Molecular Analysis of the *MN1* Oncogene and the Leukemia-Associated Fusion Gene *MN1-TEL*

Magda A. Meester-Smoor

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Moleculaire analyse van het *MN1* oncogen en het leukemie-geassocieerde fusiegen *MN1-TEL*

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Promotor:
Prof.dr. E.C. Zwarthoff

Overige leden:
Prof.dr. R. Fodde
Prof.dr. I.P. Touw
Prof.dr. J.W. Oosterhuis

yesterday a child came out to wonder
caught a dragonfly inside a jar
fearful when the sky was full of thunder
and tearful of at the falling of a star

.

then the child moved ten time round the seasons
skated over ten clear frozen streams
words like 'when you're older' must appease him
and promises of someday make his dreams

and the seasons they go round and round
and the painted ponies go up and down
we're captive on the carousel of time
we can't return we can only look
behind from where we came
and go round and round and round
in the circle game

sixteen springs and sixteen summers gone now
cartwheels turn to car wheels thru the town
and they tell him 'take your time, it won't be long now
till you drag your feet to slow the circles down'

so the years spin by an now the boy is twenty
though his dreams have lost some grandeur coming true
there'll be new dreams, maybe better dreams and plenty
before the last revolving year is through

Contents

LIST OF ABBREVIATIONS

aa	amino acids	DR	direct repeat
ABL	Abelson murine leukemia viral oncogene homolog 1	DRRF	dopamine receptor regulating factor
		E	embryonic day
Akt	v-akt murine thymoma viral oncogene homolog 1	E2F2	E2F transcription factor 2
		EMSA	electrophoretic mobility shift assay
ALL	acute lymphoid leukemia	ERG	ETS related gene
AML	acute myeloid leukemia	ERK	extracellular signal-regulated kinases
AML1	acute myeloid leukemia 1	ETO	Eight twenty one protein
AP-2	activator protein-2	ETS	E26 transformation-specific
aRNA	amplified RNA	Ets-1	E26 transformation-specific homolog 1
ATRA	all trans retinoic acid		
BAALC	brain and acute leukemia, cytoplasmic	ETV6	ets variant 6
		EVI1	ecotropic viral integration site 1
BAC	bacterial artificial chromosome	EWS	Ewing sarcoma breakpoint region 1
BCL2	B-cell CLL/lymphoma 2	FACS	fluorescence-activated cell sorting
BCR	breakpoint cluster region	FLI1	Friend leukemia virus integration 1
BM	bone marrow	GAPDH	glyceraldehyde-3-phosphate dehydrogenase
BMI1	B lymphoma Mo-MLV insertion region 1 homolog		
		GEO	gene expression omnibus
CALM	calmodulin 1	GFP	green fluorescent protein
CBF	core binding factor	GLI	glioma-associated oncogene
CBFbeta	core binding factor, beta subunit	GMP	granulocyte/monocyte progenitor
CDKN2C	cyclin-dependent kinase inhibitor 2C	GN-CSF	granulocyte-macrophage colony stimulating factor
cDNA	complementary DNA		
CDX2	caudal type homeobox 2	GSH2	GS homeobox 2
CHIC2	cysteine-rich hydrophobic domain 2	HDAC	histone deacetylase
ChIP	chromatin immunoprecipitation	HE	hematoxylin-eosin
CLL	chronic lymphoid leukemia	HLH	helix loop helix
CLP	common lymphoid progenitor	HLTF	human T-cell leukemia virus enhancer factor
CMP	common myeloid progenitor		
CN-AML	cytogenetically normal acute myeloid leukemia	HLXB9	Homeobox protein HB9
		HOX	homeobox
COMMD3	COMM domain containing 3	HSC	hematopoietic stem cell
DBD	DNA binding domain	ID1	inhibitor of DNA binding 1
db	database	IGF	insulin-like growth factor
DHRS9	dehydrogenase/reductase (SDR family) member 9	IGFBP5	insulin-like growth factor binding protein 5
Dlx2	distal-less homeobox 2	IL-3	interleukin 3
DMEM	Dulbecco's Modified Eagle's Medium	ITGAM	integrin alpha M
DMSO	dimethylsulfoxide	Jak	Janus kinase
DNA	deoxyribonucleic acid	kb	kilobase
DNAJC1	DnaJ (Hsp40) homolog, subfamily C, member 1	kDa	kiloDalton
		L(3)-MBT	lethal (3) malignant brain tumor

LSC	leukemia stem cells
LT	long-term
MDS2	myelodysplastic syndrome 2 translocation associated
MEIS1	myeloid ecotropic viral integration site 1 homolog
MEK	mitogen-activated protein kinase or extracellular signal-regulated kinase
MEP	megakaryotic/erythoid progenitor
miRNA	microRNA
MLL	myeloid/lymphoid or mixed-lineage leukemia
MMLV	moloney murine leukemia virus
MMP	multipotent progenitor
MN1	meningioma 1
MPD	myeloproliferative disorder
mRNA	messenger RNA
MSCV	murine stem cell virus
MSV-LTR	moloney sarcoma virus long terminal repeat
Msx1	msh homeobox 1
MYH	myosin heavy chain
NCoA3	nuclear receptor coactivator 3
N-CoR	nuclear receptor corepressor
NDRG1	N-myc downstream regulated 1
NF2	neurofibromin
NIH	National Institute of Health
NK	natural-killer
NKI-CMF	Netherlands Cancer Institute Central Microarray Facility
NPM	nucleophosmin
NUP98	nucleoporin 98kDa
Oct-1	octamer-binding transcription factor 1
OSTL	opposite six-twelve leukemia gene
Osx	zinc finger protein osterix
p14/ARF	p14 alternate reading frame
PAX5	paired box gene 5
PBS	phosphate-buffered saline
PCR	polymerase chain reaction
PDGFR	platelet-derived growth factor receptor, beta polypeptide
Pg	progesteron
PI3K	phosphoinositide-3-kinase
PLZF	promyelocytic leukemia zinc finger protein
PML	promyelocytic leukemia
PolR2A	RNA polymerase II, subunit A
PR	progesteron receptor
PRC	polycomb-repressive complex
PRE	progesteron-responsive element
qPCR	quantitative PCR
RA	retinoic acid
RAC3	ras-related C3 botulinum toxin substrate 3
RANKL	receptor activator of nuclear factor kappa B ligand
RAR	retionoic acid receptor
RIF1	nuclear receptor-interacting factor 1
RNA	ribonucleic acid
RT-PCR	reverse transcriptase PCR
RUNX	runt-related transcription factor
RXR	retinoid X receptor
SCC25	squamous cell carcinoma 25
seq	sequence
SIP1	mothers against decapentaplegic homolog (SMAD)-interacting protein 1
SMMHC	smooth muscle myosin heavy chain
SMRT	silencing mediator for retinoid and thyroid hormone
SPAG6	sperm-associated antigen 6
SRC	v-src sarcoma (Schmidt-Ruppin A-2) viral oncogene homolog
ST	short-term
STL	six-twelve leukemia gene
TAD	transactivating domain
Tbx22	T-box 22
TEL	translocation ETS leukemia
tet	tetracyclin
TGFbeta	transforming growth factor beta
TMPRSS2	transmembrane protease, serine 2
TRTC	tyrosine kinase receptor C
TSA	trichostatin A
TTL	tubulin tyrosine ligase
U2OS	U2 osteosarcoma
UCSD	University of California, San Diego
VDR	vitamin D receptor
Zeb2	zinc finger E-box binding homeobox 2

CHAPTER 1

Introduction

NORMAL HEMATOPOIESIS AND LEUKEMIA

Hematopoiesis

The word hematopoiesis comes from the ancient Greek; *haima* translates to 'blood' and *poiesis* means 'to make': hematopoiesis describes the formation and development of bloods cells. Different types of blood cells are recognized. Red blood cells, the erythrocytes, are important for transport of oxygen throughout the body, white blood cells, B and T-lymphocytes, are crucial for the immune response and granulocytes and monocytes are important for responses against infections. Platelets, which form clots to stop bleeding, are also formed by hematopoiesis.[1]

Hematopoiesis is an intriguing process that continuously supplies the organism of new blood cells for its complete lifespan. In humans, the first primitive blood cells are formed in the blood island of the yolk sac (2 to 8 weeks of development).[2] This is followed by blood cell formation in the fetal liver and the spleen. At five months of development the bone marrow is the site of production of blood cells and it continues to do so throughout life.

The cells from the hematopoietic system are generated from the self-renewing hematopoietic stem cells (HSC) that reside mainly in the bone marrow.[3,4,5] HSCs are divided in long-term (LT)-HSC and short-term (ST)-HSC (Figure 1). LT-HSC are slow-dividing cell populations that contain self-renewing cells that sustain long-term hematopoiesis, divide a-symmetrically, and form ST-HSC. These cells are faster-dividing cell populations with limited self-renewal potentials and are more committed, ready-to-differentiate and produce the progeny of the different lineages. ST-HSC differentiate into nonself-renewing multipotent progenitors (MPP). This cell type differentiates into two oligolineage progenitors, the common myeloid progenitor (CMP) or common lymphoid progenitor (CLP). T-cells, natural killer (NK)-cells and B-cells are formed from the CLPs. CMPs differentiate into either megakaryotic/erythoid progenitors (MEP) that produce erythrocytes and platelets or granulocyte/monocyte progenitors (GMP). Dendritic cells are produced either from CMPs or CLPs.

Each blood cell type is produced in different numbers, and this production is regulated strictly to meet the changes needed. The regulation is controlled by extracellular signals, such as interleukin-3 (IL-3) and granulocyte-macrophage colony stimulating factor (GM-CSF). These signals are passed on within the cell by signaling cascades, including the Jak-Stat pathway, the Raf/MEK/ERK pathway and the PI3K/Akt pathway.[6,7] At the end of these pathways are many transcription factors that control the expression of genes.[8,9] Each differentiation step requires the up and downregulation of a specific set of genes.
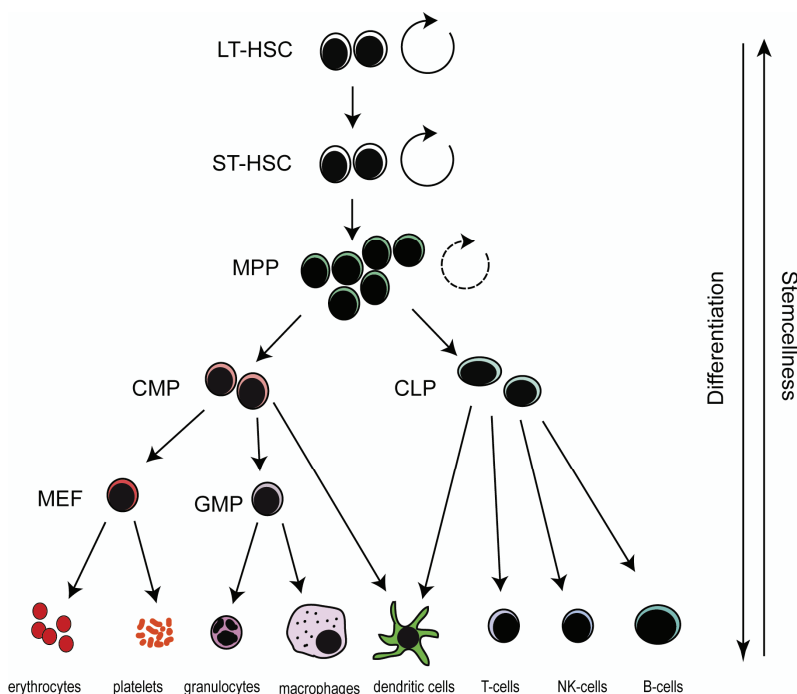
Figure 1. Cell lineages in hematopoiesis. Long-term (LT)-HSCs contain self-renewing cells and short-term (ST)-HSC consist of more commited cells that will differentiate into multipotent progenitor population (MPP). These cells differentiate into the so-called oligolineage-restricted progenitors, the common myeloid progenitor (CMP) and the common lymphoid progenitor (CLP). Het CLPs give rise to indicated cells. Dendritic cells are formed from either CMPs or CLPs. The CMPs differentiate to megakaryocytes-erythrocytes progenitors (MEPs) and the granulocyte/monocyte progenitors (GMPs). These progenitors give rise to the mature cell types indicated. This figure is a simplified version of the one that appeared in Passegue et al.[5]

Leukemia

In leukemia, the differentiation and proliferation of blood cells is disturbed and cells accumulate in a pre-mature state and will overgrow the normal cell populations in the blood. Symptoms of leukemia include low red blood cell count (anemia), low platelet levels (thrombocytopenia) and low white blood cell count (neutropenia), leading to fatigue, easy bleeding, and failure to fight infections respectively.[10] The main subgroups of leukemia are classified according to the cell lineage involved (myeloid or lymphoid) and the development of the disease (chronic or acute).

The incidence rate for leukemia is 15 per 100.000 for the US.[11] It accounts for 3% of all newly diagnosed cancer patients but still is among the ten leading cancer types for both males and females. The majority of patients are diagnosed with chronic lymphoid leukemia (CLL, 35% of cases) or acute myeloid leukemia (AML, 30% of cases). The remainder of the

patients has acute lymphoid leukemia (ALL) or chronic myeloid leukemia (CML) and other leukemia subtypes.

Leukemias, and cancers in general, develop from a single cell that acquires multiple genetic and epigenetic changes, such as translocations (see below), pointmutations, amplifications and aberrant methylation patterns. These changes usually affect the self-renewal and proliferation capacity of the cell. Within a tumor, not all cells are capable of initiating and sustaining tumor growth *in vivo*. Only cancer-initiating cells, also called cancer stem cells, have the capacity to do so.[12] In case of leukemia this was already recognized in 1994; transplantation of only a small subset of cells with stem cell like properties was able to engraft recipient mice.[13] Leukemia is thus organized as a hierarchy similar to normal hematopoiesis: leukemic cells are sustained by a small number of leukemia stem cells (LSCs). These LSCs can arise from primitive stem cells with multilineage potential or from progenitors that are already differentiated into the lymphoid or myeloid pathway. The majority of adult leukemia cases arise from the more primitive stem cells whereas childhood leukemias usually have their origin in the lymphoid and myeloid-restricted progenitor cells.[14]

CHROMOSOMAL ABNORMALITIES IN LEUKEMIAS

Translocations

In the early days of cytogenetics, it was already recognized that leukemias frequently are associated with the presence of translocations or other chromosomal abnormalities. In 1960, a minute chromosome that was associated with chronic granulocytic leukemia has been reported on a meeting in Philadelphia by Nowell and Hungerford.[15] Later it was referred to as the philadelphia chromosome and turned out to be caused by a translocation between chromosome 9 and 22.[16] In 1983 and 1984 it was shown that the genes *BCR* and *ABL* are involved.[17,18]

Up to 65% of acute leukemias harbor a translocation. At the genetic level two types of translocations are recognized. Either a translocation causes deregulation of a gene by placement of the gene behind controlling elements of another gene (Figure 2A), or translocations create fusion genes by linking the exons of two genes that result in the production of fusion proteins (Figure 2B). The first type of translocations mostly involves rearrangements with immunoglobulin or T-cell receptor genes and the adjacent gene is upregulated. In the second type of translocations, many different classes of genes are involved. The majority, however, are transcription factors, tyrosine kinases, *HOX* genes, and nuclear receptors and growth factors. The fusion proteins display altered functions and

disturb pathways that are normally tightly regulated in steady-state hematopoiesis. One of the hallmarks of cancer is that cells acquire multiple characteristics during its development and that multiple changes in the genome are required to obtain these characteristics.[19] This is also true for leukemias; besides a translocation, many more cooperating gene mutations are required that finally give the leukemic cells the proliferative advantage in combination with the impaired differentiation.[20]
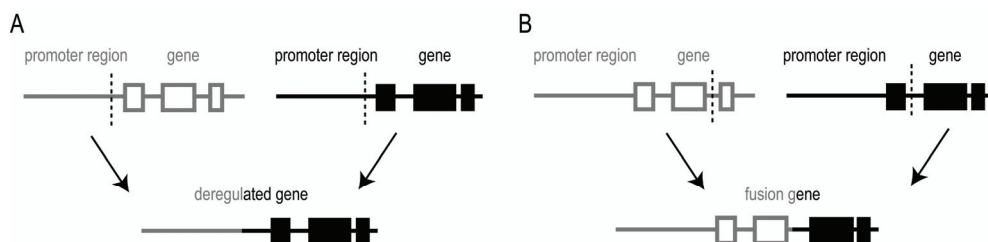

Figure 2. Two major types of translocations are recognised in hematological disorders. (A) The exon structure of a gene is not disturbed but the gene is deregulated by the translocation. The promoter regions of immunoglobulin or T-cell receptor genes are usually involved in these translocations. (B) A translocation results in fusion of genes. One gene contributes the promoter and 5' exons and another gene, derived from the other chromosome, contributes the 3' exons. Exons are incidated with a box, dashed line indicates the breakpoint.

Translocation networks and distribution of translocations

Some translocations have been diagnosed in leukemias show a high degree of specificity between the partner genes, for example the *BCR* and *ABL* translocation. Other translocations seem to be less specific for the partner that is involved. An example is translocations including the *MLL* locus. The *MLL* gene has been shown to translocate to over 20 different partners. The translocation networks for translocations found in hematological diseases are displayed in Figure 3. It shows that the number of key players is limited and this points to common mechanisms for leukemogenesis. The next subchapter discusses the most common translocations and the mechanisms involved in oncogenesis. The occurrence of these different translocations is not evenly distributed among the patients diagnosed with leukemia. Some translocations account for over 10% of all patients diagnosed with a particular leukemia subtype whereas others are only found in one or two patients. A list of the most commonly found mutations in acute leukemias in children and young adults is listed in Table 1. In this group of patients the *AML1-ETO* and *CBFβ-MYH11* fusion genes have been detected in 12% of AML cases each. In addition, 20% of ALL cases have been diagnosed with a TEL-AML1 fusion.[21]
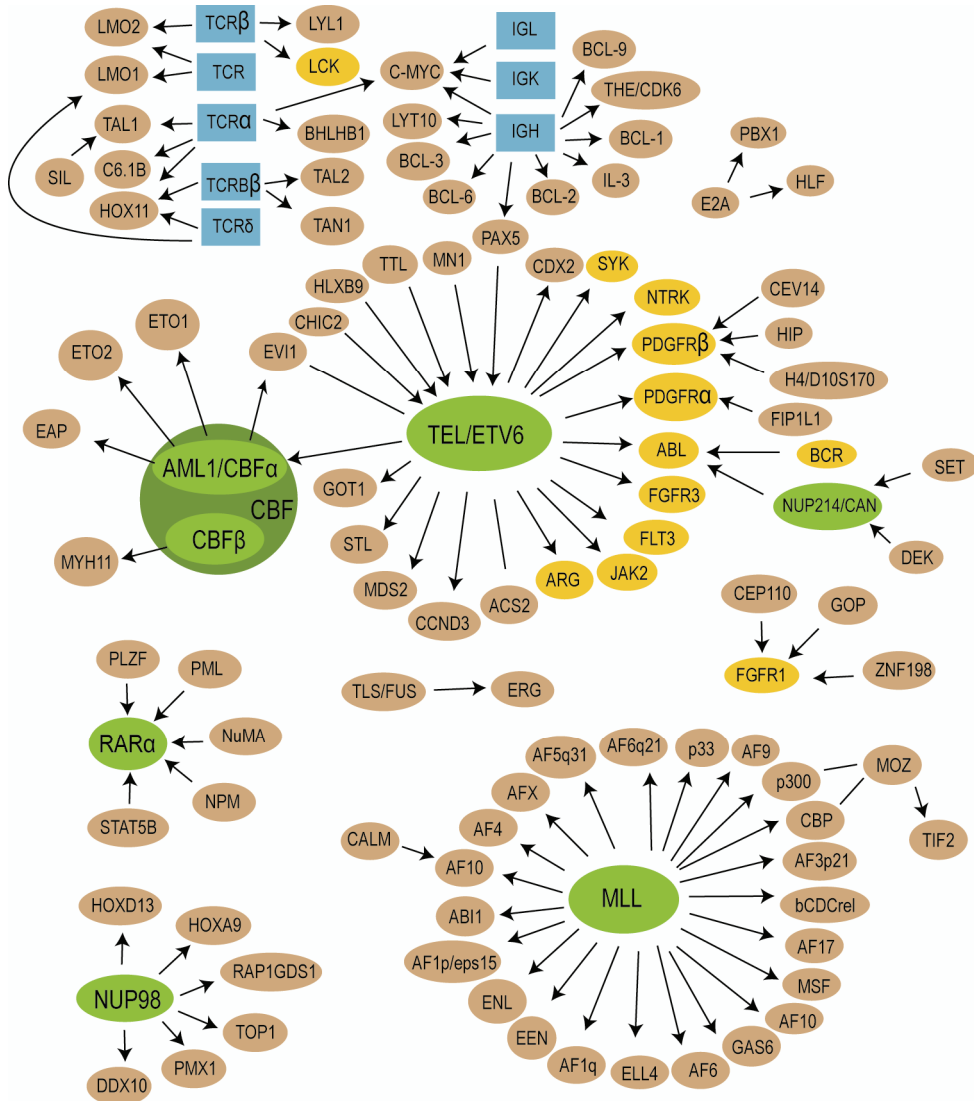
Figure 3. Translocation networks in hematological disorders. Genes that are involved in a translocation are connected by a line or an arrow. The direction of the arrow indicates the direction of the fusion. For instance, the *MN1* and *TEL* genes are fused and the arrow points towards *TEL*. This means that the 5' part of the fusion gene consists of *MN1* sequences and the 3' part are *TEL* sequences. A line instead of an arrow indicates that it is unknown which one of the two possible combinations of gene fusions is leukemogenic. A blue box indicates that solely the regulatory sequences and no coding sequences of the gene are involved in the translocation, usually leading to upregulation of the translocated gene. Yellow indicates genes with tyrosine kinase activity. Green indicates the major players in the network. Information about translocations is gathered together from several review articles and chapter 5 of the book 'Leukemia'.[21,22,23,24,25,26,27,28,29]

