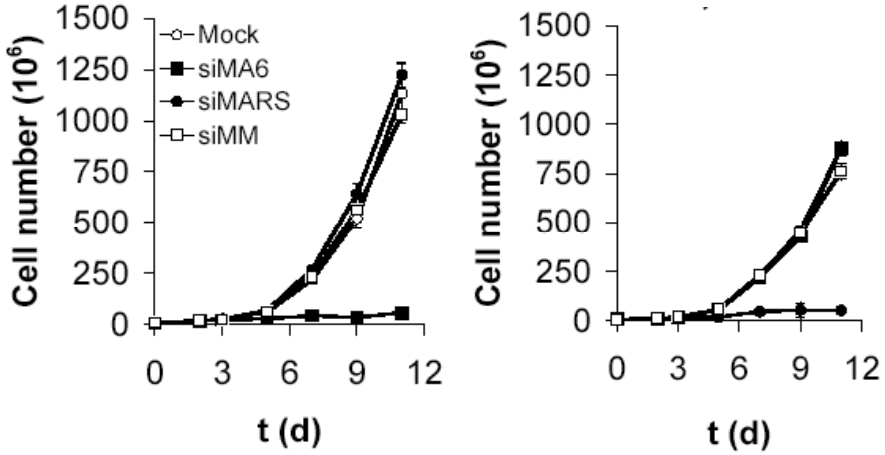


Fig. 12 Growth curves of siRNA-treated t(4;11) cell lines.

Cells were electroporated every second day with 500 nM siRNA. Two independent experiments yielding very similar results were performed, one representative experiment is

shown. Raw data were obtained by photometrical measurements. For determining the corresponding cell numbers the standard dilution curve was used. Error bars indicate standard deviations.

IV.6.3 Depletion of *MLL-AF4* affects cell-cycle progression

The reduced proliferation of t(4;11)-positive cells upon *MLL-AF4* depletion was paralleled by changes in the cell cycle distribution. During a time course of 10 days with repetitive MLL-AF4 siRNA electroporation, the fraction of S phase cells decreased in both SEM and RS4;11 cells from 50 % to 30 % and 20 %, respectively, with a concomitant increase in the fraction of G0/G1 phase cells (Fig. 13). Notably, siMA6 affected cell cycle distribution only in SEM cells, whereas siMARS caused those changes only in RS4;11 cells. Thus, depletion of *MLL-AF4* negatively interferes with the progression of t(4;11)-positive cells from G1 to S phase.

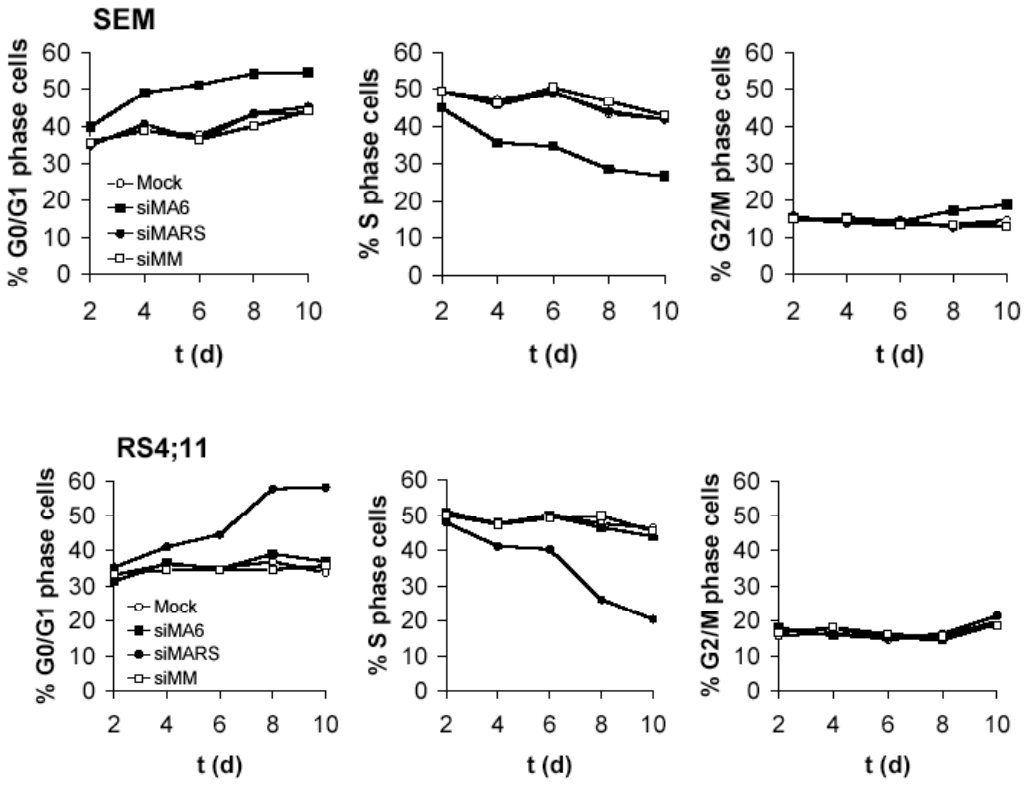


Fig. 13 Effects of MLL-AF4 siRNAs on the cell-cycle distribution of SEM and RS4;11 cells.

The graphs show the percentage of cells in the indicated cycle phase. Cell cycle distribution was determined by flow cytometry at the indicated days using cells from the time course experiments shown in Fig. 12. Two independent experiments yielding very similar results were performed, one representative experiment is shown.

IV.6.4. Decrease in expression of *HOX* genes

Expression of MLL oncoproteins including MLL-AF4 is associated with increased expression of several *HOX* genes, including *HOXA9* and the homeotic gene *MEIS1* (Yeoh et al. 2002; Ferrando et al. 2003; Rozovskaia et al. 2003; Quentmeier et al. 2004). In acute lymphoblastic leukemia (ALL) lines containing rearranged *MLL* genes, *HOXA7*, *HOXA9* and *MEIS1* levels are higher compared to ALL lines with wildtype *MLL* (Armstrong et al. 2002). Therefore, the expression of these three homeotic genes in dependence on the MLL-AF4 level was examined. After two consecutive transfections of SEM cells with the MLL-AF4 siRNA siMA6, *HOXA7*, *HOXA10* and *MEIS1* mRNA levels decreased by 60%, and *HOXA9* levels by 40 % (Fig. 14). Thus, MLL-AF4 causes an increased expression of these homeobox genes.

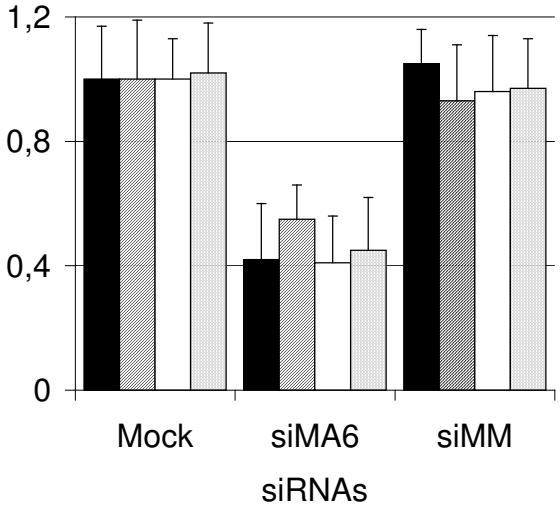


Fig. 14 MLL-AF4 suppression inhibits *HOXA7*, *HOXA9*, *HOXA10* and *MEIS1* gene expression

Total RNA was isolated 48 hours after the second electroporation with 500 nM of the indicated siRNA and analyzed by real-time RT-PCR. The average of at least three independent

experiments in triplicates is shown here. The error bars indicate standard deviations. The decrease of all 4 genes upon MLL-AF4 suppression is statistically significant ($P < .05$).

IV.6.5. Onset of myeloid differentiation

MLL fusion genes, such as *MLL-ENL*, have been shown to interfere with hematopoietic differentiation in a *HOXA9* and *MEIS1*-dependent fashion (Zeisig et al. 2004). Furthermore, absence of *HOXA7* and *HOXA9* expression results in B-cell development even in the presence of MLL-AF4 (Bertrand et al. 2003). Therefore, the decreased *HOXA7*, *HOXA9* and *MEIS1* expression upon MLL-AF4 suppression might result in an, at least partially, reactivated hematopoietic differentiation. Expression of *CD133* (Prominin), a marker for hematopoietic stem and early progenitor cells, correlates with mixed lineage leukemia (Armstrong et al. 2002). Moreover, *CD133* expression is controlled by the methylation status of a CpG island (Shmelkov et al. 2004), raising the possibility that *CD133* is a direct target gene of MLL-AF4. The consequences of MLL-AF4 depletion on the expression of *CD133* in SEM cells was analyzed. Inhibition of *MLL-AF4* expression resulted in twofold reduced *CD133* mRNA levels and in a more than twofold reduced surface expression of *CD133* (Fig. 15), which may indicate the onset of hematopoietic differentiation.

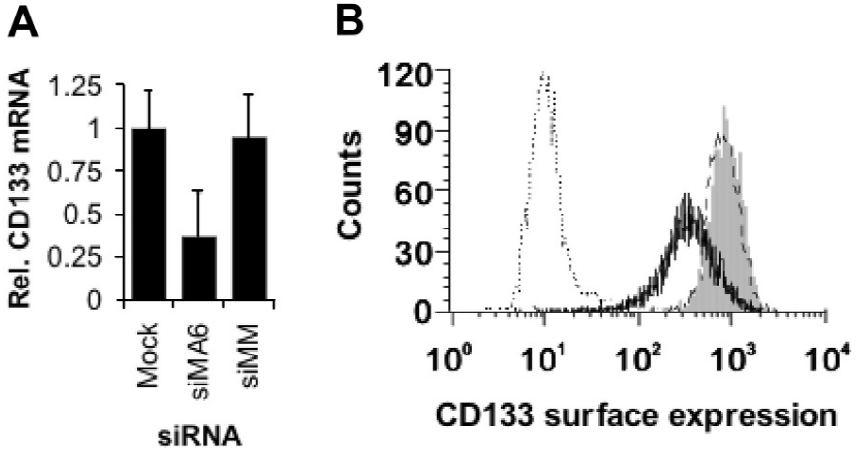


Fig. 15 MLL-AF4 depletion facilitates hematopoietic differentiation.

A. Reduction of *CD133* mRNA levels upon MLL-AF4 suppression. The average of at least three independent experiments in triplicates is shown here. The error bars indicate standard deviations between three experiments. The reduction of *CD133* transcripts is significant ($p < 0.005$). B. MLL-AF4 siRNAs diminish CD133 surface expression; gray peak indicates mock-

transfected cells; solid line, siMA6-transfected cells; dotted line, isotype control. Two independent experiments yielding very similar results were performed, one representative experiment is shown.

IV.6.6 Suppression of MLL-AF4 induces apoptosis in t(4;11)-positive cells.

Cell cycle analysis of SEM and RS4;11 cells revealed that the continuous depletion of MLL-AF4 for ten days raised the number of sub-G1 cells tenfold compared to controls, indicating an increased amount of apoptotic cells (Fig. 16).

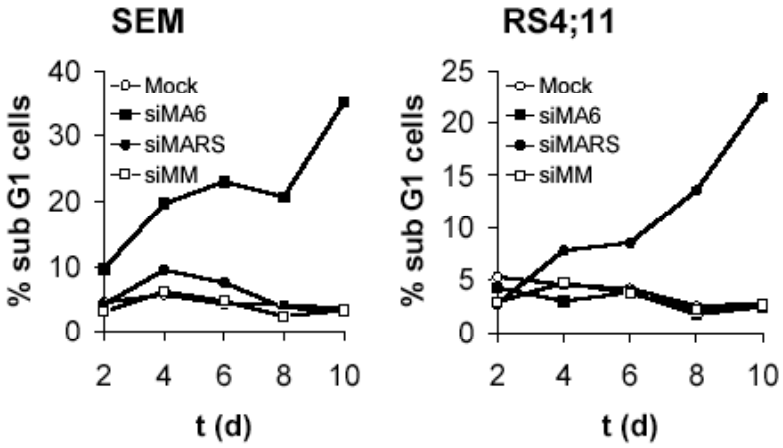


Fig. 16 Effects of MLL-AF4 suppression on the fraction of sub-G1 cells.

Cells obtained from the time courses shown in Fig. 12 and 13 were analyzed for DNA content by flow cytometry. Two independent experiments yielding very similar results were performed, one representative experiment is shown.

Staining with annexin V and propidium iodide also demonstrated for SEM cells a threefold increase in apoptotic cells upon suppression of MLL-AF4 (Fig. 17). The almost inactive siRNA siMA13 (see Fig. 1) only marginally affected the amount of apoptotic cells suggesting a direct correlation between the extent of MLL-AF4 depletion and rate of apoptosis.

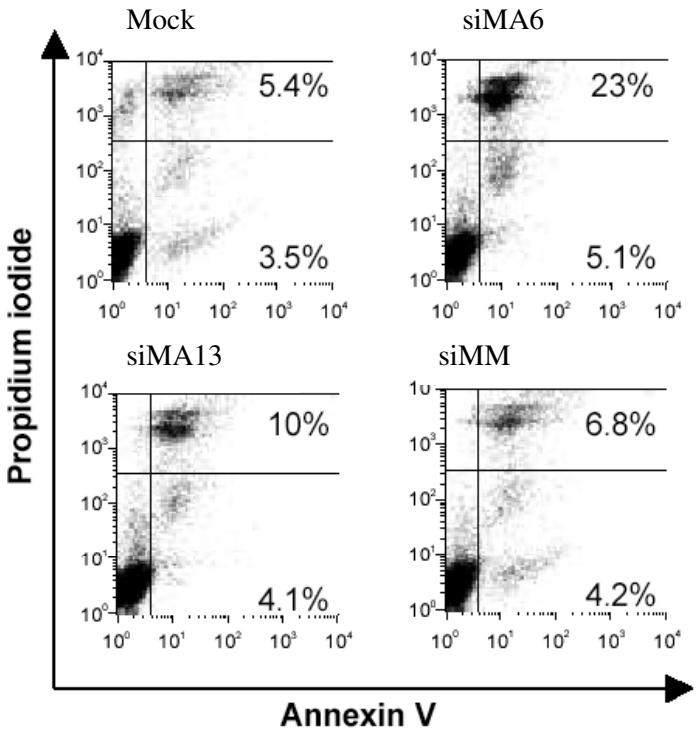


Fig.17. Annexin V staining of SEM cells.

Annexin V-positive SEM cells were quantified by flow cytometry 4 days after second electroporation with 500 nM of the indicated siRNA. The percentages of annexin V and annexin V/propidium iodide-positive cells are given in the corresponding quadrants. Two independent experiments yielding very similar results were performed, one representative experiment is shown.

Proteolytic activation of caspase-9, caspase-3 and decreased amounts of the anti-apoptotic protein BCL-XL accompanied the siMA6-mediated induction of apoptosis.

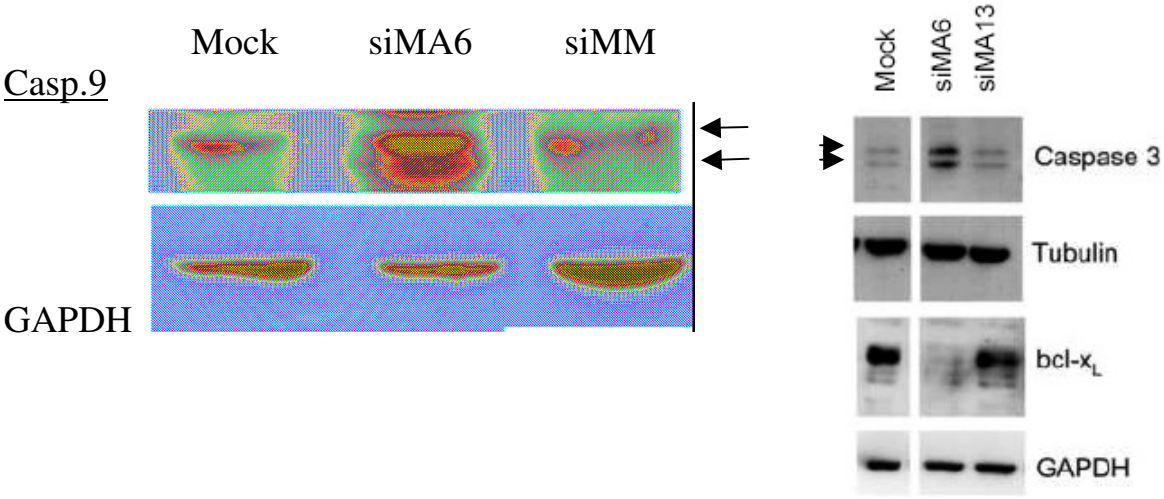


Fig. 18 MLL-AF4 suppression triggers caspase-3 and caspase-9 activation

and diminishes BCL-XL protein levels.

Immunoblots show BCL-XL and proteolytically activated caspase-3 (right panel) and caspase-9 (left panel). The products of proteolytic cleavage of caspase 9 (34 and 37 kDa) and caspase 3 (17 kDa and 19 kDa) are indicated by arrows. Tubulin and GAPDH served as loading controls. Two independent experiments yielding very similar results were performed, one representative experiment is shown.

IV.6.6.1 Analysis of apoptosis induction upon MLL-AF4 depletion

Analysis of intrinsic signaling pathways of apoptosis showed that MLL-AF4 depletion induces the expression of the proapoptotic genes of apoptotic peptidase activating factor-1 (*APAF-1*) and septin 4 (*SEPT4*). In both cell lines, SEM and RS4;11, levels of *APAF-1* and *SEPT4* transcripts were raised four and nine fold, respectively (Fig. 19).

