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The role of the leukemia-associated ETO homologue repressors in hematopoiesis

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The role of the leukemia-associated ETO homologue repressors in hematopoiesis

Doctoral Thesis
by

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Original Papers

I. Lindberg SR, Olsson A, Persson A-M, Olsson I. *Interactions between the leukaemia-associated ETO homologues of nuclear repressor proteins.* Eur J Haematol, 2003. **71**(6): p. 439-47[1].

II. Lindberg SR*, Olsson A*, Persson A-M, Olsson I. *The Leukemia Associated ETO Homologues Are Differently Expressed During Hematopoietic Differentiation.* Experimental Hematology, 2005. **33**(2): p 189-98[2].

* These authors contributed equally to this work

III. Olsson A, Olsson I, Dhanda RS. *Transcriptional repression by the leukaemia-associated ETO-homologue MTG16 and the interacting co-repressors SIN3B and N-CoR.* **Submitted for publication**

IV. Olsson A, Lindberg SR, Olsson I, Mulloy J. *AML1-ETO inhibits expression of the leukemia associated ETO-homologue repressor protein MTG16.* **Manuscript**

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Abbreviations and explanations

AE-cells	AML1-ETO expressing cells
ALY	Always early protein
AML	Acute Myeloid Leukemia
AML1	Acute Myeloid Leukemia 1
AML1-ETO	Acute Myeloid Leukemia 1-Eight Twenty One
Bcl-6	B-Cell lymphoma 6
CBF	Core Binding Factor
CBFA2T	CBFα subunit 2 translocated to
CBFβ-MYH11	Core Binding Factorβ-Myosin Heavy chain 11
CBP	CREB binding protein
CEBPα	CCAAT/enhancer binding protein
C-fos	Cellular oncogene fos
CM-cells	CBFβ-MYH11 expressing cells
CML	Chronic Myeloid Leukemia
COS7	Monkey kidney cell line
EPO	Erythropoietin
ETO	Eight Twenty One
FACS	Fluorescence activated cell sorting
FLT3	Fms-related tyrosine kinase 3
GATA-1	GATA-binding protein 1
G-CSF	Granulocyte-colony stimulating factor
Gfi-1	Growth factor independence-1
GM-CSF	Granulocyte monocyte-Colony Stimulating Factor
GST	Glutathione S-transferase
HDAC	Histone deacetylase
HEB	HeLa E-box binding protein
HEL	Human Erythro Leukemia
HOX	Homeobox protein
HSC	Hematopoietic Stem Cell
HSPC	Hematopoietic stem progenitor cell
IL-3	Interleukin-3
IP	Immunoprecipitation
LEF	Lymphoid enhancer binding factor
M-CSF	Monocyte-colony stimulating factor
MDS	Myelodysplastic syndrome
MEF	Myeloid Elf-1-like Factor
MPO	Myeloperoxidase
MPRO	Murine neutrophil progenitor cell line
MTG16	Myeloid translocation gene on chromosome 16

MTG8	Myeloid translocated gene 8
MTGR1	Myeloid translocation gene related 1
MYND	Myeloid Nervy Deaf
NB4	Human promyelocytic cell line
N-CoR	Nuclear Co-repressor
NHR	Nervy Homology region
P14ARF	p14 alternate reading frame?
PKA RII	type II c-amp dependent protein kinase
PLZF	Promyelocytic leukemia zinc finger
PST	Proline serine threonine rich region
RARα	Retinoic acid receptor α
RAS	Retrovirus associated DNA sequences
RHD	Runt Homology Domain
Runx1	Runt-related transcription factor 1 (=AML1)
SCL	Stem cell leukemia hematopoietic transcription factor (=TAL1)
SIN3	SWI-Independent 3
SMRT	Silencing Mediator for Retinoic acid and Thyroid hormone receptors
TA	Transcriptional activation domain
TAF	TATA binding protein Associated Factor
TAL1	T-cell acute lymphocytic leukemia 1
TCRβ	T-cell receptor β
U937	Human monocytic cell line

Introduction

Leukemia pathophysiology

Hematopoiesis is the process of blood formation. Billions of blood cells are manufactured every day to supply the body with oxygen, to defend against infections and to participate in coagulation. All blood cells are produced in a hierarchical manner from a very small number of hematopoietic stem cells. Stem cells have the ability to selfrenew and to produce multipotent progenitors, which have lost the selfrenewal ability but are able to proliferate and develop into the different blood-cell lineages. Hematopoiesis secures a continuous production of effector cells both for oxygen supply and for innate and specific immunity.

Neoplasias of the blood forming organs are classified as acute and chronic leukemias that are further divided into myeloid and lymphoid categories. Common for acute myeloid leukemia is that the normal production of blood cells is disrupted by a clonal expansion of malignant cells that typically are arrested in differentiation and are more or less resistant to apoptosis. This results in an accumulation of immature blast-like cells. In contrast, cronic leukemia cells, for example in CML, show continuous hematopoietic differentiation. The patients with CML may have few symptoms at an early stage, whereas acute leukemia is a rapidly progressing disease and will result in fatality if left untreated [1].

In solid tumors an array of heterogeneous genetic aberrations have been reported, consisting of chromosomal deletions and translocations, gene amplifications and point mutations [2]. In leukemia, an acquired chromosomal translocation is a typical genetic aberration [3]. More than 300 chromosomal translocations have been described in leukemia, but much fewer leukemic phenotypes have been defined. This indicates that different chromosomal translocations may have similar phenotypes perhaps reflecting similar involvement of cellular signaling pathways for proliferation, differentiation and viability [4]. A chromosomal translocation can cause a loss of function from haploinsufficiency or a gain of function through a chimeric protein with novel properties.

In lymphoid malignancies an Ig gene or a T cell receptor gene is often rearranged to become a proto-oncogen. This typically leads to overexpression of the gene that is under control of the Ig or TCR promoter [5]. In CML, a gene for a tyrosine kinase (ABL) is fused to a gene with an oligomerization domain (BCR) [6, 7]. Oligomerization of the gene product constitutively activates the tyrosine kinase, which gives a cellular growth advantage. In AML, chromosomal translocations commonly affect transcription factor genes whose protein products are necessary for normal hematopoiesis of one or more lineage [8]. Genes for transcriptional co-activators may also be involved in chromosomal translocations [9-11]. In AML, single translocations may impair differentiation but are probably not sufficient to be leukemogenic by themselves. Further activating mutations for instance in genes for tyrosine kinases such as FLT3, c-kit and RAS are believed to contribute to the pathogenesis [12-16]. Transcription factor genes frequently involved in AML are genes for core binding factor (CBF), retinoic acid receptor (RARα) and the HOX family [17]. The gene for the AML1 transcription factor, which is part of the CBF complex, is the most frequently disrupted gene in AML. The translocation between chromosome 8 and 21 [t(8;21)] results in the chimeric gene AML1-ETO. AML with this aberration is most often subtyped as FAB AML M2, which is characterized by a partial block at the myeloblastic stage of differentiation. ETO was identified from the cloning of t(8;21) in 1992 [18]. The AML1-ETO fusion protein retains a 5' region of the AML1 transcription factor and almost all of the nuclear ETO phosphoprotein [18, 19].

AML1 and AML1-ETO

AML1 (Runx1) belongs to the AML family of transcription factors together with AML2 and AML3. AML1 is primarily expressed in cells of the hematopoietic system and has been shown to be crucial for definitive hematopoiesis [20, 21]. AML1 regulates genes for hematopoietic growth factors such as those for GM-CSF, IL-3 as well as genes for hematopoietic granule proteins such as MPO and neutrophil elastase [22-24]. AML1 is the α-subunit of the so-called CBF complex, which also contains a β-subunit CBFβ. The Runt Homology Domain (RHD) of AML1 binds both to DNA

and CBFβ. The binding of CBFβ increases the affinity of AML1 for DNA. Furthermore, the distal part of AML1, containing the transcriptional activation domain, recruits coactivators such as p300, CBP, MOZ and ALY [25-28]. The RHD of AML1 is preserved in AML1-ETO whereas the transcriptional activation domain of AML1 is lost (Figure 1) [29].