Table 1. Chromosomal abnormalities found in acute leukemias in children and young adults*

ALL			AML		
abnormality	gene(s) involved		abnormality	gene(s) involved	
t(12;21)	TEL-AML1	20%	t(8;21)	AML1-ETO	12%
11q23	MLL fusions	6%	inv(16)	CBFβ-MYH11	12%
t(1;19)	E2A-PBX1	5%	t(9;11)	MLL-AF9	7%
14q11	TCRαδ	4%	17q21	RARα	7%
t(9;22)	BCR-ABL	4%	3q26	EVI1	3%
7q35	TCRβ	3%	t(3;5)	NMP-MLF1	1%
8q24	MYC	2%	t(6;9)	DEK-CAN	1%
t(17;19)	E2A-HLF	1%	t(16;21)	FUS-ERG	1%
normal karyotype		30%	t(7;11)	NUP98-HOXA9	1%
rare translocations		25%	normal karyotype		19%
			rare translocations		36%

Normal karyotype refers to leukemias that lack identifiable gene abnormalities.
*This table is deduced from a figure that appeared in Look et al (1997)[19]

MAJOR PLAYERS OF THE TRANSLOCATION NETWORK

AML1 and CBFβ

The t(12;21) is found in approximately 20% of all pediatric patients and it was shown to occur in most cases already before birth.[30,31] The translocation generates the TEL-AML1 fusion protein, also known as ETV6-RUNX1.[32,33] AML1 is the DNA binding subunit of the heterodimeric transcription factor core-binding factor (CBF). The other regulatory subunit is CBFβ. The heterodimeric transcription factor complex controls important developmental decisions by regulating a number of hematopoietic-specific genes, including *IL-3* and *GM-CSF*. Disturbance of this complex by the fusion proteins deregulates the differentiation and proliferation of the hematopoietic cells.[29,34] The *TEL* gene encodes a DNA binding transcriptional repressor that recruits corepressors and histone deacetylases (HDACs). The *TEL* gene is more extensively discussed at the end of this introduction. The N-terminal and central domains of TEL are fused to the C-terminal DNA binding domain of AML1, also called RUNT domain, that is also important for dimerization with CBFβ. Evidence is provided that TEL-AML1 can still bind to promoter and enhancer regions of AML1-target genes and that the TEL moiety of the fusion protein recruits co-repressors.[35,36,37,38] The fusion protein, however, still needs the binding to the CBFβ subunit of CBF for leukemogenesis as has been shown in mice studies by generating point mutations in the RUNT domain of TEL-AML1.[39] The recruitment of corepressors by the TEL-AML1 fusion leads to repression of CBF-target genes that are normally activated by CBF. Expression of the non-translocated *TEL* allele is frequently absent in ALL cases that are diagnosed with a *TEL-AML1* fusion.[40] This is caused either by 12p deletions or by other non-cytogenetically detectable

mutations.[41,42,43] This latter phenomenon is not unique for *TEL-AML1* cases: also one-third of AML patients have deficient TEL expression.[44] The absence of TEL protein suggests that this is an important step and contributes to leukemogenesis.

Another translocation in which the AML1 gene takes part is the t(8;21) frequently detected in AML patients. The translocation fuses *AML1* to *ETO,* a gene encoding a transcription factor. Also this translocation may be of prenatal origin.[45,46] In contrast to the TEL-AML1 fusion, the N-terminal part of AML1-ETO is provided by *AML1* sequences. The breakpoint within the *AML1* gene, however, is much further C-terminally compared to the t(12;21) and as a consequence, AML1 again contributes the RUNT domain of AML1 to the fusion protein. The ETO moiety of the fusion protein contributes, similar to the mechanisms described for TEL-AML1, the sequences that recruits corepressors to the promoters that are normally stimulated by the AML1-CBFβ heterodimer.[47,48,49] One of the targets of AML1-ETO that was recently identified is the microRNA (miRNA) miR-233, the activity of which is linked to differentiation of myeloid precursors. The expression of this miRNA is silenced by the fusion protein, thereby inhibiting the differentiation of myeloid precursors as seen in AMLs.[50]

CBFβ, the non-DNA binding regulatory subunit of CBF, is involved in a frequently occurring inv(16), which is also associated with AML. Molecular cloning has revealed that *CBFβ* is fused to the *smooth muscle myosin heavy-chain* gene, *MYH11*. The same fusion can occur as a result of translocation between two chromosomes 16. The exact mechanism by which the fusion protein CBFβ-SMMHC fusion protein (this nomenclature refers to the protein, whereas MYH11 refers to the gene) performs it leukemogenic activity is largely unknown. Molecular studies have shown that also this fusion recruits repressor proteins to the promoters that are regulated by the AML1-CBFβ heterodimer.[51] Animal studies have shown that secondary mutations are obligatory. Expression profiling of leukemic cells from patients with an inv(16) have shown that the *MN1* gene is frequently upregulated. Mice studies have confirmed that overexpression of *MN1* is an important second hit for the leukemogenesis by the inv(16) fusion protein.[52]

MLL fusions

The *MLL* gene is localized on chromosome 11q23 and many translocation partners have been described for this gene. The multiplicity of the partner genes has raised questions about the role of these genes in the leukemic process. All fusion genes, however, create in-frame fusions at the mRNA level, making it plausible that the fused parts contribute to leukemogenesis. The mere absence of C-terminal sequences of the MLL gene is likely not sufficient to obtain leukemogenic properties.[24] The *MLL* gene is important for normal

hematopoiesis as has been shown in *Mll*-null mice. These mice have defects in yolk sac hematopoiesis.[53] The MLL protein is a histone methyl transferase that regulates, amongst others, the expression of *HOX* genes. *HOX* genes were shown to be expressed in hematopoietic precursors, with preferential expression in self-renewing HSCs, and are downregulated following differentiation.[54] The *MLL* fusion proteins upregulate *HOX* genes, resulting in a block in hematopoietic differentiation, especially in the myeloid lineage.[34,55] Recently it has been shown that MLL also indirectly influences expression of *HOX* genes by regulating the expression of miRNA mir-196b. This miRNA is located within the HoxA cluster and targets several *HOX* genes. Upon differentiation of hematopoietic cells, miRNA-196b is downregulated. *MLL* fusions were shown to upregulate this miRNA and this suggests an additional mechanism whereby the increased expression of this miRNA contributes to leukemia development by MLL fusions.[56]

Retinoic acid receptor (RAR)α translocations

The most frequent and well studied translocation from this subgroup is de t(15;17) that generates the *PML-RARα* fusion gene. RARα is a member of the nuclear receptor family and binds as a heterodimer with the retinoid x receptor (RXR) to DNA response elements. The heterodimer binds the ligand *all-trans* retinoic acid (RA) using its ligand binding domain and stimulates or represses transcription by the recruitment of cofactors when bound to its responsive elements in the DNA.[57] RA, a derivative of vitamin A, is essential during vertebrate development[58] and stimulates the differentiation of normal hematopoietic progenitors.[59] The fusion protein PML-RARα retains the ligand binding domain of RARα but, in the presence of physiological levels of RA, co-repressors bind to the fusion protein that result in repression of target genes that are normally activated by RARα.[60] Several classes of genes are targets of RARα, including differentiation regulators and chromatin modifiers[61] and miRNA genes.[62] Repression of these miRNA genes results in increased expression of *HOX* genes that keeps cells in a non-differentiated state by promoting self-renewal and blockage of differentiation.[62]

Molecular studies have shown that high levels of RA displaces the repressor complexes from the fusion protein and subsequently activates genes required for the differentiation of cells in myeloid lineage. These days, patients diagnosed with a *PML-RARα* fusion are successfully treated with pharmalogical levels of RA.[63,64] Unfortunately, another *RARα* fusion, *PLZF-RARα* does not respond to RA treatment.[65] Although this translocation also results in the recruitment of corepressor complexes to the RAR-responsive elements,

these complexes are not released by high doses of RA, because the PLZF moiety itself has the capacity to bind corepressors, whereas this is not the case for PML.[66]

The TEL (ETV6) gene translocations

About 20 partners for the TEL (ETV6) gene have been described so far. One of the translocations, the *TEL-AML1* fusion gene, was described before. A large group of TEL partners including *PDGFRβ, JAK2, ABL, FGFR3, TRKC* and *ARG* encode tyrosine kinases.[67,68,69,70,71,72] The fusion proteins all contain the HLH domain of TEL that provides the TEL protein the interface for dimerization with itself. The TEL moiety of the fusion protein enables dimerization and this constitutively activates the tyrosine kinases, leading to activation of downstream pathways. This finally results in loss of differentiation and stimulation of proliferation of hematopoietic progenitors.

Another group of translocations involving the *TEL* gene are more diverse and a common mechanisms seems to be the induced ectopic expression or overexpression of either the translocated gene or genes in the vicinity of the translocations. The translocation with the *BTL* gene was shown to produce a small fusion protein, but rather the ectopic expression of the homeobox gene *GSH2*, which is located near the breakpoint on chromosome 4 is thought to be critical for leukemogenesis.[73] Translocations with *MDS2* and *STL* result in fusions that contain only 4 amino acids (aa) from the coding sequences of the genes. Overexpression of neighboring genes, such as *E2F2* (near *MDS2*) and *OSTL* (on antisense strand of *STL* gene) are most likely the main pathogenic mechanisms in these translocations. Also the translocations with *EVI1* and *CDX2* are thought to rely on this mechanism. The *EVI1* and *CDX2* fusions with *TEL* only harbor a very small part of TEL and ectopic expression of *EVI1* and *CDX2* is thought to contribute to leukemogenesis rather than the properties of the fusion protein.[74,75,76]

The last group of translocations is rather small and the mechanism has been detected in both the *MN1-TEL* and the *PAX5-TEL* fusion genes. Both fusion genes carry the C-terminus of TEL that contains the DBD domain and it is conceivable that the PAX5-TEL fusion, like the MN1-TEL fusion, binds to *ETV6*-target genes and deregulates their expression.

Altogether, the many different fusions detected with the *TEL* gene show that *TEL* is a major player in leukemogenesis. Translocations involving the *TEL* gene activate partner kinases, upregulate genes close to the translocation breakpoint, or create fusion genes with unique properties. Additionally to these translocations, the non-translocated *TEL* allele is frequently inactivated by deletions or pointmutations. This observation supports that *TEL*

also functions as a tumor suppressor gene. The inactivation of *TEL* in combination with the specific characteristics of the translocation defines the subtype of leukemia that is found in patients.

Since the *MN1* gene is the major topic of this thesis, the remainder of the introduction will focus on the *MN1* gene and the leukemogenic fusion protein MN1-TEL.

THE MN1 GENE

Conservation, expression and regulation.
The *MN1* gene is located on chromosome 22q11.2 and is comprised of two large exons of 4.7 kb (5' end) and 2.8 kb (3' end). Homologues of *MN1* have been found in more than ten species, including mouse (*Mus musculus*), duck-billed platypus (*Ornithorhynchus anatinus*), puffer fish (*Tetraodon nigroviridis*) and horse (*Equus caballus*). On Northern blots, homologues in fruit fly (*Drosophila melanogaster*) and frog (*Xenopus laevis*) have been detected.[77]

Northern blots from different tissues with *MN1* probes showed the existence of two transcripts. The largest transcript (8 kb) has been detected in several tissues, including brain, heart, skeletal muscle and liver. In skeletal muscle, a smaller band of 4.5 kb has been detected, possibly representing alternative splicing of the transcript in this tissue.[77] Expression of *MN1* has also been detected in the hematopoietic system [52] and in hair follicle cells.[78] The majority of cell lines of different tissue origins have no detectable levels of *MN1* expression. Kuang and coworkers have performed promoter methylation studies in more than twenty AML and acute lymphoid leukemia (ALL)-derived cell lines and showed that the *MN1* promoter is highly methylated in both types of cell lines.[79] The only two cell lines in our laboratory to date exhibiting *MN1* expression are two osteosarcoma-derived cell lines: U2-osteosarcoma (U2OS) and MG63.

The promoter of the *MN1* gene is not well mapped and pathways that regulate *MN1* expression are largely unknown. The synergy between MN1 and the vitamin D3/RX receptor dimer (discussed below) led to the identification of vitamin D3 as a regulator of the *MN1* gene.[80] Expression profiling of vitamin D-treated SCC25 cells (tong keratinocytes) has confirmed this finding.[81] Upregulation of *MN1* is reported in response to TGFβ in epithelial cells of different origin, including skin keratinocytes, long epithelial cells and breast cancer cells.[82,83] The synthetic androgen R1881 induced *MN1* in breast cancer cells (MDA-MB-453 cells),[84] but not in hair follicle cells.[78] A genome-wide mapping of polycomb-target genes has revealed that MN1 is a target of both polycomb-repressive complex 1 (PRC1) and PRC2.[85]

The coding sequence of the MN1 gene harbors two CAG repeats encoding glutamines (Q). The largest glutamine stretch is 28Q and is encoded by CAG codons interspersed by four single CAA codons. CAG repeats of this size can be subjected to instability and repeats of extreme lengths are associated with neurodegenerative diseases, such as Huntington's disease.[86] The length of the largest glutamine repeat within the MN1 gene varies in the population, but only three alleles have been found, differing 3 CAG codons in length.[77] The repeat is stable upon transmission to offspring and no expansions of the glutamine repeat have been reported in patients suffering from neurodegenerative disorders or other pathology (unpublished data).

Translocations involving the MN1 gene

A balanced translocation (4;22) present in a meningioma, a benign brain tumor, has led to the identification of the MN1 gene. The breakpoint within the gene is located in the first exon, thereby disrupting the MN1 open reading frame. No other meningiomas samples have been found with large deletions or translocations within the MN1 gene region. Also the lengths of the large CAG repeats within the coding region of the MN1 gene have been analyzed in meningioma samples and were of normal length. A causal relationship between MN1 expression and meningioma development has thus not been proven to date. Notably, MN1 expression in meningiomas varies greatly from tumor to tumor.[77]

More than ten leukemia patients have been diagnosed with a t(12;22) within their leukemic cells.[27,87,88,89,90,91,92] A majority of these patients have been shown to exhibit a fusion gene involving the MN1 and TEL (ETV6) genes. In addition, several patients display this translocation after chemo and radiation therapy directed against solid tumors elsewhere in the body.[87,88] The amount of patients analyzed, however, is too small to conclude that the t(12;22) is therapy-induced. TEL is translocated to over 20 partners in hematological disorders as discussed before (see Figure 3). Two leukemia cell lines, MUTZ-3 and UCSD/AML1 are known to harbor a t(12;22), creating only in UCSD/AML1 the MN1-TEL fusion. The breakpoint within the TEL gene in MUTZ-3 lies in the 5' untranslated region, ruling out the possibility of an MN1-TEL fusion. The translocation in MUTZ-3 inactivates one copy of the TEL gene and the haploinsufficiency of TEL expression is considered to have a pathogenetic role in AML.

The MN1 protein is a transcriptional cofactor

The gene encodes a protein of 1319 amino acids (aa) in which the second exon accounts for only 59 aa. The protein does not show any homology to other proteins and besides the

glutamine-rich regions that point to a function in transcription regulation, no obvious structural domains have been detected. Indeed the MN1 protein was found to be able to stimulate transcription. It has been extensively studied on a viral promoter and deletion mutants have shown that transcription stimulating activity is located in the N-terminal region of the MN1 protein.[93] On this viral promoter, it functions as a cofactor for the nuclear receptor dimer of RAR and RXR. Retinoic acid (RA), as discussed above, is a regulator of differentiation in various stages of embryogenesis and hematopoiesis and MN1, as a cofactor, is thought to modulate RA pathways. Binding studies have shown that MN1 does not bind the receptors directly.[93] It confers activation by synergizing with p300 and RAC3, both known coactivators of nuclear receptors (Figure 4A and chapter 4).[94] p300 possesses histone acetyltransferase activity and the acetylation of histones is associated with a relaxed chromatin configuration. This is thought to facilitate transcription factor access to DNA, resulting in a higher transcription activity.[95] RAC3 is a member of the SRC family of nuclear receptor coactivators and is also known as nuclear coactivator 3 (NCoA3).[96] Expression profiling studies have uncovered that MN1 has a more widespread effect that is not limited to nuclear receptors. It can both stimulate and repress transcription with and without the involvement of RA (Figure 4A,B, and D; see chapter 5).[97] Several of the genes identified in this study as MN1-target genes are known players in leukemogenesis, such as *BMI1* and *MEIS1*. Another interesting target of MN1 is *DHRS9*, which is involved in the synthesis of retinoic acid. RA stimulated the expression of *DHRS9* whereas MN1 blocks this stimulatory effect of RA. This interference of the RA-regulated expression of *DHRS9* by MN1 might be involved in the differentiation block apparent in leukemias.

Additionally, Sutton and coworkers have identified MN1 as a cofactor for the vitamin D receptor (VDR).[80] This nuclear receptor, like RAR, forms a dimer with RXR and has a dual function; it both inhibits proliferation and stimulates differentiation. MN1 is able to stimulate VDR-driven transcription and combining MN1 with SRC-1 or SRC-2, two coactivators of VDR, results in a synergistic activation (Figure 4C).

Transcription-stimulation of MN1 can also occur via CACCC-rich elements in promoters. We have studied this mechanism on a natural target of MN1, the *insulin-like growth factor binding protein 5 (IGFBP5)* gene (see chapter 3).[98] MN1 does not bind directly to the CACCC element within the promoter and transcription factors linking MN1 to the CACCC sequence are currently unknown (Figure 4E).

Post-translational modifications of proteins, such as phosphorylation and methylation are important mechanisms to modulate the activity or localization of proteins in cells. Phosphorylation is carried out by kinases and occurs at serine, threonine or at tyrosine

residues. Interestingly, Beausoleil and coworkers identified that MN1 is phosphorylated at the evolutionary conserved serine at aa position 1081.[99] Another large-scale study, aimed at identifying proteins that are methylated at arginine residues, identified that MN1 is methylated around position 943.[100] It is yet to be discovered whether these phosphorylated and methylated residues are important for controlling the activity of MN1.
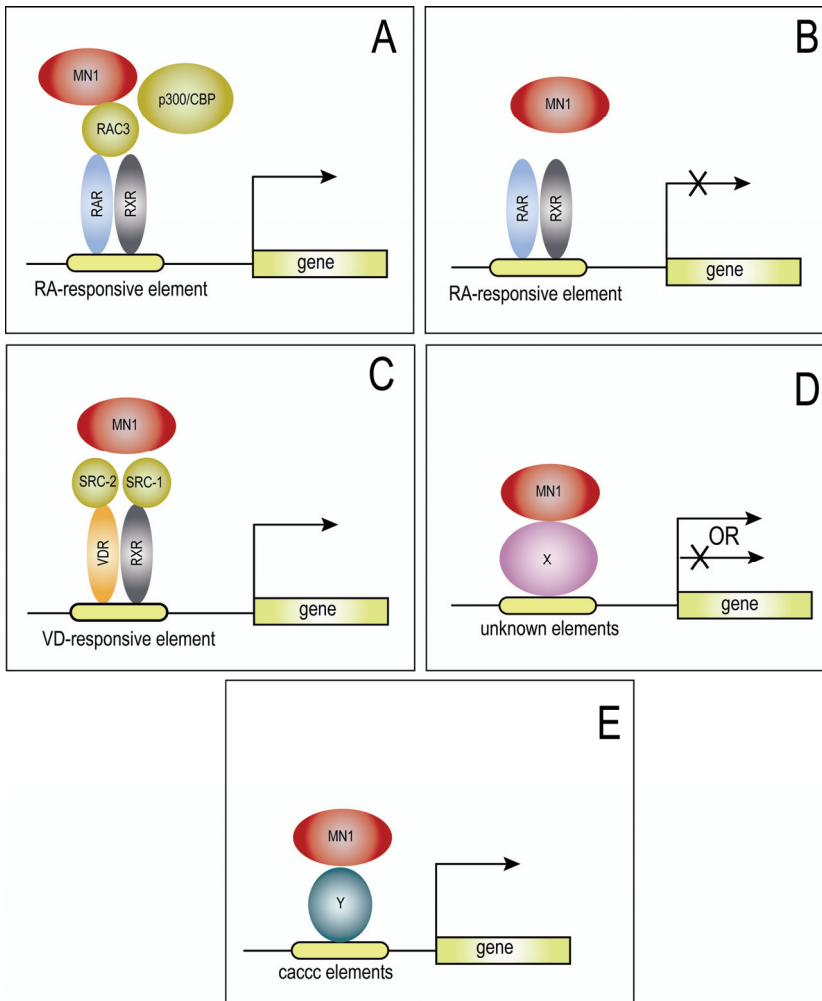
Figure 4. MN1 is a transcriptional cofactor. (A) MN1 is a cofactor that stimulates RAR/RXR-mediated transcription together with p300/CBP and RAC3. (B) MN1 can also inhibit RAR/RXR-mediated transcription. Co-acting proteins are unknown. (C) Together with SRC-1 and SRC-2, MN1 enhances VDR/RXR-mediated transcription. (D) MN1 can both stimulate and inhibit transcription. (E) Indirect binding of MN1 has been proven on the *IGFBP5* promoter. The CACCC-binding transcription factor is unknown (indicated with Y).