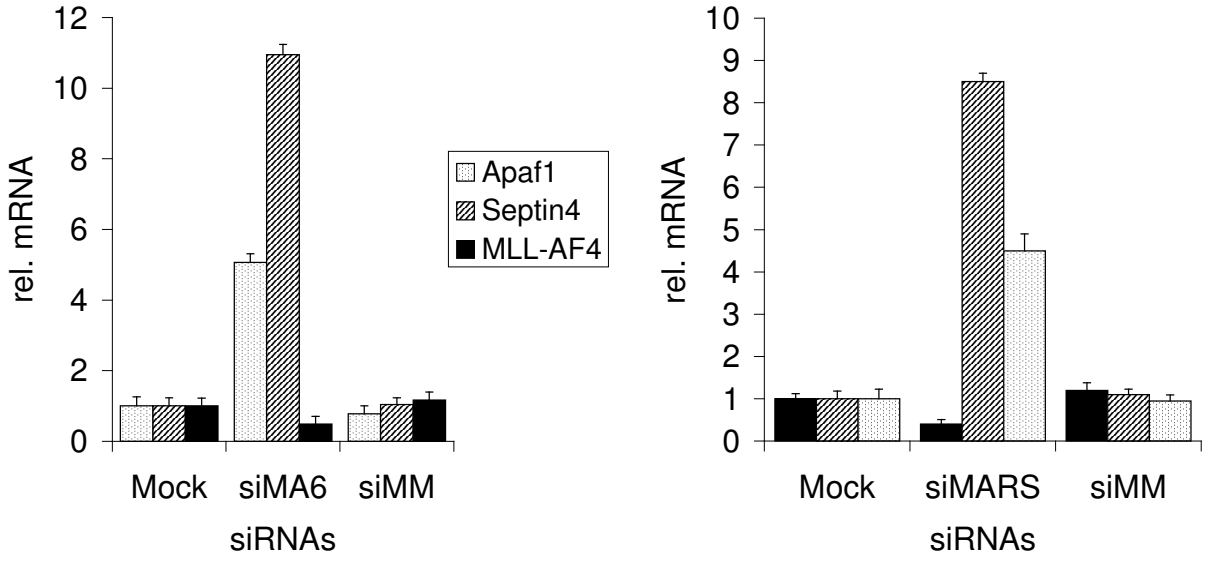
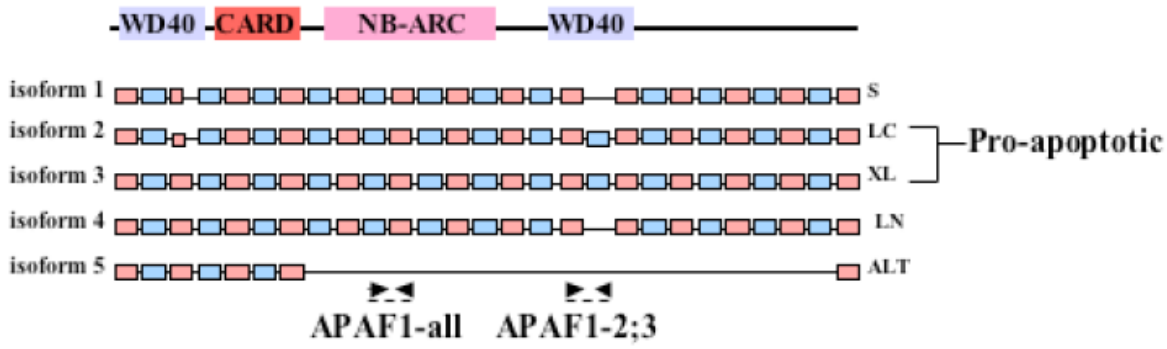


Fig. 19 MLL-AF4 depletion induces expression of the proapoptotic genes APAF-1 and SEPT4.

Real-time RT-PCR analysis was performed with RNA, isolated 2 days after 3 electroporations of SEM (left panel) and RS4;11 (right panel) cells. For detection of *APAF1* and *SEPT4* were used APAF1-all and SEPT-all primers, respectively (scheme 10 and 11). Two independent experiments yielding very similar results were performed, one representative experiment is shown. The error bars show standard deviations.

Interestingly, these two genes could be expressed via several transcripts generated by alternate splicing, which encode different isoforms. In the case of *APAF-1*, there are 2 transcripts, lacking C-terminal or all WD40 repeats considered to be anti-apoptotic (isoforms 1 and 4 on the scheme 8) and 2 isoforms (variant 2 and 3), which were shown to be pro-apoptotic.



Scheme 10. Exon structure of the *APAF-1* gene.

To distinguish the neighboring exons, they are depicted in blue/ red colors. The arrows show the positions of forward and reverse primers. The alternative names of the isoforms are given on the right side of the diagram. The primers "APAF1-all" were used for the detection of all isoforms, except ALT. The primer pair "APAF1-2;3" was used for the detection of the splice variants LC and XL, which are considered to be pro-apoptotic.

Taqman analysis of the same RNA from Fig. 19 revealed that depletion of MLL-AF4 in SEM cells resulted in increased expression of pro-apoptotic transcripts 2 and 3 (Fig. 20).

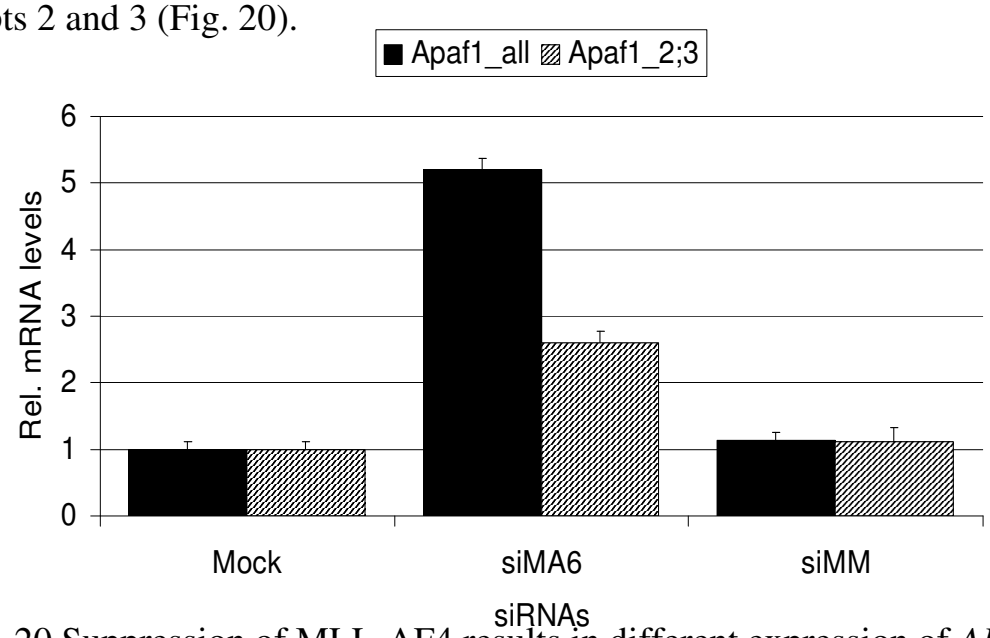


Fig. 20 Suppression of MLL-AF4 results in different expression of *APAF-1* splice variants.

RNA was isolated 2 days after the third electroporation and the primers were specifically designed to discriminate between different splice isoforms. Two independent experiments in triplicates yielding very similar results were performed, one representative experiment is shown. The error bars show an error progression between gene of interest and *GAPDH* as a housekeeping gene used for normalization.

APAF-1 DNA promoter methylation was demonstrated in different types of leukemia, suggesting that this epigenetic modification might contribute to the inactivation of *APAF-1* expression (Fu et al. 2003). Therefore, SEM cells were treated with 5-Aza-2´-deoxycytidine (5-Aza-dC) (Lyons et al. 2003), an inhibitor of DNA methyltransferases, for 4 days and the level of *APAF-1* mRNA expression was studied with real-time RT-PCR using „APAF1-all" primers. As it is shown on the Fig. 21, inhibition of CpG methylation by 5-Aza-dC lead to the increased levels of *APAF-1* mRNA, proving that DNA methylation, at least partially, affects the *APAF-1* transcription levels.

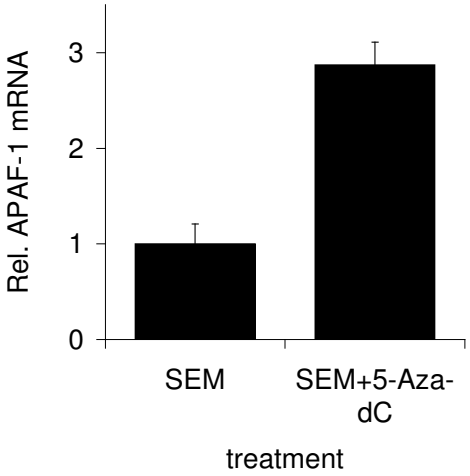


Fig. 21 Treatment with 5-Aza-dC induces levels of *APAF-1* transcription.

Total RNA was isolated 4 days after continuous treatment with 5-Aza-dC. Taqman analysis was performed using "APAF1-all" primer pair. Two independent experiments in triplicates yielding very similar results were performed, one representative experiment is shown. The error bars indicate standard deviations.

Since the methylation of CpG in the region between +87 and +128 of the *APAF-1* gene was almost exclusively observed in *APAF-1* defective cell lines, this region of the promoter was analyzed in SEM cells.

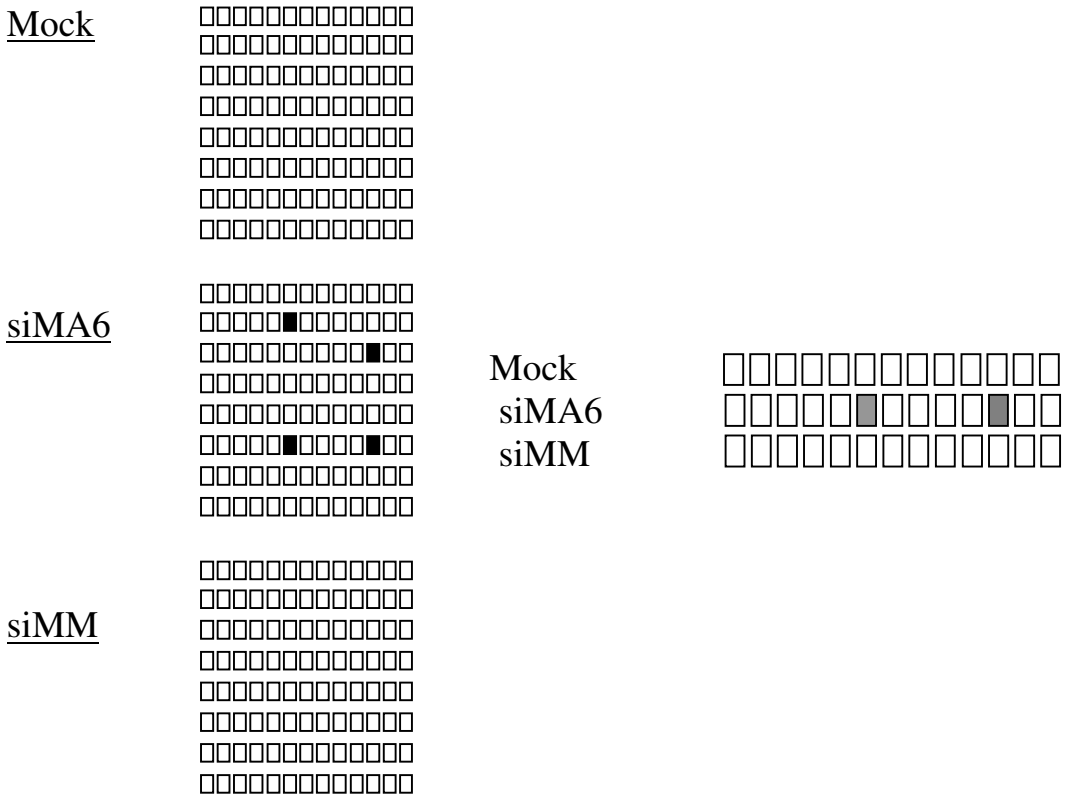


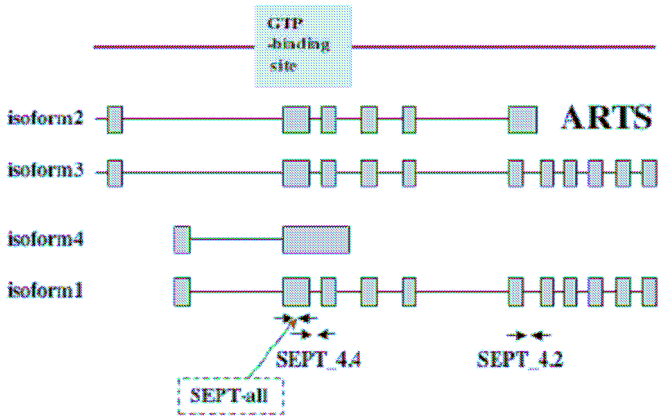
Fig. 22 Methylation analysis of the *APAF-1* promoter.

Eight clones per sample are shown, where each square represents one CG dinucleotide. White squares designate unmethylated status of every CG dinucleotide, black squares show methylated pattern. On the right panel a summarized status of the clones is presented, white squares represent 100% lack of methylation; grey squares represent-40-80% lack of methylation.

The cells were electroporated with mock, siMA6 or siMM, and the genomic DNA after bisulfite treatment was cloned in TA vector and sequenced. As it is shown on the Fig. 22, the methylation status of this particular region appeared to be hypomethylated and was not substantially affected in the cells with depleted MLL-AF4.

Similarly to the *APAF-1* gene, there are also several splice variants expressed from *SEPT4* gene. One particular transcript 2 of this gene, also called *ARTS*, was described to be lost in the majority of ALL patients (Elhasid et al. 2004) (scheme 11). High levels of this mitochondrial protein were shown to sensitize cells towards apoptosis, and its intracellular levels are regulated through ubiquitin-mediated pathway of degradation (Lotan et al. 2005). The loss

of ARTS provides a selective advantage for cells to escape apoptosis, thereby contributing to their transformation to malignant lymphoblasts.



Scheme 11. Exon structure of *SEPT4* gene.

The alternative names of the isoforms are given on the right. The primer pair "SEPT_all" was used for the detection of all variants of *SEPT4* gene. The primers "SEPT_4.4" were used for the detection of the isoform 4 of *SEPT4* gene; "SEPT_4.2" – isoform 2; "SEPT_4.1;3"-for the detection of the isoforms 1 and 3.

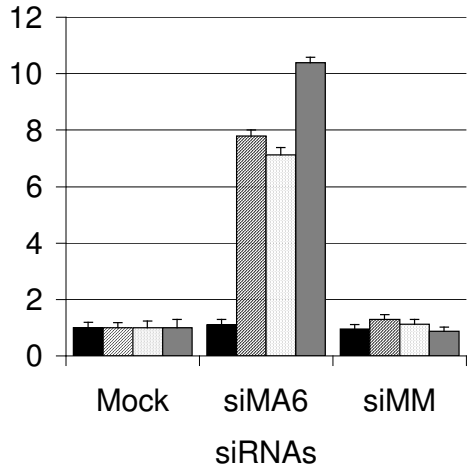


Fig. 23 Suppression of MLL-AF4 results in different expression of *SEPT4* splice variants.

RNA was isolated 2 days after third electroporation and the primers were specifically designed to discriminate between different splice isoforms. Two independent experiments in triplicates yielding very similar results were performed, one representative experiment is shown. The error bars show standard deviations.

To test whether MLL-AF4 depletion results in different expression of *SEPT4* splice variants, real-time RT-PCR was performed using primers SEPT,

SEPT_4.2 and SEPT_4.4 (scheme 9). As it is seen on the Fig. 23, suppression of MLL-AF4, also in the case of *SEPT4* gene, results in different expression of its splice variants.

It was demonstrated that in patients with different hematological malignancies, DNA methylation process contributes to the loss of *ARTS* expression (Larisch 2004). Therefore, the levels of *ARTS* mRNA were detected after the treatment of cells with the inhibitor of DNA methyltransferase - 5-Aza-2´ deoxycytidine (5-Aza-dC). Taqman analysis using "SEPT-all" primers revealed that 4 days after the treatment with 5-aza-dC, the mRNA level of *ARTS* is increased (Fig. 24). Thus, *ARTS* expression, at least partially, may be controlled by DNA promoter methylation.

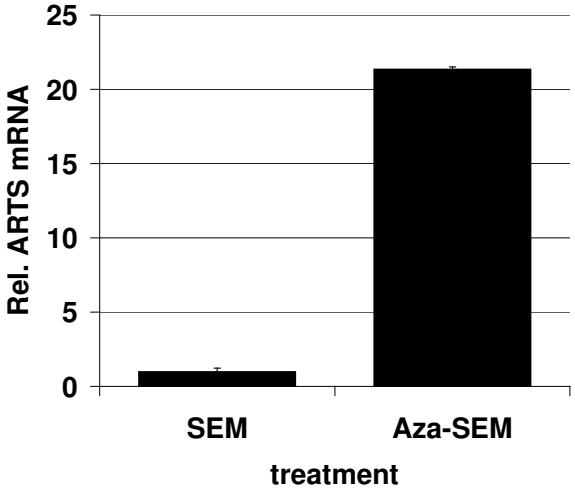


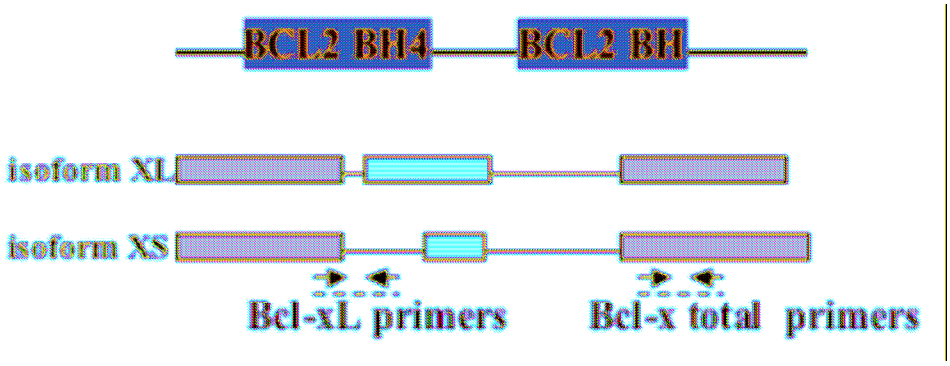
Fig. 24 Treatment with 5-Aza-dC induces the level of *ARTS* transcription.

Total RNA was isolated after 4 days of continuous treatment with 5-Aza-dC and Taqman analysis was performed using "SEPT-all" primer pair. Two independent experiments in triplicates yielding very similar results were performed, one representative experiment is shown. The error bars show an error progression between gene of interest and *GAPDH* as a housekeeping gene used for normalization.

The BCL-XL protein encoded by the *BCLX* (*BCL2L1*) gene belongs to the BCL-2 protein family (Thomadaki et al. 2006). The proteins encoded by this gene are located at the outer mitochondrial membrane, and have been shown to regulate outer mitochondrial membrane channel (VDAC) opening. VDAC regulates the mitochondrial membrane potential, and thus controls the

production of reactive oxygen species and release of cytochrome C by mitochondria, both of which are the potent inducers of cell apoptosis (Shibue et al. 2006). Two alternatively spliced transcript variants, which encode distinct isoforms, have been reported. The longer isoform (*BCL-XL*), containing a BH4 motif, required for anti-apoptotic activity, acts as an apoptotic inhibitor. The shorter form (*BCL-XS*), lacking the BH4 domain, acts as an apoptotic activator (scheme 12) (Kim 2005).

The levels of BCL-XL protein diminish upon MLL-AF4 depletion (Fig. 18). Similar analysis of the transcription level of different *BCL-X* splice variants demonstrated that the reduction of the BCL-XL protein is accompanied with the concominant decrease of mRNA level of this particular splice variant (Fig. 25).



Scheme 12. Exon structure of *BCL-X* gene.

The isoforms nomenclature is shown on the left. BCL-x total primers were used for the detection of both splice variants, whereas primer pair BCL-xL was used for the detection of *BCL-XL* splice isoform.