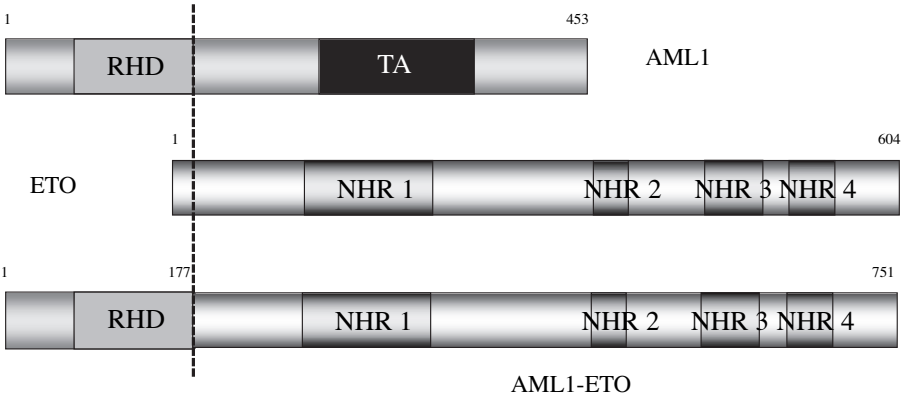

Figure 1. Functional domains of AML1, ETO and AML1-ETO (resulting from the translocation t[8;21]). The RHD is the DNA binding region, TA is the Transactivation Domain and the NHRs are the Nervy Homology Regions, which mediate interactions with transcription factors and co-repressor molecules. The AML1-ETO retains the 5' region of AML1 including the DNA-binding RHD, and almost the whole sequence of ETO except for the most amino-terminal 30 amino acids.

The ETO homologues

ETO (also designated MTG8 or CBFA2T1) belongs to a family of evolutionary conserved proteins. ETO is a nuclear phosphoprotein, which can become part of a co-repressor complex. Two homologues to human ETO have been found, **MTGR1** and **MTG16**. MTGR1 was identified as a binding partner to AML1-ETO [30]. MTG16 was identified from a gene rearrangement in the chromosomal translocation t(16;21), which encodes an AML1-MTG16 fusion protein [31, 32]. This fusion protein is present in a minority of patients with therapy related leukemia and MDS. All three

ETO homologues are closely related. ETO has 61% and 67% homology with MTGR1 and MTG16 respectively, whereas MTGR1 shares 54% homology with MTG16 [30, 32].

Expression

Human ETO is found for example in brain, heart, thymus, preadipocytes and neuronal cells [33-36]. Originally Erickson *et al* detected ETO in megakaryocytes and CD34+ cells [37]. Miyoshi *et al* reported a lack of ETO expression in hematopoetic cells [19]. A surprising finding was that gene targeting of ETO in mice is associated with reduced postnatal viability due to abnormal development of the midgut. However, no defects in hematopoiesis were noted in the knockout mice [38]. In contrast to ETO, MTGR1 is ubiquitously expressed in human cells [33, 39]. But similar to the ETO gene knockout, the MTGR1 gene knockout is also associated with a malformed gut structure [40]. More specifically, a gradual loss of goblet-, Paneth- and enteroendocrine-cells is noted. The MTGR1 knockout mouse is smaller than normal [40]. MTG16 is expressed in most tissues investigated including the major hematopoietic lineages [39, 41]. However, human kidney cells seem to lack MTG16 [32].

Splice forms

ETO consists of 13 exons transcribed into the alternatively spliced transcripts ETOa and ETOb, which differ at the 5' end. They code for proteins of 577 and 604 amino acids respectively [36]. The MTGR1 gene has 15 exons transcribed into MTGR1a and MTGR1b, which yield proteins of 575 amino acids and 604 amino acids respectively [33]. The MTG16 gene has 13 exons, which encode two transcripts, MTG16a and MTG16b, which code for 653 amino acids and 567 amino acids respectively [32].

Structure

The ETO homologues have four evolutionary conserved regions also present in the *drosophila* protein nervy [30, 42]. These are termed Nervy Homology Regions (NHR) 1-4 (Figure 2). NHR1 resembles TAF110 and is also referred to as the TAF domain. NHR2 contains an amphiphatic helical domain important for oligomerization [30].

NHR3 is referred to as the nervy domain. NHR4, also termed MYND, contains zinc finger motifs [30, 42].

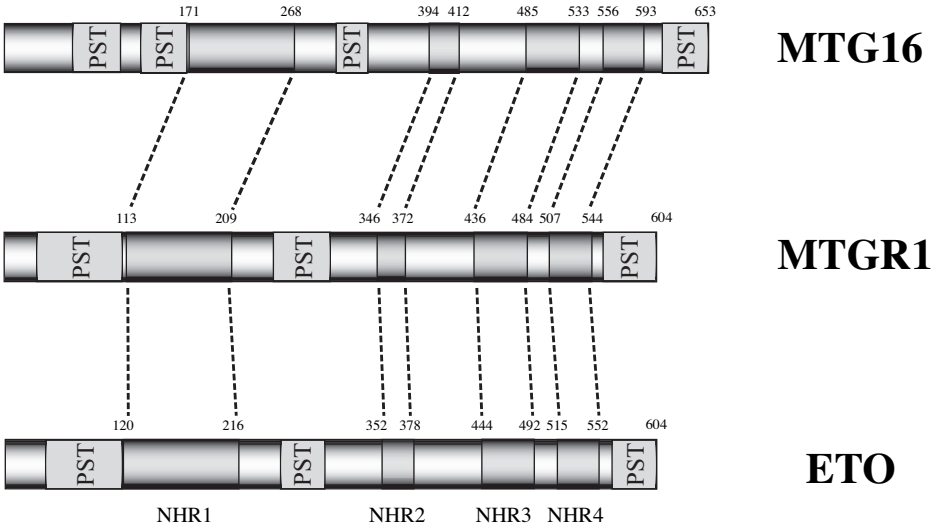

Figure 2. Organisation of the ETO homologues showing the evolutionary conserved domains NHR 1-4. PST= Proline, Serine Threonine rich region.

The region between NHR1 and NHR2 contains a nuclear localization signal necessary for nuclear import [43]. The amphiphatic NHR2 mediates homo- and hetero-dimerization between the homologues [30]. The ETO region from NHR2 to NHR4 associates with co-repressors such as mSIN3a, N-CoR, SMRT, which recruit HDACs 1-3 [40, 44-48]. The assembled complex inactivates the chromatin by HDAC-mediated deacetylation of histones (Figure 3).

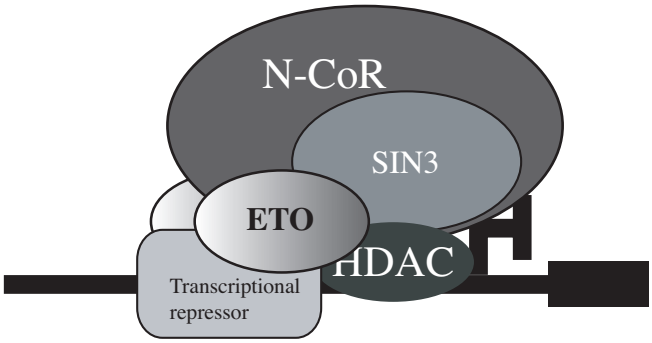

Figure 3. Assembly of an ETO complex leads to repression. ETO binds a sequence specific transcriptional repressor and dimerizes. Binding of co-repressors mSIN3 and N-CoR leads to HDAC recruitment and transcriptional inactivation.

Subnuclear localization

ETO shows a punctate subnuclear pattern [43, 49, 50], but can be detected in the cytoplasm too [35, 51]. MTGR1 is found in the nucleoplasm, while MTG16a is located in the nucleolus [52]. MTG16a forms a complex with either ETO or MTGR1 as detected by immunofluorescent microscopy [52]. Therefore, MTG16a may drag ETO and MTGR1 from the nucleoplasm into the nucleolus. This could lead to lack of ETO and MTGR1 in the nucleoplasm with decreased repression as a consequence. Furthermore, the resulting transfer of ETO homologues into the nucleolus could lead to increased repression at this location. In addition, an ETO homologue present in excess could sequestrate co-repressor molecules, thus blocking the ability of repression of the other homologue due to lack of co-repressor elements.

Transcriptional repression

The fact that the ETO homologues form complexes with the co-repressors N-CoR and SIN3, suggests a repressor function [44, 47]. However, the lack of DNA binding domains indicates that the ETO homologues do not act directly as repressors themselves. Rather, they could act as adapters between transcriptional repressors and co-repressors. They would do this by binding to DNA-specific repressors and recruit

co-repressors and HDAC to form a complex that mediates chromatin silencing by histone deacetylation.