Mn1's role in embryogenesis

Whole mount in situ hybridization on mice embryos of various developmental stages has shown that Mn1 is first detected at embryonic day (E) 9.5. Expression has been detected in the midbrain and hindbrain tissues and in craniofacial mesenchyme. At day E10.5 *Mn1* was highly expressed in various structures of the developing face, the developing somites and limb buds. Strong *Mn1* mRNA expression persisted in the brain, in particular in the ventricular zone.[101]

Mn1 knockout mice display severe defects in the bone structures of the skull (see chapter 2) and, in addition, these mice developed a cleft palate, an abnormality that is not compatible with life for newborn mice.[102] Nearly all bones within the skull arise by intramembranous bone formation (i.e. the formation of bone within the flat surface), showing an essential role for Mn1 in this process. In relation to the observations in *Mn1* knockout mice, it is notable that *MN1* expression is found in two osteosarcoma cell lines. The exact origin of these cell lines (from membranous or endochondral bones), however, is not clear.

Liu and coworkers have extended these studies focussing on the function of Mn1 in the developing palate in the *Mn1* knockout mice strain.[101] During normal development, palatal shelves elevate and grow together to fuse and form the roof of the mouth cavity. *Mn1* displayed differential expression within distinct regions of the developing palate. In *Mn1*$^{-/-}$ mice the palatal shelves failed to elevate and fuse and increased apoptosis and reduced proliferation has been detected. Mice studies have identified a set about 10 genes with functions in palatal development. Of those, only *Cyclin D2* and *Tbx22* had greatly reduced expression levels in the palatal structures of the *Mn1*$^{-/-}$ mice and, in wildtype animals, the expression patterns of the genes resembled that of *Mn1* in the developing palate. Both genes are putative downstream targets of Mn1 in the process of palatal outgrowth. Cyclin D2 is an important cell cycle regulator. *Tbx22* is the murine homologue of the human X-linked cleft palate gene. Reporter gene studies using the *Tbx22* promoter have demonstrated that indeed Mn1 is capable of upregulating *Tbx22* levels. Interestingly, various CACCC elements, similar to the ones that were identified as the MN1-target sequence within the *IGFBP5* promoter, are present in the mouse and human TBX22 promoters. It remains to be investigated which other players are involved in this novel molecular pathway regulating murine palatal development.

The group of MacDonald has investigated the role of Mn1 on osteoblast function using the *Mn1* knockout mice. In 2005 they have described the upregulation of MN1 in osteoblasts by vitamin D.[80] The *Mn1* knockout mice provided this research group with a valuable tool to extend these studies. Recently an article was published describing that calvarial osteoblasts

derived from *Mn1* knockout mice were shown to have reduced differentiation, impaired proliferation and produced less mineralized matrix.[103] Normal bone growth is a continuous process of bone formation by osteoblasts and the resorbtion of bone by osteoclasts. Osteoblasts secrete essential factors, like RANKL, a member of the tumor necrosis factor cytokine family, and M-CSF to regulate osteoclasts. Expression of both *RANKL* and *M-CSF* was lower in *Mn1* knockout osteoblasts and reporter gene constructs with the *RANKL* promoter were used to prove that Mn1 stimulates the *RANKL* promoter. Altogether, these results show that Mn1 is important for appropriate osteoblast proliferation and differentiation and regulates osteoclast function through stimulation of *RANKL* gene transcription.

MN1 expression in primitive, undifferentiated cells of different origin
Within the hematopoietic system many different lineages and progenitor cells have been characterized (Figure 1). Wagner *et al* has isolated the long-term hematopoietic stem cells (LT-HSC) by using surface marks CD34+/CD38- and cultured these for several days. Subsequently they have isolated the slow-dividing, more primitive fraction of this pool of cells. Expression profiling has showed that *MN1* is expressed in hematopoietic stem cells and expression is higher in slow-dividing cells, primitive cell populations, which are characterized by asymmetric division and self-renewal.[104]

Heuser *et al* isolated similar bone marrow fractions, although the additional step to isolate slow dividing cells was not included in the isolation procedure. Highest *MN1* expression was detected in the CD34+/CD38- fraction of cells, which represent a pool of cells with LT-HSCs. *In vitro* differentiation of early hematopoietic, stem cell-like cells show that *MN1* was downregulated by day 4, whereas CD34, the marker for hematopoietic stem/progenitor cells, was only downregulated by day 8.[105] Also culturing CD34+ cells in lineage-unspecific cytokines supporting proliferation of early-stage progenitors can not sustain the expression of *MN1*. MN1 function might therefore be most important for uncommitted progenitors.

In addition to the HSC pool, distinct progenitor populations are recognized within the bone marrow that give rise to the different blood cell lineages (Figure 1). Carella *et al* has sorted mouse bone marrow in five different fractions, representing hematopoietic stem cell (HSC), common myeloid progenitor (CMP), common lymphoid progenitor (CLP), granulocyte/ monocyte progenitor (GMP) and megakarocytes-erythrocytes progenitor (MEP). *MN1* expression has been detected in both HSC en GMP fractions, showing highest expression in the GMP fraction. No expression was detected in CLP and MEP and CMP fractions.[52] The CMP is a cell type in between the HSCs and GMPs. There are two possible

explanations for the lack of expression in the CMP fraction. (1) MN1 expression is switched off by transition from HSC to CMPs and subsequently switched on in GMPs. (2) The signal obtained in the HSC pool is derived from 'contaminating' GMP cells within this fraction (personal communication, G. Grosveld, Memphis). This latter explanation could also be true for the two experiments described above. The expression of MN1 in the myeloid lineage, however, is evident and is in line with the fact that leukemias in which *MN1* is overexpressed are all of myeloid origin. In conclusion, *MN1* expression is found in progenitors of myeloid origin and possibly in early stem cells, suggesting that *MN1* expression is important for maintaining the non-differentiated state and possibly involved in self-renewal of cells. Overexpression of *MN1*, as found in leukemias, contributes to maintain the primitive, undifferentiated state.

Expression of *MN1* is not restricted to the hematopoietic system and a similar role for MN1 in maintaining the primitive state of cells in other tissues is possible. This hypothesis is supported by the finding that *MN1* is also enriched in neuronal stem cells[106] and in Wilms' tumor.[107] Wilms' tumor arises from very primitive, embryonic cells that are arrested in differentiation and were originally destined to become kidney cells.

A genome-wide mapping of polycomb-target genes has identified that the promoter of MN1 is occupied by polycomb-repressive complex 1 (PRC1) and PRC2.[85] This observation seems to be in contrast to above results, since these complexes are usually considered as being transcriptional repressors that are required for the maintenance of stem cell populations. Additional research is necessary to understand the regulation of MN1 by polycomb complexes.

MN1 expression in AML and ALL cases

Over the last years, numerous expression profiling studies, aiming at classifying different subtypes of AML, have discovered that *MN1* expression is altered in different leukemia subtypes. Most profound is the overexpression of *MN1* detected in inv(16) AML.[52,108,109,110] All patients investigated in these studies display overexpression of *MN1* and mice studies, discussed below, have shown this is an important independent step in the development of leukemia by the inv(16) fusion protein.[52]

Response to chemotherapy in AML patients is difficult to predict and Heuser *et al* conducted a study to identify an expression profile that can discriminate good from poor responders.[111] A profile of CD34+ bone marrow cells, representing the progenitor/stem cell population, was included for comparison. Blasts of poor response patients displayed a profile that was highly similar to that of the CD34+ cells, indicating that these leukemic cells

retained or regained properties of self-renewal. The smallest discriminating set of genes that was able to discriminate good from poor responders consisted of 11 genes and included *MN1*. *MN1* expression was found to be higher in poor response patients.

Later studies by Heuser *et al* focused on a subgroup of AML patients: cytogenetically normal acute myeloid leukemia (CN-AML). In this group of patients high *MN1* expression was found to be an independent predictor for poor response to chemotherapy, relapse-free survival and overall survival.[105] Langer *et al* have confirmed the prognostic value of *MN1* expression in an independent group of 119 CN-AML patients.[112]

High *BAALC* expression is an independent predictor for poor outcome in CN-AML patients and expression profiling has shown that high *BAALC* expression is correlated to high *MN1* expression.[113] Consistent with this observation is that *NPM1* mutations in AMLs, which are associated with a favorable prognosis, were shown to correlate with lower *MN1* expression.[114]

Occasionally, AML patients are treated with RA in addition to standard chemotherapy. RA stimulates differentiation of hematopoietic cells and a substantial part of patients benefit from this treatment. Molecular studies have revealed that MN1 is a cofactor for RA-induced expression as discussed above. A retrospective study has been performed to evaluate whether *MN1* expression levels and RA-related treatment outcome are associated. Indeed, high *MN1* expression in patients with AML at diagnosis was associated with resistance to RA-related treatment. Only patients with low *MN1* expression benefited from addition of RA to standard chemotherapy.[115,116]

Haferlach and coworkers performed expression profiling on 937 AML and ALL patients in order to classify known leukemia subgroups like inv(16) and *MLL* rearrangements. The profiling enabled them to separate all subgroups from each other and sets of genes were identified that could discriminate these subgroups. The *MN1* gene belongs to a set of genes that can identify AMLs that are characterised by *MLL* rearrangements (11q23).[117] Surprisingly, instead of being upregulated, *MN1* is downregulated in this group of AMLs (personal communication, T. Haferlach). Downregulation of *MN1* expression, by means of promoter methylation, was also detected in the majority of ALL samples and ALL-derived cell lines.[79]

On the whole, the above observations emphasize MN1 as an important player in leukemogenesis and prove that *MN1* expression levels are important. High *MN1* expression levels are unfavorable for AML patients. Either *MN1*-upregulation in several myeloid leukemias or downregulation of *MN1* expression levels in leukemias of lymphoid origin (and the 11q23 subgroup of AMLs) contributes to leukemogenesis.

In recent years, the interest in microRNAs (miRNAs) as an alternative way to regulate gene expression, has been increasing. It has become evident that specific miRNA signatures exist for different tumor types and that altered miRNA expression contributes to carcinogenesis. Several leukemia-related fusion proteins were shown to deregulate the expression of miRNA genes.[56,62] Langer *et al*. has shown that high *MN1* expression in CN-AMLs is associated with a specific miRNA signature consisting of at least 15 miRNAs.[112] Interestingly, there is a negative correlation of miR-16 with high *MN1* expression. This miRNA targets the *BCL2* gene and in case of high MN1 expression, an indirect upregulation of *BCL2* is thus expected. BCL2 has proven anti-apoptotic functions and is frequently overexpressed in leukemia.[118] The indirect upregulation of *BCL2* by means of downregulating miR-16 might be one of the leukemogenic pathways involved in the leukemias that overexpress *MN1*. This is strengthened by the observation of Garzon *et al* who has described the upregulation of miR-16 in response to retinoic acid-induced differentiation of human acute promyelocytic leukemia.[119]

MN1 overexpression in mouse bone marrow

Forced overexpression of *MN1* in bone marrow (BM) stimulates the outgrowth of myeloid cells. Cells rapidly proliferate and acquire a growth advantage over the non-transduced cells. Mice receiving transplants of these *MN1*-overexpressing BM cell lines rapidly develop myeloproliferative disease (MPD). MPD is a condition slightly different from AML in which myeloid cells are highly proliferative but still differentiate into mature cells. Both the *in vivo* and *in vitro* experiments show that MN1 stimulates the proliferation of myeloid cells, but does not block their differentiation.[52] The growth stimulatory effects of MN1 in bone marrow cells is opposite to the inhibition of proliferation in osteosarcoma cells, as described by Sutton *et al*,[80] showing that MN1 can have opposite effects depending on the cell type in which it is expressed. Contradictory to this latter result is the decreased proliferation that is detected in calvarial osteoblasts derived from the *Mn1* knockout mice.[103]

Co-expression of the inv(16) fusion protein Cbfβ-SMMHC and MN1 in bone marrow causes AML in mice within 5 to 8 weeks after transplantation, whereas Cbfβ-SMMHC expression alone does not result in hematopoietic disease.[52] Furthermore, *MN1* is not upregulated by the fusion protein itself. Altogether, this shows that MN1 is an important independent step in the development of leukemia caused by inv(16).

Heuser and coworkers have performed similar mouse experiments, but the outcome was slightly different. In contrast to the above described mouse models, mice transplanted with *MN1*-transduced BM did develop an AML phenotype. *In vitro* experiments have shown

that MN1 both stimulates proliferation and blocks differentiation. The blocking of differentiation depends on the transcriptional repressor activity of MN1, whereas the promotion of self-renewal and proliferation is carried out by the stimulatory properties of MN1.[116] One of the differences between these two separate mouse experiments is the promoter that controls the expression of *MN1* in the BM cell lines. Heuser *et al* have used the spleen focus forming virus promoter, whereas Carella *et al* have used the murine stem cell virus (MSCV) promoter to control expression of *MN1*. Possibly the timing or level of expression of MN1 differs in these experiments. Also a slight difference in the subset of cells that express MN1 might account for the differences observed.

Other studies that have provided evidence for the involvement of Mn1 in the development of leukemias of different origin are based on retroviral insertional mutagenesis. CALM-AF10 and NUP98-HOXD13 are two fusion proteins that are formed by leukemia-causing translocations (2;11) and (10;11). Transgenic mice expressing either one of the fusion proteins develop leukemia, although in both mouse models the penetrance is not 100%.[120,121] To identify genes that might collaborate with the fusion proteins, retroviral insertional mutagenesis has been performed. Integration of the retrovirus accelerated leukemogenesis in both mouse models and viruses were shown to be integrated adjacent to well known leukemia-associated genes, such as *Meis1* and *Evi1*, proving the validity of this method. In both mouse models, insertions near the *Mn1* locus have been found.[122,123,124] In NUP98-HOXD13 mice, the level of *Mn1* expression has been analyzed and all mice with integrations near the *Mn1* gene showed upregulation.[124]

THE LEUKEMOGENIC FUSION PROTEIN MN1-TEL

t(12;22)(p13;q11) in leukemia

The t(12;22)(p13;q11) is only found in a minority of AML patients. The translocation fuses the first exon of *MN1* to exon 3 of the *TEL* (*ETV6*) gene, resulting in a fusion protein that consists of almost the entire *MN1* coding region (aa 1 to 1260) and aa 56 to 452 of the TEL protein. The reciprocal product of this translocation, *TEL-MN1*, is only 113 aa long and has been detected in patient material.[88] Biological relevance for this reciprocal product has not been shown, however. Another patient has been described in which the breakpoint in the TEL gene is located in intron 3. This translocation results in an in-frame fusion of the first exon of *MN1* with aa 110 to 452 of TEL.[87]
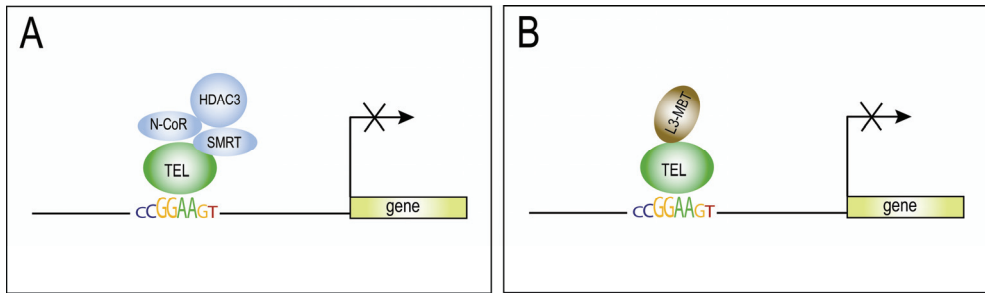
Figure 5. TEL is a sequence-specific transcriptional repressor. (A) TEL represses transcription by the recruitment of complexes containing N-CoR , SMRT, and HDAC3. (B) Another route of repression is by a histonedeacetylase-independent mechanism and involves the recruitment of L3-MBT, a polycomb-group protein. TEL-responsive element is decuded from data that appeared in thesis of A.Buijs.[143]
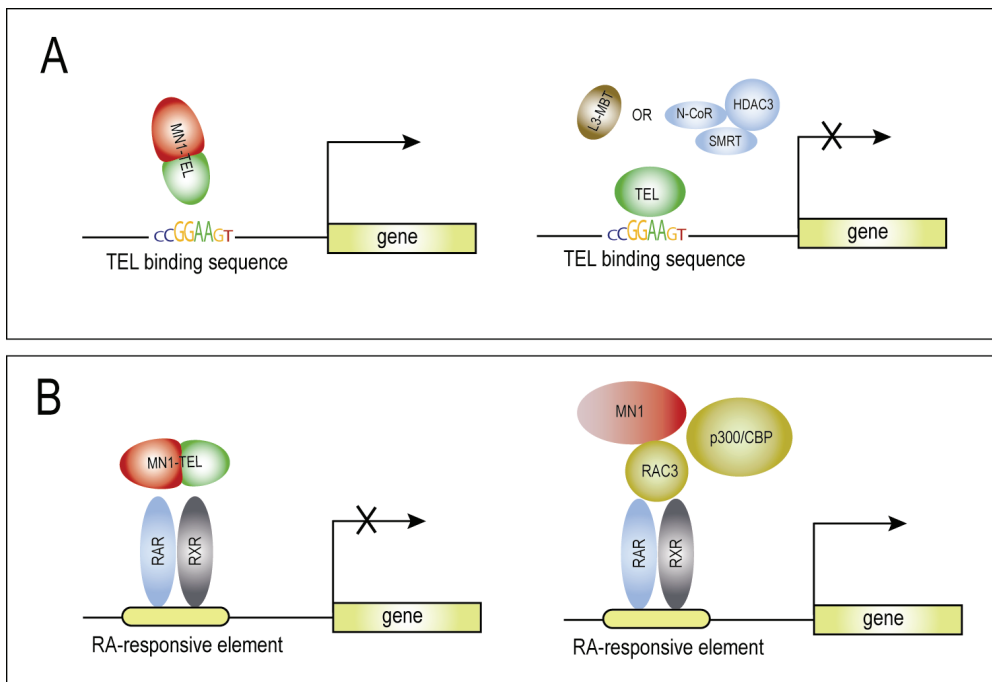
Figure 6. The fusion protein MN1-TEL influences the normal functions of TEL and MN1. (A) MN1-TEL has been shown to bind to TEL binding sequence. It interferes with the normal function of TEL by stimulating genes that are normally repressed by TEL. Normal function of TEL is shown on the right. (B) MN1-TEL is a dominant-negative mutant of MN1 and inhibits RAR/RXR-mediated transcription. Normal function of MN1 as a cofactor for RAR/RXR is shown on the right.

TEL is a sequence specific repressor

TEL, also known as ETV6, is a transcriptional repressor that binds to sequence specific factors that consists of 26 members. Most of them are transcriptional activators instead of

repressors, like TEL.[125] Other members of this family include the TEL2, ETS1 and PU.1 proteins. ETS family members are important regulators in various biological processes, including cellular proliferation, differentiation, hematopoiesis, apoptosis, metastasis, angiogenesis and transformation.[126] Many ETS family members were shown to be involved in different cancer types, including breast cancer and prostate cancer.[127] An example is the ETS family member ERG; the *TMPRSS2-ERG* fusion has been detected in up to 70% of prostate cancer.[128,129] TEL is expressed throughout the body including the hematopoietic system and is crucial for HSC maintenance, as has been shown in *Tel* knockout mice.[130] These mice have been shown to die due to yolk sac angiogenesis. Chimeras with *Tel*$^{-/-}$ ES cells have shown that Tel function is also essential for the establishment of hematopoiesis of all lineages in the bone marrow during normal transition of hematopoietic activity from fetal liver to bone marrow.[131] Members of the ETS family are highly homologous in their helix-loop-helix (HLH) and DNA binding domains (DBD). Most members of the family bind to a core DNA sequence consisting of GGAA. [126] The surrounding sequences define the binding specificity for the different members of the family.[132] The most common identified TEL-responsive element is CCGGAAGT. The HLH domain provides the TEL protein an interface for homodimerisation with other TEL proteins and heterodimerisation with other members of the family.[125,133] After binding to its responsive sequence, corepressors N-CoR and SMRT are recruited to TEL and bind to the region between the HLH and DBD domains. Subsequently, HDAC3, a histone deacetylase, is recruited.[134] Removal of histone acetyl groups by HDAC3 will compact the DNA around the histones, making the DNA less accessible for transcription initiation complexes (Figure 5A). Another route of transcriptional repression for TEL is carried out by the binding to L(3)MBT polycomb group protein.[135] This mechanism of repression has been shown to be independent of histone deacetylation and is thought to facilitate long-term repression of TEL-target genes (Figure 5B). Post-translational modicifations of TEL involve phosphorylation[136,137,138,139] and sumoylation[140,141] and regulate the activity of the protein.