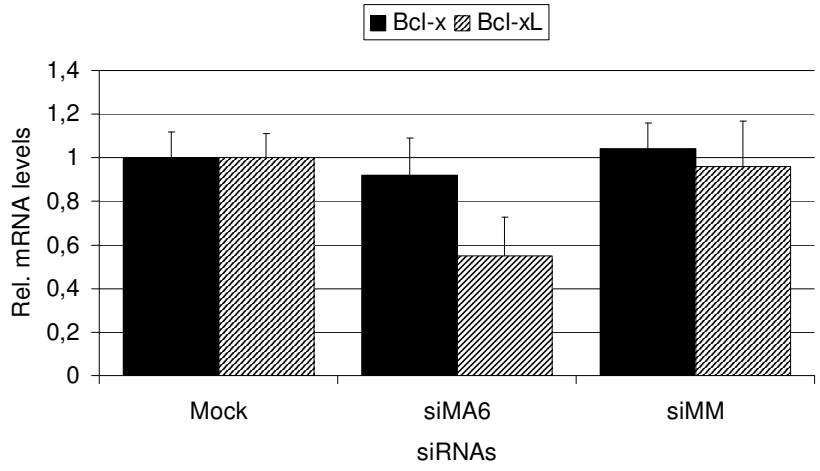


Fig. 25 Suppression of MLL-AF4 results in different expression of *BCL-X* splice variants.

RNA was isolated 2 days after third electroporation and the primers were specifically designed to detect *BCL-XL* isoform. Two independent experiments in triplicates yielding very similar results were performed, one representative experiment is shown. The error bars show standard deviation. The reduction of *BCL-XL* transcripts are statistically significant ($p < 0.01$).

IV.7 Characterization of MLL-AF4 target genes

Real-time RT-PCR analysis showed that the suppression of MLL-AF4 also inhibits expression of *HOXA7*, *HOXA9* and *MEIS1*. Therefore, as a next step in investigating the pathways involved in MLL-AF4-mediated leukemogenesis, the siRNAs targeting these genes were synthesized. Another member of MLL-associated complex, menin, was also examined. This protein, encoded by *MEN1* gene, was shown to be able directly bind to the MLL^N subunit and to participate in the activation of *HOXA9* transcription (Martin et al. 2003). siRNA targeting *MEN1* was also included in these sets of experiments.

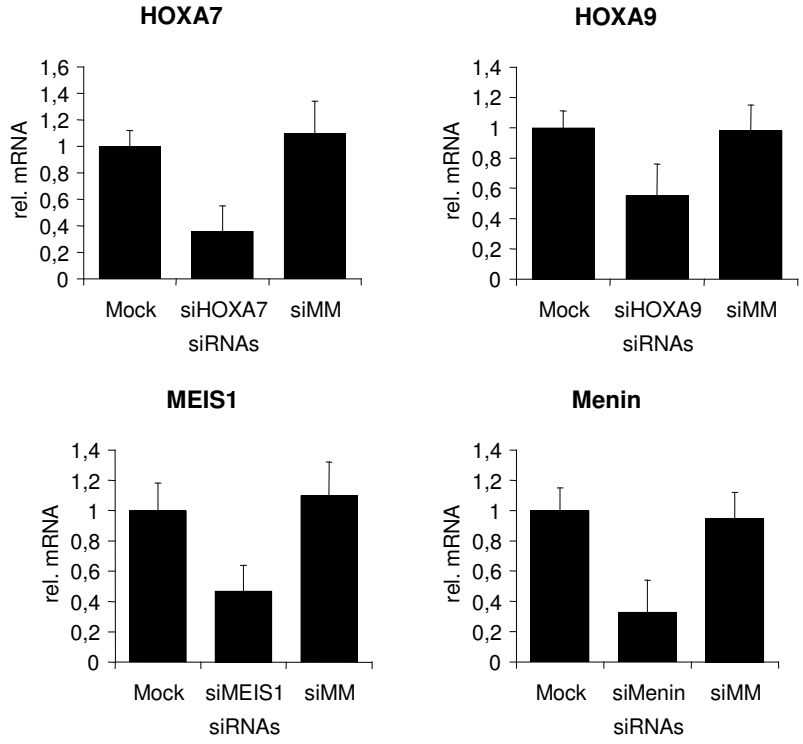


Fig. 26 Activity of siRNAs against *HOXA7*, *HOXA9*, *MEIS1* and *MEN1* in SEM cells.

Total RNA was isolated 24 h after electroporation with indicated siRNAs. For each diagram, the average of at least three independent experiments in triplicates is shown here. The error bars demonstrate standard deviations.

To examine the relevance of these siRNAs for leukemic colony formation , the SEM cells were transfected with indicated siRNAs followed by incubation in semisolid medium. After 2 weeks, the number of colonies was counted and normalized to the mock-transfected cells. As it is seen from Fig. 27, the depletion of HOXA7 gave a similar effect as a suppression of MLL-AF4, namely reduction of colonies more than 4-fold. The down-regulation of HOXA9 and menin with siRNAs caused insignificant decreases in colony number. siRNA-mediated suppression of MEIS1 did not affect colony formation of SEM cells.

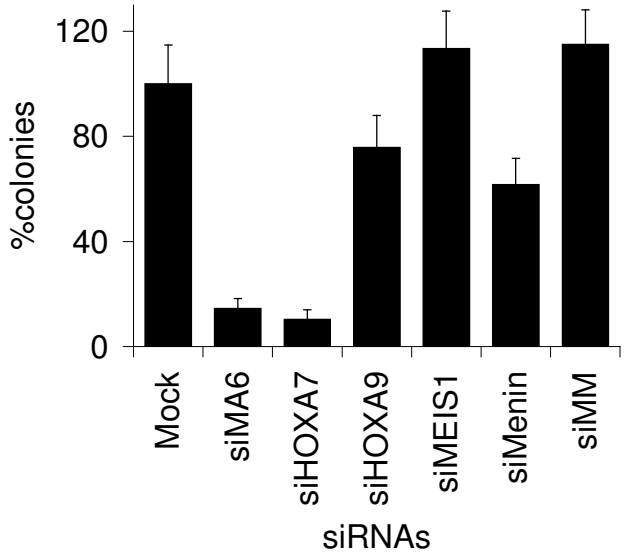


Fig. 27 Colony formation of SEM cells treated with designated siRNAs.

The average of at least three independent experiments in quadruplicates is shown here. The error bars indicate standard deviations between three experiments. The reductions of colony formation by siMA6 and siHOXA7 are statistically significant ($p < 0.005$), for siHOXA9 and siMenin the *P* values are larger than 0.05 (not significant) according to the unequal unpaired student t-test.

To analyze changes in the cell cycle distribution, SEM cells were electroporated twice with the set of siRNAs and then analyzed on the FACS. Here, in agreement with a colony formation assay, siHOXA7 like siMA6, had also an effect on the cell cycle distribution, an increase of G0/G1 phase and reduction of S phase. siHOXA9 and siMEIS1 did not affect cell proliferation according to the FACS analysis, whereas depletion of menin also inhibited cell

cycle progression from G0/G1 to S phase.

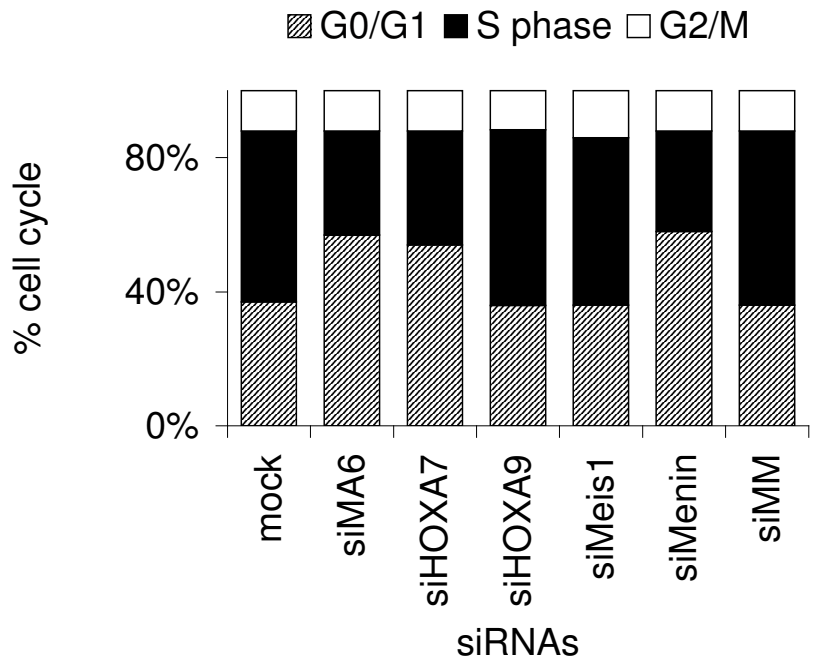


Fig. 28 Cell cycle distribution 2 days after 2nd electroporation of SEM cells with indicated siRNAs.

Three independent experiments yielding very similar results were performed, one representative experiment is shown. The decrease of S phases in the cells, treated with siMA6, siHOXA7 and siMenin, is statistically significant ($p < 0.01$ according to the unequal unpaired student t-test).

Cell cycle analysis of SEM cells, treated with the siRNAs, revealed that depletion of the indicated genes for longer time raised the number of sub-G1 cells more than 10-fold compared with the controls, indicating an increased amount of apoptotic cells. Similar to the reduced proliferation caused by siMA6, siHOXA7 and siMenin, an induction of apoptosis in the same populations could be observed. The suppression of MEIS1 and HOXA9 did not cause any induction of apoptosis (Fig. 29).

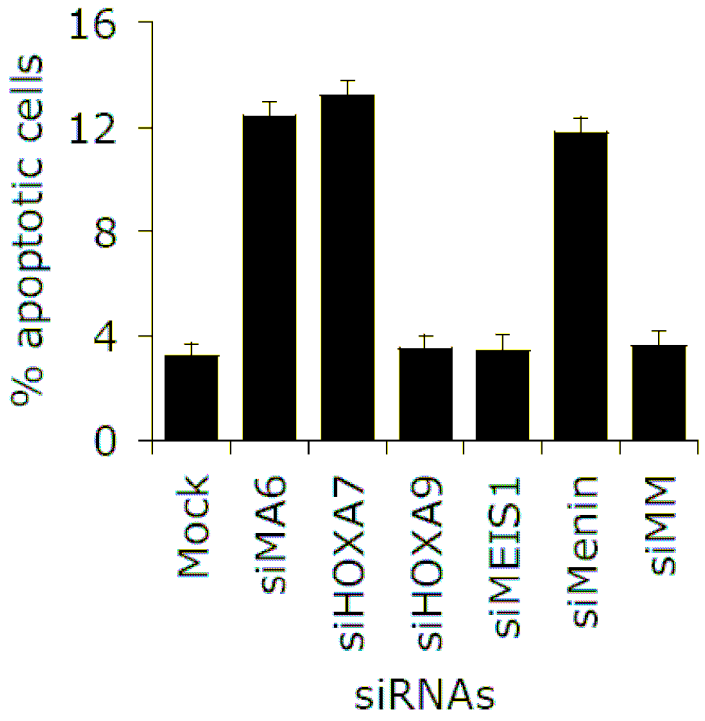


Fig. 29 sub-G1 cell population of the SEM cells, treated with indicated siRNAs.

FACS analysis was performed 2 days after 2nd electroporation. The average of at least three independent experiments in triplicates is shown here. The error bars show standard deviations.

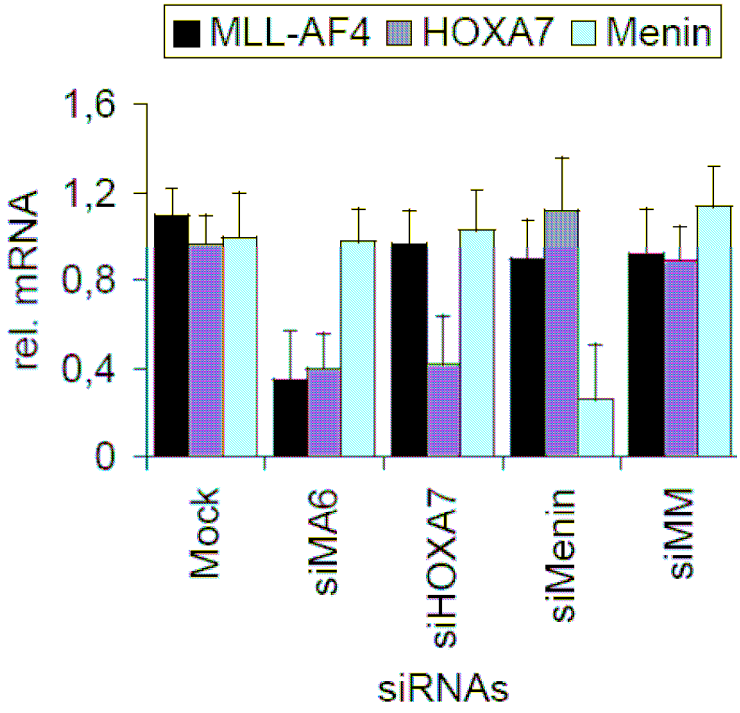


Fig. 30 Taqman analysis of *MLL-AF4*, *HOXA7* and *MEN1* expression after siRNA treatment.

Total RNA was isolated 2 days after 2nd electroporation. The average of at least three independent experiments in triplicates is shown here. The error bars show standard deviations.

In conclusion, two siRNAs – siHOXA7 and siMenin – resulted in the almost similar phenotypical changes as the treatment with siMA6. Since siMA6-mediated suppression of MLL-AF4 leads to the downregulation of HOXA7, but not of Menin, siHOXA7 was taken for further experiments for the analysis of HOXA7 as a potential mediator of MLL-AF4-mediated leukemogenesis (Fig. 30).

IV.8 MLL-AF4 interferes with the telomerase activity

IV.8.1 Effects of *MLL-AF4* and *HOXA7* depletion on *hTERT* mRNA

As it was already mentioned, menin was shown to be a component of MLL supercomplex, which associates with its N-terminal subunit. One of the target gene, which is directly regulated by Menin, is the gene coding for the human telomerase reverse transcriptase-*hTERT* (Lin et al. 2003). Analysis of the mRNA levels of *hTERT* in cells with depleted MLL-AF4 showed that downregulation of HOXA7 as well as MLL-AF4 with siHOXA7 and siMA6, respectively, lead to the suppression of the *hTERT* mRNA levels (Fig. 31). Furthermore, a reduction of *MLL-AF4* and *HOXA7* expression was also paralleled by the downregulation of *hTERT* mRNA in RS4;11 cells (Fig. 31).

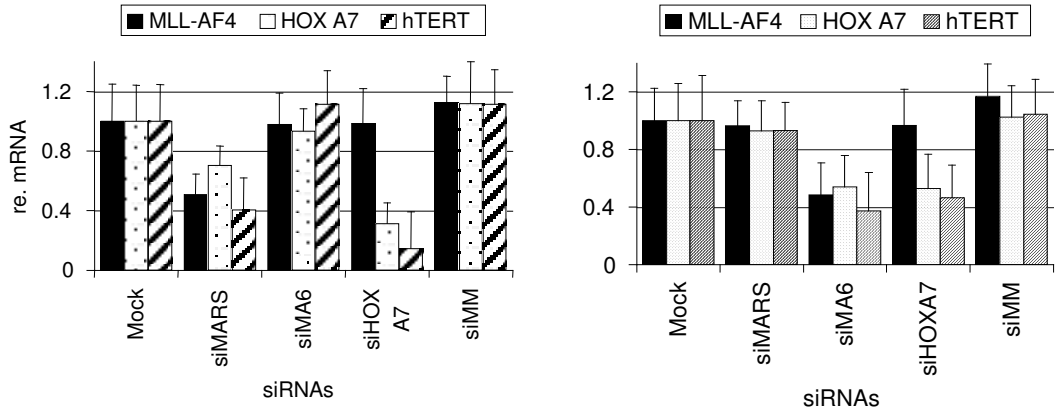


Fig. 31 Suppression of MLL-AF4 and HOXA7 in SEM cells (right) and RS4;11 (left) results in the decreased expression of *hTERT* mRNA.

Taqman analysis was performed 2 days after 2nd electroporation. Three independent experiments in triplicates yielding very similar results were performed, one representative experiment is shown. The error bars show an error progression between gene of interest and *GAPDH* as a housekeeping gene used for normalization.

IV.8.2 Inhibition of telomerase activity

Telomerase is a multimeric complex, consisting of several components, such as RNA template, *hTERC*, reverse transcriptase, hTERT, and some additional small proteins. A rate-limiting factor in the proper function of telomerase is the expression of *hTERT* gene. The observed decrease in *hTERT* mRNA upon depletion of MLL-AF4 and HOXA7 suggested that the telomerase activity is also affected after the siRNA treatment.

There are several methods to measure telomerase activity. The commonly used telomeric repeats amplification protocol (TRAP) assay allows amplification of the telomerase reaction product by PCR (Saldanha et al. 2003). The classical variant of the method involves analysis of ^{32}P-labeled reaction products by polyacrylamide gel electrophoresis and the telomerase activity is considered from band intensities on gel patterns. The black arrows on the Fig. 32 show amplified telomeric repeats separated on the sequencing gel. The intensity of these bands represent the comparative telomerase activity. TRAP assay of the SEM cells, treated with mock or designated siRNAs for different periods of time, showed a decrease of the telomerase activity in the cell lysates with depleted MLL-AF4.

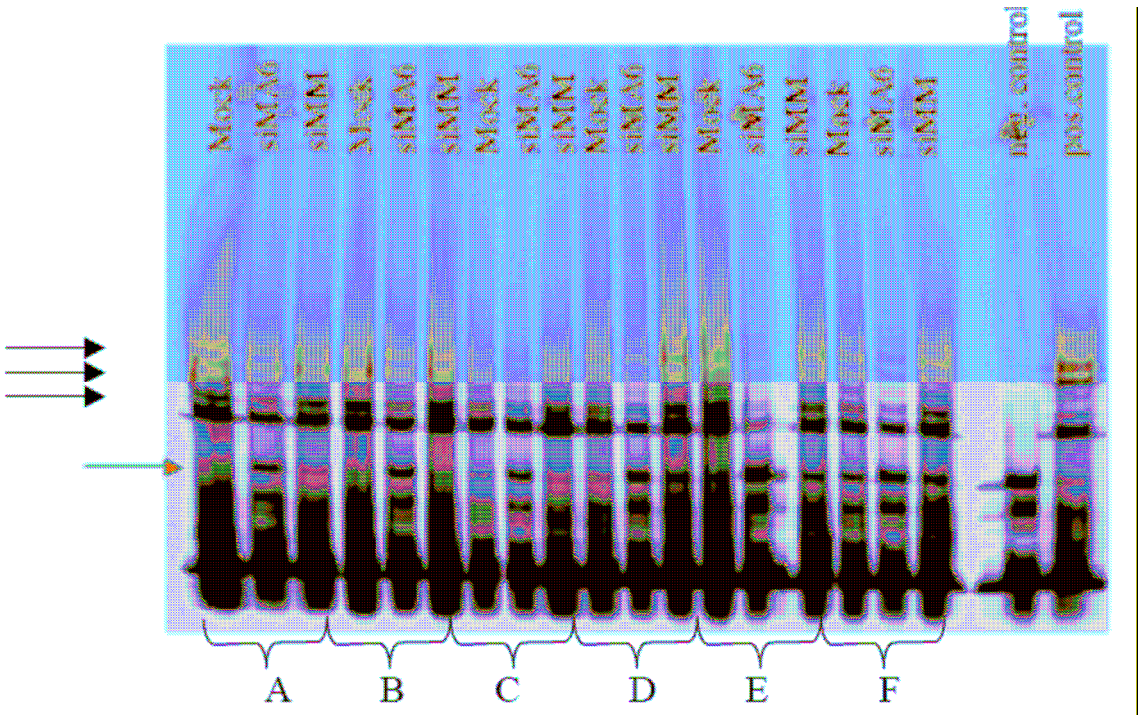


Fig. 32 Telomeric Repeats Amplification Protocol (TRAP) assay of the cells treated with mock or designated siRNAs.