ETO can interact with a number of nuclear proteins. Among known interacting proteins is PLZF, which is a nuclear repressor protein causing growth suppression and differentiation inhibition of hematopoietic cells [53] (Figure 4). The PLZF-induced repression is enhanced by ETO. Furthermore, ETO and MTG16 both bind the transcription factor Gfi-1 [50], which is assumed to regulate differentiation and survival in megakaryocytic and erythroid cells [54, 55]. ETO also associates with the basic helix-loop-helix transcription factor HEB [56] which is involved in regulation of T and B lymphocyte differentiation [57]. ETO represses HEB through blocking the binding of the activator molecules p300/CBP [56]. Furthermore, ETO can bind to and enhance the repression of the antiapoptotic Bcl-6 molecule [58]. ETO is also associated with the regulatory subunit of type II c-AMP dependent protein kinase, which phosphorylates target genes in the centrosome-Golgi area [59]. In addition, ETO can associate with the transcriptional repressor Atrophin-1, implicated in neurodegenerative disease [60]. The functional domains of the ETO homologues that interact with specific nuclear proteins are shown in Figure 4. Taken together, the ETO homologues are known to mediate repression of genes that are mostly involved in proliferation and differentiation. Therefore, the ETO homologues might be important in control of lineage choice, differentiation and proliferation. Additional ETO homologue interacting proteins are likely to be discovered.

Presence in multi-protein complexes

The ETO homologue interacting proteins N-CoR and SIN3 are both large proteins that are found in multiprotein complexes with repressor activity. Each complex is associated with HDAC activity [61, 62].

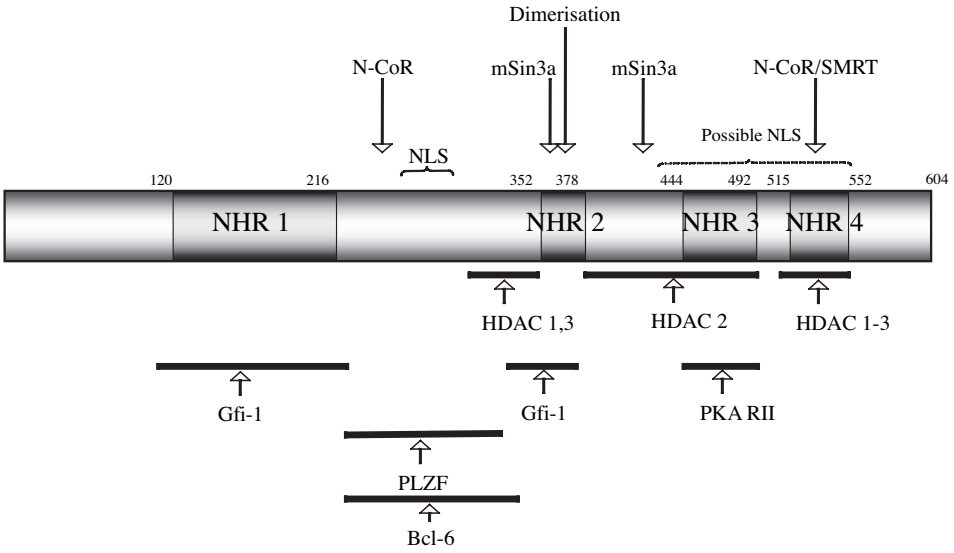

Figure 4. Schematic view of the functional NHR domains of ETO and known interacting proteins. Lines show the ETO regions necessary for the indicated interactions.

SIN3 is the structural center in a core complex that may contain many additional components such as HDAC1, HDAC2, RbAp46, RbAp48, SAP30, SAP18 and Sds3 [61, 63, 64]. The HDACs mediate the enzymatic repressor activity [63], whereas the other core proteins seem to stabilize the SIN3–HDAC interaction [65], mediate contact with the nucleosome [66] and increase the stability of the complex [67, 68]. The core complex can bind to and repress transcription factors such as Mad-Max [69] and UME6 [70]. The SIN3 core complex is also able to indirectly confer repression to a number of nuclear hormone receptors by recruiting N-CoR and SMRT that act as bridges between the core complex and the transcription factor [71-73]. Apart from associating with the SIN3 core complex, N-CoR and SMRT are found in complexes with different compositions that also have HDAC activity [62, 74-76]. Not only that, HDACs are also found in other co-repressor complexes such as NURD and CoREST-HDAC [65, 77-79].

Thus, the ETO homologues have an ability to bind both SIN3, N-CoR and HDACs, all with the ability to confer repression. The combinatorial possibilities of

these proteins in complexes are vast and the complexity of the co-repressor machinery has not fully been resolved. The role of the ETO homologues in this machinery remains to be further determined.

AML1-ETO

Clinical features

Some clinical features of t(8;21) AML are summarized in Table 1. Leukemic blasts of t(8;21) leukemia have characteristic morphological features corresponding to the M2 subgroup of the French American British (FAB) classification. Large blasts with strong peroxidase reaction are typical. Furthermore, the blasts have cytoplasmic vacuoles, many azurophil granules and needle shaped Auer rods. In addition, dysmorphic features of erythroid and megakaryocytic cells are frequent as well as hypergranular neutrophils. This gives a trilineage dysplasia with MDS-like cells. Eosinophilia is frequent. Phenotypically, the blasts are CD34+, but they often show expression of the B-cell marker CD19 without other B-cell antigens or immunoglobulin gene rearrangements. Sometimes the NK-cell marker CD56 is also expressed [80, 81].

AML1-ETO clinical characteristics	
Cell morphology	MPO positive blasts
	Auer rods
	Large cytoplasmic vacuoles
	Dysgranulopoiesis
	Eosinophilia
Additional features	Granulocytic sarcoma (10%)
Phenotypic features	CD34+
	CD19+
	CD56+
Additional chromosomal aberrations	X-,Y-
	Del9q

Table 1. Clinical characteristics of t(8;21) leukemia.

Transcriptional activity

AML1-ETO retains the DNA binding part of AML1, whereas the transactivation domain is deleted and replaced by ETO. It seems that AML1-ETO deregulates a number of genes by repression and possibly by transcriptional activation. Some of the target genes for AML1-ETO are normally regulated by AML1, while others are not. Thus, in one case AML1-ETO is thought to have a major dominant negative effect on AML1 target genes as suggested in Figure 5a. Among such genes are those for GM-GSF, TCRβ enhancer, C-fos and p14ARF [82-86].

However, AML1-ETO does not only affect transcription directly by binding to DNA. It may also interfere with transcription indirectly through protein-protein interactions. In this case, AML1 normally binds a transcription factor X for gene A without binding directly to DNA (Figure 5b). AML1-ETO may compete out AML1 and take its place on the transcription factor. This will result in indirect AML1-ETO-mediated transcriptional repression instead of normal AML1/X-mediated activation. This fits several examples of AML1-ETO-mediated repression. One example is abrogation of CEBPα transcriptional activation during which AML1-ETO interferes with the binding of AML1 to CEBPα [87]. This results in a decreased expression of CEBPα and inhibition of granulopoiesis [88]. Another example is displacement of AML1 and the co-activator c-jun from PU.1 [89] leading to downregulation of PU.1 target genes. Similarly, AML1-ETO competes with AML1 in its binding for MEF, which normally activates the IL-3 gene transcription synergistically with AML1. The interaction between AML1-ETO and MEF leads to IL-3 promoter repression [90, 91].

In addition to transcriptional repression, AML1-ETO may also show transcriptional activation. In this case (Figure 5c), AML1 normally binds to a gene B without transcriptional activation. But AML1-ETO may replace AML1 in binding to the promoter, which leads to transcriptional activation. Thus, AML1-ETO would activate gene B by recruitment of co-activators to the promoter. One example of this is activation of the anti-apoptotic gene BCL-2 after binding of AML1-ETO to the AML1 consensus sequences of the BCL-2 promoter [92].