Leukemogenic mechanisms of MN1-TEL

The *MN1-TEL* fusion encodes a protein that combines the transcription activating domains (TAD) of MN1 with the DBD of TEL. Although most of the HLH domain is present in the fusion protein, it is not functional.[142] The presence of a TAD and DBD suggests that the MN1-TEL protein functions as an altered transcription factor that will stimulate genes that are normally repressed by TEL. Expression profiling of MN1-TEL-overexpressing cells have shown that MN1-TEL can indeed stimulate genes that are normally regulated by ETS

transcription factors (Figure 6A and chapter 5).[97] The list of genes, however, is very small and the most noticeable activity of MN1-TEL that has been detected in this study is the inhibition of the stimulatory effects of RA on a large set of genes. Furthermore, MN1-TEL is unable to stimulate a large set of genes that are induced by MN1. The interplay between MN1 and MN1-TEL on RAR/RXR-mediated transcription has been studied in more detail using the MSV-LTR (see chapter 4).[144] These studies have also shown that MN1-TEL represses RAR/RXR-mediated transcription and exhibits dominant-negative effects on both MN1 and RA (Figure 6B).

MN1-TEL expression in mouse BM

Kawagoe *et al* have inserted the MN1-TEL fusion protein in the *Aml1* locus in mice.[145] *Aml1* is expressed in primitive progenitors of both myeloid and lymphoid lineages. BM cell lines derived from these mice have shown that MN1-TEL, expressed in both lineages, stimulates the proliferation and blocks the differentiation of both myeloid progenitors and T lymphocytes. In humans, *MN1-TEL* is under control of the *MN1* promoter. The *MN1* promoter is activated in the myeloid lineage and this determines that in t(12;22) patients the myeloid lineage and not the lymphoid lineage is affected by expression of MN1-TEL.

Mice expressing *MN1-TEL* from the endogenous *Aml1* promoter do not develop AML, but T-lymphoid tumors are found. In some mice expansion of myeloid progenitors has been detected.[146] MN1-TEL thus requires secondary mutations to cause AML in these mice. *HOXA9 is* an important transcription factor for normal myeloid proliferation and differentiation and elevated expression is frequently observed in myeloid leukemia, including two MN1-TEL AML patients. Transduction of *HOXA9* in BM of mice causes AML and the co-expression of *HOXA9* and *MN1-TEL* accelerated the onset. Expression profiling of tumor material of these mice have shown that *N-Myc* is highly upregulated and contributes to the AML pathogenesis in these mice. In contrast to MN1-TEL expression from the *Aml1* locus, expression of MN1-TEL in mouse BM using a viral transduction system is sufficient to cause an AML phenotype.[147] This might be explained by the much higher expression level of MN1-TEL generated by viral transduction.

OUTLINE OF THIS THESIS

In order to unravel the function of the *MN1* oncogene and the molecular mechanisms involved in its normal function and its oncogenic activity, different research lines were conducted over the last years in our laboratory. The fusion gene *MN1-TEL* has also been studied within these research projects.

An important tool to study the function of a gene is the generation of a knockout mice strain. By homologous recombination *Mn1* null mice were created and in **chapter 2** the phenotype of these mice has been described. We have performed Caesarean sections to obtain embryos of various embryonic stages, ranging from embryonic day 8.5 to 18.5. The embryos were extensively studied using macroscopy, microscopy and whole-embryo bone and cartilage stainings (alizarin red and alcian blue stainings). Perinatal death of the *Mn1*$^{-/-}$ mice has prevented us from studying the effect of omission of *Mn1* in adult mice. *Mn1* heterozygotes were alive after birth and were followed for 30 months in order to obtain data about long-term effects of the presence of only one functional *Mn1* gene.

Additional molecular studies on the MN1 protein have focused on its ability to stimulate transcription. Although MN1's involvement in transcription regulation had been recognized soon after cloning, the MN1-responsive elements remained largely unknown. An oligo selection assay was performed to identify these DNA sequences. As described in **chapter 3** several DNA elements were identified. The coordinated expression of *MN1* and *IGFBP5* in meningioma samples has prompted us to screen the regulatory sequences of the *IGFBP5* gene for MN1-responsive elements. We have cloned several regions of the *IGFBP5* promoter in luciferase reporter plasmids en have tested the ability of MN1 and MN1-TEL to activate the cloned promoter regions in transient transfections in two different cell lines. In similar assays we tested previously described stimulatory reagents of the *IGFBP5* promoter is combination with MN1 and MN1-TEL. Electrophoretic mobility shift assays were performed to prove the binding of MN1 to distinct regions within the promoter.

The fusion gene *MN1-TEL* harbors large sequences of both partner gene *MN1* and *TEL*. The DNA binding ability of the TEL moiety strongly suggests that MN1-TEL functions as a deregulated transcription factor that binds to elements that are normally bound by TEL. Additionally, MN1-TEL could possibly influence the normal function of MN1, independent of its DNA binding capacity. Research described in **chapter 4** has addressed this latter question. We have used a viral promoter that harbors many transcription factor-binding sites, including a RAR/RXR-responsive element that is highly activated by MN1. We have studied the activity of MN1-TEL on this element and the cooperation of MN1 and MN1-TEL with cofactors p300/CBP and RAC3. The TEL moiety of the MN1-TEL fusion protein

contains a strong DNA-binding domain that creates a complex situation in which MN1-TEL can influence the normal function of both TEL and MN1. The use of a point mutant of MN1-TEL that can not bind to DNA has enabled us to investigate the function of MN1-TEL in relation to MN1 without interference of the DNA binding properties of the TEL moiety.

The interest in the *MN1* gene has increased over the last years because of expression-profiling studies showing that this gene is upregulated in several subtypes of leukemia. The most important are the (inv)16-diagnosed patients and the cytogenetically normal AML patients. In the latter group, upregulation of *MN1* is associated with worse survival and poor response to treatment. Additionally, a study has described that a high *MN1* expression level in AML patients is a predictor for resistance to RA therapy and this is explained by the fact that MN1 functions as a cofactor, both inhibitory and stimulatory, for RAR. Identification of genes that are regulated by MN1 without or in combination with RA provides tools to further study the pathways underlying MN1-involved leukemic processes. In **chapter 5** we have described the establishment of leukemic cell lines with inducible expression of *MN1* or *MN1-TEL*. Expression profiling of these cell lines under various conditions, i.e. with and without expression of MN1 or MN1-TEL at various time points after induction in the presence or absence of RA, has allowed us to identify the genes that responded to any of the conditions tested. Quantitative reverse transcriptase PCRs were performed to verify the effects observed in the expression arrays. Several interesting candidates were identified that have shed light on the molecular pathways involved in leukemias in which MN1 and MN1-TEL are involved.

In **chapter 6** the papers presented in this thesis are discussed in relation to recent literature. Furthermore, it provides leads for future research on MN1 and MN1-TEL.

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CHAPTER 2

Targeted disruption of the *Mn1* oncogene results in severe defects in development of membranous bones of the cranial skeleton

Magda A. Meester-Smoor,[1] Marcel Vermeij,[1] Marjolein J.L. van Helmond, [1] Anco C. Molijn,[1] Karel H.M. van Wely,[1] Arnold C.P. Hekman,[1] Christl Vermeij-Keers,[2] Peter H.J. Riegman,[1] Ellen C. Zwarthoff[1]

[1]*Department of Pathology, Josephine Nefkens Institute, Erasmus MC, Rotterdam, The Netherlands*
[2]*Department of Plastic and Reconstructive Surgery, Erasmus MC, Rotterdam, The Netherlands*

ABSTRACT

Fusion of the *MN1* gene to *TEL* (*ETV6*) results in myeloid leukemia. The fusion protein combines the transcription activating domain of MN1 and the DNA binding domain of TEL and is thought to act as a deranged transcription factor. In addition, disruption of the large first exon of the *MN1* gene is thought to inactivate MN1 function in a meningioma. To further investigate the role of *MN1* in cancer, we generated *Mn1* knockout mice. $Mn1^{+/-}$ animals were followed for 30 months, but they had no higher incidence of tumor formation than wild type littermates. *Mn1* null mice, however, were found to die at birth or shortly thereafter as the result of a cleft palate. Investigation of newborn or embryonic day 15.5 (E15.5) to E17.5 null mice revealed that the development of several bones in the skull was abnormal. The affected bones are almost exclusively formed by intramembranous ossification. They are either completely agenic at birth (alisphenoid and squamosal bones and vomer), hypoplastic or deformed (basisphenoid, pterygoid, and presphenoid), or substantially thinner (frontal, parietal and interparietal bones). In heterozygous mice hypoplastic membranous bones and incomplete penetrance of the cleft palate were observed. We conclude that Mn1 is an important factor in development of membranous bones.

INTRODUCTION

The *MN1* gene, localized on human chromosome 22, was cloned by our research group in 1995 as a candidate gene for sporadic meningioma, a benign brain tumor arising from the arachnoidal cap cells found on the surface coverings (called meninges) of the brain.[1] In a meningioma, the *MN1* gene was found disrupted by a balanced translocation (4,22)(p16;q11). The breakpoint of the translocation lies within the first exon of the *MN1* gene, and the (truncated) protein was not detected. However, no mutations or deletions of the *MN1* gene were found in other meningiomas; thus the causative relationship between MN1 and meningiomas remains unclear. Subsequently, the *MN1* gene was found to play a role in acute myeloid leukemia (AML).[2] The translocation (12;22)(p13;q11) creates a fusion between the *MN1* and *TEL* (or *ETV6*) genes, resulting in the *MN1-TEL* gene. The TEL protein is a member of the ETS family of transcription factors and contributes its C-terminal DNA binding domain (DBD) to the fusion protein MN1-TEL. The MN1 protein donates 1259 amino acids, 95% of its entire length, to the fusion protein. The fusion protein MN1-TEL has transforming activity on NIH3T3 cells and most likely acts as a deregulated transcription factor. MN1-TEL may adhere to genes via the TEL moiety and activate these genes whereas they are normally controlled by the repressor TEL.[3]

The MN1 protein comprises 2 exons and encodes a protein of 1319 amino acids. The amino acid sequence shows no homology to other proteins or with specific domains with known functions. However, several proline/glutamine-rich regions and a polyglutamine stretch are present and point to a function in transcription regulation. We have shown previously that MN1 activates the transcription activity of the Moloney sarcoma virus long terminal repeat (MSV-LTR) and comprises multiple transcription activating domains.[3] We recently described that MN1 can act on the MSV-LTR (and other promoters)(unpublished results) as a transcription coactivator in retinoic acid receptor (RAR)-retinoid-X-receptor (RXR)-mediated transcription leading to a synergistic induction of expression when MN1 and the RAR-RXR ligand retinoic acid (RA) are combined. Furthermore, we have shown that there is an interplay between MN1 and p300 and RAC3, both known coactivators of retinoic acid receptors.[4]

Hybridization of a cDNA probe to a blot containing DNA of a number of different species shows signals in species as evolutionarily distant as *Xenopus laevis* and *Drosophila melanogaster*, proving conservation of MN1 in evolution.[1] The identity/similarity between human and murine MN1 proteins is 93%. Database searches reveal homologues of MN1 in mice (*Mus musculus*) and puffer fish (*Tetraodon nigroviridis*). The puffer fish MN1 homolog

shows an identity/similarity of 64% with the human gene; the structure of the gene however is different. The gene consists of at least four exons; two additional small introns of 16 and 32 bp are present in the region covered by exon 1 in humans and mice. Surprisingly, in puffer fish, MN1 has less pronounced proline/glutamine-rich regions and lacks the large glutamine stretch (28Q) present in human and mice half way in the protein. The importance of the glutamine stretch is, however, underlined by the fact that the puffer fish MN1 protein contains a large glutamine stretch (15Q) at a more N-terminal position, which is completely absent in humans and mice.

To determine the physiological significance of MN1 *in vivo*, we disrupted the *Mn1* gene in mice. The majority of heterozygous mice (*Mn1*^{+/-}) showed no obvious defects and appeared to have a normal lifespan and tumor incidence was not altered when compared to wild-type littermates. In *Mn1* null mice (*Mn1*^{-/-}) cranial bone development is severely affected with several bones being completely absent or hypoplastic. In addition, null mice have a cleft palate and, due to these defects, do not survive beyond day 1 after birth. A minor percentage of heterozygotes also die due to a cleft palate. Closer examination of *Mn1*^{+/-} mice revealed that they have an intermediary deficiency in cranial bone formation.

MATERIALS AND METHODS

Construction of targeting vector

A cosmid clone was isolated from C129 cosmid library[5] containing the mouse *Mn1* gene using a human MN1 cDNA probe C.[1] A *Bam*HI/*Eco*RI fragment containing exon 1 was cloned in a targeting vector pMC1neo poly(A).[6] The *neomycin* resistance gene (*neo*) was inserted into exon 1 of the *Mn1* gene using *Bam*HI/*Xho*I sites. The *neo* gene is controlled by the herpes simplex virus promoter, and the transcriptional orientation is opposite to the orientation of the *Mn1* gene (Figure 1A).

Generation of Mn1^{+/-} and Mn1^{-/-} mice

The targeting vector was electroporated into strain 129 embryonic stem (ES) cells and G418 resistant clones were isolated. G418 resistant clones were checked for the presence of the *neo* gene at the correct location using Southern blot hybridisation on *Nco*I digested DNA and a genomic probe upstream of the targeting construct. Targeted ES cells with correct integration of the *neo* gene and with the normal number of chromosomes (40) were injected into blastocysts of C57BL/6 mice. The blastocysts were injected into (strain FVB) foster mothers, and chimeras were obtained. Chimeric, transmitting males were crossed with

normal FVB females for 10 generations to produce an inbred strain. Mice were genotyped by Southern blots or by PCR using three primers. Primer MuMn1-F (5'-CTTTGGGGGCAACTTCGGTGG-3') is a forward primer localized within exon 1 upstream of the inserted *neo* gene. Primer MuMn1-R (5'-CTCCAGACCCACAGGCATC-3') is a reverse primer located in the part of exon 1 deleted by the integration of the *neo* gene and thus primes only on the wild-type *Mn1* allele, creating a 627 bp product in combination with MuMn1-F. The third primer is Neo-R (5'-GATGCAATGCGGCGGCTGCA-3') and in combination with primer MuMN1-F detects the mutant allele resulting in a PCR product of 528 bp.

Examination of embryos, and newborn and adult mice: macroscopy, microscopy and alizarin red/alcian blue staining

To obtain embryos of different stages of development (embryonic day 8.5 (E8.5) to E18.5), cesareans were performed on pregnant females. The day when a vaginal plug appeared was designated as E0.5. Extensive macroscopic and microscopic analysis was performed on embryos and newborn mice. Embryos were weighed and lung, heart, pancreas, spleen, liver, stomach, intestine, bladder, sex organs, thymus, and glands were dissected and macroscopically examined. They were fixed by immersion in 4% buffered formalin and embedded in paraffin. In addition, complete embryos were weighed, fixed, decalcified using EDTA for 5 days and embedded in paraffin. Four-micrometer sections were deparaffinized in xylene, rehydrated and hematoxylin-eosin (HE) stained and microscopically examined. Alizarin red/alcian blue staining of complete embryos or newborn mice was performed using standard methods as described by Kaufman.[7] Briefly, complete embryos were deskinned, and organs removed and fixed in 80% EtOH (2 days), dehydrated in 96% EtOH (2 days) followed by aceton (2 days), stained with alizarin red/alcian blue at 37°C for 2 days and subsequently cleared using 1% KOH for several days. Finally, the specimens were stored in glycerol.

RESULTS

Mn1+/- mice have a normal lifespan

In order to generate *Mn1* knockout mice, a targeting construct was generated (Figure 1A). Disruption of the *Mn1* gene at the *Bam*HI site results in a truncated protein of 121 amino acids, deleting over 90% of the protein, including the transcription activating domain and the glutamine stretch. Chimeras successfully transmitted the targeted allele to the germ line,

and after crossing, mice harboring only one functional *Mn1* gene were obtained (*Mn1*$^{+/-}$). Mice were genotyped using either Southern blot hybridisation of *Nco*I-digested tail DNA (Figure 1B) or by PCR using 3 primers simultaneously in one reaction (Figure 1C). Because of the involvement of the *MN1* gene in AML and its putative role in sporadic meningiomas, we determined whether the *Mn1*$^{+/-}$ genotype resulted in excess tumor formation or other forms of pathology. To this end, heterozygous males (n=143) and females (n=95) and wild type siblings (127 males and 93 females) were followed for 30 months. No significant differences were observed in lifespan of *Mn1*$^{+/-}$ or *Mn1*$^{+/+}$ animals. Autopsies were performed on 47 *Mn1*$^{+/-}$ and 37 *Mn1*$^{+/+}$ animals that either died spontaneously or that were killed because of apparent lack of well-being. No significant differences in number of tumors between heterozygous or wild-type controls were observed. Macroscopic and microscopic examination of heart, lungs, kidney, spleen, liver and muscle also revealed no abnormalities.
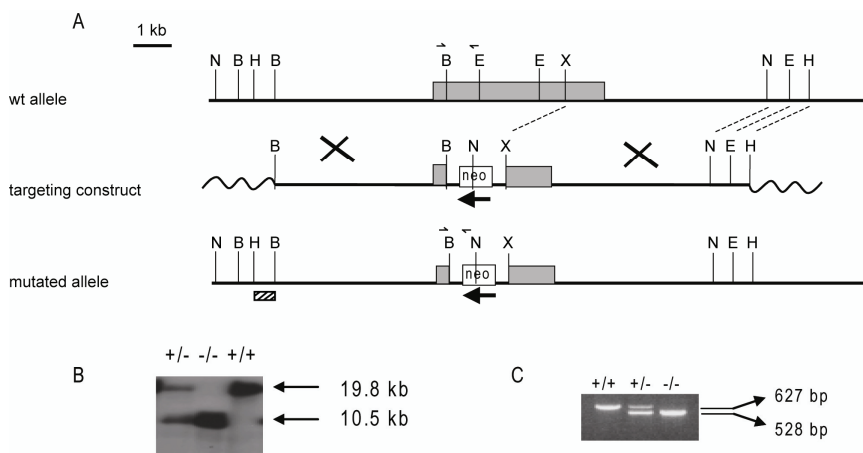
Figure 1. Targeted disruption of the mouse *Mn1* gene. (A) Restriction enzyme map showing relevant sites (N, *Nco*I; B, *Bam*HI; H, *Hin*dIII; E, *Eco*RI; X, *Xho*I) in the first exon of *Mn1* (top), the structure of the targeting vector (middle), and the targeted gene (bottom). The transcriptional orientation of the *neomycin* resistance gene (*neo*) is indicated with an arrow. The probe used for Southern blot hybridisation, a 0.6 kb *Hin*dIII/*Bam*HI fragment, is indicated as a hatched box. Primers are indicated with arrowheads. (B) Southern blot analysis of *Nco*I-digested genomic DNA of a mouse homozygous for the wild-type allele (+/+), a heterozygous mouse (+/-) and a mouse homozygous for the mutated allele (-/-). Wild-type (wt) and targeted alleles show bands of 19.8 and 10.5 kb, respectively, after hybridization with the indicated probe (hatched box). (C) PCR genotyping of mice using one forward primer and two reverse primers (sequences mentioned in Materials and Methods). The wt allele gives a band of 627 bp; the mutant product detected with the reverse primer located in the neomycin resistance gene is 528 bp.

Mn1 null mice and a minor percentage of Mn1 heterozygotes die shortly after birth due to a cleft secondary palate

Crosses between $Mn1^{+/-}$ mice failed to produce viable $Mn1^{-/-}$ mice. However, examination of embryos at various developmental stages (E8.5 to E18.5) showed that $Mn1^{-/-}$ mice were present in numbers compatible with normal Mendelian inheritance (Table 1).