The cell lysates were prepared: **A.**1 day after 2nd electroporation **B.**2 days after 2nd electroporation **C.**3 days after 2nd electroporation **D.**1 day after 3rd electroporation **E.**2 days after 3rd electroporation **F.**3 days after 3rd electroporation. As a negative control the lysates of the telomerase activity-negative cell line A431 were used. As a positive control, the telomerase activity-positive lysates of the human leukemia cell line HL60 were taken. The red arrow indicates the band corresponding to the primer dimers, whose appearance serves as additional control of the decreased telomerase activity. This analysis was performed once. The experiment was done in the Lab of Prof. U. Klotz in Robert-Bosch Krankenhaus, Stuttgart.

The method, described above has some disadvantages, primarily connected with the ^{32}P handling and quantification of the band intensities, which is tedious and often depends on the operator. Recently, a modification of the TRAP assay was introduced, which allows to detect and exactly quantify the telomerase activity in the samples using Taqman, called RQ-TRAP (Wege et al. 2003). Incorporation of SYBR-Green into amplified products of telomerase activity allows to detect and exactly quantify the products of RQ-TRAP reaction, using the same principle. In consistence with the TRAP assay shown above, RQ-TRAP analysis revealed that the treatment of not only SEM cells, but also RS4;11 cells with homologous MLL-AF4 siRNAs leads to the decrease of the telomerase activity in both types of cells.

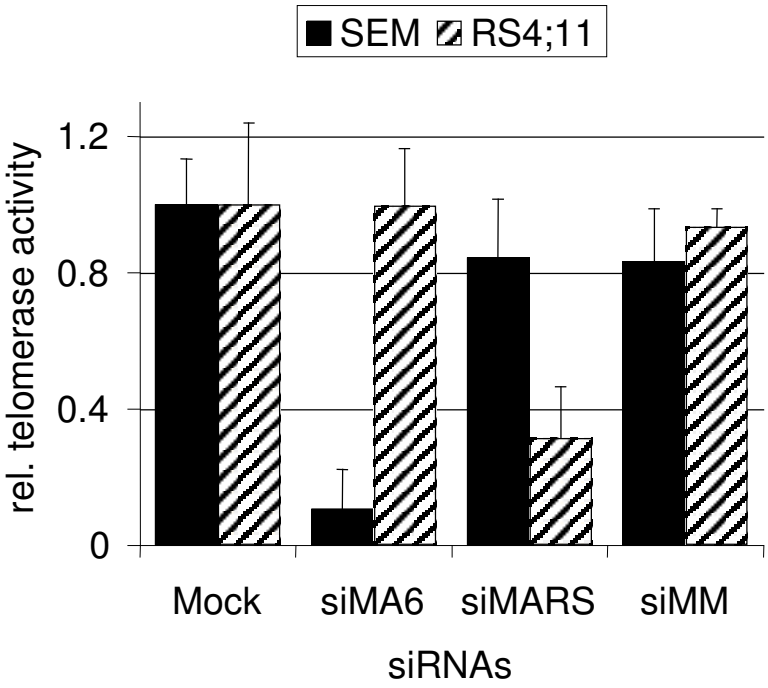


Fig. 33 Telomerase activity of the SEM and RS4;11 cells, treated with indicated siRNAs.

Telomerase analysis was performed by Taqman on the 3rd day after 3 electroporation. The average of at least three independent experiments in triplicates is shown here. The error bars indicate standard deviations.

IV.8.3 Changes in the methylation status of *hTERT* promoter upon MLL-AF4 and HOXA7 depletion

Telomerase activity is known to be regulated mainly at the level of transcription of the *hTERT* gene, but the exact molecular mechanism underlying the tumor-specific expression of telomerase remains unclear. Clusters of CpG dinucleotides, located within the *hTERT* promoter, are the targets for DNA methylation. The presence of abundant CpG sites in the *hTERT* promoter region has triggered an increasing interest in examining the possible role of DNA methylation in regulation of *hTERT* transcription in normal and cancer cells. Using a bisulfite genomic DNA sequencing method, several regions of the *hTERT* promoter were analyzed in mock-, siMA6, siHOXA7 or siMM-treated SEM cells. The genomic DNA after bisulfite treatment is quite fragile; therefore, the promoter region of the *hTERT* was chopped into several parts for the

amplification with the specific primers. The region I between –384 bp and –161 bp is shown on the Fig. 34. There are 10 clones per sample; every square represents one CG dinucleotide.

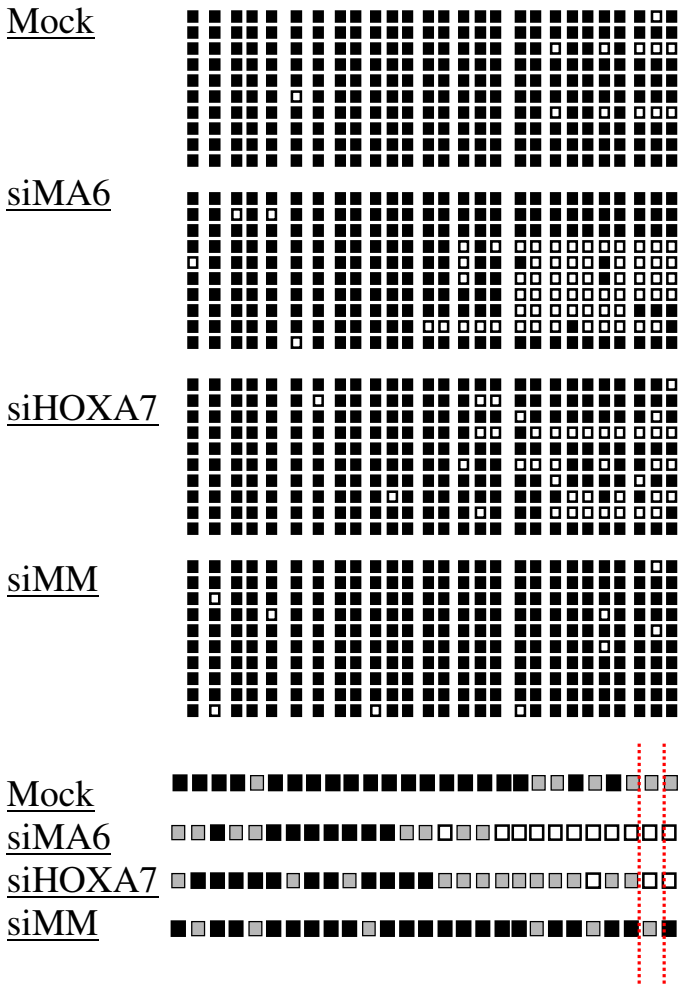


Fig. 34 CpG methylation analysis of the *hTERT* promoter region from –384 bp to –161 bp.

The analysis of genomic DNA was performed 3 days after 3 electroporation of SEM cells with mock or indicated siRNAs. Low panel, summarized presentation, where every square represents an average of ten clones. Black squares-100% hypermethylation; grey squares-90-60% hypermethylation; white squares-50-10% hypermethylation. Two red lines on the low panel designate the CG dinucleotide, which is incorporated into one of the E boxes in *hTERT* promoter.

There was substantial decrease in the methylation status of the *hTERT* promoter in the cells with depleted MLL-AF4 and, to the lesser extent, with HOXA7. Unfortunately, I could not analyze the adjacent interval between –161 bp to –62 bp. The step of the amplification of bisulfite-treated genomic DNA was not successful. The reason could be an extremely GC-rich region of the

promoter.

The region II spans from –62 bp +233 bp and the results of the methylation analysis are shown on the Fig. 35. Ten clones per sample are represented on this figure, each square indicates one CG dinucleotide. This region of the promoter appeared to be unmethylated in mock-, siMA6- and siMM-treated cells, whereas in siHOXA7-transfected cells some CG sites were methylated.

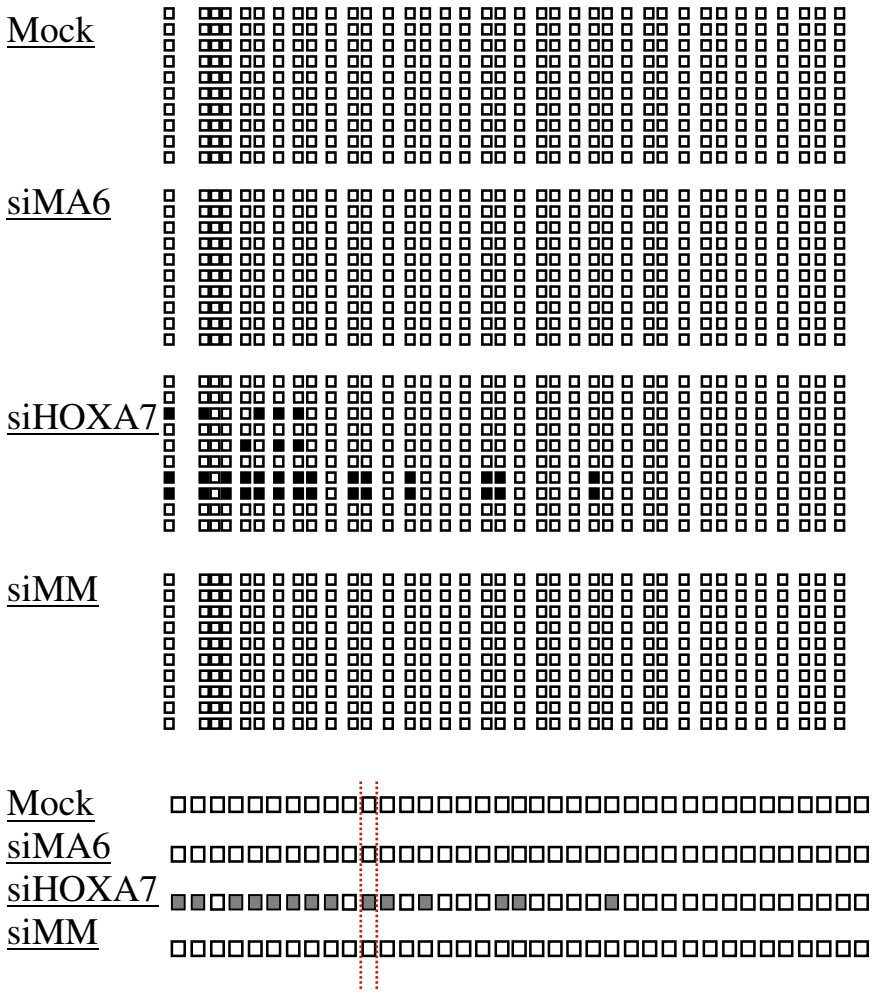


Fig. 35 CpG methylation analysis of the *hTERT* promoter region from –62 bp to +233 bp.

The analysis of genomic DNA was performed 3 days after 3rd electroporation of SEM cells with indicated siRNAs. Lower panel, summarized presentation, where every square represents an average of ten clones. Black squares-100% hypermethylation; grey squares-90-40% hypermethylation; white squares-30-10% hypermethylation. Two red lines on the lower panel show proximal E box within *hTERT* promoter.

IV.8.4 Status of c-MYC phosphorylation upon MLL-AF4 downregulation.

The alterations in the methylation pattern of the *hTERT* promoter surround its E box sequences, which are the binding sites for c-MYC (Dang et al. 1999). This is probably one of the most studied transcription factors, which recognizes E boxes, directly binds and in complex with Max activates *hTERT* transcription.

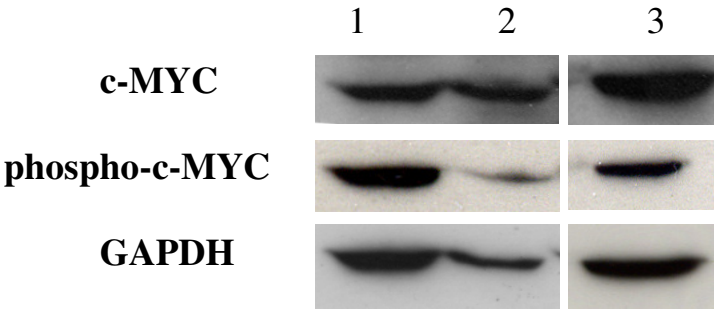


Fig. 36 Western Blot analysis of p-MYC and c-MYC 2 days after 3rd electroporation with designated siRNAs.

The cells were electroporated with: 1- mock; 2-siMA6; 3-siMM. One membrane was used for detection of, firstly, phospho-c-MYC, then, c-MYC and, finally, GAPDH for loading control. One representative experiment is shown here, the experiment was repeated twice.

The steady-state levels of c-MYC in the cell are regulated through ubiquitin-mediated degradation (Sears 2004). The signal for the ubiqutination is a phosphorylation of c-MYC in the position Ser 58. The levels of phospho-c-MYC and c-MYC were detected with western blot. The phospho-c-MYC (Thr58/Ser62) antibody detects endogenous levels of c-MYC singly phosphorylated at threonine 58 or doubly phosphorylated at threonine 58 and serine 62. It does not detect c-MYC singly phosphorylated at serine 62. As it is seen from the Fig. 36, there is less phosphorylated c-MYC in the cells with depleted MLL-AF4, whereas unmodified levels stay unchanged.

IV.9. Treatment of t(4;11) cells with demethylating agent 5-Aza-2´-deoxycytidine

5-Aza-2´-deoxycytidine (5-aza-dC) is a well-known inhibitor of DNA methyltransferases (Stresemann et al. 2006). Due to its chemical similarities to the cytosines, 5-aza-dC is able to incorporate into the DNA strands during replication and is recognized by DNA methyltransferases. However, attempted transfer of the methyl groups to 5-aza-dC covalently traps DNMTs to newly synthesized DNA. This sequestration ultimately depletes cellular stores of DNMTs, which results in the widespread genomic hypomethylation (Esteller 2006). The antitumor activity of this substance occurs due to the induction of methylation-regulated tumor-suppressive pathways (Klose et al. 2006). This drug was recently clinically approved for treating of myelodysplastic syndrome (Decitabine).

IV.9.1 Titration of the 5-aza-dC response in SEM cells

To follow the changes in the gene expression and methylation status of the promoters of several genes, a titration of 5-Aza-dC was performed. For that, to the culture medium of SEM cells a various concentrations of 5-aza-dC were added and every day substituted with the fresh dilution, due to the instability of the substance and high proliferation rate of these cells. The cellular response was followed as changes in the cell cycle distribution and induction of apoptosis, since many leukemic cell lines were shown to undergo apoptosis within the first 4 days of treatment.

On the Fig. 37, the cell cycle analysis and an induction of apoptosis using 4 different 5-Aza-dC concentrations are shown. As the concentration of 20 μM appeared to cause the most prominent effects on the cell cycle distribution and the induction of apoptosis, this concentration was subsequently used in the following experiments.

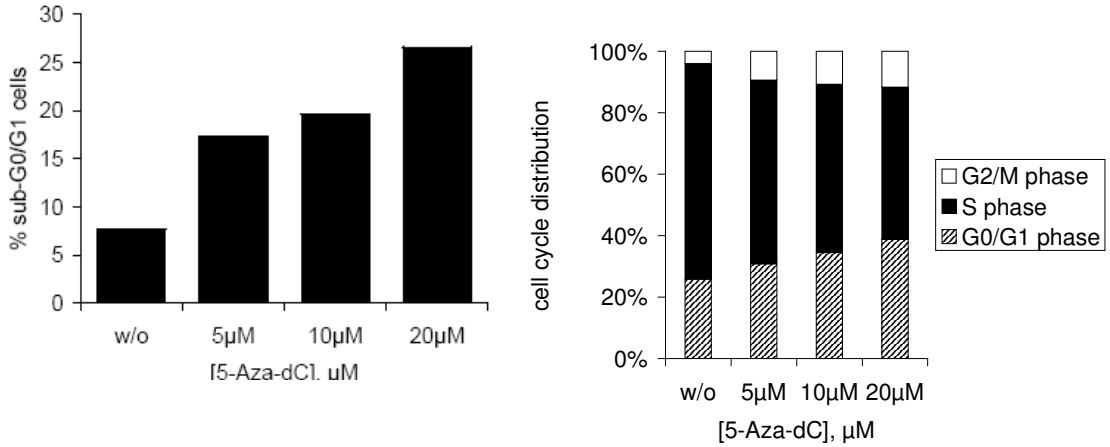


Fig. 37 Titration of 5-Aza-dC response in SEM cells.

On the right panel, cell-cycle distribution is shown after continuous treatment with different concentrations of 5-Aza-dC for 4 days. On the left, sub-G1 cell population measured 4 days after continuous treatment with different 5-Aza-dC concentrations of SEM cells. The experiment was performed once.

IV.9.2 Effect of 5-Aza-dC treatment on the gene expression of *hTERT*, *HOXA7* and other genes

To test whether expression of putative target genes of MLL-AF4, is, at least partially, regulated by DNA methylation, a quantitative RT-PCR analysis was performed.

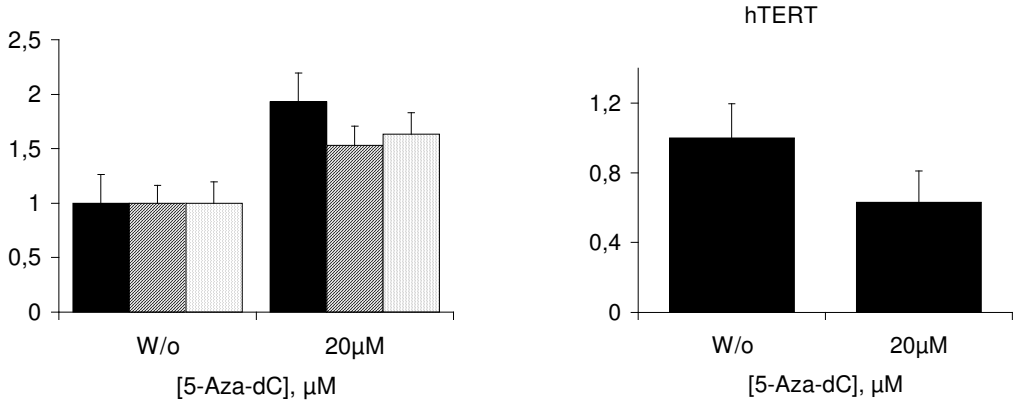


Fig. 38 Real-time RT-PCR analysis after 5-Aza-dC treatment.

Levels of *CD133*, *HOXA7* and *HOXA10* expression are shown on the left and levels of *hTERT* mRNA on the right. Total RNA was isolated 4 days after continuous incubation with 20µM 5-Aza-dC. On the left, one representative experiment is shown here, the experiment was repeated twice in triplicates, yielding similar results. The error bars show standard deviations. The increases in expression are statistically significant ($p<0.05$) On the right, the average of at

least three independent experiments in triplicates is shown. The error bars indicate standard deviations. The decrease of hTERT expression is statistically significant ($p<0.05$).