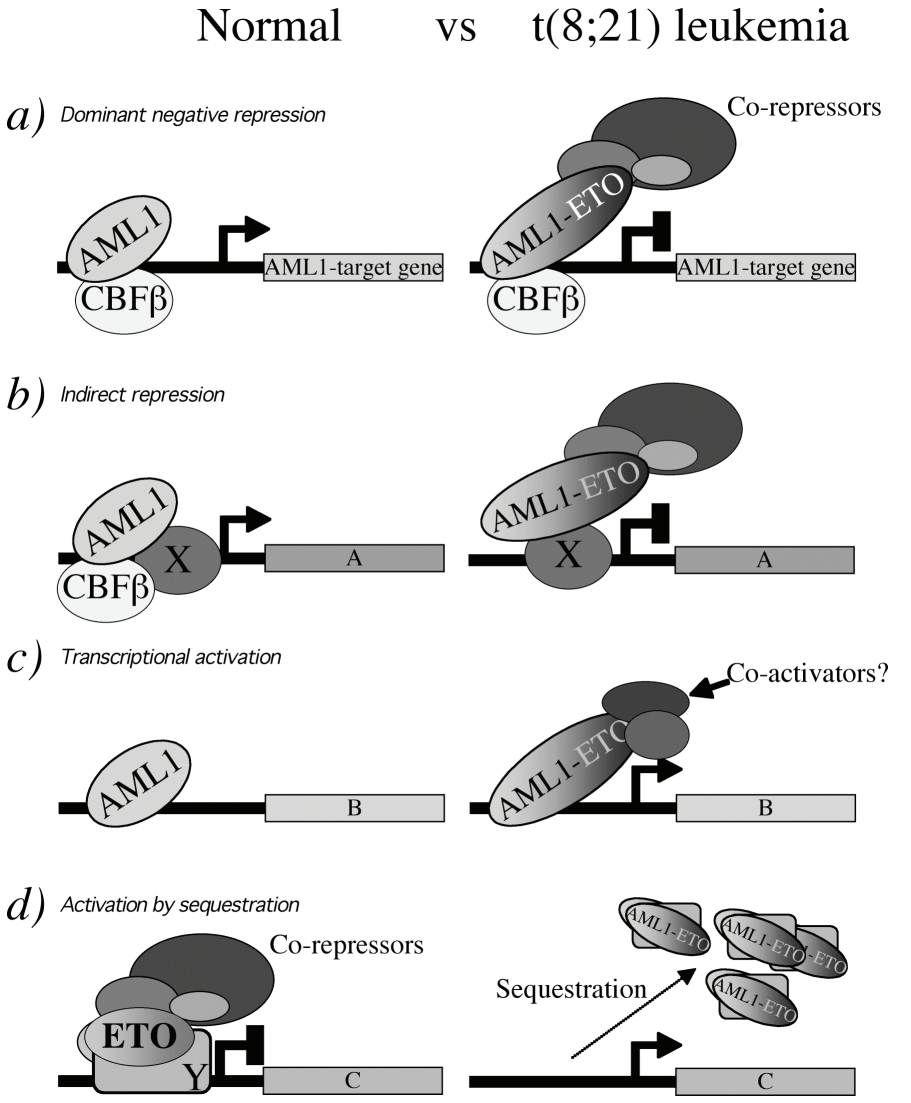

Figure 5. Modes of AML1-ETO-mediated interference with gene transcription. Normal AML1 actions are shown to the left and the suggested corresponding AML1-ETO actions to the right. *a).* Dominant negative effect – AML1-ETO directly interferes with AML1 by competitive binding to the promoter. *b).* Indirect repression – AML1-ETO replaces AML1 and abrogates synergy between AML1 and the transcriptional activator X. AML1-ETO binding leads to transcriptional inactivation of gene A. *c).* Transcriptional activation – AML1 alone has no transcriptional activity on gene B whereas AML1-ETO binding leads to recruitment of co-activators. *d).* Transcriptional activation – AML1-ETO sequestrates the transcriptional repressor Y from the ETO repressor complex leading to transcriptional activation of gene C.

Another example of AML1-ETO-mediated transcriptional activation is depicted in Figure 5d. In this case, a transcriptional repressor (Y) tethered to DNA normally binds ETO leading to gene repression. However, an abundance of AML1-ETO may sequestrate the repressor (Y) and displace it from the promoter. As a result, the sequestration of Y by AML1-ETO may lead to transcriptional activation of Y target genes. This mode of action has been shown for PLZF, which is sequestrated by AML1-ETO, leading to lack of repression [53].

It is important to consider that the support for the models in Figure 5 is based on *in vitro* studies with gene reporter systems. The fact that AML1-ETO binds and affects transcription of genes *in vitro* is not questioned but the *in vivo* relevance of these findings is yet to be determined. For instance, the *in vitro* studies are based on overexpression, which can produce interactions that rarely occur *in vivo*. Furthermore, gene expression *in vivo* is more complex and affected by, not one, but many different factors. Endogenous factors can be displaced in favor of the over-expressed protein. In addition, cell specific factors may influence transcription giving different transcriptional settings *in vitro* and *in vivo*.

Many genes involved in regulation of hematopoiesis are affected by AML1-ETO through interference with AML1-dependent and AML1-independent transcription. In conclusion, AML1-ETO can repress transcription directly by binding to DNA and indirectly by interfering with the binding of transcription factors to the promoters or the assembly of co-activators. Furthermore, AML1-ETO can also activate transcription.

Cell biology

AML1-ETO-mediated inhibition of granulocytic differentiation has been demonstrated both in leukemic cell lines and primary hematopoietic cells [93-96]. Inhibition of granulocytic differentiation has been reported in mouse myeloid precursor L-G and 32D cell lines and in the human monoblastic U937 cell line [30, 93-95]. Erythroid differentiation was impaired by AML1-ETO in the human erythroleukemia K562 cell line and the murine erythroleukemia cell line MEL [97, 98]. Thus, the AML1-ETO effect in leukemia cell lines primarily seems to be differentiation inhibition. This effect

may be cell type specific as AML1-ETO has been observed selectively to inhibit granulocyte and erythroid differentiation [93, 94, 97, 98].

AML1-ETO enhanced proliferation in the murine L-G and 32D cell lines upon addition of G-CSF [30, 95]. However, AML1-ETO reduced proliferation in the human U937 cell line [94]. In contrast to AML1-ETO, AML1-MTG16 inhibited proliferation in 32D cells [99]. In fact, the MTG16 moiety of the fusion protein was responsible for the proliferation inhibition. Thus, the pro- or anti-proliferative effect of AML1-ETO seems to be dependent on cell type.

In primary hematopoietic cells, retroviral expression of AML1-ETO inhibits proliferation of committed progenitors and promotes proliferation of more primitive progenitors [100, 101]. Interestingly, transduced hematopoietic progenitor cells remain CD34+ and show enhanced self-renewal. The longevity of the cultures correlates to the proportion of CD34+ cells, which remain constant until the cells go through senescence. The AML1-ETO expressing progenitor cells have a restricted capacity for multi-lineage differentiation and are able to differentiate into monocytes and B-cells only [102] but not into granulocytes [96]. It has been known that the expression of a single ETO NHR4 domain in human progenitor cells inhibits granulocytic but not monocytic differentiation [103]. This observation enforces the importance of the ETO partner for the biological effects of the fusion protein AML1-ETO. The fusion protein can also suppress erythropoietic differentiation of progenitor cells [104]. Thus AML1-ETO-expressing proerythroblasts have an ability to expand more than control cells. Furthermore, the early erythroid differentiation is disrupted although the cells retain an ability to differentiate partially in response to EPO. In addition to that, the differentiated erythroid cells have a more immature profile than the corresponding normal cells. In conclusion, the data from cell lines and hematopoietic progenitor cells indicate that AML1-ETO selectively inhibits granulocytic and erythrocytic differentiation, and that the ETO part of AML1-ETO plays a major role in inhibition of differentiation.

It is learned from gene targeting that AML1 is necessary for blood formation [20, 21]. In support of this, AML1-ETO “knock in” mice lack fetal liver hematopoiesis and show midgestational lethality due to brain hemorrhage, traits similar to those of AML

gene targeting in mice [105]. Furthermore, the hematopoietic AML1-ETO "knock in" cells have features of dysplastic multilineage hematopoietic progenitors with increased self-renewal capacity [106]. Conditional AML1-ETO "knock in" mice show normal hematopoiesis apart from a promotion of self-renewal in progenitor cells, but they do not develop leukemia [107]. However, when these mice are treated with mutating agents, they develop AML. This indicates that a second hit is required for leukemogenicity [108]. Importantly, AML1-ETO in itself may not be leukemogenic, but it may prolong the lifespan of cells to make it possible to acquire additional mutations (second hits) that are necessary for oncogenicity. In fact, activating mutations in tyrosine kinases FLT3, KIT and NRAS have been found in 28% of patients with t(8;21) [109]. It is of special interest that an AML1-ETO mutant lacking the ETO domains NHR3-4 was found to be a more potent leukemogenic inducer in transgenic mice than AML1-ETO [110]. A similar form of AML1-ETO lacking NHR4 is found as a splicing variant and is expressed in both AML1-ETO containing cell lines and in patients with t(8;21) [111]. Together, these data indicate that a second hit can in fact occur within AML1-ETO itself or in proteins and pathways dependent on the NHR3-4 domains of ETO.