TABLE 1. Genotyping of embryos of different embryonic stages

Embryonic stage	No.(%) of embryos with genotype		
	+/+	+/-	-/-
E 8.5	0	3	4
E 10.5	4	11	1
E 12.5	4	11	11
E 14.5	4	8	4
E 15.5	1	6	1
E 16.5	4	2	3
E 17.5	2	5	3
E 18.5	3	5	8
Total	22 (20)	51 (47)	35 (32)

The $Mn1^{-/-}$ or $Mn1^{+/-}$ embryos of various developmental stages did not differ from their wild-type siblings in weight, length and external appearance. These mice were examined microscopically and no abnormalities were observed in any of the internal organs including lung, heart, thymus, liver, kidney, pancreas, adrenal glands and sex organs. Subsequently, a new series of breedings were closely monitored at the moment of delivery. A 10h observation of newborn pups showed that a subset of pups died immediately following birth because they failed to start breathing, apparently because of breathing difficulties. A second group of pups did not suckle (empty stomachs), were lethargic, became cyanotic and subsequently died. Genotyping of all dead pups showed that most of these pups (83%) are homozygous mutants. To our surprise, the remaining group of dead pups (17%) was genotyped as heterozygotes (Table 2). We first examined whether the lungs of the *Mn1* null mice displayed any abnormalities. Lungs of *Mn1* null mice and wild-type animals were examined by light and electron microscopy and were found to be normal (data not shown). Subsequently, total RNA was isolated from lungs of postnatal day 0 (P0) $Mn1^{-/-}$, $Mn1^{+/-}$ and

wild-type animals. The mRNA levels of *surfactant A, B and C*, factors known to be crucial for proper lung function, were analysed using quantitative reverse transcription-PCR and again no differences were found (data not shown). Subsequently, a closer macroscopic examination of the head region of newborn mice revealed that all dead mice had a cleft secondary palate. In mice, this defect is not compatible with life. The cleft secondary palate was clearly visible on inferior views of the cranial base of the heads (Figure 2A). Wild-type pups showed normal fusion of the palatal shelves while mutant mice do not. Three out of 21 (15%) heterozygotes also had a cleft secondary palate, but the defect was less severe when compared to null mice: the palatal shelves were closer to each other in transversal direction leaving a cleft of only about half the distance when compared to homozygous *Mn1* knockouts. The palatal shelves in affected heterozygotes did form small rugae and were fused with the primary palate. Figure 2B and C show coronal sections of heads of newborn embryos at different positions. In wild-type animals the palate (p) has been formed correctly (Figure 2B, left panel). In null mice (*Mn1*$^{-/-}$) the palatal shelves (ps) had developed (right panel of Figure 2B) but were far apart and had not fused, resulting in a connection between the nasal and oral cavities. A cleft palate is frequently seen in mouse knockout models, and in some models this is accompanied by agenesis of molars.[8,9] In *Mn1*$^{-/-}$ mice, however, molars of both upper and lower jaw were correctly formed (Figure 2C, left and right panels).

TABLE 2. Close examination of newborn pups

Status of newborn pups	No (%) of pups of genotype			Total (%)
	+/+	+/-	-/-	
Total	8 (18)	21 (48)	15 (34)	44 (100)
Viable	8	18	0	26
Dead[a]	0 (0)	3 (17)	15 (83)	18 (100)

[a] pups either died directly after delivery or within the first 10 hours

Skulls of mutant mice at E15.5 to E18.5 display many bone abnormalities

The process of palate formation is complex and depends on the correct position and formation of bone and soft tissue structures within the skull.[10,11] In order to visualize bone formation in the skull, wild-type, heterozygous and homozygous mice at different stages of development (E15.5 to E18.5) were deskinned and stained with alizarin red and alcian blue.
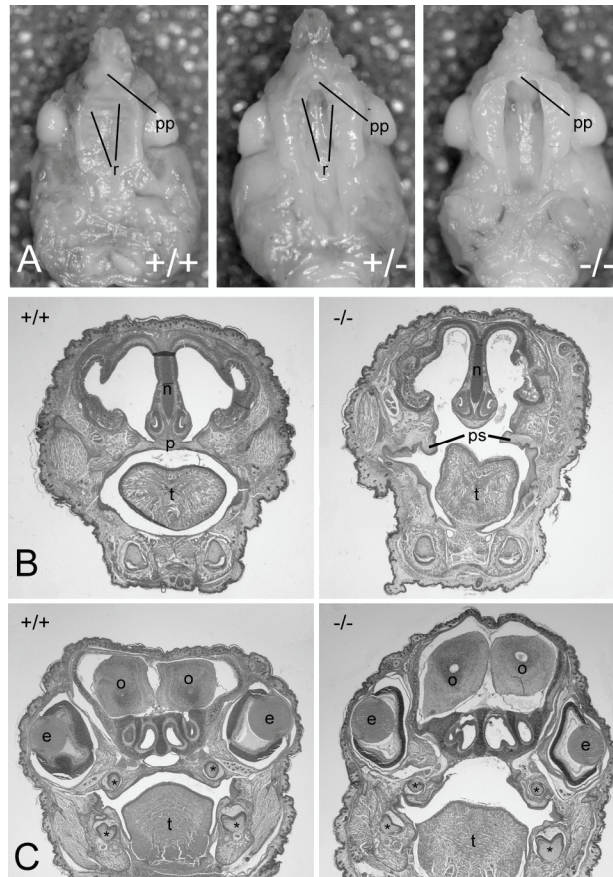
Figure 2. Inferior views and histological sections of heads of newborn pups. (A) Normal palate with rugae (r) in a wild-type (wt;+/+) animal (left panel), and cleft secondary palate in a *Mn1*+/- and *Mn1*-/- animals (middle and right panels). Small rugae on both sides of the cleft are seen in the heterozygote. pp, primary palate. (B and C) Coronal sections showing the correctly formed palate (p) in wt animals (left panels). Palatal shelves (ps) are not fused in *Mn1*-/- animals (right panels). Molars in maxilla and mandible are indicated with asterisks. e, eyes; o , olfactory lobe; n, nasal septum; t , tongue.

Figure 3A shows inferior views of the skulls from which the mandibles were removed. At E15.5 the palatal shelves have just fused in wild-type mice (+/+) and bone formation took place from both maxillary and palatine shelves. The increase in bone size within the palate is clearly visible at stage E16.5 (top, middle panel), and at stage E17.5 the bone structures from both sides had formed sutures (top, right panel). The *Mn1*-/- embryos had a cleft secondary palate and the bone growth within the palatal shelves (both maxillary and palatine) is hypoplastic at all stages (Figure 3A, bottom panels). Many additional abnormalities were obvious in the skulls of *Mn1*-/- mice. Several bone structures were absent (agenic) or were severely hypoplastic. In the E17.5, *Mn1*+/+ skull, the alisphenoid (a) and

squamosal (s) bones and the vomer (v) in a wild-type sibling are indicated. These bones were completely absent in the $Mn1^{-/-}$ mouse (Figure 3A, bottom, -/-). Furthermore, the presphenoid (ps) and basisphenoid (bs) were hypoplastic and the pterygoid bones (pt) were not formed correctly. The heterozygous $Mn1$ mice (Figure 3A, middle panels) showed intermediate defects. In the palate, bone structures in both the maxillary and palatine shelves are hypoplastic. In addition, bones that were agenic or hypoplastic in null mice (basisphenoid, alisphenoid and squamosal bones, and vomer) were also clearly hypoplastic in $Mn1^{+/-}$ mice when compared to wild-type mice. The $Mn1^{+/-}$ mice of E15.5 and E16.5 had a closed palate; the $Mn1^{+/-}$ mouse of E17.5 had a cleft palate. These results show that the heterozygous $Mn1$ mice display an intermediate phenotype when compared to $Mn1$ null mice. This suggests that the extent of the defect may be related to dosage of the Mn1 protein.

We also stained heads of E18.5 mice to study the bone structures of the skull at the moment close to delivery. The dorsal view of the skull shows the normal development of the supraoccipital bone in a wild-type mouse (Figure 3B, top). Bone formation starts in two ossification centers at E17 and fusion takes place at around P0. The formation of this bone in the $Mn1^{-/-}$ mouse is delayed (Figure 3B, bottom). Heterozygous mice (+/-, middle panel) had a normally developing supraoccipital bone. Figure 3C shows a detail of the E18.5 skull in lateral view. The alisphenoid and squamosal bones were agenic in the $Mn1^{-/-}$ mouse (lower panel). The differences between wild-type (+/+) and heterozygotes (+/-) at this stage were less pronounced, compared to differences seen at E15.5 and E16.5. At E18.5 the squamosal and alisphenoid bone were hypoplastic in heterozygotes (+/-) and the suture formation between these bones is in a less advanced stage. The bone within these structures is less dense, and the foramen ovale (for the mandibular nerve) and the foramen rotundum (for the maxillary nerve) are clearly larger, altogether indicating a delayed development of both bone structures in the $Mn1^{+/-}$ mouse. A closer look at the skulls of $Mn1$ knockout mice and wild-type mice gave us the impression that all flat bones forming the roof of the skull (frontal bone, parietal and interparietal bone) were thinner in $Mn1^{-/-}$ mice.

Figure 3. Alizarin red/alcian blue staining of skulls. (A) Inferior views of skulls (mandibles removed) of E15.5, E16.5 and E17.5 mouse embryos. Wild-type (+/+), heterozygote $Mn1$ (+/-) and homozygote $Mn1$ (-/-)mice are shown in upper, middle and lower panels, respectively. Within E17.5, the affected bones in $Mn1^{-/-}$ and $Mn1^{+/-}$ mice are indicated. In the E17.5 $Mn1$ null mouse skull bones are indicated for orientation purposes. (B) Dorsal view of skulls of E18.5 mice showing the exoccipital and supraoccipital bones. Arrows indicate the delay in formation of the supraoccipital bone in the $Mn1^{-/-}$ mouse. (C) Detail of lateral view of E18.5 mice. The frontal direction of the skull is on the right side of the photographs, cranial base is on the bottom side of the photographs. Note the agenesis of alisphenoid and squamosal bones in the $Mn1^{-/-}$ mice. All animals of equal developmental stage are littermates. bs, basisphenoid; ps, presphenoid; p, palatine shelf; m, maxillary shelf; a, alisphenoid bone; pt, pterygoid bone; s, squamosal bone; so, supraoccipital bone; pm, premaxilla; z, zygomatic bone; mx, maxilla; t, tympanic ring; b, basioccipital bone; e, exoccipital bone; fo, foramen ovale; fr, foramen rotundum; v, vomer.
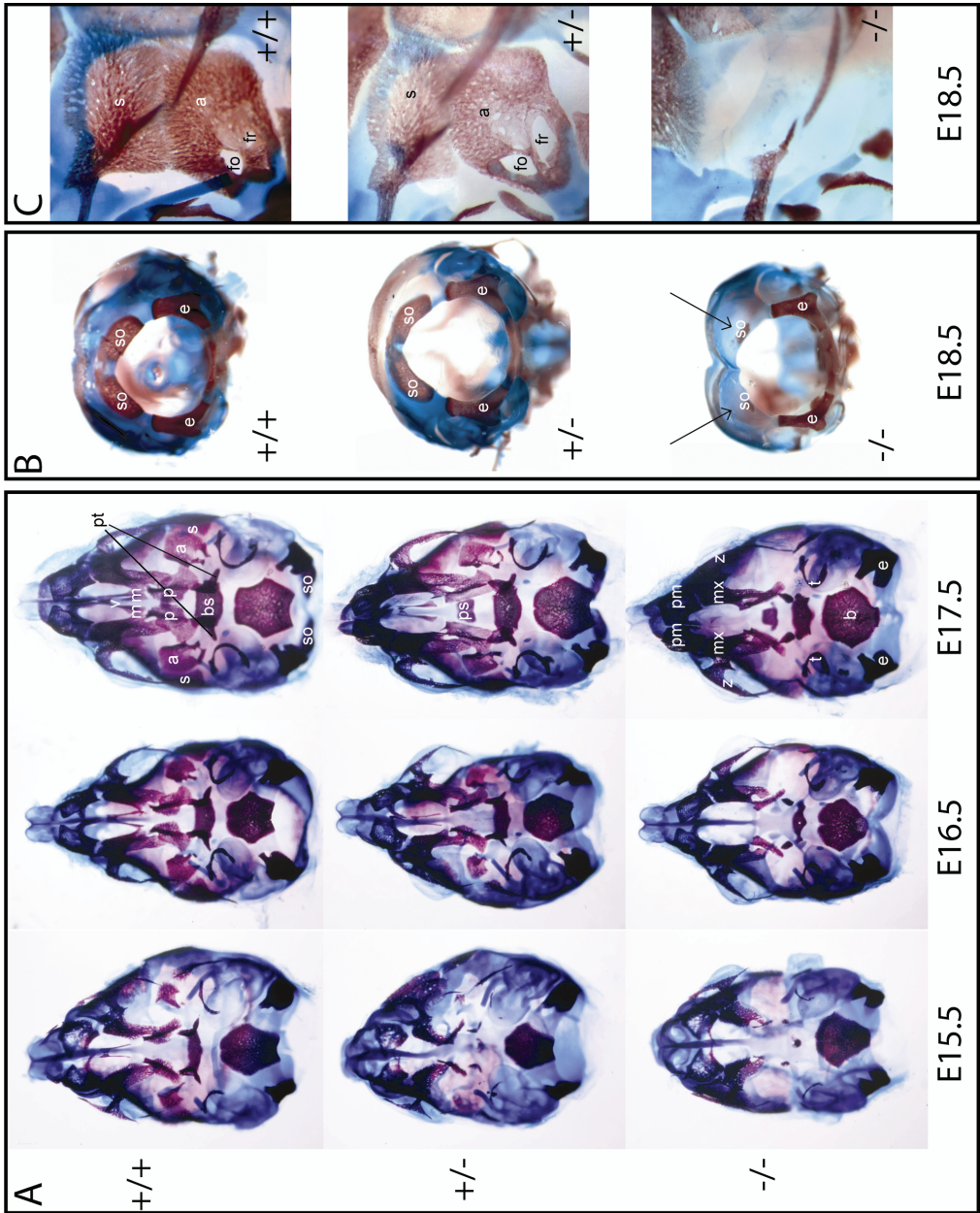
Staining of these bones was clearly less pronounced, and after processing the heads, the skulls from null mice appeared extremely fragile and compressible when compared to wild-type littermates. We performed micro-computerized tomography (CT) scans on skulls of wild-type and *Mn1* null mice (E18.5) and these confirm this observation: CT scans on skulls of a wild-type animal were readily obtained, but scans of skulls of *Mn1*^{-/-} mouse were of poor quality, because of very thin frontal, parietal and interparietal bones (data not shown). A summary of all skull bones affected and not affected in *Mn1*^{+/-} and *Mn1*^{-/-} mice is shown in Table 3.

TABLE 3. Overview of bones within the skull of E18.5 *Mn1* mice

Bone(s)	Result[a] for mice of genotype	
	+/-	-/-
Maxilla	n	n
Mandible	n	n
Hard palate, maxillary	hy	hy
Hard palate, palatine	hy	hy
Premaxilla	n	n
Zygomatic	n	n
Squamosal	hy	ag
Tympanic ring	n	n
Frontal	n	hy
Pterygoid	hy	hy
Parietal	n	hy
Vomer	hy	ag
Interparietal	n	hy
Nasal bone	n	n
Supraoccipital	n	hy
Exoccipital	n	n
Alisphenoid	hy	ag
Basioccipital	n	n
Basisphenoid	hy	hy
Presphenoid	hy	hy
Maleus, incus, stapes	n	n

[a] n, normal development; hy, hypoplastic; ag, agenic.

No abnormalities were observed in the appendicular skeleton, axial skeleton (below the skull), or internal organs of Mn1$^{-/-}$ and Mn1$^{+/-}$ mice

Skeletons of mice of different stages of embryonic development (E15.5 to E18.5) were stained with alizarin red/alcian blue and close observation did not reveal any abnormalities besides the abnormalities in the skull. All limbs were formed correctly, and no delay of endochondral bone formation was observed in *Mn1*$^{-/-}$ or *Mn1*$^{+/-}$ compared to wild-type animals (data not shown). Analysis of *MN1* expression in human tissues showed that *MN1* is expressed in many tissues but at low levels with higher expression in skeletal muscle,[1] kidney and striatum (putamen and caudate nucleus, unpublished results). The extensive analysis of many organs in the P0 *Mn1*$^{-/-}$ mice, however, revealed no abnormalities in these tissues or organs, indicating that *Mn1* plays no critical role in their development. Since *Mn1* null mice are not viable, the role of *Mn1* in normally functioning skeletal muscle, kidney and striatum has not been investigated in this mouse model.

DISCUSSION

In this study we show that Mn1 plays a pivotal role in the development of membranous bones in the skull during embryogenesis. In null mice, agenesis is observed for the alisphenoid and squamosal bones, and vomer. Pterygoid, presphenoid, and basisphenoid bones are hypoplastic and the supraoccipital bone is delayed in formation. Furthermore, the parietal, frontal, and interparietal bones forming the roof of the skull are thinner and less mineralized. Bones not affected include the mandible, maxilla, and the nasal bones. In heterozygous *Mn1*$^{+/-}$ mice these developmental defects are less pronounced with normal mineralization of frontal, parietal and interparietal bones of the skull and formation of the supraoccipital bone and the vomer. The squamosal and alisphenoid bones are formed but are hypoplastic and less mineralized and contain larger foramina. This suggests that there is a dosage effect of the Mn1 protein on cranial bone development. Incomplete penetrance is found for cleft secondary palate, a phenomenon seen in many mouse models with cleft secondary palate. Loss of one functional copy of the *Mn1* gene is compatible with a normal lifespan and does not lead to excess tumor formation or other pathologies.

Mineralization by osteoblasts occurs by two mechanisms: endochondral and intramembranous ossification.[7] During endochondral ossification, precursor cells condense in areas destined to become bone, forming cartilage that will be replaced by bone material at a later stage. This mechanism is used for most of the vertebrate skeleton, including limbs, ribs, and part of the base of the skull. In the skull the parietal, interparietal, and frontal bones

as well as the alisphenoid, pterygoid, squamosal, and palatal bones (from maxillary and palatine shelves) are formed by intramembranous ossification, which involves the condensation of precursors cells and the direct transition to differentiated bone cells, without an intermediate cartilage template formed. In mice, the remainder of the skull, including maxilla and mandible, is formed by a combination of the two processes. Thus nearly all bones affected in $Mn1^{-/-}$ mice are formed by the process of intramembranous ossification, and consequently it appears that $Mn1$ plays a pivotal role in the formation of membranous bones during embryogenesis. The supraoccipital bone, which is delayed in its formation in $Mn1$ null mice, is formed by endochondral ossification. The supraoccipital bone is formed late during embryonic development (E17.5) and processes involving the onset of bone formation are unknown. Possibly, the formation of bone structures in the vicinity of the supraoccipital bone, known to be affected in the $Mn1$ knockout mice, influences the onset of ossification. The question remains whether all bone structures formed by intramembranous ossification are affected in $Mn1$ null mice. The mandible and maxilla are formed by a combination of the two processes, and an impaired mineralization is difficult to detect in these structures by alizarin red/alcian blue stainings.

Since MN1 is able to function as a transcriptional coactivator, an interplay between MN1 and other transcription factors regulating intramembranous ossification is plausible. Many transcription factors known to have a function in bone formation, like *Cbf-β, Osx,* and *Runx*2,[12,13,14] are controlling osteoblast differentiation in both intramembranous and endochondral ossification. Cleidocranial dysplasia, an autosomal dominant skeletal disease in humans, is caused by mutations in the *RUNX2* gene.[15] Haploinsufficiency of the transcription factor causes skeletal abnormalities of both membranous en endochondral bones in patients, including enlarged calvaria with open fontanelles and short stature. Two isoforms of Runx2, I and II, have been found.[16] Separate promoters control the expression of the two isoforms, resulting in two proteins with only minor differences in the N-terminus. By generating selective *Runx*2-*II*-deficient mice, the separate functions of the two isoforms were studied. In *Runx2-II* null mice, intramembranous ossification was not affected, suggesting that it is the Runx2-I isoform that is responsible for proper development of membranous bones. Runx2-I and II are subunits of the heterodimeric transcription factor PEBP2/CBF.[17] The C-terminal region of Runx2 is involved in interaction with various transcription factors, coactivators, and corepressors. Since Mn1 is known to act as a transcriptional coactivator in RA-mediated transcription, it is possible that Mn1 acts as a coactivator in Runx2-I-related transcriptional regulation.

In humans no syndromes are known that resemble the abnormalities seen in *Mn1* knockout mice. Defects in membranous bones are rare; only 2 papers describe families with delayed intramembranous ossification.[18,19] During infancy, these patients show a complete lack of ossification of the calvarial bones forming the roof of the skull resulting in a soft skull. In adults, the cranial vault is ossified but is deformed because of delayed ossification. The temporalis (squamosal) and sphenoid bones are not severely affected and no cleft palate is observed in these patients. One of the families had a translocation (2;3)(p15;q12). The translocation breakpoints and the localization of *MN1* in the human genome (chromosome 22), together with the differences in defects observed, rule out the possibility that *MN1* is the mutated gene in these families. The gene mutated in these families may only be involved in intramembranous ossification of a subset of the calvarial bones forming the roof of the skull.

A short administration of RA to pregnant mice carrying embryos of E10, results in abnormalities showing similarities to the ones observed in *Mn1* knockout mice. At birth these mice show a failure of ossification at P0 of the parietal and interparietal bones and reduction in size of the alisphenoid and squamosal bones.[20] Biochemical experiments in our group have shown that MN1 can act as a transcription coactivator in RAR-RXR-mediated transcription.[4] Thus, since MN1 enhances RAR function, one would expect that obliteration of Mn1 has the opposite effect from a high, non physiological, dose of the RAR ligand RA. However, it is known that both long-term excess but also deficiency of vitamin A in the diet of a pregnant mouse causes a broad range of abnormalities in the offspring[21,22] and that the abnormalities in both diet conditions show great similarities. This shows that a shift in the balance of RAR-RXR-related processes results in similar defects. A more subtle short-term administration of RA at E10 or the lack of Mn1 also results in similar defects, indicative of an interplay between Mn1 and RA in the processes involving the correct formation of the affected bones.