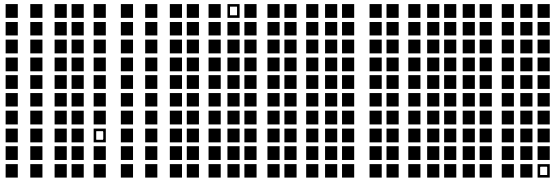
As it is demonstrated on Fig. 38, the levels of *HOXA7*, *CD133* and *HOXA10* expression were upregulated upon 5-Aza-dC treatment when compared to the untreated cells. The level of *hTERT* expression, in contrast, was reduced.

Downregulation of *hTERT* mRNA correlates with the findings on the Fig. 31, that demethylation of *hTERT* promoter leads to the silencing of its expression. Such a correlation was not described or analyzed for *HOXA7*, but according to the RT-PCR analysis, treatment with 5-Aza-dC leads to the increase of *HOXA7* expression. It indicates that DNA methylation may play a role in the regulation of *HOXA7* transcription.

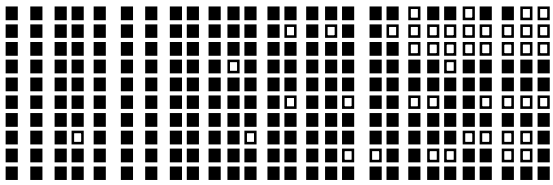
IV.9.3. 5-Aza-dC treatment results in a similar pattern of *hTERT* promoter methylation

Methylation analysis of *hTERT* promoter in the cells treated with 20 μM 5-Aza-dC revealed a similar methylation pattern of the promoter region I, which was also paralleled with the decrease of *hTERT* expression. On Fig. 39, 10 clones per sample are demonstrated, representing the methylation status of the region I of *hTERT* promoter.

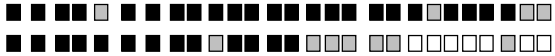
SEM



SEM+5-Aza



SEM



SEM+5-Aza

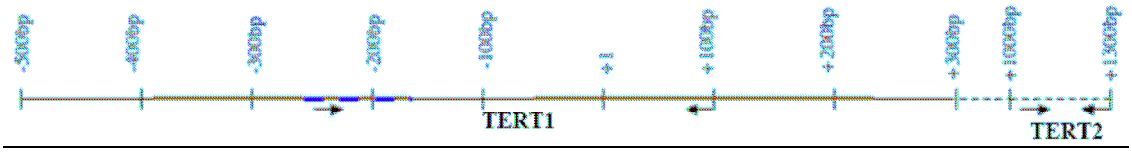
Fig. 39 CpG methylation analysis of mock or 5-Aza-dC – treated SEM

cells.

10 clones per sample were analyzed and every square on the diagram show separate CG dinucleotide. Low panel, summarized presentation, where every square represents an average of ten clones. Black squares-100% hypermethylation; grey squares-90-60% hypermethylation; white squares-50-10% hypermethylation.

IV.10. HOXA7 directly binds *hTERT* promoter

Based on the observed data, it could be concluded that HOXA7 directly participates in the regulation of telomerase activity in SEM cells. The Hox proteins were described as a transcriptional factors, which regulate numerous pathways during developmental and normal cellular processes (Eklund 2006). Nothing is known about the target genes of HOXA7. To test the possibility that HOXA7 directly binds to the DNA sequences of hTERT promoter a chromatin immunoprecipitation (ChIP) analysis was performed. For that, a flag-tagged cDNA of *HOXA7* (a gift from R. Slany) or flag-tagged cDNA of a transcription factor *FHL2* (a gift from S. Raimundo), taken as a negative control, were cloned in pMCSV vector. The SEM cells were transfected with cDNA of either *HOXA7* or *FHL2* containing vector and 18 hours later the cells were treated with formaldehyde for a cross-linking of DNA. For the pull-down of immunocomplexes, the α-Flag antibodies were used. The final DNA, recovered after precipitation, was analyzed with two different sets of primers (scheme 13). As it is seen on the Fig. 40, the final PCR analysis after ChIP revealed, that HOXA7 directly binds to the *hTERT* promoter (TERT1 primers on Fig. 40 and schemes 8 and 13). The primers surrounding the region of the *hTERT* gene between +1000 bp and +1300 bp, did not give any amplification product, what shows that precipitation was specific and there is no HOXA7 binding 1 kb downstream from transcriptional start point.



Scheme 13. Graphical presentation of the hTERT gene and its promoter.

The transcription start point is marked with +1. Red lines show the regions, where the methylation analysis was performed. The blue dashed line designate that part of the I region, which pattern was shown to be at mostly changed upon MLL-AF4 depletion (Fig. 34).

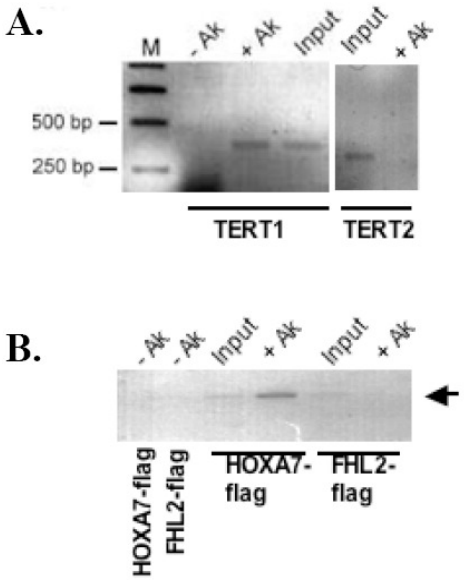


Fig. 40 PCR after ChIP analysis with the cells, transiently transfected with pMCSV-HOXA7-flag and pMSCV-FHL2-flag.

The result of two independent experiments are shown on the panel A and B. Panel A: M-Marker; lane 1-no antibody; lane 2- input; lane 3- +antibody; lane 4- input; lane 5-+antibody. Panel B: lane 1-no antibody (cells, transfected with *HOXA7*-flag); lane 2- no antibody (cells, transfected with *FHL2*-flag); lane 3- input; lane 4-+antibody; lane 5-input; lane 6-+ antibody. The primer pair TERT1 gives an amplicon of 330 bp, which is positioned within the region I of *hTERT* promoter (see scheme 13) (the product of an expected size is shown with the arrow). The primer pair TERT2 results in the amplicon of 300 bp, which surrounds the region around 1kb downstream from the transcriptional starting point (scheme 13) (the PCR product of expected size is shown with the arrow).

IV.11 MLL-AF4 depletion affects leukemic engraftment of t(4;11) positive cells

Leukemic cell growth in *SCID* mice has been shown to be associated with high-risk B-ALL (Uckun et al. 1995). Therefore, we used an t(4;11)-*SCID* mouse model to ask, whether siRNA-mediated depletion of MLL-AF4 affects leukemic engraftment and the development of leukemia *in vivo* (Pocock et al. 1995). For that, SEM cells were electroporated twice with mock or 500 nM siMA6 /siMM. On day 4, the cells were counted with trypan blue, and $20x10^6$ of viable cells were injected in each mouse. There were 8 mice per each group, female, all being around 2 months old. Intraperitoneal transplantation of either mock- or control siRNA-treated SEM cells into *SCID* mice resulted in a 100% leukemia-associated mortality within 70 days post transplantation with a median survival of 52 days (Fig. 41). Xenotransplantation of MLL-AF4-depleted SEM cells yielded a median survival of 82 days and an overall survival of 38% at day 125 ($p < 0.01$).

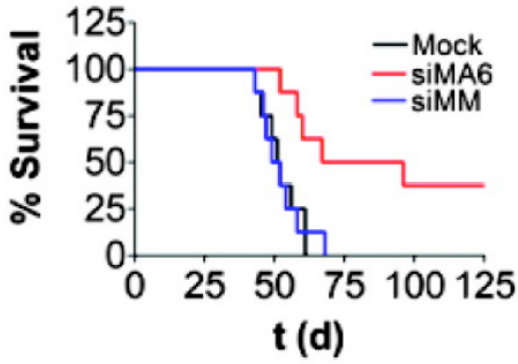


Fig. 41 MLL-AF4 suppression diminishes leukemic engraftment.

Kaplan-Meyer survival curves of SCID mice that received a transplantant of SEM cells. Prior to transplantation, SEM cells were electroporated twice with the indicated siRNAs. Red line show the survival curve of mock group, pink – of siMM and blue – siMA6. Two mice of siMA6 group was killed for the control at day 131, the last mouse of siMA6 group was also killed for the control at day 228, all of them being completely healthy.

Animals succumbing the disease showed ovarian tumors, massive leukemic blast infiltration in bone marrow (Fig. 42), spleen and liver (Fig. 43) and concomitant hepatosplenomegaly (Fig. 44), whereas the organs of surviving

animals of the siMA6 group showed no signs of leukemic infiltration up to 228 days after transplantation. In conclusion, siRNA-mediated suppression of MLL-AF4 reduced the leukemic engraftment of t(4;11)-positive cells in xenotransplanted *SCID* mice.

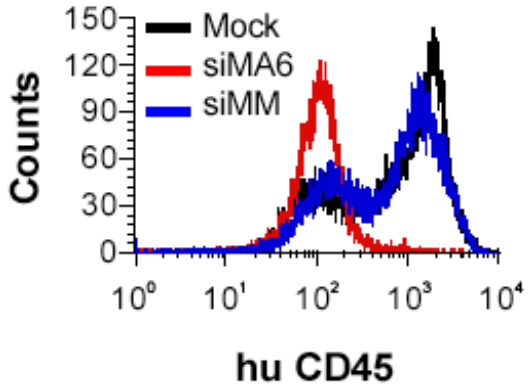


Fig. 42 FACS analysis of bone marrow.

Bone marrow cells of animals were stained with α-human CD45 antibody and analyzed by flow cytometry.

For the histology analysis, the organs of killed mice were isolated, fixed in formalin and after dehydratation were cut on 0,5 µm slices. Histological analysis of the liver slices show huge amount of infiltrated lymphoblasts (blue circles) in the „mock“ and „siMM“ groups, whereas in „siMA6“ mice, no blast infiltration was detected. Similarly, spleens of sick mice („Mock“ and „siMM“) show homogeneous, unstructured tissue, massively penetrated with leukemic blasts; spleen slices of „siMA6“ group, in opposite, demonstrate highly structured, heterogeneous tissue pattern without any infiltration.

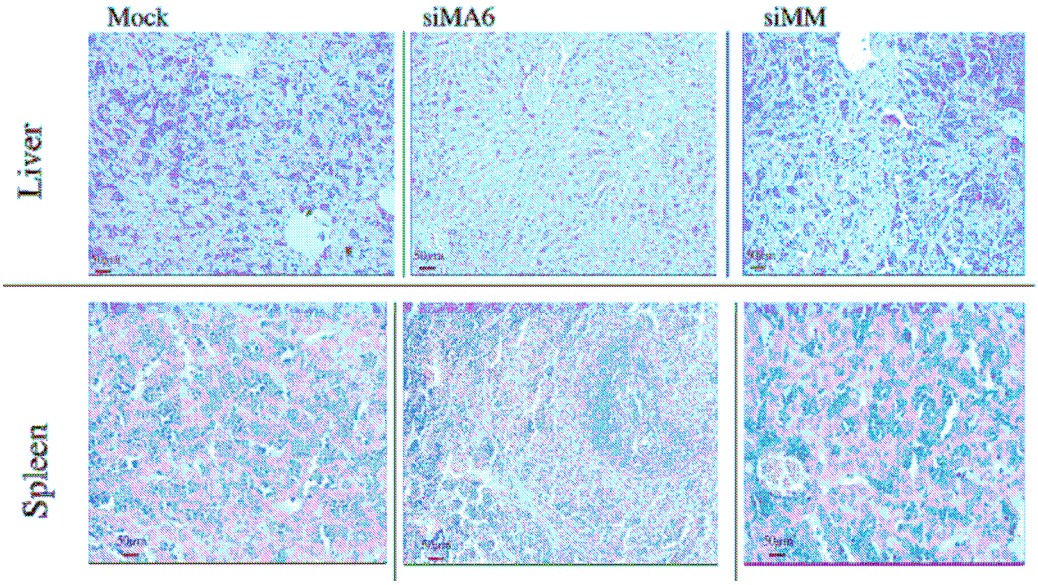
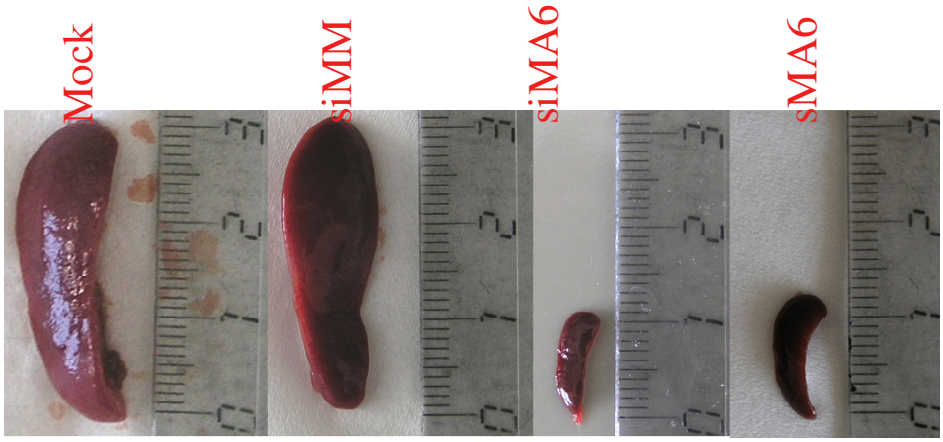


Fig. 43 Liver and spleen histologies.

Mice that received a transplant of mock or siMM-pretreated cells were moribund at the time of analysis. The animals that received a transplant of siMA6-pretreated cells were killed 131 and 228 days after transplantation without any sign of leukemia-associated morbidity.

Directly after preparation of the animals, the organs were isolated, weighted and photographed. Whereas the histological analysis of the liver slices showed massive blast infiltration in this organ, the sizes of livers of transplanted mice among different groups were found to be not significantly changed (Fig. 43). However, the sizes of spleens of the mice, which got the cells transfected with mock or siMM, were enormously increased. The spleen weight values of the siMA6 group mice were 10-fold lower in comparison with the control groups.

A



B

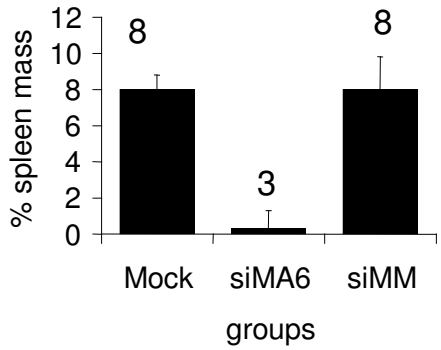


Fig. 44 Comparison of the organs of the mice from different groups.

A. Photograph of the spleens directly after preparation of (from left to right) mock, siMM and two siMA6 mice died 131 and 228 days respectively. B. Percent of the spleen (left) or liver (right) mass from the total body weight. On the left diagram, the numbers show how many mice per group were taken for the statistical calculation. On the right graph, error bars show standard deviation between weight values of eight animals per group. The differences in spleen masses are significant ($p<0.005$), whereas the liver sizes differ not significantly ($p>0.05$).

V. DISCUSSION

V.1 Targeting MLL-AF4 with siRNAs

The role of MLL-AF4 in the acquirement and maintenance of leukemic phenotype is not clearly defined. To address this question, an RNA interference approach to suppress *MLL-AF4* expression in t(4;11)-positive leukemic cell lines SEM (Greil et al. 1994) and RS4;11 (Stong et al. 1985) was developed.

To interfere with *MLL-AF4* expression, a scan of the mRNA junction site with 15 different siRNAs was performed. By shifting the siRNA target site by single nucleotide intervals, major changes in the extent of mRNA reduction were observed (Fig. 1). Similar findings have been reported by Holen and co-workers targeting the human coagulation trigger tissue factor (Holen et al. 2002). One reason for these changes might be the choice of siRNA strand being incorporated into the RNA-induced silencing complex (RICS). Recently, several reports suggested that the siRNA strand with the lower thermodynamic stability is favoured to become part of RISC (Khvorova et al. 2003; Reynolds et al. 2004; Ui-Tei et al. 2004). However, any correlation between the sequences of active MLL-AF4 siRNAs and the relative 5'-terminal stabilities was not detected (Thomas et al. 2005). This discrepancy might be explained by the unusual design of most of MLL-AF4 siRNAs, which contain only one 3'-overhang and one blunt-ended terminus.

The electroporation protocol used in this study requires higher siRNA concentrations than siRNA lipofection with cationic lipids (Grunweller et al. 2005). However, in contrast to lipofection, the suspension of cells is diluted twenty fold after electroporation. Thus, the amount of siRNA per cell is comparable or even lower than that used for the lipofection of, for instance, the leukemic cell line K562 with BCR-ABL siRNAs (Wilda et al. 2002; Zhelev et

al. 2004). The siRNA concentrations used for the electroporation of SEM cells to suppress MLL-AF4 are fivefold higher than those required for AML1/MTG8 suppression (Martinez et al. 2004), but are comparable to those used to inhibit *BCR-ABL* expression (Scherr et al. 2003; Wohlbold et al. 2003). Thus, the MLL-AF4 siRNA concentrations used here are well within the range of siRNA concentrations applied in comparable experimental settings.

When comparing the specificity of two most efficient siRNAs, siMA3 and siMA6, only the latter diminished *MLL-AF4*, but neither *MLL* or *AF4* mRNA levels (Fig. 4). SiMA6 inhibited only the clonogenicity of SEM, but not of 6 other leukemic cell lines including two t(4;11)-positive lines expressing different *MLL-AF4* variants than SEM (see "siRNA sequences" in Methods section). In contrast, siMA3 affected the clonogenic growth of 5 other leukemic lines in addition to SEM, which correlates with a reduction of the unfused *AF4* wild type transcript caused by this siRNA. One possible reason for these differences in specificity might be the different target site locations of the two siRNAs. The antisense strand of more specific siMA6 binds with 13 nucleotides to the *AF4* part and with 10 nucleotides to the *MLL* part of the fusion transcript, whereas the antisense strand of siMA3 binds with 16 nucleotides to the *AF4* part (Table 1). Since a stretch of 15 complementary nucleotides can be sufficient to induce RISC-mediated RNA cleavage (Czauderna et al. 2003), the wild type *AF4* transcript might be a direct target of siMA3-mediated degradation. This assumption is further supported by the observed twofold reduction of *MLL-AF4* mRNA variant present in RS4;11 cells (Table 1), since the *AF4* part of this fusion variant is identical with that of the *MLL-AF4* variant expressed in SEM cells (Scheme 9). Thus, when compared to siMA3, the superior specificity of siMA6 may be due to the more central location of the *MLL-AF4* fusion site within its target sequence; the complementarities to the unfused wild type mRNAs might be too short for efficient gene suppression.

In RS4;11 cells, siMARS targets e10-e4 variant of *MLL-AF4* transcript.