Though not fully leukemogenic by itself, AML1-ETO expressing cells have traits constituting a cell prone to become leukemogenic. A leukemic cell is "immortalized", immature and differentiation inhibited. Hematopoietic progenitor cells expressing AML1-ETO are not immortalized but immature and inhibited in differentiation [102].

Aims of the present investigation

The overall aim of this thesis is to shed light on how AML1-ETO might be leukemogenic. AML1-ETO requires the ETO moiety to be able to interfere with transcriptional regulation [30, 112]. Figure 6 is a summary of the cell biology of the AML1-ETO discussed in the previous section. It emphasizes changes of gene regulation in hematopoietic stem cells leading to a partial differentiation block. It also emphasizes the AML1-ETO-mediated growth arrest that has to be overcome by "second hits" for leukemia to occur. In addition, this model suggests that activation of

anti-apoptotic genes contributes to the leukemic phenotype. Furthermore it poses our question of the ETO homologue participation in t(8;21) leukemia. This thesis focuses on the normal function of the ETO homologues in hematopoiesis with the aim to understand their involvement in leukemia.

(I) The first aim was to biochemically characterize the interactions between the ETO-homologues to determine whether they can cooperate.

(II) The second aim was to elucidate the role of the ETO homologues in the different hematopoietic lineages. If the ETO homologues are involved in hematopoietic differentiation, the AML1-ETO differentiation block might be caused by AML1-ETO interfering with normal ETO homologue function.

(III) The third aim was to examine the transcriptional repressor activities of the ETO homologues. More specifically, the aim was to investigate deletion of functional domains in MTG16 and to study the relation between MTG16 and co-repressors such as SIN3B and N-CoR.

(IV) The fourth aim was to investigate whether AML1-ETO can influence the expression of the ETO homologues. If AML1-ETO affects the ETO homologues, this could be a contributing factor to the AML1-ETO phenotype and help to elucidate the role of the ETO homologues in hematopoiesis.

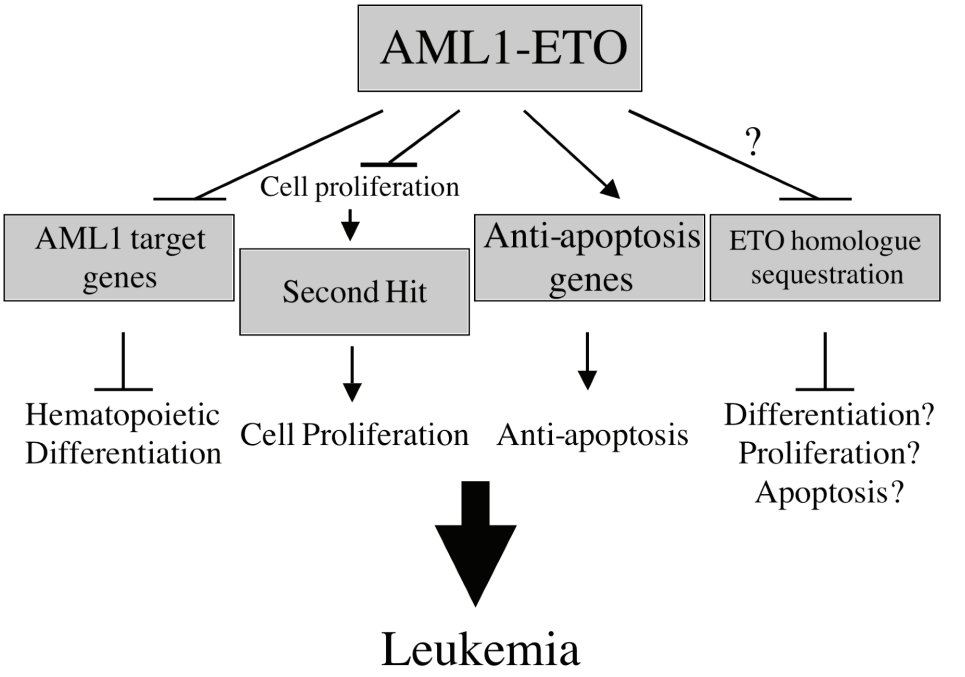

Figure 6. A hypothetic model for the transforming properties of AML1-ETO as a basis for this thesis. The impact on AML1 target genes, the importance of second hits, activation of anti-apoptotic pathways and stimulation of proliferation are emphasized.

Experimental considerations

Cell models

Cell lines are easily available and offer high reproducibility. However, cell lines contain multiple genetic aberrations and deviate in differentiation, proliferation and apoptosis programs as compared to primary cells. This can obscure the properties that are studied. Primary cells are preferable if available. However, primary cells are not available in infinite numbers, often have a short life span and are difficult to keep in culture. Both primary cells and hematopoietic cell lines such as U937, NB4, HEL and MPRO were used in this thesis for studies of ETO homologue interactions, prevalence and expression during hematopoiesis. The monkey derived COS7 and the human 293T

cell lines were used in transient transfection experiments. AML1-ETO transduced HSPCs were also used.

Gene transfer

DNA can be transferred to cells by dendrimer-based transfection, electroporation or retroviral transduction. Dendrimer-based systems offer a quick and convenient way of DNA transfer. The dendrimers form a positively charged complex with DNA, which is taken up by endocytosis, and the DNA is translocated to the nucleus. This system is useful for transient transfection of adherent cells and has low cytotoxicity. Electroporation offers a way of transfecting cells in suspension. Passive integration of DNA into the genome during mitosis is necessary for stable transfection. Electroporation requires a large number of cells due to substantial cell death. Delivery of DNA with retrovirus yields higher efficiency than electroporation but is more elaborate. Retroviral gene transfer has high efficiency because the delivery mode is non-toxic and genomic integration is supported. Most retroviruses need cell division for gene integration so quiescent cells are not suitable.

Transcriptional assays

A transcriptional reporter consists of a promoter linked to a reporter gene that codes for a protein that can be quantified. The amount of reporter protein is proportionally related the activity of the promoter. This system is used to study the transcriptional effects of promoter binding proteins. However, the ETO homologues appear not to have DNA-binding capacity. Therefore, the ETO homologue genes have to be fused to an inert promoter-binding protein for transcriptional studies. We utilized the 5xGAL4-*tk*-luc system, which consists of a reporter vector, where a herpes virus thymidine kinase (*tk)* promoter drives luciferase expression that can be assayed in a luminometer. Five GAL4 sequences are situated upstream of the promoter. The gene for an ETO homologue is ligated to the gene for a GAL4 binding domain. This construct generates a fusion protein of which the GAL4-binding domain binds to the GAL4 sequence of the reporter plasmid. Thereby, the ETO homologue becomes localized to the promoter

and the transcriptional effects of the ETO homologue can be measured. In this way it is possible to measure transcriptional effects of non-DNA binding proteins such as an ETO homologue. Transcriptional reporter systems expressed transiently in cell lines give sensitive, rapid and reproducible results. A drawback is that the promoter activity can be influenced by endogenous transcription factors and co-factors.

PCR

Reverse transcriptase PCR

RT-PCR is used for detection of specific mRNA transcripts. The method is based on exponential amplification of DNA. First, the mRNA is copied to cDNA by reverse transcriptase using random hexamer primers. Then the cDNA is denatured at 95°C to separate the two strands. After cooling, primers are annealed to a specified site on each strand. By use of Taq DNA polymerase, tolerant to high temperature, DNA is extended from the primers at 60°. The newly synthesized DNA is denatured at 95°C and used for new rounds of synthesis. After 20-40 rounds, the PCR product is analyzed by agarose gel electrophoresis.

Quantitative real time PCR

Quantitative real time PCR (Q-PCR) is used to quantitate mRNA transcripts. Compared to Northern blotting, Q-PCR requires less cells and takes less time but needs more optimizing. Each amplification cycle exponentially increases the DNA product by a factor of 2. As the amount of DNA doubles with each PCR cycle, there is a 2x2 increase after two cycles, and 2x2x2 increase after three cycles (the 2^n rule) and so forth. However, the reaction levels out with time due to polymerase inactivation and inhibition of primer-template annealing.