Another mouse model showing striking similarities to the bone abnormalities observed in *Mn1*^{-/-} mice is the *Dlx2*^{-/-} mouse.[9] *Dlx2* is a homeobox gene controlling the patterning of cells within the first and second proximal branchial arches starting around E8.5. Bones absent in the *Mn1* knockout mice are also agenic in the *Dlx2*^{-/-} mice (squamosal and alisphenoid bones). Furthermore, both mouse models show abnormalities of basisphenoid and pterygoid bones, and a cleft of secondary palate is observed in 80% of *Dlx2* knockout mice. However, there are also notable differences. The vomer is not affected in *Dlx2* knockout mice, and the supraoccipital bone is not delayed in formation. In addition, there is no evidence for impaired mineralization of parietal, frontal and interparietal bones of the skull. In *Dlx2*^{-/-} mice abnormalities in the inner ear, forebrain, and enteric nervous system are

observed, but we have no evidence that any of these structures is affected in $Mn1^{-/-}$ mice. In conclusion, defects in $Mn1^{-/-}$ and $Dlx2^{-/-}$ mice overlap but there are also deficiencies associated with the lack of each gene separately.

The most obvious external abnormality observed in the $Mn1^{-/-}$ and in a minor percentage of $Mn1^{+/-}$ mice is the cleft secondary palate. The formation of the palate is a complex process, and a cleft secondary palate has been observed in a number of transgenic mice[8,9,22,23,24,25,26] and in a spontaneous mutant mice strain, the Twirler mice.[27] Cleft secondary palate in these mice is caused by a variety of defects, including a deformed tongue ($Ryk^{-/-}$ mice),[23] shortened mandibles ($Msx1^{-/-}$ mice),[8] a wider midface (Twirler mice),[27] malformation of the inner ear region ($Hoxa-2^{-/-}$ mice),[26] agenesis of numerous bones of the lateral skull ($Dlx2^{-/-}$ mice),[9] and a failure to fuse the patatal shelves ($TGF-\beta3$ $^{-/-}$ mice).[24,25] Besides the $TGF-\beta3$ knockout model, the palatal defects in these mice can be attributed, at least partially, to the malformations in the vicinity of the palatal shelves that cause mechanical hindrance in positioning, elevation, and fusion of the shelves. The cleft palate observed in $Mn1^{-/-}$ mice is most likely a secondary effect of missing bones in the vicinity of the palatal shelves, although we can not rule out the possibility that Mn1 plays an intrinsic role in palatal development.

We conclude that loss of the $Mn1$ gene is not compatible with life, that Mn1 plays an essential role in intramembranous bone formation and that the lack of proper development of these parts of the cranial skeleton results in a cleft secondary palate. The $Mn1$ knockout mouse is the first mouse model that selectively affects membranous bones and therefore presents an excellent model to study this complex process of bone formation.

ACKNOWLEDGEMENTS

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CHAPTER 3

The MN1 oncoprotein activates transcription of the IGFBP5 promoter through a CACCC-rich consensus sequence

Magda A. Meester-Smoor,[1] Anco C. Molijn,[1] Yixian Zhao,[1] Nicole A. Groen,[1] Cora A.H. Groffen,[1] Merel Boogaard,[1] Diny van Dalsum-Verbiest,[1] Gerard C. Grosveld[2] and Ellen C. Zwarthoff[1]

[1]Department of Pathology, Josephine Nefkens Institute, Erasmus MC, Rotterdam, The Netherlands,
[2]Department of Genetics and Tumor Cell Biology, St. Jude Children's Research Hospital, Memphis, TN, USA.

ABSTRACT

The IGF-binding protein (IGFBP) family consists of six proteins that are expressed and secreted in different tissues. The proteins are regulators of physiological processes throughout the body by modulating the activity of IGF-I and IGF-II. In this article, we describe the coordinated expression of IGFBP5 and MN1 in meningiomas. MN1 is a transcriptional coactivator and we show that MN1 stimulates the IGFBP5 promoter in Hep3B cells. A CACCC-containing sequence, located 140 bp upstream of the transcription start site of the promoter, is required for MN1 action. This sequence matches with the CACCCAC consensus sequence that was selected in an oligonucleotide selection assay performed for MN1. The CACCC element has also been shown to be important for induction of the IGFBP5 promoter by retinoic acid (RA) and progesterone (Pg). We were unable to confirm the effect of Pg on the promoter in Hep3B and U2-osteosarcoma cells regardless of the presence of MN1. On the other hand, we show that induction of the promoter by RA depends on co-expressed MN1 in Hep3B cells. MN1-TEL, a leukemia-related fusion protein containing parts of the *MN1* and *TEL* (*ETV6*) genes, is capable of stimulating the *IGFBP5* promoter but is unable to cooperate with RA in Hep3B cells. This suggests that the effects of RA can be negatively affected in leukemias caused by MN1-TEL.

INTRODUCTION

The insulin-like growth factor-binding proteins (IGFBPs) belong to a protein family with six members and are expressed and secreted in a tissue and developmental stage specific manner. The proteins bind the small effector molecules IGF-I and IGF-II, and act as carrier proteins. IGFs are the most important regulators of mammalian growth and development and mediate the effects of growth hormone. The six IGFBP members are highly homologous, but each has distinct functions and biochemical properties. Dependent on the particular IGFBP and the type of tissue, the effect on IGF action can be inhibitory or stimulatory. IGFBPs can also have IGF-independent effects on cell growth. These effects are exerted by binding of IGFBPs to non-IGF molecules, that are either present outside or inside the cell.[1,2,3] IGFBP5 is the most conserved member of the protein family and plays an important role in mediating IGF effects, particularly in bone. IGFBP5 binds IGF-I and is regulated by proteolysis, glycosylation and phosphorylation. It binds extracellular matrix components in osteoblast cell cultures and potentiates IGF function by positioning it in the vicinity of the cell membrane.[4,5] In cultured osteoblasts and muscle cells both inhibitory and stimulatory effects of IGFBP5 on IGF function have been described. Transgenic mice overexpressing IGFBP5 have osteopenia, lower body weight, retarded muscle development and lower female fertility.[6,7] Within different tissues, there is a tight balance of IGFBP5 expression and disregulation of this equilibrium is thought to be a cause of disorders such as osteoporosis and renal osteodystrophy.[8,9] Other physiological processes in which IGFBP5 is involved are mammary gland involution, follicular development in the ovary and kidney physiology.[3]

Expression of IGFBP5 is stimulated by IGF-1 through the phosphatidylinositol-3 kinase pathway.[10] Transcription factors that have been shown to affect the IGFBP5 promoter are the progesterone receptor (PR),[11] the retinoic acid receptor (RAR),[12,13] the glucocorticoid receptor,[14] activator protein-2 (AP-2)[15] and Myb.[16] Furthermore, prostaglandin E2, parathyroid hormone, and osteogenic protein-1 have been described to regulate the expression of IGFBP5.[17,18,19]

Several studies have associated IGFBP5 expression with cancer. IGFBP5 was shown to be able to stimulate growth of prostate cancer cells *in vitro* and tumor growth *in vivo.*[20,21] Both inhibition and induction of apoptosis have been described in breast cancer cells *in vitro*.[22,23,24] Furthermore, IGFBP5 was shown to inhibit the proliferation of cervical carcinoma cells and osteosarcoma cells.[25,26] This study is, among others, based on our unpublished findings that IGFBP5 and the transcriptional coactivator MN1 are co-expressed in

meningiomas, a benign brain tumor arising from the arachnoidal cap cells. This suggests that expression of the two proteins is related.

The *MN1* oncogene was cloned and described by our group in 1995 on the basis of its involvement in a t(4;22) found in a meningioma.[27] The protein consists of 1319 amino acids and has a nuclear localization. The primary protein sequence shows no homology to other proteins, but homologues of MN1 are found in mice (*Mus musculus*) and puffer fish *(Tetraodon nigroviridu*s), proving conservation of MN1 in evolution.[28] Extensive searches in domain databases did not reveal any specific domain within the protein. The proline/glutamine-rich regions in the sequence suggested a function in transcription and indeed, we have shown that MN1 activates the transcription activity of the Moloney sarcoma virus long terminal repeat (MSV-LTR)[29] MN1 can synergize on this promoter with transcription mediated by the RAR - retinoid X receptor (RXR) heterodimer in the presence of the RAR-RXR ligand RA. We have also shown that MN1 binds p300 and RAC3, both known coactivators of RARs.[30] *MN1* is fused to the *TEL* (*ETV6*) gene as a result of the t(12;22) in acute myeloid leukemia. The MN1-TEL fusion protein is able to transform NIH3T3 cells *in vitro* and causes leukemia and lymphoid tumors in mice.[29,31,32]

Since *Mn1* null mice die shortly after birth as the result of a cleft secondary palate, *MN1* is an essential gene.[28] Interestingly, the development of several bones in the skull of these mice is abnormal. The affected bones are almost exclusively formed by intramembranous ossification. They are either completely agenic at birth or substantially thinner. Endochondral bone formation is normal. Thus, Mn1 plays a crucial role in the formation of the membranous bones in the skull during mammalian development. This is further supported by Sutton *et al*. They have described the inhibition of osteoblastic cell proliferation by MN1.[33]

In this report we further investigated the role of MN1 in regulating transcription. Because MN1 has transcription-activating domains, we were prompted to investigate whether it can bind DNA and function as a transcription factor. We show that MN1 activates the IGFBP5 promoter, that this effect is exerted through CA-rich elements present in the proximal promoter, and that the activation of the promoter by RA is dependent on the presence of MN1 in the cells used in this study. These findings also explain the coordinated expression of MN1 and IGFBP5 in meningiomas.

MATERIALS AND METHODS

Oligonucleotide-binding selection

The method used has been described previously[30] and is a modified version of that described by Blackwell and Weintraub.[34] Oligonucleotides for selection contained a central 15-nucleotide random sequence flanked by two constant regions of 20 bp. Cellular extracts from MN1-expressing HtTA cells (cells stably transfected with a tetracyclin-conrolled activator),[30] were incubated with the oligonucleotides, and a monoclonal antibody against MN1 called 2F2,[29,30] was used to precipitate protein-DNA complexes. Bound DNA was amplified using the constant flanking sequences and used for further selection/enrichment. After five rounds of selection, PCR fragments were cloned and sequenced.

Northern blot hybridisation

Total RNA was isolated from primary meningioma tumors using guanidinium thiocyanate method.[35] Electrophoresis, blotting and hybridisation were carried out using standard procedures as described previously.[27] For detection of the *MN1* transcript, a 0.6 kb *Bam*HI cDNA fragment was used. For IGFBP5 we used a 0.9 kb murine cDNA covering the entire coding region.[36] The coding regions of murine and human IGFBP5 are 91% identical. As a control for RNA loading we used a 0.8 kb *Eco*RI-*Pst*I fragment derived from the coding region of the human glyceraldehyde-3-phosphate dehydrogenase (GAPDH) cDNA. Hybridisation signals were quantified using a phosphorImager (GE Healthcare, Uppsala, Sweden). Hybridisation signals for *MN1* and *IGFBP5* were corrected for background and *GAPDH* expression, and the resulting values were plotted against each other.

Cloning of IGFBP5 promoter and generation of deletion/mutation constructs

PCR was performed on bacterial artificial chromosomes (BAC) clone PR11-506C8[37] containing the *IGFBP5* promoter. The forward primers are localized at position -1111 (5'-CAGGTACC-CTAGTGGCATGATTCGGTTC-3') and -204 (5'-CGGGTACCGGAGGAGGGC-GCTGTTCAGG-3') of the promoter with respect to the transcription start site and each contains a *Kpn*I site for cloning purposes. The reverse primer at position +1645 contains a *Bam*HI site (5'-GCGGATCC-CAGGAGAGCGAGAGTGCAGG-3'). The resulting PCR products were cloned in pGL2basic and sequenced. A *Kpn*I-*Xma*I fragment (-204 to + 119) from pGL2 BP5 –204 +738 was subcloned in pGL3basic, resulting in pGL3BP5 –204 +119. The mutants mPR, dCA, dGT and dCAdGT were generated using the QuikChange Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA, USA). The mPR mutant is as described

by Boonyaratanakornkit *et al.*.[11] The CA-rich element at positions −147 to −133 (CCCCACCCCCACCCC) is mutated in CCCCACCaaaACCCC. The dCA mutant lacks the entire CA-rich element (from positions −147 to −133), and the dGT mutant lacks the complete GT-rich element localized at positions −87 to −78 (GGGTGTTGGG). The dCAdGT mutant lacks both elements. All plasmids were sequenced to confirm the different deletions/mutations.

Cell culture and transfections

Hela-derived HtTA cell lines and 3T3 Gene-Switch cell lines were generated and cultured as described previously.[30] In 3T3 Gene-Switch cell lines expression of HA-tagged-MN1 and HA-tag (as control) were induced overnight with mifepristone (10^{-8}M) and used to generate cell lysates for electrophoretic mobility shift assays (EMSAs). Transient transfections were performed using hepatocarcinoma cell line 3B (Hep3B)[38] or U2-Osteosarcoma cells (U2OS). U2OS cells were obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA) and are known as HTB-96 in this collection. Hep3B was maintained in α-minimum essential medium (α-MEM) supplemented with 5% fetal calf serum (FCS), 2 mM L-glutamine, 1000U/l penicillin and 1mg/l streptomycin at 37°C with 5% CO_2 and U2OS cells were cultured in Dulbecco's Modified Eagle's medium (DMEM) supplemented with 10% FCS, 1000U/l penicillin and 1mg/l streptomycin at 37°C with 5% CO_2. The day before transient transfections cells were seeded in 24-well plates (0.7×10^5 cells/well). On the day of transfection, medium was changed and, if indicated in figure legends, progesteron (Pg (Q2600), Steraloids, Newport, RI, USA) or all-*trans* RA (R2625, Sigma-Aldrich, St.Louis, MO, USA) was added at the desired concentration (Pg, 10^{-7}M; RA, 10^{-9}M). Transfections were carried out using FuGENE 6 Transfection Reagent (Roche Applied Science, Basel, Switzerland) according to the manufacturer's recommendations. The MN1 expression plasmid, pMN50, has been described previously,[30] the expression plasmid for PR (pCMV3.1 PR-β) and the PR-responsive luc (PRE2-luc) reporter were obtained from G.Jenster (Department of Urology, Erasmus MC, Rotterdam, The Netherlands).[39] Expression plasmid pcDNA3 TEL was generated by cloning the insert of pSCTOP TEL[29] (*Hind*III-*Not*I digestion) into pcDNA3.1. pcDNA3 MN1-TEL was made by cloning the C-terminal region of MN1-TEL (pSCTOP MN1-TEL, *Sfi*I-*Not*I digestion[29]) into pMN50. For a transfection experiment, 375ng of expression plasmid and 25ng of reporter plasmid were used. For each experiment, the total amount of transfected DNA and the molar ratio of CMV promoter were kept constant by the addition of puc6 plasmid DNA. After incubation for 48 h, cells were lysed and luciferase activity was measured on a Fluoroscan Ascent FL luminometer (Thermo Electron

Corporation, Waltham, MA, USA). Transfection efficiency was monitored regularly by adding a *Renilla* luciferase construct or a LacZ internal control plasmid (pcDNA3.1 HisLacZ). Transfection efficiency was shown not to vary within an experiment (data not shown). Each experiment was done at least thrice in triplicate.

Real-time PCR (Taqman analysis)

RNA was isolated from 3×10^5 cells using the Rneasy mini kit (Qiagen, Venlo, the Netherlands) according to manufacturer's recommendations. RNA (3µg) was reverse transcribed using 300 U Moloney Murine Leukemia Virus (M-MLV) reverse transcriptase (Invitrogen, Carlsbad, CA, USA) and 750ng random hexamers. cDNA (12.5 ng) was analysed for the expression of *RNA polymerase II, subunit A* (*PolR2A*), *MN1* and *IGFBP5* by real-time PCR using SYBR green PCR assay (Applied Biosystems, Foster City, CA, USA) and an ABI Prism 7700. Levels of expression for *MN1* and *IGFBP5* were calculated relative to PolR2A level in the sample using the observed threshold cycle. Two separate RNA samples of cell lines were analysed. Primers (7.5pmol used for each PCR): PolR2A-F: 5'-CGGATGAACTGAAGCGAATG-3'; PolR2A-R: 5'-GAGTCCACAAGCAGTTTGG-3'; MN1-F: 5'-TCGCTGATGGCAGAGCACAG-3; MN1-R: 5'-GTCATTCAAGTTAGGGCAG-3'; IGFBP5-F: 5'-CGGGGGAGCCGAGAACAC-3'; IGFBP5-R: 5'-GGCGCTGGCTGGAGTCGG-3'.

Electrophoretic mobility shift assay (EMSA)

Lysates were made of mifepristone (10^{-8}M)-induced 3T3 cell lines (expressing hemaglutinin (HA)-tagged MN1 and the HA-tag alone as a control) using EMSA lysis buffer (20 mM HEPES pH 7.9, 10 mM KCl, 1 mM $MgCl_2$, 300 mM NaCl, 0.1% Triton X-100, 20% glycerol, 0.5mM dithiotreitol (DTT) and protease inhibitor cocktail Complete (Roche, Germany)). Cells were disrupted in a cuphorn sonifier. Protein concentration was measured and 20ug of protein was used for each EMSA experiment. Protein lysates were incubated with 10,000 cpm ^{32}P-labeled double-stranded oligo in 10 mM Hepes pH 7.9, 60 mM KCl, 4 % Ficoll 400, 1 mM DTT, and 1 mM EDTA at room temperature for 30 min. Double-stranded oligos used for EMSA: IGFBP5-CA: 5'-CCTCTCCCCACCCCCACCCCGTGTG-3'; IGFBP5-GT: 5'-GAGTTGGGTGTTGGGAAGCT-3'; Selex-78: 5'-GACCACCCACGTTGGCTCC-3'. Samples were analysed on a 4% acrylamide gel (37,5 : 1; acrylamide: bisacrylamide) in 0.5x Tris-boric acid-EDTA (TBE) at 15mA. After electrophoresis, the gel was transferred to paper and dried. Bands were quantified using a Typhoon 9200 scanner (GE Healthcare, Uppsala, Sweden).

RESULTS

Oligonucleotide selection experiments reveal that MN1 recognizes a CACCC-rich sequence

We have shown previously that MN1 can activate transcription from the MSV-LTR, and that MN1 contains a transcription-activating domain near its N-terminus.[30,40] In order to investigate whether the MN1 protein can bind to DNA and whether it recognises a specific DNA element, we performed an oligonucleotide selection assay. To this end, an

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Oligo ...GACNNNNNNNNNNNNNNNNNCTC....


              CACCCACGTTGGCTC*
              CACCCACCACGCCTG
              CACCCACCGCGATTC
              CACCCACCACGTCTC
              CACCCACCATGATAC
              CACCCACGTAGATAC
              CACCCACGAAGCGAC
              CACCCACGAAGCTGC
              CACCCACGATGGCTC
              CACCCACAGTGTACC
              CACCCACAAAGTACC
              CACCCACGGCGAAC
              CACCCACGAAGTCCC
              CACCCACAACGTAAC
              CACCCACAAAGTACC
              CACCCACGAAGACTC
              CACCCACCATCGAGC
              CACCCACGAAGTCCC
           ACCACCCACCTTGCCC
              CACCCAAGTTGATCC
              CACCCAGGTAGTGAC
           ACACCACCCCAGCCC
      CCCCCATGACGCCACC
              CACCAGGTACACCTC
             CACCCCGTCGTGACT
              CACACACTAAGCGGC
              CACACACGACGTGTCAC
              CACAAAACCTTGCAC
              CACAAATGGACTGTC
          CGCCACATATAAATC
              CACAATGCCCCCCAC
              CACAGTATATGAGCC
              CACTCGTAACTCCTT
             CCACTCGTTGGCCC

Consensus     CACCCAC
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Figure 1. Oligonucleotide selection assay. To establish the consensus sequence CACCCAC, 34 oligonucleotides from the selection assay were used. The numbers indicate the percentage of oligonucleotides containing each nucleotide. The oligonucleotide indicated with an asterisk was used for the bandshift assay (figure 4).

oligonucleotide pool was synthesized with a random core of 15 residues with 20 residues of known flanking sequence on both sides. The pool was incubated with a lysate obtained from an HtTA HeLa cell line in which MN1 expression was induced by omitting tetracycline from the medium.[30] Oligonucleotides binding to MN1 were precipitated from the mixture using the MN1 monoclonal antibody 2F2. These oligonucleotides were amplified using primers annealing to the sequences flanking the random core. This selection procedure was repeated five times. The resulting oligonucleotides enriched for MN1-binding were cloned and the insert sequence of 54 different plasmids was determined. A CACCC-rich sequence was found in 34 plasmid inserts from which a consensus-binding sequence CACCCAC was deduced (Figure 1). In seven plasmids, a sequence AGGTCAaAGGTCA resembling a RAR-RXR DR1-binding site was observed. The RAR/RXR-responsive elements were shown to be responsible for a synergistic induction of expression by RA and MN1 in an earlier paper.[30] The remaining 13 inserts contained random sequences.