This siRNA also contains 13 nucleotides targeting *AF4* part of the fusion transcript, which is identical between SEM and RS4;11 cells, and 10 nucleotides covering *MLL* part (scheme 9). Nevertheless, siMARS provided any effects on the downregulation of *AF4* neither in SEM nor in RS4;11 cells. Comparing siMARS with siMA3, it could be seen that 13 nucleotides within *AF4* part of siMARS are too less for its mRNA degradation whereas 16 nucleotides of siMA3 appeared to be enough. Therefore, siMA3 was considered to be unspecific towards targeting of *MLL-AF4* whereas siMARS was used as a specific and active siRNA in downregulation of *MLL-AF4* in RS4;11 cells.

In a set of "cross-over" experiments siMA6 and siMARS were applied in both cell lines for the mutual control (Fig. 5, Fig. 9, Fig. 12, Fig. 13). These experiments showed that only the siRNA with perfect homology to the fusion breakpoint site was active in the corresponding cell line, whereas not-perfectly homologous siRNA did not have any effects on the downregulation of *MLL-AF4* expression. Furthermore, it was demonstrated that the phenotypical effects evoked by the application of MLL-AF4 siRNAs could be attributed specifically to the depletion of corresponding *MLL-AF4* transcript.

siMA13 exhibited an anti-clonogenic effect in three different leukemic cell lines including RS4;11 (Table 2). Despite a stretch of 17 nucleotides being homologous to the MLL part of the fusion transcript, siMA13 did not reduce the levels of wild type *MLL* mRNA in any of the cell lines tested. Recently, it has been demonstrated that siRNAs may inhibit protein synthesis without degrading the corresponding transcripts (Saxena et al. 2003). Thus, it could not be excluded that siMA13 either modulates MLL translation thereby reducing MLL protein levels or exert some unspecific off-target effects (Scacheri et al. 2004).

An important point of concern for any siRNA application is the specificity of siRNAs and the possible occurrence of off-target effects. For instance, siRNAs and shRNAs may induce a limited interferon response leading to nonspecific changes of gene expression patterns (Bridge et al. 2003; Sledz et al.

2003; Kim et al. 2004). One possible readout for interferon response is an increase in *STAT1* and *OAS* mRNA levels (Sledz et al. 2003). However, none of the MLL-AF4 siRNAs tested caused a significant change in *STAT1* or *OAS1* expression (Fig. 6) suggesting that these siRNAs do not induce a substantial interferon response. Transfection with polyIC, a strong inductor of interferon response, increased *OAS1* and *STAT1* transcript levels, demonstrating that interferon response pathways can be induced in these leukemic cells.

Because of their high specificity, the MLL-AF4 siRNA siMA6 (for targeting e9-e4 variant in SEM cells) and siMARS (for downregulating of e10-e4 variant in RS4;11 cells) and the mismatch control siRNA siMM were chosen for further experiments to prove the significance of *MLL-AF4* expression for the maintenance of leukemic phenotype.

V.2 Clonogenicity, proliferation and cell cycle distribution

Experimental models using ectopic expression of *MLL-AF4* in, for instance, Drosophila showed that *MLL-AF4* expression leads to a retarded cell cycle and larval lethality (Muyrers-Chen et al. 2004). Furthermore, ectopic expression of *MLL-AF4* in the myelomonocytic leukemia cell line U937 inhibits proliferation and cell cycle progression and is associated with an increased rate of apoptosis (Caslini et al. 2004). In contrast, t(4;11)-positive ALLs are very resistant against induction of apoptosis (Kersey et al. 1998; Dorrie et al. 1999). Additionally, t(4;11)-positive leukemic cell lines such as RS4;11, MV4;11 or SEM engraft very efficiently and give rise to a rapid development of aggressive leukemias in xenotransplantation models (Pocock et al. 1995). Therefore, the anti-proliferative and pro-apoptotic effects observed upon ectopic *MLL-AF4* expression may either be outweighed by other mutations such as FLT3 overexpression (Ferrando et al. 2003), or *MLL-AF4* expression might become dispensable in the final stages of leukemogenesis.

This latter possibility, however, is rather unlikely, since siRNA-mediated depletion of *MLL-AF4* inhibited the clonogenicity and proliferation and decreased the fraction of S-phase cells of SEM and RS4;11 cells upon treatment with the corresponding siRNAs. The diminishment of clonogenicity, reduction of proliferation and inhibition of the cell cycle transition from G0/G1 to S phase can be only attributed to the suppression of the corresponding fusion transcript, since the siMA6 and siMARS mutually control themselves. Transfection of primary human hematopoietic CD34+ cells did not affect the number of GEMM or GM colonies, which is an essential control in case of therapeutical application.

The treatment of SEM cells with the siRNA targeting menin resulted in a slight reduction of a colony formation, but provided prominent changes in the cell cycle distribution and induction of apoptosis (Fig. 27, Fig. 28, Fig.29). This discrepancy could be attributed, on the one hand, to the different cell density and the duration of *MEN1* depletion. The cells, plated in methylcellulose for colony formation assay after electroporation, are very diluted. Every single cell has to initiate the proliferation by its own, without the help of neighboring cells, which usually produce a number of a growth factors exerted in culture medium. The lack of these extracellular factors could be responsible for the difference between cell cycle analysis in suspension culture and colony formation in semi-solid medium. Furthermore, the cells for colony formation assay were electroporated only once, while cell cycle analysis was performed after double electroporations. Probably, downregulation of the *MEN1* for more than 4 days is necessary for detection of changes in colony formation. On the other hand, lack of effect of the menin depletion on the colony formation could be due to the irrelevance of menin for an initiation of proliferation and cellular renewal. Meanwhile, menin appears to be important for the maintaining of ongoing processes, such as proliferation and apoptosis. Depletion of MLL-AF4, in contrast, affects clonogenicity as well as proliferation and apoptosis, which

demonstrates the significance of MLL-AF4 for both processes of the initiation and maintaining of the proliferation.

V.3 Decrease in *HOX* gene expression and onset of myeloid differentiation

As it was mentioned in the introduction, the overexpression of *HOX* genes, in particular *HOXA7*, *HOXA9*, *HOXA10* and *MEIS1*, is a common mechanism observed with MLL-associated leukemias (Quentmeier et al. 2004). The suppression of *MLL-AF4* was paralleled with a decreased expression of these homeobox genes, suggesting that *MLL-AF4* is the cause of their upregulated transcription in leukemia.

The absence of *HOXA7* and *HOXA9* expression results in the development of B cells even in the presence of MLL-AF4 (Ayton et al. 2003). Therefore, the observed reduction in *HOXA7*, *HOXA9* and *MEIS1* expression upon MLL-AF4 depletion might result in an, at least partially, reactivated hematopoietic differentiation. Or, vice versa, the onset of differentiation could be a reason for a decrease of expression of these homeobox genes. Gene expression profiling data, obtained by two independent groups, show an increased amounts of *CD133* transcript in the cells with MLL rearrangements (Armstrong et al. 2002; Zeisig et al. 2003). The antigenic profile of SEM cells is CD133+/CD34-, which might be more immature than the CD133+/CD34+ unique myeloid cell line MUTZ-2, expressing *CD133* (Hu et al. 1996). Therefore, the diminished expression of *CD133* upon siRNA treatment suggests that interfering with MLL-AF4 functions may, at least to a limited extent, support hematopoietic differentiation (Fig. 15). Moreover, *CD133* expression is controlled by the methylation status of a cytosine-phosphate-guanosine (CpG) island (Shmelkov et al. 2004), which additionally indicates that *CD133* could be a direct target gene of MLL-AF4.

V.4 Induction of apoptosis

Apoptotic cell death serves as a major mechanism for the precise regulation of cell numbers and as a defence against unwanted and potentially dangerous cells (Meier et al. 2000). The regulation of apoptosis is fundamental to hematopoietic homeostasis. Aberrant apoptosis has been associated with development of hematological malignancies (Hanahan et al. 2000). Therefore, from the therapeutical point of view, induction of apoptosis, observed upon MLL-AF4 suppression, has an important application for the treatment of t(4;11) leukemias.

Two separable caspase activation pathways have been characterized (Green 2000). The extrinsic pathway is initiated by activation of transmembrane death receptors (CD95, TNF receptor, and TRAIL receptor) to trigger membrane-proximal (activator) caspases (caspase-8 and -10), which in turn cleave and activate effector caspases such as caspase-3 and -7 (Sheikh et al. 2004). This pathway can be regulated by c-FLIP, which inhibits upstream activator caspases, and inhibitor of apoptosis proteins (IAPs), which affect both activator and effector caspases (Kataoka 2005). The intrinsic pathway requires disruption of the mitochondrial membrane and the release of mitochondrial proteins including Smac/DIABLO, HtRA2, and cytochrome c. Release of cytochrome c and the production of reactive oxygen species from the outer mitochondrial membrane is controlled by the group of BCL proteins, in particular by BCL-X protein (Kim et al. 2006). Cytochrome c, in turn, functions with APAF-1 to induce activation of caspase-9, thereby initiating the apoptotic caspase cascade, while Smac/DIABLO and HtrA2 bind to and antagonize IAPs (Hill et al. 2003).

The t(4;11) leukemias are characteristically resistant to conventional chemotherapeutic treatments operating through the induction of apoptosis. The mechanisms leading to increased cell survival and resistance to cell death in these leukemias are largely unknown. It was shown, for instance, that t(4;11)

lines SEM, RS4;11, and MV4;11 expressed low levels of *CD95* and were completely resistant to CD95-mediated death (Dorrie et al. 2002). Interferon-gamma (IFNγ), a potent inhibitor of hematopoiesis, acts in part by upregulating *CD95* and sensitizing cells to CD95-induced apoptosis. Although addition of IFNγ markedly upregulated *CD95* expression in t(4;11)-positive cell lines, there was only a slight increase in sensitivity to CD95-mediated cell death after treatment with IFNγ (Dorrie et al. 1999). The reason for that could be abundant levels of antiapoptotic proteins BCL-2 and BCL-XL, known to inhibit CD95-signaling, which are present in the cells carrying t(4;11) (Findley et al. 1999). Resistance of the leukemic blasts to CD95-mediated cell death and the failure of IFNγ to substantially sensitize the CD95-signaling pathway contribute to the highly malignant phenotype of t(4;11) leukemias.

Nevertheless, specific suppression of MLL-AF4 resulted in the induction of apoptosis of both SEM and RS4;11 cells. The almost inactive siMA13, which reduces the target *MLL-AF4* mRNA by 10 %, only marginally affected amount of apoptotic cells, suggesting a direct correlation between the extent of MLL-AF4 depletion and the rate of apoptosis (Fig. 1 and Fig. 17). Proteolytic activation of caspase-9, and consequently caspase-3, and decreased amounts of the antiapoptotic protein BCL-XL accompanied the siMA6-mediated induction of apoptosis, suggesting the triggering of the mitochondria-mediated pathway of the programmed cell death (Fig. 18). In addition, siRNA treatment could sensitize cells towards induction of an extrinsic apoptotic pathway, which was not tested yet. These findings are supported by the recent studies of Srinivasan, who showed that the peptide-mediated disruption of the interaction between MLL-AF4 and AF9 triggers apoptosis in t(4;11) leukemia cell line (Srinivasan et al. 2004).

Coexpression of the homeobox protein MEIS1 and either HOXA7 or HOXA9 is characteristic for many types of AML (Armstrong et al. 2003). Although *MEIS1* can be overexpressed in bone marrow cells, it is incapable of

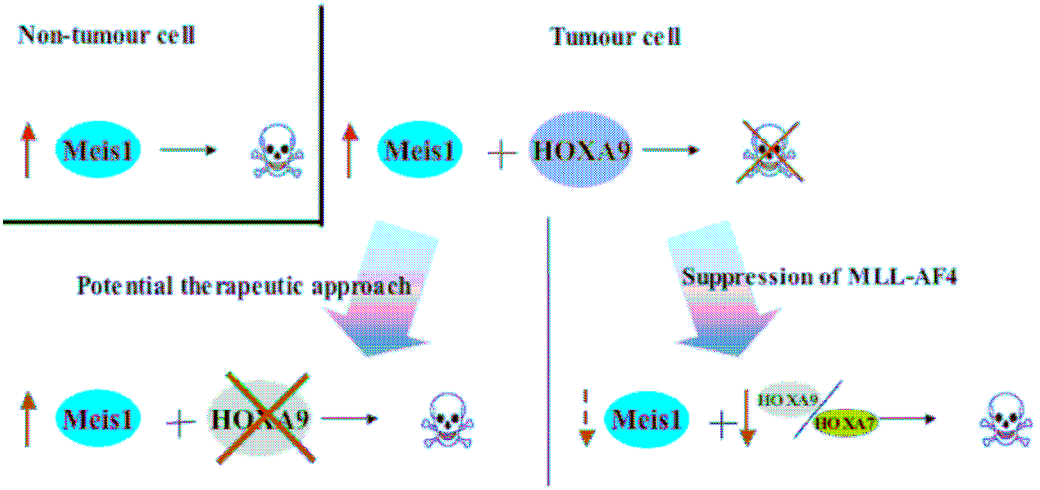
mediating their transformation (Zeisig et al. 2004). While overexpressing *HOXA9* alone transforms murine bone marrow cells, concurrent *MEIS1* overexpression greatly accelerates oncogenesis. Moreover, MEIS1-HOXA9 cooperation suppresses several myeloid differentiation pathways (Martin et al. 2003).

The *MEIS1* overexpression strongly induces apoptosis in a variety of cell types in vitro via caspase-dependent processes (Wermuth et al. 2005). However, coexpression of *HOXA9* with *MEIS1* suppresses this apoptosis and provides protection from several apoptotic inducers. Thus, *MEIS1* and *HOXA9* coexpression provide a selective advantage during oncogenesis permitting cells to accumulate additional growth stimulatory mutations without undergoing the risk of triggering the cell's apoptotic machinery. The authors present a potential therapeutic target for treating tumors exhibiting upregulated *MEIS1/HOXA9* expression. Namely, targeting *HOXA9* expression while leaving *MEIS1* expression intact may eliminate the advantage of *HOXA9* co-expression, leaving only MEIS1 apoptotic effects (scheme 14).

Presumably, a similar picture is observed in the case of *MLL-AF4* inhibition. The siMA6 treatment leads to the decreased expression of *HOXA9* and, to a lesser extent, of *MEIS1*. The reduction of *HOXA9*, however, in this case could be sufficient for the disturbance of the balance in HOXA9-MEIS1 tandem, whereas the levels of *MEIS1* stay over a particular threshold to be able to trigger cell death (Scheme 14).

However, targeted suppression of *HOXA9* with the siRNA did not result in the induction of apoptosis in SEM cells (Fig. 29). The reason for that could be an insufficient degree of the expression downregulation or substitution of HOXA9 with another member of the homeobox family, like HOXA7 or HOXA10, which overtake transiently its cellular functions. The latter possibility is quite likely, since depletion of HOXA7 with siRNA indeed resulted in the induction of apoptosis, which suggests that the tandem MEIS1-HOXA9 could

also exist in the form of MEIS1-HOXA7 (Fig. 29). Nevertheless, the lack of HOXA9 could be compensated with the functions of HOXA7 whereas HOXA9 was not able to substitute HOXA7. Downregulation of MEIS1 did not result in the induction of apoptosis, which proves that the presence of MEIS1 over a particular threshold is important for triggering MEIS1-dependent cell death.



Scheme 14. Interconnection of MEIS1 and HOXA9 expression with the induction of apoptosis.

Briefly, in a vast majority of normal cells overexpression of *MEIS1* (shown with red arrow) leads to the apoptosis (skull). In tumour cells, overexpression of *MEIS1* in tandem with *HOXA9* blocks this induction of apoptosis (red cross). MLL-AF4 depletion leads to the decreased levels of *HOXA9*, *HOXA7* and to a less extension of *MEIS1*, which could serve as a trigger of the cell death.

The detailed view on the induction of apoptosis upon MLL-AF4 depletion in both cell lines revealed an increase of expression of two other genes, which function primarily interferes with the cell death: *APAF-1* and *SEPT4*.

The *APAF-1* gene, which is under transcriptional regulation of E2F-1, encodes a cytoplasmic protein that initiates a caspase cascade leading to apoptosis (Furukawa et al. 2002). This protein contains several copies of the WD-40 domain, a caspase recruitment domain (CARD), and an ATPase domain (NB-ARC) (Scheme 10). Upon binding cytochrome c and dATP, this protein forms an oligomeric apoptosome. The apoptosome binds and cleaves caspase 9 preproprotein, releasing its mature, activated form. Activated caspase 9 stimulates the subsequent caspase cascade that commits the cell to apoptosis

(Zou et al. 1999). Strikingly, the DNA methylation silencing is a mechanism of the inactivation of *APAF1* in acute leukemia (Furukawa et al. 2005).

Recently, several *APAF-1* isoforms, generated by the alternative splicing mechanism, have been identified in tumor cell lines (Fu et al. 2001). The major RNA form, which was found to be expressed in all tissues, is the *APAF-1XL* isoform (Scheme 10), containing both the NH2-terminal and COOH-terminal WD40 repeats (Twiddy et al. 2004). Another identified isoform, *APAF-1LN*, contains NH2-terminal insert but lacks an additional C-terminal WD-40 repeat. Functional analysis of all identified *APAF-1* isoforms demonstrated that only those with the additional C-terminal WD-40 repeat were able to bind cytochrome c, subsequently self-associate, bind procaspase 9 and form active APAF-1 oligomers. The NH2-terminal WD-40 domain is not required for this activity. In this aspect, some of the isoforms are able to trigger caspase-dependent apoptosis and are considered as pro-apoptotic. The other isoforms, possessing CARD domain for procaspase binding but lacking binding moiety for cytochrome c (isoforms 1 and 4 on the scheme 10), are not able to form active apoptosomes and, therefore, thought to play an anti-apoptotic role by trapping procaspases without subsequent sequestration (Benedict et al. 2000).

Consistent with these observations, the expression analysis of pro-apoptotic *APAF-1* isoforms with specific primers showed that siMA6-mediated induction of apoptosis resulted in the increased expression of these particular *APAF-1* splice variants. It could be assumed that the increase of the total mRNA of *APAF-1* is caused by the induced levels of the pro-apoptotic transcripts of *APAF-1*. The differences in the induction can be attributed to yet not identified pro-apoptotic splice variants.

The structural feature of the *APAF-1* promoter suggests that methylation of CpG island serves as the major mechanism of transcriptional regulation of *APAF-1*. Indeed, it was shown that *APAF-1* expression is successfully restored in many melanoma and leukemia cell lines by the treatment with 5-Aza-2´-

deoxycytidine, an inhibitor of DNA methylation (Lyons et al. 2003; Furukawa et al. 2005).

The whole CpG island of *APAF-1* gene spans from –776 bp to +185 bp. In particular, hypermethylation between +79 bp and +168 bp downstream of ATG preferentially corresponded with *APAF-1* silencing (Fu et al. 2003; Furukawa et al. 2005). Analysis of the methylation status in mock- or siMA6-treated SEM cells between +79 bp and +168 bp revealed that this region is hypomethylated. These findings do not agree with the analysis of the same region in K562 cells (Fu et al. 2003). Additionally, there were no changes in methylation pattern of *APAF-1* promoter evoked by the suppression of MLL-AF4, in spite of the observed induction of *APAF-1* transcription.