By labeling the PCR product with fluorochromes, the exponential amplification can be measured. Sybr Green becomes fluorescent after intercalation with double stranded DNA. It is easy to use but can react with unspecific PCR products and primer-dimers. In contrast, the oligo-nucleotide Taqman probe anneals only to the specific PCR product and has both a fluorochrome and a quencher attached. Thus, the

dye will not become fluorescent until the DNA polymerase elongates over the annealed probe leading to digestion of the probe and release of the fluorochrome that becomes active. Therefore, the Taqman method is more specific than the Sybr Green.

The PCR kinetics are important for quantitation. Initially the PCR is non linear but after a few cycles enough template has accumulated for linear amplification following the 2^n rule, before the reaction reaches a plateau phase (Figure 7). Quantification of cDNA is done during the linear phase (Figure 7) when a threshold is set. The threshold cycle (C_t) is the point where the amplification curve crosses the threshold. Assuming efficient PCR, two samples that are separated by 2 cycles will have a fourfold difference in transcript levels. However, because of target secondary structures, nucleotide composition, primer design etc., the PCR seldom amplifies according to the 2^n rule. A standard curve is constructed from a DNA of known concentration to calculate the true amplification efficiency. The C_t values of the standard curve is used create an equation from which the actual transcript level of the sample can be calculated.

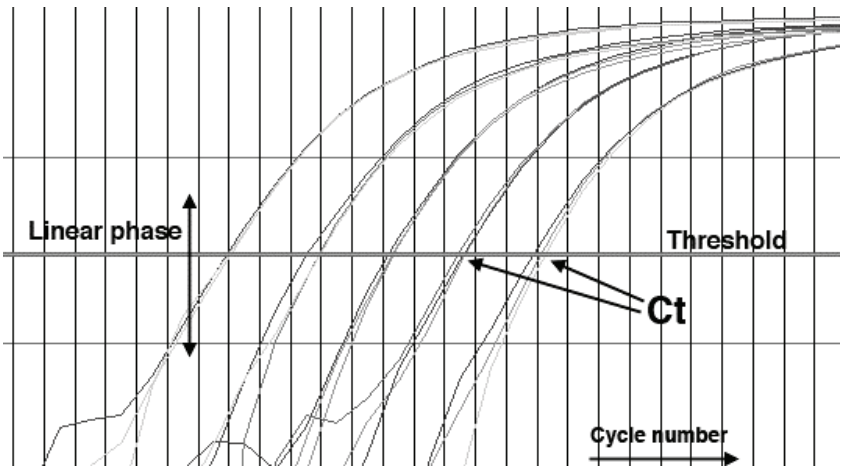

Figure 7. Amplification of a standard curve. Samples are separated with a 5-fold dilution, giving rise to a difference of approximately 2.3 cycles. A threshold is set within the linear amplification phase. The C_t is the point where the amplification curve crosses the threshold and this is used for calculation of the amplification efficiency.

An internal standard (loading control) is needed to adjust for differences between samples in regard to amount, tissue type, differentiation status and methodological error margins. House-keeping gene transcripts assumed to have constant expression such as that for GAPDH, 18S, β-actin, β_2-microglobulin or tubulin are often used as an internal standard to make samples comparable. In this work, a suitable internal standard was sought for differentiating U937 cells. Ribosomal RNA 18s was unsuitable because of too high a level since the internal standard should be within approximately 5 PCR cycles of the target gene. The transcripts for GAPDH and HPRT were found to fluctuate during cell differentiation. β_2M was chosen as the internal standard because it displayed low variation during cell differentiation and showed consistent levels when different experiments were compared. In primary cells, c-abl was found to be an appropriate internal standard.

IP-Western

In IP-Western, solubilized protein is precipitated with a specific antibody and separated by SDS-PAGE followed by Western blotting with a specific antibody. IP-Western can be used to detect interactions between two proteins by co-precipitation. An antibody against one protein is used for IP and an antibody against the other for subsequent Western blotting. Proteins that are too miniscule to be detected by direct Western blotting can be detected by IP-Western. By using the same antibody, the protein is enriched by IP and detected by Western blotting. Quantitative comparisons cannot be made by IP-Western since it is not possible to include an internal loading control.

Results/Discussion

Interactions between the ETO homologues (I).

We showed that all ETO homologues were able to interact with each other as well as with AML1-ETO. The coiled-coil oligomerization domain NHR2 was required for homo- and hetero-interactions. Furthermore, our results suggested that the NHR3

region was also required for ETO-MTG16 interaction. A region within NHR3 might be responsible for a direct contact of ETO with MTG16 or NHR3 might normally bind a stabilizing protein that fortifies an ETO-MTG16 interaction.

The size of complexes differed between mutants lacking the functional domains NHR1-NHR4 as shown by gel chromatography. Thus, 600 kDa *wt* ETO-containing complexes were detected. However, ETO lacking NHR2 was not detected in a complex but detected as a 70 kDa monomer. This clearly demonstrated the importance of an intact NHR2 coiled-coil domain for oligomerization. ETO lacking NHR1 or NHR4 formed larger complexes than those formed by *wt* ETO. In accordance with this observation, an AML1-ETO splicing variant lacking NHR4 was found to form multimers instead of dimers [111]. The larger complex size is unlikely to be due to a different complex composition since the deletions would rather result in an inability to bind proteins. Instead, it is possible that the larger complex size is due to an abnormal conformation of the deletion mutants leading to aggregation.

Upon ectopic ETO homologue expression in COS cells, complex formation was observed between all the homologues. However, we failed to detect ETO homologue interactions *in vivo* probably because of a too low endogenous concentration of the proteins. Therefore, endogenous hetero-dimerization between the ETO homologues remains to be shown. If homo-dimerization between the ETO homologues occurred naturally, then ETO dimers could be present in one kind of complex and MTG16 dimers in another kind of complex. If so, ETO homologues might form specific repressor complexes and thus repress individual target genes. If, on the other hand, hetero-dimerization occurred naturally, then different ETO-homologues would be present in the same complex to a high extent. It is possible that certain combinations of ETO-homologues would bind one repressor, while other combinations of ETO homologues would bind another repressor. Furthermore, a specific combination of ETO homologues might determine the level of repression as different homologues may affect the co-repressor recruitment differently. This is supported by the fact that ETO binds to one subset of HDAC whereas MTG16 binds another subset [44]. The subnuclear compartmentalization of the ETO homologues is also important for interactions. ETO and MTGR1 normally reside in the nucleoplasm, whereas MTG16a

resides in the nucleolus [52]. If MTG16a associates with ETO or MTGR1 it could drag them to the nucleolus leaving the nucleoplasm ETO homologue-free. This might lead to increased or decreased repression in the nucleolus and the nucleoplasm respectively. Consequently, various combinations of homologues might affect the repression activity differently.

Expression of ETO homologues during hematopoietic differentiation (II).

MTG16 and MTGR1 are ubiquitously expressed in tissues and cell lines [32, 33]. In contrast, the expression of ETO is more restricted. Of all leukemic cell lines examined, ETO was expressed only in the erythroid cell line HEL indicating restricted hematopoietic expression.

MTG16 protein decreased in U937 cells during granulocyte-like differentiation but not during monocyte-like differentiation suggesting lineage-specific regulation. Also MTG16 transcript levels decreased during granulocyte-like differentiation in these cells. These findings suggest a role for MTG16 in granulocytic differentiation.

A more complete and relevant transcript expression profile of the ETO homologues was achieved by examination of FACS-sorted hematopoietic cells and differentiating CD34+ bone marrow cells. MTG16 transcript levels decreased 5-10 fold during both granulocytic and erythroid differentiation. Furthermore, low MTG16 transcript levels were observed in the FACS-sorted differentiated cells compared to the CD34+ progenitor cells with a strong inverse correlation between the MTG16 expression and the degree of differentiation. This could mean that in progenitor cells, MTG16 is either required for commitment to differentiation or is inhibiting differentiation. In the latter case, MTG16 needs to be downregulated for differentiation to progress. However, support for the former idea comes from the finding that overexpression of MTG16 relieves the block of granulocytic differentiation caused by AML1-ETO [103]. Thus, ectopic expression of AML1-ETO in myeloblastic 32D cells inhibited terminal differentiation, but MTG16 overexpression relieved the differentiation block. Therefore, MTG16 could be part of a repressor complex that antagonizes maintenance of progenitor cell status and thereby promotes commitment

to differentiation. As we have observed that AML1-ETO can bind MTG16 **(I)**, it seems plausible that AML1-ETO-mediated sequestration of MTG16 would lead to a possible shortage of MTG16 in repressor complexes. If MTG16 were required for initiation of differentiation of the progenitor cell, such sequestration would impair differentiation. Alternatively, MTG16 could be required for proliferation of progenitor cells. As an example, N-terminally deleted MTG16 but not *wt* MTG16 was shown to inhibit G-CSF-dependent proliferation in myeloblastic 32D-cells [99]. This suggests that N-terminally deleted MTG16 has lost a regulatory capacity and constitutively represses proliferation. The higher level of MTG16 seen in progenitor cells may be consistent with a role in proliferation at this stage.