We have shown previously that MN1 and RAR-RXR can synergistically induce expression from the MSV-LTR, a viral promoter that contains a RA-responsive element.[30] To investigate the effect of the CACCC element on expression, an oligonucleotide containing two of the CACCC consensus sequences was inserted into a reporter vector in front of a TATA box. However, when MN1 cDNA was co-transfected with the reporter, we were unable to establish an effect on expression of this synthetic promoter.

Coordinated expression of MN1 and IGFBP5 in meningiomas

In a previous study we showed that *MN1* expression varies considerably between meningiomas.[27] We also investigated a putative role for the IGF pathway in these benign tumors. These experiments showed that there was a correlation between the expression of *MN1* and *IGFBP5*. Figure 2A shows a representative northern blot containing RNA from ten meningiomas hybridized with probes against *IGFBP5* (top panel), *MN1* (middle panel) and control *GAPDH* (bottom panel). Quantification of expression of *MN1*, *IGFBP5* and *GAPDH* using a phosphorImager revealed that expression of the *MN1* gene correlated with expression of the *IGFBP5* gene in 18 out of the 20 meningiomas tested, with high expression of *MN1* occurring in the same tumors that showed high expression of *IGFBP5*. Figure 2B is a graphical representation of all the meningiomas tested; the Pearson correlation coefficient (r) calculated is 0.765 (P value <0.0001 at significance level 0.01). These results suggest a functional relationship between the expression of these genes.
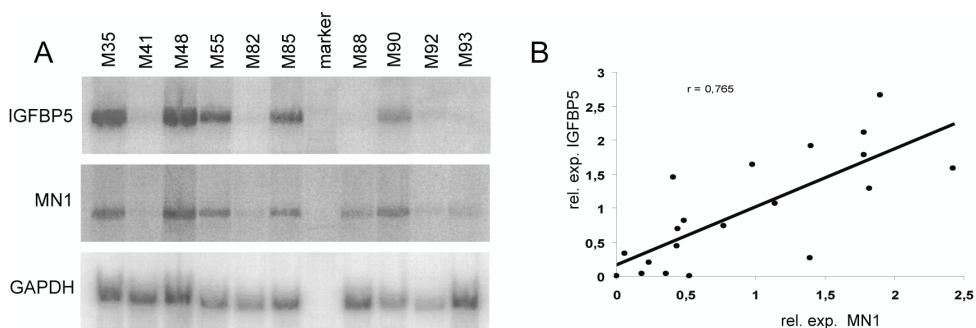
Figure 2. Coordinated expression of *MN1* and *IGFBP5* in meningiomas. (A) The northern blot, containing 20 ug of each RNA sample, was hybridised to probes for *IGFBP5* (top panel), *MN1* (middle panel) and *GAPDH* (lower panel). (B) Correlation of *MN1* and *IGFBP5* expression in 20 meningiomas. Expression of *IGFBP5* and *MN1* was calculated in relation to *GAPDH* using phosphorImager data. r, Pearson correlation.

MN1 stimulates expression of the IGFBP5 gene through CA- and GT-rich elements

Inspection of the promoter of the *IGFBP5* gene showed that it contains a CA-rich element that may present a possible natural target for regulation of expression by MN1. Another element is GT-rich, and is nearly the reverse sequence of the consensus sequence picked up by the oligonucleotide selection assay. Both the elements are located just upstream of the transcription start site: (-146) CCCCACCCCACCC (-132) and (-86) GGTGTTGGG (-77).

In order to establish whether MN1 induces expression of IGFBP5, we cloned the promoter in front of a luciferase reporter (outline of the constructs is shown in Figure 3A). Transient transfections were carried out using an MN1 expression construct. For these experiments, we used the Hep3B hepatocarcinoma cell line, in which there is no expression of the endogenous *MN1* gene. Figure 3B shows that MN1 induces the *IGFBP5* promoter about sixfold, and that the smaller promoter construct spanning nucleotide (nt) -204 to nt 738 is sufficient for induction by MN1. Deletion of nucleotide +119 to +738 did not influence the induction of MN1 (data not shown).

To investigate the role of the CA-rich and/or GT-rich elements in MN1-induced expression, the sequences were deleted from the shortest *IGFBP5* promoter construct. Both single and double mutants (lacking both CA en GT-rich elements) were generated. The effect of the mutations is shown in Figure 3C. MN1 was able to induce the *IGFBP5* promoter in a dose-dependent manner, and deletion of both elements negatively affected the
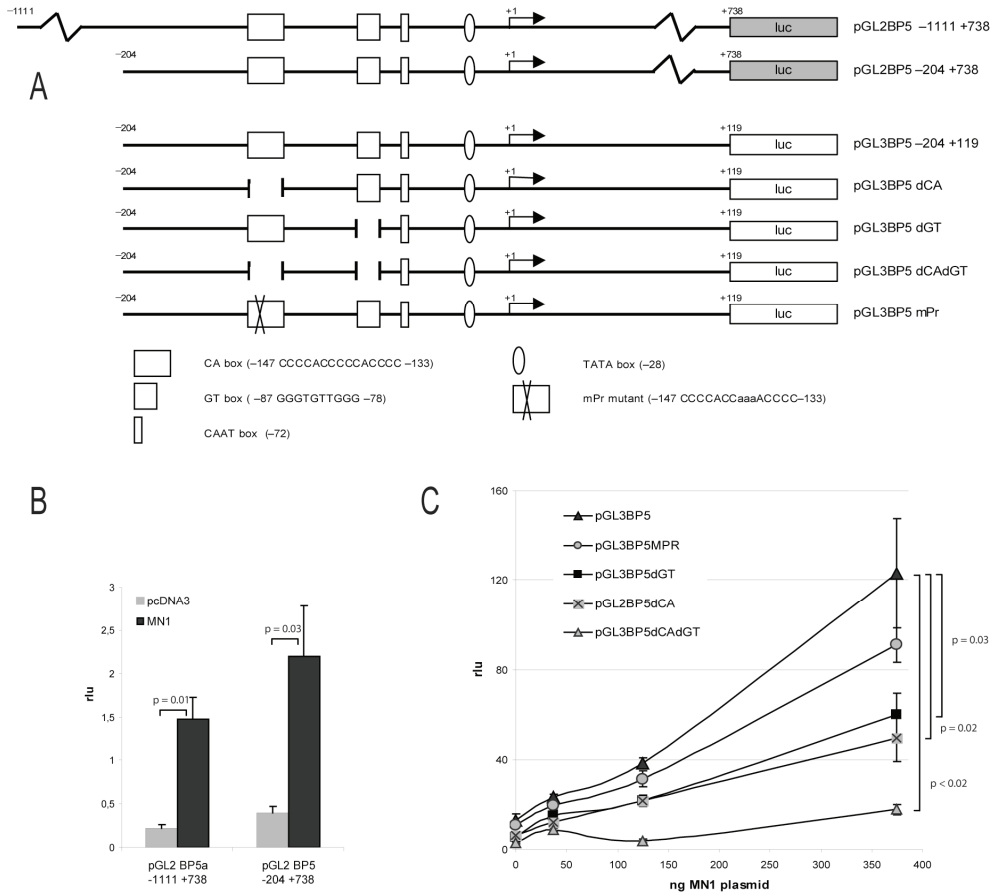
Figure 3. MN1 stimulates the *IGFBP5* promoter through CA-rich elements. (A) Schematic representation of luciferase reporter constructs of the *IGFBP5* promoter. Numbers indicate nucleotide positions relative to the transcription start site (+1, indicated with an arrow). The constructs of the *IGFBP5* promoter contain +738 or + 119 basepairs of untranslated exon 1. The constructs used for Figure 3B are cloned in pGL2basic. The constructs used for Figure 3C are cloned in pGL3basic. This vector contains a modified coding region in the luciferase gene, resulting in an enhanced luciferase activity. (B) In Hep3B cells, 25 ng of reporter plasmids and 120 ng of MN1 expression plasmid or pcDNA3 was transfected. MN1 stimulates the *IGFBP5* promoter and -204 bp of the promoter is sufficient for induction by MN1. (C) In Hep3B cells, 25 ng of reporter plasmid and increasing amounts of MN1 expression plasmid were transfected. The promoter is stimulated in a dose-dependent manner and the mutated promoters are stimulated less efficiently. Values shown are the mean of triplicate wells $\pm$ S.E., except for Figure 3C (mean of two separate RNA samples analysed $\pm$ S.E.). *P* values are shown for significant changes (two-tailed Student's *t*-test). rlu, relative light units.

induction of the *IGFBP5* promoter by MN1. When both elements were deleted, promoter activity was almost completely abolished. In the literature, the CA-rich element is described as a PR-sensitive element.[11] A mutant used by the authors of this article in which 3 C's were exchanged with A's was no longer able to confer PR activity to the *IGFBP5* promoter. We

used this mutant to investigate a possible interplay between MN1 and PR. Figure 3C shows that the Pr mutation construct (mPr) is also stimulated less efficiently by MN1. On the basis of these experiments, we conclude that MN1 is able to efficiently induce the *IGFBP5* promoter and that the CA-rich and GT-rich elements are involved in MN1-induced expression.

MN1 binds indirectly to CA-containing oligonucleotides

The finding that MN1 recognizes a CACCC sequence in the oligonucleotide selection experiments and is able to activate the *IGFBP5* promoter containing similar elements prompted us to investigate whether MN1 binds to the CA and GT-rich DNA elements in a bandshift assay. To this end, an NIH3T3 cell line was used in which expression of HA-tagged MN1 can be induced by addition of mifepristone. Lysates of HA and HA-MN1 cell lines were incubated with ^{32}P-labeled double-stranded oligonucleotides containing the CA consensus sequence (as shown in figure 4A; selex 78) and CA and GT containing oligonucleotides derived from the IGFBP5 promoter sequence (see Materials and methods section and Figure 4B for specific sequences). The results of the mobility shift assays are shown in Figure 4. The CA-containing selex oligonucleotide (selex 78) was found to bind to four separate protein complexes (lane 4, complexes numbered I-IV). Figure 4B is a graphical representation of the binding. The four complexes are distinguishable as four peaks. The CA oligonucleotide derived from the *IGFBP5* promoter (IGFBP5-CA) binds two complexes, similar to complexes I and II detected with selex 78 oligonucleotide (lane 1). Although the sequence of the GT oligonucleotide is not exactly complementary to the CCACCC consensus sequence, the IGFBP5-GT oligonucleotide binds two complexes similar to complexes III and IV. Close examination of the sequences of the selex 78 and the IGFBP5-GT oligonucleotide reveals that the oligonucleotides share the GTTGG sequence: most likely, this represents the sequence responsible for the complexes binding to both oligonucleotides.

Binding specificity was established by competition with cold oligonucleotides (lane 3, 6 and 9). In the presence of HA-MN1, the relative intensities of complexes I and II change and complex II is now the major complex for bandshifts with both selex 78 and IGFBP5–CA oligonucleotides experiments (lane 2 and 5; indicated with arrows in Figure 4A and B). Possible explanations are that the newly formed complex with HA-MN1 migrates at the same position as the complex II that is seen in the absence of MN1, or alternatively, that MN1 stimulates the formation of complex II, for instance by increasing the recruitment of

proteins, such as p160 and p300 that bind to MN1. No influence of HA-MN1 was detectable on the complexes binding the IGFBP5-GT oligonucleotide.

In order to establish whether binding of MN1 was direct or indirect, mobility shift assays were performed with MN1 produced through *in-vitro* transcription-translation. No binding of MN1 to the oligonucleotides was found (data not shown). Thus, we conclude that MN1 recognizes CACCC-rich sequences indirectly, presumably by binding to a CACCC-specific transcription factor.
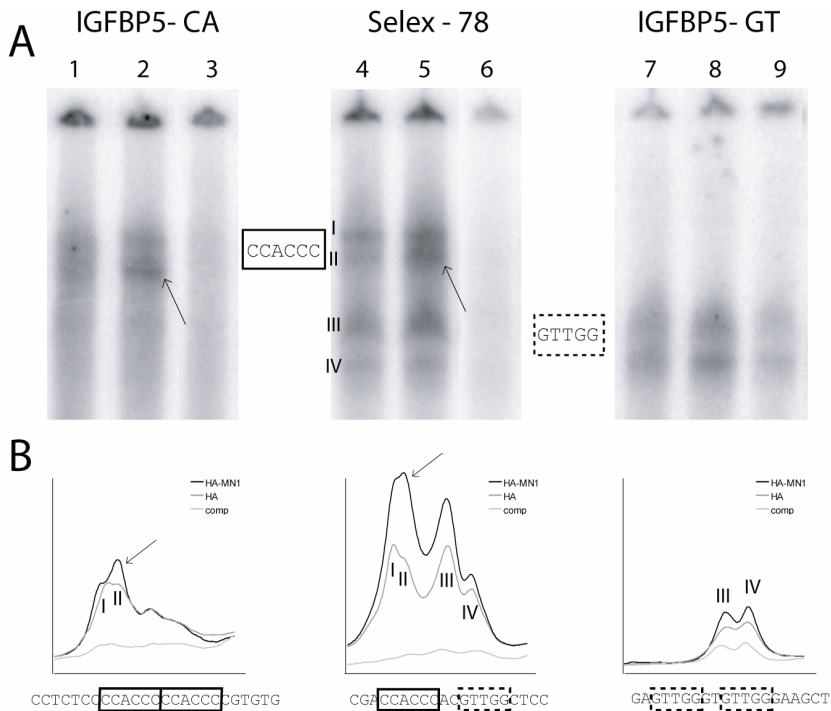
Figure 4. Bandshift assay using the selected CACCCAC sequence and IGFBP5-derived oligonucleotides. (A) Autoradiograms of bandshift assays using three different oligonucleotides incubated with total lysates of cell lines expressing HA-tagged MN1 and the HA-tag alone as a control. Four protein complexes binding the selex78 oligonucleotide are indicated with roman numbers I-IV. Lanes 1, 4, and 7: shifts observed with control lysate of HA-expressing 3T3 cell line. Lanes 2, 5 and 6: shifts observed with lysate of HAMN1 expressing cell line. Arrows indicated the more intense band appearing in lanes 2 and 5. Lanes 3, 6 and 9: competition experiment using cold oligonucleotides (100 x molar excess). (B) Graphic representation of bandshift assays. Peaks are indicated for protein complexes I, II, III and IV. More intense bands are indicated with an arrow.

The progesterone receptor (PR) does not stimulate the IGFBP5 promoter in Hep3B cells and U2 Osteosarcoma cells, and the effect of MN1 is not affected by PR

It has previously been shown by Boonyaratanakornkit et al.[11] that the PR can stimulate the *IGFBP5* promoter in the presence of its ligand, progesterone. Although no canonical PR-responsive elements are present in the IGFBP5 promoter, Boonyaratanakornkit et al. showed that induction by PR is mediated by the CACCC element in the promoter. Since MN1 also uses this element to induce to *IGFBP5* promoter, and can collaborate with another nuclear receptor, the RAR-RXR heterodimer,[30] we sought to investigate the effect of a combination of PR and MN1 on this promoter. To this end, we co-transfected MN1 and PR expression constructs and the IGFBP5 reporter into Hep3B cells. The result of this experiment is shown in Figure 5A. Whereas MN1 efficiently induced the *IGFBP5* promoter as shown before, we were unable to find induction of the promoter by PR. Neither did different concentrations of PR result in induction of the *IGFBP5* promoter in Hep3B cells (data not shown). Co-transfection of MN1 and PR did not increase the induction of the *IGFBP5* promoter by MN1.

Since the experiments described by Boonyaratanakornkit *et al*. had been carried out in human U2OS cells, we repeated the experiments in this cell line. U2OS cells express MN1 endogenously as is shown in Figure 5C and, as a consequence, no effect of transfection of MN1 is obvious (Figure 5B). Figure 5C also shows that MN1 and IGFBP5 are both expressed in U2OS cells but not in Hep3B cells. In contrast to Boonyaratanakornkit *et al.*, we found inhibition of the *IGFBP5* promoter by the PR in U2OS cells rather than stimulation (Figure 5B). As a control for PR activity we established that PR in the presence of ligand but not in its absence could induce a luciferase reporter driven by two copies of the canonical progesteron-responsive element (PRE, Figure 5D). The results are shown for U2OS cells and were similar in Hep3B cells.

The IGFBP5 promoter can also be induced by MN1-TEL, and induction by MN1 can be enhanced by RA in Hep3B cells

Because of the effect of IGFBP5 in various forms of cancer, we next sought to investigate whether the leukemia-associated MN1-TEL fusion protein is also capable of induction of the *IGFBP5* promoter. Figure 5E shows that MN1TEL is indeed able to induce the *IGFBP5* promoter. It has also been described that RA can induce IGFBP5 in neuroblastoma cells and other cell types.[12,13] Since MN1 is known to collaborate with the receptors of this ligand, RAR-RXR, by inducing expression from the MSV-LTR,[30] we investigated the effect of RA on the *IGFBP5* promoter in the presence or absence of MN1. In Hep3B cells, that express all

RARs and RXRs,[41] we observed that addition of RA alone is insufficient to stimulate the promoter. In the presence of MN1, however, a clear induction was found (Figure 5E). Thus, in Hep3B cells, induction of the *IGFBP5* promoter with RA requires expression of MN1.
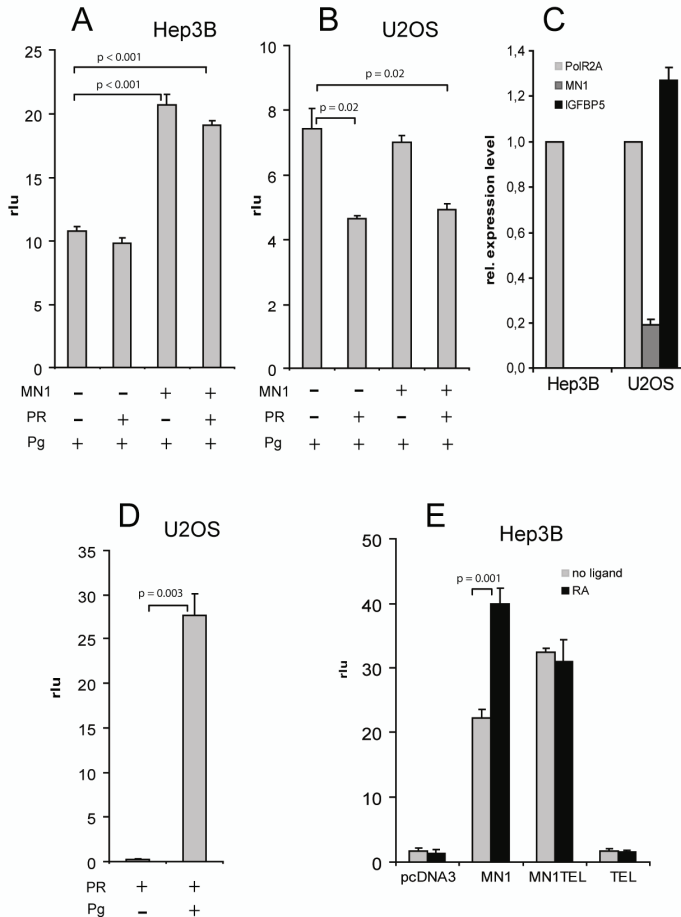
Figure 5. The PR does not stimulate the *IGFBP5* promoter; RA induction of the *IGFBP5* promoter depends on the presence of MN1. (A) Expression plasmids for MN1 (125 ng) and PR (250 ng) were transfected together with pGL3 BP5 -204 +119 in Hep3B cells in the presence of progesterone (Pg; 10^{-7}M).The PR has no effect on the transfected promoter. (B) Transfection performed in U2OS cells (as described for 5A). The PR does not stimulate transcription from the transfected *IFGBP5* promoter in U2OS cells. (C) Endogenous expression of *MN1* and *IGFBP5* in Hep3B and U2OS cells as determined by real-time PCR. Expression of MN1 and IGFBP5 was observed in U2OS cells, whereas Hep3B had no expression of either of the genes. (D) Control for the effect of progesterone. pPRE-luc contains two copies of the canonical PR responsive element and, when co-transfected with PR, is efficiently induced in the presence of Pg. (E) In Hep3B cells, 25 ng reporter plasmid (pGL3 BP5 -204 + 119) and 375 ng of expression plasmid for MN1, MN1-TEL and TEL were transfected in the presence or absence of retinoic acid (RA; 10^{-9}M). RA can stimulate the *IGFBP5* promoter only in the presence of MN1. MN1-TEL cannot replace MN1 in this process. TEL does not have any effect on the *IGFBP5* promoter. Values shown are the mean of triplicate wells $\pm$ S.E. *P* values are shown for significant changes (two-tailed Student's *t*-test). rlu, relative light units; RA, retinoic acid; Pg, progesterone; PR, progesterone receptor.

Since we previously found that the fusion protein MN1-TEL can induce expression, but is not capable of synergising with RA in the case of the MSV-LTR (van Wely *et al*., submitted), we investigated whether the same was true for the *IGFBP5* promoter. In analogy to our results with the MSV-LTR, the effect of MN1-TEL was not enhanced in the presence of RA. We also found that the fusion partner TEL had no effect on the *IGFBP5* promoter (Figure 5E). If induction of the *IGFBP5* promoter by RA was always dependent on co-expressed MN1, the sole addition of RA to U2OS cells would be sufficient to induce the *IGFBP5* promoter, since these cells express MN1 endogenously. Transfection of the IGFBP5 reporter construct in U2OS cells and incubation with RA, however, did not result in increased expression from the promoter. We conclude that there was no induction of the IGFBP5 promoter by RA alone either in Hep3B or in U2OS cells and that expression of MN1 in Hep3B cells is required for RA to have an effect on the *IGFBP5* promoter.