It is worthwhile to mention that in the melanoma and leukemia cell lines studied, the presence of methylation within the +79 bp - +168 bp region, correlating with the silencing of *APAF-1*, was always accompanied with the overexpression of *DNMT1* (Fu et al. 2003). Nothing is known about the levels of *DNMT1* expression in SEM cells. And, besides that, it can not be excluded that in t(4;11) cells another mechanism of methylation-dependent transcription regulation of *APAF-1* gene is present and the methylation status of another part of CpG island is crucial for its regulation. The induction of *APAF-1* transcription after 5-Aza-dC treatment supports the idea that DNA methylation has an impact on the level of *APAF-1* mRNA expression in SEM cells (Fig. 21). Alternatively, since it is known that APAF-1 protein levels are controlled at the post-transcriptional level (Fu et al. 2003) and taking into account unchanged pattern of methylation upon MLL-AF4 depletion, the differences in the expression levels of *APAF-1* isoforms could be explained by interfering of MLL-AF4 with the mechanism of alternative splicing. However, this hypothesis was not yet tested and the influence of MLL-AF4 on the generation of alternatively spliced variants of APAF-1 gene is not clear.

The *SEPT4* gene is a member of the *Septin* gene family of nucleotide

binding proteins, originally described in yeast as cell division cycle regulatory proteins (Paavola et al. 1999). *Septins* are highly conserved in yeast, Drosophila and mouse and appear to regulate cytoskeletal organization. Alternative splicing of this gene results in four transcript variants which encode different isoforms (Scheme 11) (Larisch 2004). One of this splice variants, called *ARTS*, deserves a particular interest. This pro-apoptotic protein, which localizes to mitochondria, is lost in the majority of acute lymphoblastic leukemia patients, but is always detectable during and after remission (Elhasid et al. 2004). It was reported that high cellular levels of ARTS protein sensitize cells toward apoptosis and its intracellular levels are regulated through ubiquitin-mediated degradation (Lotan et al. 2005). ARTS induces apoptosis by antagonizing IAPs, including XIAP (Gottfried et al. 2004) and, similar to the *APAF-1*, the process of DNA methylation contributes to the loss of its expression (Elhasid et al. 2004).

Analogously to *APAF-1*, suppression of MLL-AF4 resulted in the increased expression of particular splice isoforms, *SEPT4.2* and *SEPT4.4* (*ARTS*). Moreover, the splice variants 1 and 3 stayed to be unaffected (scheme 11 and Fig. 23). It suggests that observed total increase of mRNA could be due to elevated mRNA levels of these variants. These two isoforms are expressed from two different promoters and the transcription of both forms was affected upon MLL-AF4 depletion. The fact that treatment with 5-Aza-dC restores transcription of ARTS in SEM cells (Fig. 24) serves as a direct hint for the, at least, partial input of DNA methylation in the regulation of *ARTS* transcription in t(4;11)-positive cells. The CpG island of this gene was not described so far, although according to the prediction of the analyzing software CpG Plot, provided by EMBL-EBI, there is a CpG island within the region –400 bp +260 bp with the estimated probability of more than 95 % (http://www.ebi.ac.uk/emboss/cpgplot/). The methylation pattern within this region of *ARTS* gene was not yet analyzed in SEM cells.

Elhasid showed that two leukemic cell lines K562 and HL60 lacking *ARTS* were resistant to the apoptotic effects of several chemotherapeutics (Elhasid et al. 2004). Ectopic expression of ARTS restored the ability of these cells to die upon drug treatment. The authors also suggested that methylation is responsible for the loss of *ARTS* in two ALL patients and myeloid leukemia cell line HL60, since alone treatment with 5-Aza-dC resulted in reappearance of ARTS protein both in HL60 cells and at remission in patients showing severely reduced levels at a disease state. In contrast, lymphocytes from healthy donors did not increase *ARTS* RNA levels upon 5-Aza-dC treatment.

The loss of pro-apoptotic genes is one of the known mechanisms by which transformed cells obtain selective advantage through reduced susceptibility to apoptotic signals. In SEM cells, the loss of two crucial proteins, which play a significant role in the induction of apoptosis, correlates with high resistance to the apoptotic stimuli. However, this apoptotic block can be reverted by either treatment with 5-Aza-dC or downregulation of MLL-AF4. Thus, MLL-AF4 contributes to the loss of expression of these two genes, possibly, through epigenetic silencing of DNA CpG island methylation or interfering with the mechanisms of alternative splicing. As it was already mentioned, *SEPT4.2* and *SEPT4.4* are expressed from two different promoters and interfering of MLL-AF4 with alternative splicing cannot be currently excluded. Similarly, downregulation of MLL-AF4 resulted in a different expression of *BCL-X* splice variant, *BCL-XL* (Fig. 25).

Recently, Milne et al could show that binding of MLL complex is not restricted to the TATA box area, where transcriptional initiation takes place (Milne et al. 2005). Instead, they found MLL spread across the whole transcriptional unit in a pattern essentially mimicking the distribution of RNA polymerase II (RNApol II) itself. In pull-down assays, they could indeed demonstrate that the C-terminal domain (CTD) of RNApol II directly interacted with MLL^N , MLL^C and menin in a complex. Moreover, genes known to be

under the control of MLL lost RNApol II binding in the cells with knocked-out *MLL* gene. In summary, the results of Milne suggest that MLL regulates gene expression at the stage of elongation. The process of elongation and splicing are coordinated and, in some cases, functionally coupled (Kornblihtt et al. 2004; Bentley 2005). Moreover, CTD of RNApol II, whose participation in key pre-mRNA processing reactions (such as capping and cleavage/polyadenylation) is well studied, was recently shown also to play an important role in alternative splicing during its transcriptional elongation "ride" along DNA (Proudfoot et al. 2002; Nogues et al. 2003; Kornblihtt 2006). In this regard, alterations in the expression of different splice isoforms of *APAF-1*, *SEPT4* and *BCL-XL* upon MLL-AF4 depletion could be explained by the direct participation and interfering of MLL-AF4 with the processes of alternative splicing.

V.5 *HOXA7* is a target gene of MLL-AF4

As it was already mentioned above, wild type MLL binds to the promoters of *HOX* genes, such as *HOXA9* to maintain their expression. These HOX proteins regulate hematopoiesis and are normally expressed only in early hematopoietic progenitors. MLL fusion proteins also directly up-regulate *HOX* expression, but in contrast to wild-type MLL, they do not allow for its normal down-regulation. This persistent expression of *HOX* genes along with expression of another up-regulated HOX co-factor, *MEIS1*, appears to be necessary and sufficient to cause leukemia (Ayton et al. 2003; Ernst et al. 2004). It was demonstrated, that MLL-ENL could not transform *HOXA9* knockout bone marrow (Zeisig et al. 2004). Furthermore, using a conditionally transforming version of *MLL-ENL*, in the Labs of R. Slany and J. Hess it was found that expression of *HOXA9* plus *MEIS1* was sufficient to completely replace the gain of function activity of MLL-ENL (Ayton et al. 2003; Zeisig et al. 2004).

Another recently identified important player and component of the MLL supercomplex is menin. It was reported to directly interact with the N-terminus of wild-type and fused MLL and to bind *HOXA9* promoter (Yokoyama et al. 2004).

The effects of siRNA-mediated suppression of *HOXA7*, *HOXA9*, *MEIS1* and *MEN1* were studied in the aspects of clonogenicity, proliferation, cell cycle distribution and apoptosis. Downregulation of HOXA7 leads to the inhibition of clonogenicity of SEM cells to the same extent as suppression of MLL-AF4. Treatment with siMenin also caused less than two-fold reduction in colony formation of SEM cells. It suggests, that menin interferes with the clonogenic capacity of these cells. However, its single suppression is not enough to achieve similar with the siHOXA7- or siMA6-mediated reduction. Nevertheless, the effects on the cell cycle distribution and induction of apoptosis were similar between treatments with siMA6, siHOXA7 and siMenin.

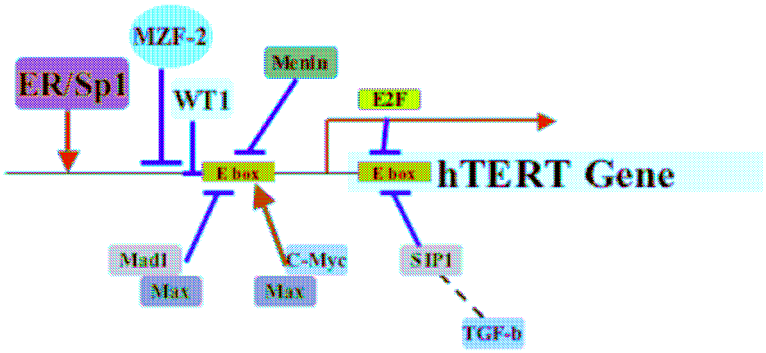
In contrast to HOXA7, depletion of MLL-AF4 does not affect transcription of *MEN1*, suggesting that menin, as an additional factor, independently contributes to the disease progression and that its activity is not directly related to the function of MLL. Instead, decrease of *HOXA7* expression, evoked by downregulation of MLL-AF4, and siRNA-mediated suppression of *HOXA7* resulted in both cases in similar findings on the inhibition of proliferation, clonogenicity and induction of apoptosis. Thus, *HOXA7* may be a downstream transcriptional target of MLL-AF4 and a mediator of MLL-AF4 activity.

V.6 Regulation of *hTERT* transcription

Telomerase activity is significantly downregulated in many somatic cells, which can be mainly attributed to the transcriptional repression of *hTERT* gene (Ahmed et al. 2003). Transcriptional control of *hTERT* has thus emerged as the

focus of regulation of telomerase activity. Characterization of the 5´-*hTERT* gene regulatory region, which is depicted on the scheme 15, reveals that it contains numerous binding sites for transcription factors. These factors are divided into two categories: repressors and activators. Repressors include Mad1, myeloid-specific zinc finger protein 2 (MZF-2), Wilms Tumor 1 (WT1) and Menin (Poole et al. 2001). Whereas menin can directly bind to *hTERT* promoter, TGF-β, through Smad-interacting protein-1 (SIP1), represses its transcription indirectly (Lin et al. 2003). Activators of *hTERT* transcription have also been identified, including c-MYC, Sp1 and estrogen receptor (ER) (Poole et al. 2001). Mad1 and c-MYC play antagonistic roles in the regulation of *hTERT*. They both bind to the consensus sequence 5´-CACGTG-3´, also called E box. In undifferentiated cells and most neoplastic and transformed cells, the levels of c-MYC protein are elevated, while Mad1 protein levels are depressed. Conversely, in differentiated somatic cells, Mad1 levels are elevated and c-MYC levels are minimal (Grandori et al. 2000).

Telomerase activity is mainly regulated at the level of transcription of the *hTERT* gene, but the exact molecular mechanism underlying the tumor-specific expression of telomerase remains unclear. There are clusters of CpG dinucleotides located within the *hTERT* promoter, which are targets for DNA methylation.

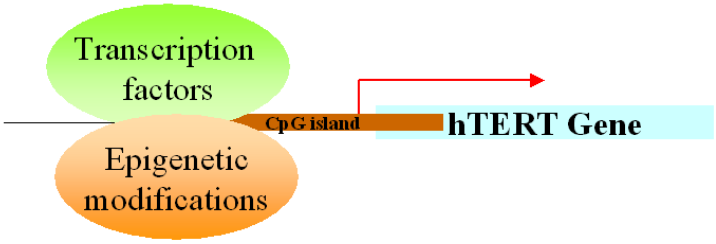


Scheme 15. Regulation of hTERT transcription by different transcription factors.

Estrogen receptor and transcriptional factor Sp1 recognize one sequence and are both activators of *hTERT* transcription (red arrow). Myeloid zink finger-2 protein (MZF-2), Wilms Tumor 1 (WT1) protein, Menin, E2F, Smad-interacting protein 1 (SIP1) are known as repressors.

Depending on the partner protein, Max in complex with c-MYC can be activator or, in dimer with Mad1, repressor of *hTERT* transcription.

Several studies have demonstrated that the extent of *hTERT* transcription depends both on the activity of different transcriptional factors as well as epigenetic modifications within the promoter (Dessain et al. 2000; Guilleret et al. 2002; Ahmed et al. 2003; Lopatina et al. 2003).



Scheme 16. Impacts of both activity of transcription factors and epigenetic modifications are equally important for the final level of *hTERT* transcription.

Generally, methylation at CpG sites within the promoter and surrounding regulatory region leads to gene silencing. The promoter regions of some tumor suppressor genes (e.g., p16 and hMLH1) become methylated during tumorigenesis, and their repression is associated with tumor- associated phenotypes, such as genomic instability and metastasis of tumor cells (Esteller 2002). The presence of abundant CpG sites within *hTERT* promoter region has triggered an increasing interest in examining the possible role of DNA methylation in regulation of *hTERT* transcription in normal and cancer cells. Using a bisulfite genomic sequencing method and a methylation-specific PCR-based assay, several groups have debated the correlation of *hTERT* promoter methylation with level of its expression (Devereux et al. 1999; Dessain et al. 2000; Bechter et al. 2002). Hypomethylation of *hTERT* promoter is seen in undifferentiated cells and untransformed cells which are hTERT-negative, suggesting that these cells have a mechanism to tightly repress *hTERT* transcription independent of promoter methylation (Dessain et al. 2000; Lopatina et al. 2003; Shin et al. 2003). Methylation of *hTERT* promoter is also

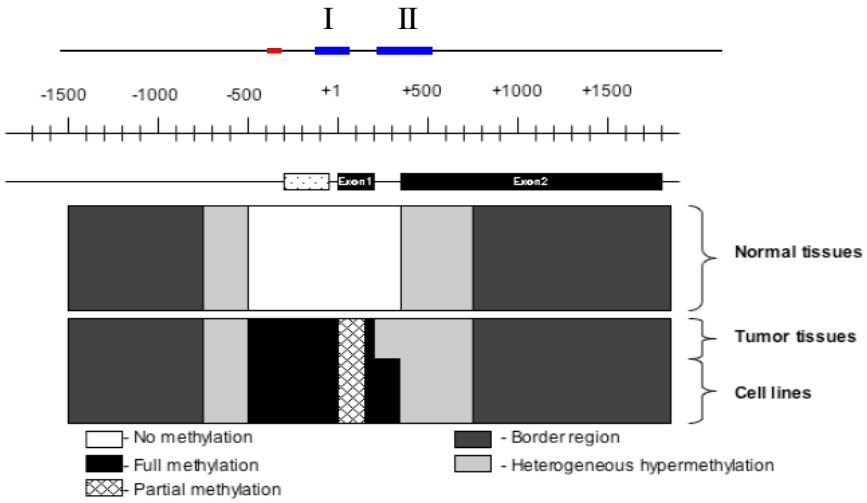
observed in differentiated and senescent cells that do not express *hTERT* (Lopatina et al. 2003; Shin et al. 2003), whereas in some transformed and neoplastic cells, *hTERT* is reactivated and transcribed regardless of its densely methylated promoter (Guilleret et al. 2002). This inconsistent correlation between *hTERT* expression and its promoter methylation may be due to the involvement of a large variety of transcription factors interacting with telomerase promoter, which determines the activity of *hTERT* depending on the final balance among all the involved factors.

V.7 MLL-AF4 interferes with the telomerase activity

Treatment of SEM cells with siMA6 or siHOXA7, and of RS4;11 cells with siMARS or siHOXA7 results in the downregulation of *hTERT* expression. This decrease was paralleled by a diminishment of telomerase activity. Notably, the telomere length in SEM cells is substantially higher compared to other leukemic cell lines (results from diploma thesis of Atila Sahinyazan, Lab of Prof. G. Fey), proving that the telomerase is very active in these cells.

Bisulfite-sequencing of the genomic DNA revealed a diminished level of methylation within the region I from –384 bp to –161 bp in SEM cells, transfected with siMA6 or siHOXA7. This interval appeared to be heavily methylated in t(4;11) cells, which is consistent with the observations from Guilleret and which is associated with high levels of *hTERT* expression. The observed correlation between methylation of *hTERT* promoter and gene expression is opposite to the classical model of regulation by DNA methylation. Guilleret et al established a detailed mapping of methylcytosines at the CpG island surround *hTERT* promoter in tissues and cells lines (scheme 17) (Guilleret et al. 2004). In telomerase-positive samples, methylation of all of the CpG sites was observed for *hTERT* promoter region (-500 bp to +1), whereas the exonic part (+1 to +450 bp) revealed an unstable methylation pattern. Comparing the

results obtained from the genomic DNA analysis of *hTERT* promoter in SEM cells, it could be concluded, that the methylation pattern of region I completely correlates with the findings of Guiliert and heavy hypermethylation within this region is associated with the high level of *hTERT* expression in mock or siMM-treated cells.



Scheme 17. Methylation analysis of hTERT promoter region by Guilleret and in SEM cells (top).

On the top, schematic representation of *hTERT* promoter, where blue lines indicate regions of promoter (I and II), which were analyzed with bisulfite-sequencing. Red line shows the predicted by GENOMATIX binding site for HOXA9/MEIS1. Below, methylation analysis of *hTERT* promoter and gene region in several tissues and cell lines (Guilleret et al. 2004).

Concerning region II, the picture is more complex. Though, there are some changes in the methylation status of *hTERT* promoter in the cells with suppressed HOXA7, this pattern does not correlate with the findings of Guiellert, who suggested that lack of methylation is associated with the reduction of expression. This inconsistent correlation between *hTERT* expression and its promoter methylation may be due to the involvement of a large variety of different transcription factors, which finally determine the level of telomerase transcription. Thus, it would be of extreme importance to fill the gap between -160 bp and -60 bp to analyze the methylation pattern of the region. Unfortunately, several attempts to get the PCR product of this interval did not lead to the success. The bisulfite-sequencing of this part of the promoter could clearly define the correlation between DNA methylation and the level of *hTERT*

transcription, especially in siHOXA7-treated cells.

Thus, the decrease of *hTERT* mRNA upon MLL-AF4 and HOXA7 depletion could be attributed to changes in the methylation pattern of its promoter region. Recently, Martin et al. showed dose-dependent transactivation by MLL-AF4 using reporter constructs containing promoter E boxes (Martin et al. 2003). Regulation of *hTERT* transcription could be, therefore, directly performed by MLL-AF4, which recognizes and binds E boxes within *hTERT* promoter. As it was already mentioned in the introduction, several studies conclude that the presence of MT domain and AT hook domain is crucial for the transforming capacity of the MLL fusions (Ono et al. 2005). Additionally, it was reported that MT domain located in the N-terminal part of MLL supercomplex as well as MLL fusions recognizes and binds non-methylated CpG islands (Birke et al. 2002). It could be speculated that the MT domain functions as a docking anchor for MLL containing complexes, targeting MLL supercomplex to E boxes within *hTERT* promoter, where its components, functioning as epigenetic histone modificators, provide a binding platform for a numerous transcription factors that in turn drive *hTERT* transcription.

Treatment of SEM cells with 5-Aza-dC lead to similar phenotypical changes as the siRNA-mediated suppression of HOXA7 and MLL-AF4, namely: decrease of proliferation and concentration-dependent induction of apoptosis. Furthermore, downregulation of *hTERT* mRNA in 5-Aza-dC treated cells was accompanied by the similar methylation pattern as in the case of siMA6 and siHOXA7 treatment. The effects provided by MLL-AF4 and HOXA7 depletion completely mimic the outcome observed by the treatment of the cells with inhibitor of DNA methyltransferases. Therefore, it could be suggested that MLL-AF4 and HOXA7 regulate their target genes through either widespread or exclusive alterations in the methylation pattern.