MTGR1 is little affected by the differentiation status of hematopoietic cells. Thus, MTGR1 transcript levels were similar in $CD34^+$ progenitor cells and in more mature bone marrow cells. Except for the ETO-homologues, other binding partners have not yet been reported for MTGR1. AML1-ETO enhanced G-CSF dependent proliferation in the murine myeloid precursor cell line L-G [30]. Co-expression of MTGR1 and AML1-ETO further augmented cell proliferation whereas MTGR1 had no effect on its own. This implies that MTGR1 acts as a "standby" repressor that has the ability to augment the activity of other ETO homologues. Further support for the involvement of ETO homologues in differentiation/proliferation comes from the finding that MTGR1 is required for normal neurogenesis in Xenopus and Chicken [113]. A putative dominant negative form of MTGR1 markedly reduced differentiation in developing neurons. Furthermore, MTGR1 gene targeting in mice show mostly undifferentiated cells in the secretory lineage of the small intestine [40]. Collectively, these results indicate that MTGR1 may play a minor role in hematopoietic regulation.

We confirmed that ETO is sparsely expressed in CD34+ hematopoietic progenitor cells as reported before [37]. Our novel interesting observation was, however, the finding of ETO transcripts in erythroid cells. ETO transcript levels were approximately 100-fold higher in erythroid bone marrow cells compared to CD34+ cells. During erythroid differentiation of CD34+ progenitor cells, ETO was upregulated transiently at the proerythroblastic stage. Erythroid differentiation is a complex process in which factors such as GATA1, PU-1 and EPO are carefully

regulated [114-116]. The upregulation of ETO in proerythroblasts suggests a role in the regulation of erythroid differentiation, and ETO might be part of a regulatory complex essential for erythropoiesis. Like ETO, the erythroid transcription factor TAL1/SCL dimerizes with E-proteins [56, 117-120]. This suggests that ETO and TAL1 may cooperate in erythroid development. In fact, the mouse MTG16 ortologue (ETO-2) has been shown to interact with TAL1 and E2A in a complex with repressor activity [121, 122]. Moreover, this complex contained the erythropoiesis factors GATA-1 and LMO-2, which further implements a role for the ETO homologues in erythropoiesis. It is possible that ETO may replace the function of ETO-2 in human erythropoiesis. Furthermore, the ETO binding partner Gfi-1 is necessary for erythropoiesis [54]. Like ETO, Gfi-1 is upregulated during erythroid differentiation and is suggested to act as a repressor at a late stage of erythroid differentiation [123]. During erythropoiesis cells go through a massive clonal expansion concomitant with differentiation. It is possible that ETO-mediated repression has a part in regulating the transition from proerythroblast to erythroblast. It remains to be determined whether the reported AML1-ETO impairment of erythroid differentiation [104] is caused by interference with ETO. AML1-ETO might sequester ETO by oligomerization in the proerythroblast thus prohibiting normal ETO function. Or, AML1-ETO might sequester ETO and maintain ETO repressor capacity when downregulation normally should occur.

Collectively, our results suggest a role for ETO homologues in hematopoiesis. The downregulation of MTG16 during both erythroid and granulocytic differentiation implies a possible connection to differentiation. Furthermore, the transient expression of ETO during erythropoiesis, suggests a functional role in this lineage.

Transcriptional repression of ETO homologues (III).

Originally ETO was found to have repressor capacity and further research on repression in this context has focused on ETO, much due to the relation with AML1-ETO. Having found that the ETO homologues are differentially expressed in hematopoiesis [39] and that MTG16 has been implicated in erythroid regulation [121,

122], we found it fit to assess MTG16's role in repression and to see whether it cooperated with the ETO homologue interacting co-repressors N-CoR and hSIN3B.

By utilizing a galactosidase-luciferase based reporter system, in which MTG16 is directed to the vicinity of a promoter, we found that MTG16 potently induced repression. This was in accordance with our expectations as similar approaches have shown similar results for ETO [48, 124]. We then focused on the importance of the functional domains of the ETO homologues, NHR 1-4, which are evolutionary conserved and thus believed to be important for the function of the ETO homologues. The NHRs are responsible for oligomerization and binding of transcriptional repressors, co-repressors and HDACs. Individual deletions of the NHRs did not alter the transcriptional repression. It was surprising that deletion of NHR2 did not decrease repression as this region binds both HDACs, SIN3 and is crucial for oligomerization [44, 46]. Thus, we hypothesized that loss of MTG16 oligomerization and SIN3 binding with diminished HDAC capacity would lead to abrogated transcriptional repression. The lack of effect when deleting the oligomerization-dependent NHR2 indicates that oligomerization is not quantitatively linked to repression. MTG16 might have retained co-repressor and HDAC binding through other NHRs. Thus deletion of a single NHR was insufficient to give an effect on repression.

ETO augments N-CoR-mediated repression together with SIN3A [125]. In contrast to ETO, murine MTG16 does not interact with mSIN3A [44]. However, the alternatively spliced SIN3B interacts with all ETO homologues including MTG16 (Dhanda RS et al., submitted for publication). This indicates that MTG16, N-CoR and SIN3B may cooperate in transcriptional repression. We found that SIN3B had repressor capacity. Furthermore, SIN3B-mediated repression was augmented by N-CoR. However, no combined transcriptional repression was observed between MTG16, N-CoR and SIN3B. Despite the lack of cooperation for repression, a physical interaction was observed between MTG16 and SIN3B. Moreover, none of the ETO homologues augmented SIN3B-mediated repression. Possibly, even though both MTG16 and SIN3B are co-repressors, their interaction might have a different consequence than the augmenting one. In "native" repression, the MTG16-SIN3B interaction could stabilize the co-repressor complex rather than activate it. Or possibly,

if MTG16 is to cooperate with SIN3B and N-CoR in repression, further yet unidentified components of the MTG16 co-repressor complex may play a role.

The ETO homologues are highly homologous. Therefore it is possible that they have redundant functions and repress the same target genes. For example, both ETO and MTG16 can bind the transcriptional repressor Gfi-1 [50] suggesting a similar function in this case. However, these observations do not rule out that the ETO homologues might have differential functions too. Some observations speak for separate functions. For example, MTGR1 was found to enhance and ETO to repress AML1-ETO mediated repression [30, 46]. However, these observations are not strictly comparable since different transcriptional assays were used in the two experiments. We observed that N-CoR does not affect MTG16-mediated repression, while a previous report has shown ETO to augment N-CoR-mediated repression [125]. This indicates differential functions for ETO and MTG16. However, the systems compared are reciprocal. Considering this, it can imply that the ETO homologues have a stronger HDAC recruiting capacity than N-CoR. Thus, when ETO binds to an N-CoR repressor complex, additional HDACs are recruited to the complex and repression is enhanced. Conversely, when N-CoR binds to an MTG16 complex, this could already be saturated with HDACs and thus repression remains unchanged. This hypothesis points to a similarity for the ETO homologues and suggests that they function primarily as HDAC recruiters in repressor complexes.

The expression of ETO homologues in leukemic cells (IV).

Human hematopoietic stem/progenitor cells (HSPCs) transduced with either AML1-ETO (AE-cells), CBFβ-MYH11 (CM-cells) or other chimeric oncoproteins have given new possibilities for determination of the role of these proteins in hematopoietic differentiation [102]. The resulting AE- and CM-cultures have longer life span than untransduced progenitor cells although the cultures do not become fully immortalized [102]. Both AE- and CM-cultures show a partial differentiation block with 30-50% of the cells remaining CD34+.