A number of different transcription factors are known to directly bind and regulate *hTERT* transcription. One of the most studied one is c-MYC, which

binds to E boxes and, in complex with Max, activates *hTERT* transcription (Dang et al. 1999). The activity of c-MYC is antagonized with MAD1, which in complex with Max represses *hTERT* expression. A distal E box lies exactly within the portion of the promoter with changed methylation pattern (Scheme 15 and Fig. 34).

In this regard, a control of c-MYC degradation by the proteosomal pathway deserves additional attention. It was reported (Yeh et al. 2004) that the phosphorylation of c-MYC protein at position Thr 58 accompanied by its dephosphorylation at position Ser 62 contributes to the degradation of c-MYC through the ubiquitin pathway. Indeed, siMA6-mediated MLL-AF4 suppression leads to the decrease of phosphorylated c-MYC, whereas its unphosphorylated form was unaffected (Fig. 36). Protein phosphatase 2A (PP2A) dephosphorylates it at position Ser 62 thereby regulating c-MYC protein stability. This enzyme was found to be a component of the MLL supercomplex, associated with its N-terminus which implies that PP2A probably also participates in the supercomplex of MLL fusions.

On the one hand, it could be speculated that the presence of MLL-AF4 leads to the functional knockout of PP2A, which results in the inappropriate regulation of c-MYC degradation and, as a consequence, to upregulated levels of c-MYC in the cells. Overexpression of c-MYC was shown to be tightly correlated with the increased expression of *hTERT* (Grandori et al. 2000). Treatment with MLL-AF4 siRNAs diminishes the level of this fusion in the cell, which allows normal-functioning MLL complex associate with PP2A and dephosphorylate c-MYC thereby tagging it for the degradation. On the other hand, as it was mentioned in the introduction, the ubiquitin-mediated degradation is deregulated in t(4;11) cells by interfering of AF4-MLL with SIAH proteins (Bursen et al. 2004). Possibly, the deregulated levels of intracellular c-MYC could be attributed to the inappropriately functioning degradation machinery.

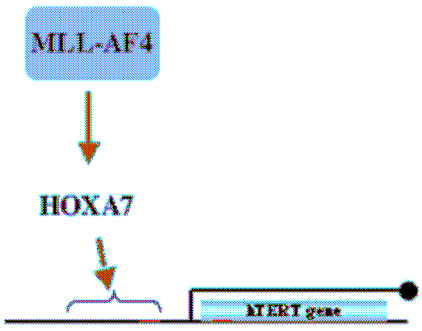
According to the ChIP analysis, HOXA7 directly binds to *hTERT* promoter. The primers used for ChIP experiment surround the region between –240 bp and +100 bp, which includes the region of altered methylation pattern. According to GENOMATIX (www.genomatix.de), there is a MEIS1/HOXA9 binding site 100 bp upstream from the region I, which was not yet analyzed with ChIP assay (scheme 13).

Not much is known about of HOX target genes. These small proteins of 20 to 30 kDa function as transcription factors, but the exact mechanism of its mediated transcription regulation has not been established so far. One scenario could be that HOXA7, by binding to some known transcriptional regulators of *hTERT* or being a part of the MLL- or some other complex, targets it to *hTERT* promoter. It could be interesting to investigate whether HOXA7 still binds *hTERT* promoter upon MLL-AF4 depletion what leads to the changes in its methylation pattern. Also, it was not yet analyzed whether MLL-AF4 itself binds *hTERT* promoter. The reason for that is lack of suitable antibodies and difficulties, which arise by cloning of the cDNA of *MLL-AF4* fusion (6 kb length).

In conclusion, the available data suggest the following scheme:

MLL-AF4 → HOXA7 → *hTERT*,

where HOXA7 serves as an intermediate of the MLL-AF4-associated regulation of *hTERT* expression.



Scheme 18. HOXA7 is a mediator of MLL-AF4 regulation of hTERT transcription.

Levels of HOXA7 are dependent on MLL-AF4 activity and depletion of HOXA7 or MLL-AF4 resulted in the similar effects on the *hTERT* expression. Moreover, according to the ChIP

analysis, HOXA7 directly binds to *hTERT* promoter. Thus, HOXA7 is a downstream target of MLL-AF4 and intermediate of the MLL-AF4-associated regulation of *hTERT* transcription.

V.8 Effects on the leukemic engraftment in vivo

Leukemic cell growth in SCID mice has been shown to be associated with high-risk B-ALL. Therefore we used a t(4;11) SCID mouse model to ask whether siRNA-mediated depletion of MLL-AF4 affects leukemic engraftment and the development of leukemia in vivo. Indeed, transient suppression of MLL-AF4 reduced the leukemic engraftment of t(4;11)-positive cells in SCID mice that received a xenotransplantant. Three of eight mice, that received xenotransplantant with depleted MLL-AF4 showed no signs of leukemia, such as infiltration of bone marrow and hepatosplenomegaly and appeared to be long-term survivors. These findings prove that even transient downregulation of MLL-AF4 interferes with the leukemic engraftment in vivo. Since the latter process crucially depends on leukemia-initiating cells, these findings may also indicate a possible function of MLL-AF4 for the persistence of this cell type. As efficient engraftment in SCID mice predicts an increased probability of relapse in patients with ALL (Uckun et al. 1995), these data suggest that interfering with MLL-AF4 functions may improve patient outcome.

V.9 Conclusions

The results obtained during the work are summarized on the scheme 19. On this scheme, the general overview of different processes, which distinguish normal cell form the leukemic cell, carrying t(4;11) translocation, is depicted. The first “kick” on the way to malignancy is the occurrence of the translocation between 4^{th} and 11^{th} chromosomes. The reciprocal translocation gives rise to two fusion proteins, whose function predestinate the fate of the cell on the way to be

malfunctioning. These two main malignant incidents lead to the numerous modifications and mutations („events“), which finally transform the cells. There are several such “events”, which are the consequences of MLL-AF4 activity. The most crucial of them are outlined below.

Genetic “hits”:

Hit 1: MLL-AF4

Chromosomal translocations of the human *MLL* gene create two reciprocal fusion genes, which encode chimeric MLL fusion proteins. The t(4;11) translocation is one of the most frequent *MLL* translocations known today (Armstrong et al. 2005). This chromosomal abnormality gives rise to two fusion genes: *MLL-AF4* and *AF4-MLL*. In this work, I investigated the role and functional importance of MLL-AF4 for the maintaining of leukemic phenotype and its influence on cell biology.

Hit 2: AF4-MLL

The impact of another product of t(4;11) translocation, *AF4-MLL*, on the growth transformation and maintenance of malignant phenotype is of critical importance. Nevertheless, there are very few studies describing the role of this derivative in the development of leukemia.

Hit 3: FLT3

Membrane-bound tyrosine receptor FLT3 was recently shown to be frequently overexpressed and/or mutated gene in ALL (Gilliland et al. 2002; Armstrong et al. 2003). Upon binding of FLT3L, wild-type FLT3 receptors dimerize and become activated by phosphorylation, positively affecting several signal transduction pathways all of which favour cell survival and proliferation (Stirewalt et al. 2003). Mutations in FLT3 appear to constitutively activate the receptor in a ligand-independent manner, promoting proliferation and survival, thus providing the leukemic cell with a growth advantage and transforming capacity.

AF4-MLL – associated "events":

Event 1: deregulation of proteosomal degradation

In the lab of R. Marschalek it was demonstrated that AF4 protein and AF4-MLL fusion protein have the capacity to transform murine embryonic fibroblasts (Bursen et al. 2004). The common sequences between AF4 and AF4-MLL are the first 360 amino acids, encoded by the first three exons of AF4 gene. This short portion of AF4 alone was able to exert oncogenic activity when transported to the nucleus. The underlying oncogenic mechanism involves interaction of N-terminal portion of AF4 with two E3 ubiquitin ligases SIAH1 and SIAH2. It was shown that AF4-MLL directly binds and traps SIAH1 proteins disabling the ubiquitin-mediated degradation in t(4;11)-positive cells (Bursen et al. 2004). These proteins belong to the group of RING Finger E3 ubiquitin ligases that specifically target their substrates for degradation at the 26S proteasome. The major function of E3 ubiquitin ligases is the control of steady-state level of several key proteins. Binding of SIAH1 to these key proteins may be compromised due to a reduction of a steady-state of SIAH1 protein levels, which are trapped in the complex with AF4-MLL. This mimics a functional inactivation of SIAH1 protein that is correlated with the deregulated functioning of cellular degradation machinery, inability to promote tumor suppression, to execute apoptosis and to cause cell cycle arrest. One of the consequences of an inappropriety working degradation machinery could be the deregulated levels of intracellular c-MYC, which additionally contributes to the malignant phenotype of t(4;11)-positive cells.

MLL-AF4-associated "events":

Event 2: blockade of apoptosis

Characteristic feature of t(4;11)-positive cells is their high resistance to many chemotherapeutics, which induce apoptosis and the lack of sensitivity towards different cell death inducers (Kersey et al. 1998; Dorrie et al. 1999).

The reason for that is an apoptotic block within these cells.

During this work it was demonstrated that MLL-AF4 plays an anti-apoptotic role in the leukemic cells. Probably by interfering with the transcription or splicing of several crucial genes, which products are primarily involved in the induction and mediation of apoptosis. Two of such a genes were identified - *APAF1* and *SEPT4*. Expression of these genes is suppressed in the presence of MLL-AF4 and is substantially upregulated upon MLL-AF4 depletion. As it is discussed above, there could be several explanations of the induction of apoptosis upon MLL-AF4 suppression.

An additional factor, which contributes to the anti-apoptotic role of MLL-AF4, is the abrogation of p53 functional activity, which is a common feature of MLL fusions-mediated leukemogenesis. It was described by Wiederschain that several frequently detected MLL fusion proteins substantially downregulate p53-mediated induction of p21, MDM2 and BAX in response to DNA damage. Furthermore, the major mechanism of the inhibitory effect of MLL fusion proteins is the reduction of p53 acetylation by p300 (Wiederschain et al. 2005). MLL chimeras, inhibiting the stress-induced p53 acetylation by p300, interfere with the cellular DNA damage defence mechanism, providing an advantage towards malignant transformation. Therefore, high resistance of t(4;11) cells towards substances, triggering different pathways of apoptosis, could be addressed to the functional role of MLL-AF4 as a mediator of an apoptotic blockade.

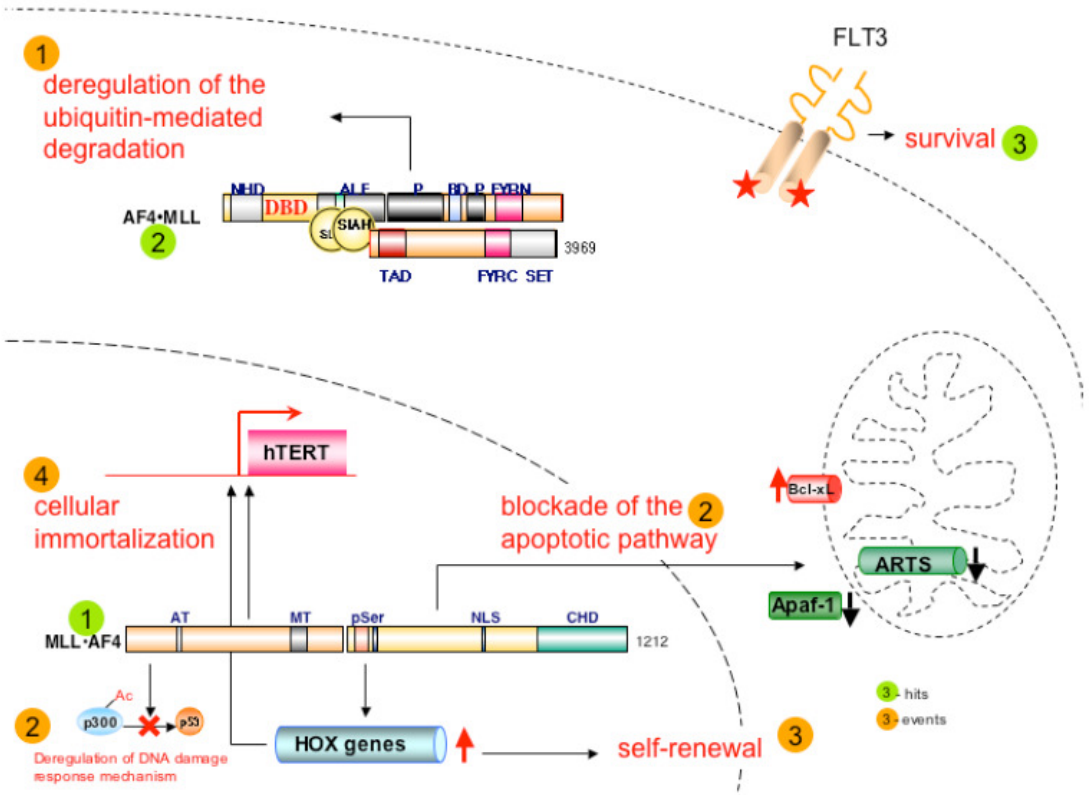
Event 3: upregulation of *HOX* genes expression

The ability of MLL to regulate *HOX* gene expression suggests that its role both in hematopoiesis and leukemogenesis may be mediated by altering patterns of *HOX* gene expression. Multiple studies have demonstrated the ability of *HOX* genes to induce leukemia in mice (Yu et al. 1995; Bertrand et al. 2003; Zeisig et al. 2004). Translocations involving *MLL* alter the expression of *HOX* genes and

this deregulation is critical for leukemogenesis.

Event 4: interference with telomerase activity

The findings, obtained during this work, show that MLL-AF4 directly interferes with the telomerase activity. Furthermore, this deregulated control of telomerase activity can be addressed to the upregulation of *hTERT* transcriptional activation. In turn, deregulated transcriptional control is mediated through the changes in the methylation status of the crucial part of the hTERT promoter. The aberrant expression of *hTERT* which leads to the constitutively active telomerase gives cell an advantage of a limitless replicative potential, which counts for one of the 6 cancer hallmarks (Hanahan et al. 2000).



Scheme 19. Summarized data of the malignant advantages provided by MLL-AF4 and AF4-MLL.

Green circles indicate genetic "hits", associated with arising of leukemia; orange circles show subsequent "events", which accumulation characterize malignant transformation. Schematic representation of the fusion proteins was borrowed from (Bursen et al. 2004).

Expression of almost all genes, affected by the decrease of MLL-AF4, was also changed upon treatment with 5-Aza-dC, demonstrating the role of DNA methylation in the regulation of their transcription. Moreover, in the case of *hTERT* gene, a direct impact of the promoter methylation on the level of *hTERT* expression, caused by the depletion of MLL-AF4, was shown. Based on these observations, it could be hypothesized, that the initial steps in MLL-AF4-driven leukemogenesis involve changes in the CpG methylation of a number of crucial genes, which subsequently leads to their aberrant expression and, as a consequence, to the acquirement of malignant transformation by the cell. This assumption could be tested, for example, by the direct identification of genomic targets of MLL-AF4 by coupling chromatin immunoprecipitation with CpG-island microarray analysis.

The data, obtained both in vitro and in vivo, have many implications for t(4;11) leukemia. Because of the exclusive expression of *MLL-AF4* in t(4;11) leukemic cells, and because of its central role in the maintenance of leukemia including a supportive function for SCID-leukemia initiating cells, *MLL-AF4* would be a very promising target for a molecularly defined treatment of this highly aggressive leukemia. Currently, there are no small molecule inhibitors available specific for this fusion protein. Here, it is demonstrated that siRNAs homologous to the fusion site efficiently suppress MLL-AF4. Moreover, two different variants of this fusion gene can be targeted with high efficacy and exclusive specificity. This specificity also proves that the observed anti-leukemic properties of these siRNAs are directly due to MLL-AF4 suppression and not to off target effects, such as unintended inhibition of other genes or induction of interferon response.

Furthermore, the successful targeting of two different *MLL-AF4* variants has implications for the treatment of possible escape mutants. RNAi resistance-conferring point mutations in the fusion site may simply be counteracted with an siRNA containing an adapted sequence. Finally, the observed reduction of

leukemic engraftment upon MLL-AF4 depletion suggests that MLL-AF4 siRNAs may also impair leukemia-initiating cell function.

Although the problem of systemic siRNA delivery to hematopoietically relevant organs is not solved yet, the present results suggest that MLL-AF4 siRNAs may provide a specific and efficient therapeutic tool for the treatment of t(4;11) ALL.

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VII. ACKNOWLEDGEMENTS

Well, this work would not be completed without the help of many people...

It is, on the first place, Olaf Heidenreich, who was not only supervisor but also an adviser, threadholder and idea-generator through the whole process. I thank you, Olaf, for your patience and participation, for the ignorance of serious mistakes that I have done. I hope I could prove you that Russian women are not only the best housewives but also not bad scientific co-workers.

I deeply thank Johann Greil. For his inexhaustible enthusiasm, constant encouragement and great support. I learned from you, Johann, how it is useful to move simultaneously in different directions in the project and how important is to be tied up with researchers from different scientific areas.

Many, many thanks to Prof. Dr. A. Nordheim for his helpful comments, suggestions and discussions during my research reports. I very much enjoyed three years spent in Molbio Team and find the diversity of the research projects within the lab of a great advantage.

A special acknowledgement goes to Prof. Dr. Rolf Marschalek for accompanying this project from the very beginning with his scientific interest and support, for helpful discussions and critical comments.

I also want to thank Heidemarie Riehle for her enormous help, her constant attendance and experienced advices.

Thousands thanks to Natalia, Meryem and Andreas. You were my best supporters and listeners through the whole period of work. Thank you a lot for your scientific and non-scientific discussions, for your sincere trust and essential help in the experiments as well as in personal concerns. It was a great honor to work with you and I hope we will keep the contact in the future.

I also would like to thank David, Anja, Nina, Tassula and Gaby for helpful comments and suggestions. Many thanks to Siggi and Bernd for their introduction into histology techniques.

I am very thankful to Bilge, Meike and Sebastian for spending much time together in „Froschraum“, discussing crucial events in the world, worthwhile-to-see-movies and news in the chess championships.

Tine and Christine, Anke, Nicole, Barbara, Stefan and Jenny I thank for funny and informative talks during the lunch time. I will really miss it. Many thanks to Heide and Elena for our, sometimes maybe too hot, discussions in growing up and educating children.

I am very grateful to my brother Vanja for his interest and concern, his way to stand by, his music and his „nothing really matters“ philosophy.

Special thanks goes to my parents, Olga Ozoline and Vladimir Arkhipov. I hope I will manage to be as good parent for my children as you are for us. Thank you for your steadfast guidance and support, for your useful advices and deep faith in all my deeds, for your understanding and love through my whole life.

Finally, I thank my wonderful husband Arkadi who observed me through these phd years, which was not always enjoyable. I owe you all my achievements, small and big victories and I hope to be able to give you back all your love, tenderness and care that you everyday gratuitously share with me.

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