The finding of similar transcript levels for MTGR1 in AE-cells, CM-cells and normal hematopoietic progenitor cells indicated that none of the fusion proteins affected MTGR1 expression. Furthermore, early or late AE cultures did not reveal any MTGR1 mRNA differences either, further emphasizing a lack of effect of AML1-ETO on MTGR1. In contrast to AE-cells, however, hematopoietic progenitor cells that had been in "mild" myeloid culture condition for three days showed a two-fold decrease of MTGR1 transcript levels. This is interpreted as an effect of differentiation of quiescent cells before loosing CD34-expression. A similar MTGR1 decrease was seen during differentiation in U-937 cells **(II)**. Possibly, MTGR1 represses a target gene involved in the initiation of differentiation. Thus, the presence of MTGR1 would inhibit differentiation and the downregulation of MTGR1 would be a prerequisite for differentiation to proceed. ETO seems to regulate adipocyte differentiation in such a manner [34]. The transcription factors C/EBP α and β regulate adipocyte differentiation. ETO can repress C/EBP α and β in preadipocytes. Upon adipocyte differentiation, the ETO expression decreases leading to C/EBP α and β upregulation, which in turn enables further differentiation.

MTG16 transcript levels were lower in AE-cells but not in CM-cells. This should not be due to a disrupted CBF-complex since this complex is disrupted in both AE- and CM-cells. The fusion proteins AML1-ETO and CBFβ-MYH11 both act as dominant negative inhibitors of CBF through AML1 and CBFβ respectively. The results therefore suggest that MTG16 is transcriptionally regulated by the aberrantly expressed ETO of AML1-ETO but not by CBFβ-MYH11. Alternatively, the different phenotypes of AE- and CM-cells might affect the MTG16 levels.

Does MTG16 affect self-renewal and/or differentiation of AE-cultures? Normal hematopoietic cells both proliferate and mature concomitantly with MTG16 downregulation [39]. The AE-cultures have enhanced proliferation capacity but are inhibited in differentiation ability. Thus, lower MTG16 transcript levels in the AE-cultures could either be correlated with enhanced proliferation or inhibited differentiation. The finding that MTG16 overexpression can induce differentiation in AML1-ETO expressing progenitor cells [103] supports the idea that MTG16 is associated with differentiation. This would imply that repressed MTG16 could have a

role for the partial differentiation block in AE-cells. Hypothetically, AML1-ETO could repress MTG16 in progenitor cells leading to differentiation inhibition of these cells.

Future perspectives

Differential expression in hematopoietic lineages as described in this thesis implies that the ETO homologues may have distinct functional properties. The fact that they have been found to bind different transcriptional repressors and co-repressors support this notion. However, the functional effects of these interactions and how they are regulated are still not fully understood. What is the mechanistic role of the ETO homologues in hematopoietic differentiation? To clarify this, additional nuclear binding partners for ETO may be sought in erythroid cells, which have a high expression of ETO and are therefore relevant in exploring the role of ETO. Potential ETO-containing nuclear complexes have been enriched by ion exchange and gel filtration chromatography followed by attempted purification by affinity chromatography on matrix-bound anti-ETO antibodies (unpublished data). Purified proteins can be analyzed by masspectrometry and identified ETO interacting proteins may be defined. The long-term AML1-ETO cultures, established from HSPCs [101] may also be used to elucidate the function of the ETO homologues. Since these cultures have a partial differentiation block and prolonged CD34+ expression they are relevant model systems for leukemic cells with AML1-ETO (*t*[8;21]). One hypothesis is that down-regulated MTG16 causes granulocytic differentiation block in leukemic cells with AML1-ETO (*t*[8;21]) **(paper IV)**. A controllable expression of MTG16 in AE-cultures might be used for exploration of this hypothesis.

What effect does AML1-ETO have on the normal interactions between the ETO homologues? Studying the sub-nuclear organization of the homologues could give answers to this question. We believe that AML1-ETO binds and sequesters the ETO homologues and that this interferes with the normal functions of the ETO homologues. AML1-ETO resides in the nucleoplasm [43] and MTG16 in the nucleolus [52]. It is plausible that AML1-ETO attracts MTG16 to the nucleoplasm like AML1-MTG16

does [52] and thus disturbs the function of MTG16. It is also possible that the co-repressor-binding domains of aberrantly expressed AML1-ETO may sequester other co-repressors such as N-CoR, mSin3a and HDACs that normally binds to the ETO homologues. If AML1-ETO interferes with either MTG16 or other co-repressors, this could dislocate and thus deregulate the normal transcriptional targets of the ETO-homologues, N-CoR, SIN3A and HDACs. This could give effects that contribute to the leukemic phenotype of AML1-ETO containing cells.

Populärvetenskaplig sammanfattning

Blodet fyller livsviktiga uppgifter i kroppen såsom syretransport och infektionsförsvar. De röda blodkropparna sköter syretransporten och de vita blodkropparna hanterar infektionsskyddet eller immunförsvaret. Blodplättar står för blodets levring vid blödning. Efterhand förbrukas blodcellerna och därför sker en kontinuerlig nybildning. Vid akut behov kan produktionen av blodceller öka mycket snabbt. De olika blodcellerna bildas från ursprungsceller (stamceller) i benmärgen vilka förökar sig samtidigt som de mognar. Det krävs ett regleringssystem för att bestämma vilka gener (arvsanlag) som ska komma till uttryck (slås på) för att styra cellväxt och utmognad. Det krävs också ett regleringssystem för att bestämma vilka gener som ska stängas av. Det sistnämnda systemet är i fokus för detta arbete vilket handlar om proteiner som stänger av gener (gen-tystare). De studerade gen-tystarna utgörs av ETO-släktet, i vilket proteinet ETO (**E**ight **T**wenty **O**ne) och ytterligare två släktingar ingår.

ETO-släktet har visat sig vara involverat vid leukemi. Vid en viss typ av leukemi har man nämligen funnit att ETO genen är förenad med en annan gen. Ett nytt protein, ett s.k. fusionsprotein, bildas istället och dess funktion är förändrad. Leukemiceller visar en störd utmognad. De förökar sig kontinuerligt men mognar inte vilket leder till en ansamling av omogna leukemiceller som inte förmår skydda mot infektioner på ett normalt sätt. Det ETO-innehållande fusionsproteinet tycks spela en roll för uppkomsten av den typiska mognadshämningen hos leukemiceller. ETO-släktets normala roll är okänd och dess inblandning i blodbildning och leukemi håller på att kartläggas.

Vi fann att ETO-släktingar både kan binda till sig själva och till varandra. Om denna korsvisa bindning sker i en normal miljö innebär detta att ETO-släktingarna kan samarbeta i deras gen-tystande aktiviteter. Dessutom fann vi att leukemispecifika fusionsproteiner också kan binda till ETO-släktingar, vilket skulle kunna störa deras gen-tystande funktioner.

ETO-släktingar binder inte bara till varandra utan ingår också i komplex med icke-besläktade proteiner som bidrar till den gen-tystande effekten. För att förstå samspelet mellan ETO-släktingar och andra proteiner i sådana komplex studerade vi den gen-tystande effekten hos somliga av de ingående proteinerna. Våra forskningsresultat antyder att ETO-släktingarna har starkt gen-tystande aktivitet jämfört med de övriga proteinerna i komplexet. ETO-släktingarna tycks förmedla och förstärka komplexets gen-tystande aktvitet.

Vi fann att ETO-släktingar produceras under blodbildningen. En av de tre ETO-släktingarna bildas särskilt under utvecklingen av de syretransporterande röda blodkropparna och inte i andra blodceller. Denna ETO-släkting kan därför ha en viktig roll i regleringen av de röda blodkropparnas bildning. Vid vita och röda blodcellers utmognad observerades för övrigt en minskning av en annan ETO-släkting. Detta kan betyda att somliga ETO-släktingar måste minska för att tillåta mognadsprocessen. Vi fann även att en av ETO-släktingarnas bildning hämmades i ett leukemi-likt system. Detta föranleder oss att tro att en minskad mängd ETO-släkting leder till att cellerna i detta system förblir omogna. Denna minskning kan vara en bidragande faktor till att leukemi utvecklas.

Detta arbete har gett en ökad förståelse för ETO-släktets roll i blodbildningen och dess koppling till leukemi. Genom samspel med andra proteiner tror vi att ETO-släktet bidrar till att bibehålla den jämvikt mellan genaktivering och gen-tystning som krävs för en normal blodbildning. En förståelse av denna jämvikt och den ojämvikt som råder vid leukemi kan i förlängningen bidra till nya idéer för behandling av blodsjukdomar.

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