DISCUSSION

The oligonucleotide selection experiments revealed a consensus sequence CACCCAC that was recognized by protein complexes containing MN1. Our data suggest that this binding is presumably indirect and through another, as yet unknown, protein or proteins. The CACCCAC sequence is present in the list of regulatory motifs in human promoters generated by the study of Xie *et al.,*[42] in which over 17,000 well-annotated genes were studied by aligning promoters and 3'UTRs of different mammalian species. The motif CACCCAC was found in reverse (GTGGGTGK) together with about 150 other short regulatory sequences. Searching the Transfac database with the motif in both orientations shows that many transcription factors, such as AP2-1, Egr-1, paired box protein (PAX)1, 5 and 9, Sp1, AP1, human T-cell leukemia virus enhancer factor (HLTF) and Dopamine receptor regulating factor (DRRF) bind to this motif (http://www.gene-regulation.com/pub/databases.html#transfac). In addition, there is an intriguing correlation between the CACCCAC-MN1 binding motif and the GACCACCCAC elements to which Glioma-associated oncogene (GLI) 1, 2 and 3 and the family members GLIS1, 2 and 3 bind.[43,44] In the sequences that were determined on the basis of the oligonucleotide selection assay, the same flanking sequence was present upstream of the conserved motif in all cases. If the motif is then extended at its 5' end with 3 nucleotides of flanking sequence, it reads GACCACCCAC. The sequence of the other flanking sequence is different for all selected sequences, suggesting that a selection has occurred for one of the

two flanking sequences in conjunction with the core motif CACCCAC, and thus that it is also possible that it a GLI family member is a target protein for MN1.

MN1 and *IGFBP5* are co-expressed in meningiomas, a benign tumor of the meninges. Considering the results presented in this work, the most likely explanation for this finding would be that MN1 induces *IGFBP5* expression in these tumors. Expression of *MN1* and *IGFBP5* between the different meningiomas differs considerably, some tumors having hardly detectable expression levels, and others having high expression. Our comparison of expression levels of *MN1* and *IGFBP5* with other characteristics of these meningiomas produced no correlation with the position of the tumor, histological subtype or grade, nor any or evidence for involvement of the NF2 tumor suppressor gene.[27,45] Sandberg Nordqvist and Mathiesen[46] studied the expression of IGFBP5 in three groups of meningiomas (classified on the basis of invasiveness) and concluded that higher expression of IGFBP5 is observed predominantly in tumors that do not invade the brain. This would correlate with our finding, since, in meningiomas, invasion of surrounding structures is extremely rare, and was certainly not seen in the meningiomas in our study. Thus, both studies suggest that expression of IGFBP5 is high in some meningiomas and low in others. Whether expression is higher than normal is not known at present. Persistent, but low-level expression of *IGFBP5* mRNA and protein has been observed in the mouse meninges,[47] but there is no data on the *IGFBP5* expression level in normal human meninges.

Boonyaratanakornkit *et al.*[11] reported previously that PR efficiently stimulated the *IGFBP5* promoter, and that the CA-rich region was responsible for this, although the sequence does not resemble a canonical PRE. They also showed that the PR does not bind the CA element directly. We investigated the relationship between MN1 and PR in the presence of Pg and we were not able to reproduce the effect of PR on the IGFBP5 promoter either in Hep3B or in U2OS cells. In both cell lines, the control PRE-driven reporter was efficiently induced by co-transfected PR after the addition of Pg. Explanations for these discrepancies are difficult to give, but may result from differences in cell lines after prolonged culturing or in culture conditions.

In the neuroblastoma cell line LAN-5, cervical carcinoma cells, human breast carcinoma cells and rat osteoblastic cells, RA was shown to stimulate the expression of *IGFBP5*.[12,13,26,48] Mutagenesis of the CACCC element decreased, but did not completely abolish induction by RA in LAN-5. This suggests that other sites for RA induction are present in the promoter.[12,13] In contrast, RA decreased *IGFBP5* levels in the prostate adenocarcinoma cell line PC-3 and in the human breast carcinoma cell line T47D.[49,50] In the Hep3B and U2OS cells studied in this work, RA had no effect on its own. We showed that

RA can cooperate with exogenously added MN1 in Hep3B cells, leading to stimulation of the promoter, but cannot do this in U2OS cells that express the protein endogenously. These results suggest that regulation of the *IGFBP5* promoter by RA occurs, but that unknown cell-dependent factors determine the outcome.

Interestingly, MN1-TEL was as efficient in activating the *IGFBP5* promoter as MN1, but this activity could not be enhanced by addition of RA. TEL on its own had no effect on the promoter. Since MN1-TEL is under control of the *MN1* promoter, its expression is probably similar to expression of *MN1*, which makes it possible that the fusion protein competes for MN1-binding partners. Its inertness in collaborating with RA is not unique for the *IGFBP5* promoter. The Moloney sarcoma virus long terminal repeat (MSV-LTR) is stimulated by both MN1 and MN1TEL; on this promoter too, MN1 can collaborate with RA, whereas MN1 TEL cannot. This property of MN1-TEL could play a role in leukemia caused by MN1-TEL.
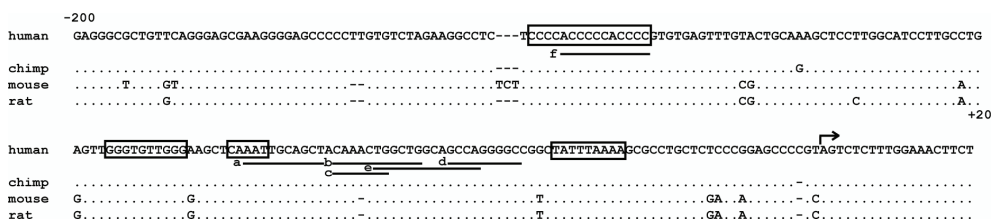
Figure 6. Alignment of the *IGFBP5* promoter in human, chimp, mouse and rat. The promoter is aligned for bp -200 to +20 relative to the transcription start site (indicated with an arrow). The CA-rich and GT-rich elements, CAAT box and TATA box are boxed. Known binding sites are indicated under the sequence with a line. a, *E*-box, CCAAT/enhances binding protein (C/EBP)-responsive element (RE); b, cortisol and osteogenic protein 1 RE; c, Myb-binding site; d, AP2-binding site; e, nuclear factor-1 (NF1)-binding site; f, MN1-RE / progesterone-RE.

No other transcription factors have been described that regulate the transcription of the *IGFBP5* gene through the regions identified as being important for MN1. The CACCCAC motif overlaps with two putative AP-2 sites but they were shown not to be used by this transcription factor. Instead, a more proximal GCCNNNGGC sequence within the promoter was shown to be the target of AP-2. Both the CA-rich and GT-rich motifs are highly conserved between species. Figure 6 shows the alignment of the *IGFBP5* promoter, which ranges from nt -200 to +20 for human, chimp, mouse and rat. Proven consensus sequences for several factors are indicated. Most of them cluster in the region between the CAAT box and the TATA box. The sequences identified here to be important for proper MN1 function are completely conserved, indicative of an important role for these regions of the promoter. Future experiments should reveal whether transcription factors known to bind CA-rich

regions, such as Sp1, GLI etc., regulate the transcription of the *IGFBP5* promoter and how MN1 is involved in this regulation.

ACKNOWLEDGEMENTS

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The MN1-TEL myeloid leukemia-associated fusion protein has a dominant-negative effect on RAR-RXR-mediated transcription

Karel H. M. van Wely,[1,3] Magda A. Meester-Smoor,[1,3] Marjolein J. F. W. Janssen,[1] Albert-Jan Aarnoudse,[1] Gerard C. Grosveld[2] and Ellen C. Zwarthoff[1]

[1]Department of Pathology, Josephine Nefkens Institute, Erasmus MC, Rotterdam, The Netherlands
[2]Department of Genetics and Tumor Cell Biology, St Jude Children's Research Hospital, Memphis, TN, USA
[3]These authors contributed equally to this work

ABSTRACT

The translocation t(12;22)(p13;q11) creates an *MN1-TEL* fusion gene leading to acute myeloid leukemia. MN1 is a transcription coactivator of the retinoic acid and vitamin D receptors, and TEL (ETV6) is a member of the E26-transformation-specific family of transcription factors. In MN1-TEL, the transactivating domains of MN1 are combined with the DNA-binding domain of TEL. We show that MN1-TEL inhibits RAR-mediated transcription, counteracts coactivators such as p160 and p300, and acts as a dominant-negative mutant of MN1. Compared to MN1, the same transactivation domains in MN1-TEL are poorly stimulated by p160, p300 or histone deacetylase inhibitors, indicating that the block of RAR-mediated transcription by MN1-TEL is caused by dysfunctional transactivation domains rather than by recruitment of corepressors. The mechanism leading to myeloid leukemia in t(12;22) thus differs from the translocations that involve RAR itself.

INTRODUCTION

The translocation t(12;22)(p13;q11) leads to the formation of an *MN1-TEL* fusion gene in acute myeloid leukemia (AML).[1] The encoded fusion protein comprises nearly all of MN1 fused to the central part and DNA-binding domain of TEL. MN1, a protein of 1319 amino acids, is a transcription coactivator of the nuclear hormone receptors for *all-trans* retinoic acid (ATRA) and vitamin D.[2,3] Targeted disruption of the *Mn1* gene causes a defect in the development of cranial membranous bones and death just after birth.[4] The *TEL* gene, also termed *ETV6*, codes for a member of the E26-transformation-specific (ETS) family of transcription factors, and is the target of many different translocations leading to leukemia.[5] Whereas TEL confers its oligomerization domain in some translocations, it contributes the ETS-like DNA-binding domain in others, thus creating chimeric transcription factors with altered properties.[6,7]

The retinoic acid receptor (RAR) is a key regulator of proliferation and differentiation of blood cell lineages. RAR forms a dimer with RXR, and recruits p160 coactivators upon binding of ATRA to its ligand-binding domain.[8] In turn, the members of the p160 family bind other factors, for example. p300/CBP. This complex of coactivators contacts the basal transcription machinery and causes histone acetylation, which is thought to facilitate access of RNA polymerase.[9] In the absence of ATRA, RAR-RXR recruits corepressors like NCoR and SMRT, which in turn recruit histone deacetylase complexes containing SIN3 and HDAC.[10] Inhibition of RAR function, for example by its fusion to promyelocytic leukemia (PML) protein, leads to downregulation of RAR-responsive genes, which results in a block in differentiation and an increase in the number of progenitor cells.[11] The transcription-repressing effect of PML-RARα relative to RARα is at least partially caused by a comparatively tight binding of corepressors.[12] It is now widely believed that fusion transcription factors can cause leukemia by altering the balance between activation and inhibition of critical target genes.

The transforming activity of MN1-TEL in fibroblasts depends on the transactivation domains of MN1 and the DNA binding domain of TEL,[13] but the fusion protein also immortalizes myeloid cells and causes AML in mice.[14,15] Although these studies have established the capacity of MN1-TEL to promote leukemia, they did not provide a molecular basis for its malignant effects. We therefore studied the fusion protein in a model system with established MN1 activity.[2] MN1-TEL acts as an altered transcription factor compared to TEL, as it stimulates transcription from a reporter carrying responsive elements normally repressed by TEL.[13] We now show that fusion to TEL also alters the properties of MN1.

Rather than stimulating RAR-mediated transcription, as does MN1, MN1-TEL inhibits RAR-mediated transcription. Moreover, MN1-TEL acts as a dominant-negative mutant, as it efficiently represses RAR-RXR- mediated transcription even in the presence of MN1. Thus, repression of RAR-responsive genes may be an important aspect of the leukemogenic activity of MN1-TEL.

MATERIALS AND METHODS

Plasmids and detection of fusion proteins

Dr. A. Zantema and Dr. G. Jenster provided expression constructs for p300 and RAC3, respectively. For expression purposes, MN1-TEL cDNA[1] was cloned into vector pcDNA3 (Invitrogen, Carlsbad, CA, USA), resulting in plasmid pcDNA3MN1-TEL. An MN1 expression construct and reporter constructs bearing the MSV-LTR and GAL4 upstream activator sequences,[2] and expression constructs for TEL and MN1-TELDBDm[1] have been described. The pGL-MSV4 construct comprises a reporter containing the 360 basepair MSV-LTR excluding all sequences upstream of the first direct repeat. In pGL-MSV45, the 5′ direct repeat of pGL-MSV4 has been replaced by the canonical DR5 sequence of the RARβ promoter.[16] Fusions to the GAL4 DNA binding domain (DBD) were constructed in plasmid pGBT9 (Clontech, Palo Alto, CA, USA) and subsequently transferred to pcDNA3 for expression in mammalian cells. Fusions to green fluorescent protein (GFP) were made by cloning the MN1 cDNA sequence into pEGFP-C3 or pEGFP-N3 (Clontech) using internal *Bam*HI or *Xho*I sites corresponding to MN1 aminoacids 48 and 1292, respectively. Correct expression was confirmed by Western blotting. The antibody to MN1 (2F2) has been described.[13] Antibodies to GAL4 (SC-510) and TEL (H-214) were from Santa Cruz Biotechnology (Santa Cruz, CA, USA), and the antibody to GFP (no. 11814460) was from Roche, Basel, Switserland.

Transient transfections

Hep3B cells were cultured in α-MEM supplemented with 5% fetal calf serum and antibiotics. For transient transfections, 8×10^4 cells were seeded per well of a 24-wells tissue culture plate. After 24h, transfections were performed using 1.0 µl FuGENE 6 (Roche) per 0.5 µg of total plasmid DNA, as recommended by the manufacturer. Where indicated, ATRA (Sigma, St. Louis, MO, USA) was added to a concentration of 1 µM, and TSA (Sigma) to the specified concentration, both from ethanol stocks. Cells were harvested 24h after transfection, lysed, and luciferase expression was assayed on a Fluoroscan Ascent FL

luminometer (Labsystems, Helsinki, Finland). In all experiments, the total amount of transfected DNA and the molar ratio of cytomegalovirus (CMV) promoter were kept constant. All transfections were performed at least three times and in triplicate, and the results proved highly reproducible. Protein expression by various fusion constructs was assayed by Western blotting of lysates of the transfected cells, and their expression level was found to be similar, confirming that transfection efficiencies were reproducible within an experiment.

Inducible cell lines and real-time quantitative PCR

U937-derived[17] stable cell lines expressing MN1 or MN1-TEL under control of the tet-off system were generated essentially as described, by selecting cells transfected with pUHD10S plasmid bearing the cDNA of the gene of interest.[18] Empty vector was used as a control. U937-derived cell lines were grown in RPMI 1640 supplemented with 10% fetal calf serum, antibiotics, 1 µg/ml tetracyclin, 0.5 µg/ml puromycin, and 100 µg/ml hygromycin. Gene expression (3 x 106 cells per sample) was induced by three washes in phosphate-buffered saline (PBS) and resuspension in 15 ml tetracyclin-free medium either without ATRA or containing 1 µM ATRA. Total RNA was extracted 24h after induction with TRIzol reagent (Life Technologies, Gaithersburg, MD, USA) and further purified using RNeasy columns with on-column DNase treatment (Qiagen, Valencia, CA, USA). Total RNA (3 µg) was converted into cDNA with the M-MLV reverse transcriptase system (Invitrogen). Real-time qPCR was performed using the cDNA equivalent of 25 ng total RNA, 330 nM of each primer, and 12.5 µl SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA, USA) in a total volume of 25 µl. *MEIS1* (ID3185) primer sequences were taken from the RTprimerDB,[19] *POLR2A* primers were provided by E. Korpershoek, and *S100A8* primer sequences were 5'-ATGTTGACCGAGCTGGAGAA-3' and 5'-CAGAATGAGGAACTCCTG GAAG-3'. Standards were prepared by fourfold serial dilutions of a pooled sample, consisting of equal amounts of cDNA from each of the MN1 and MN1-TEL inducible cell lines. Quantitative RT-PCR was performed in duplicate for each biological duplicate, on an ABI 7700 (Applied Biosystems), annealing at 60°C and measuring at 72°C.

RESULTS

MN1-TEL does not synergize with RAR-RXR

Both MN1 and MN1-TEL stimulate transcription from the murine sarcoma virus long terminal repeat (MSV-LTR), and MN1 synergizes with RAR-RXR, p160, and p300 in the activation of

this promoter.[2,13] The MSV-LTR comprises multiple transcription factor binding sites including a direct repeat 5 (DR5) ATRA-responsive element[2] and several binding sites for ETS transcription factors.[20] To determine whether MN1-TEL synergizes with RAR-RXR when stimulating the MSV promoter, we co-transfected human Hep3B cells with the MSV1 reporter and an MN1-TEL expression construct. Co-transfections were carried out either in the absence or presence of ATRA, thus activating the endogenous RAR-RXR.[21] MN1 and TEL cDNA expression constructs were used as controls, and expression was verified by Western blot analysis (Figure 1A and B). MN1 stimulated transcription of the MSV-LTR and synergized with RAR-RXR after addition of ATRA (Figure 1C). The transcriptional repressor TEL[22] inhibited transcription from the MSV-LTR (Figure 1D). MN1-TEL also stimulated transcription, albeit less efficiently than MN1 (Figure 1E). Addition of ATRA independently
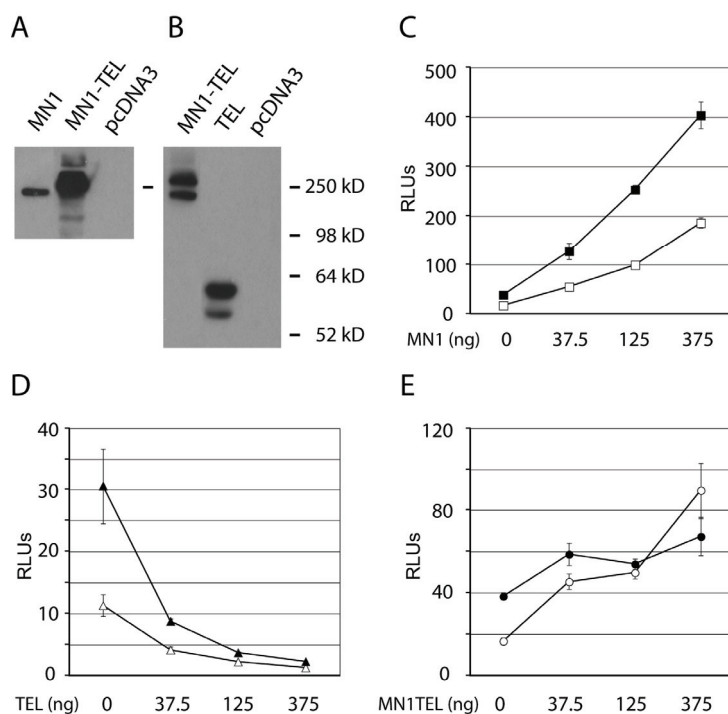
Figure 1. MN1-TEL does not synergize with RAR-RXR when stimulating the MSV-LTR. Expression constructs were transfected into Hep3B cells, and Western blot analysis using antibodies to MN1 (A) or TEL (B) showed comparable levels of MN1, MN1-TEL and TEL. The MSV1 reporter was co-transfected with the indicated amounts of MN1 (C), TEL (D), or MN1-TEL (E) expression construct in the absence (open symbols) or presence (closed symbols) of ATRA. Whereas MN1 synergizes with RAR-RXR in activation of the MSV-LTR, MN1-TEL activates transcription but does not synergize with RAR-RXR. TEL suppresses the MSV-LTR.

stimulated transcription, but the stimulation by ATRA was progressively lost in the presence of higher concentrations of MN1-TEL. Thus, in contrast to MN1, MN1-TEL does not synergize with RAR-RXR.

To analyse whether MN1-TEL exerts a similar effect on endogenous genes as it does on the MSV-LTR, we determined whether MN1 and MN1-TEL differentially regulate *MEIS1* and *S100A8* (*MRP8*), two RAR target genes involved in differentiation of hematopoietic cells.[23,24,25] *MEIS1* and *S100A8* mRNA levels in U937 cells expressing MN1 or MN1-TEL under the control of the tet-off system were determined by real-time quantitative reverse transcription-polymerase chain reaction (RT-PCR) (Figure 2). The results were normalized using the mRNA levels of RNA polymerase 2 and presented relative to those in the presence of the tet-repressor. Compared to basal expression levels, both ATRA and MN1 individually induced *MEIS1* and *S100A8*. MN1 expression in the presence of ATRA resulted in a synergistic induction. U937 cells expressing MN1-TEL failed to induce *MEIS1* and *S100A8*, and did not synergize with ATRA. MN1-TEL behaved similar to the control, showing a slight reduction of *MEIS1* and *S100A8* stimulation by ATRA upon tetracyclin release. The failure of MN1-TEL to synergize with RAR-RXR is thus a more general phenomenon and not restricted to the MSV-LTR.
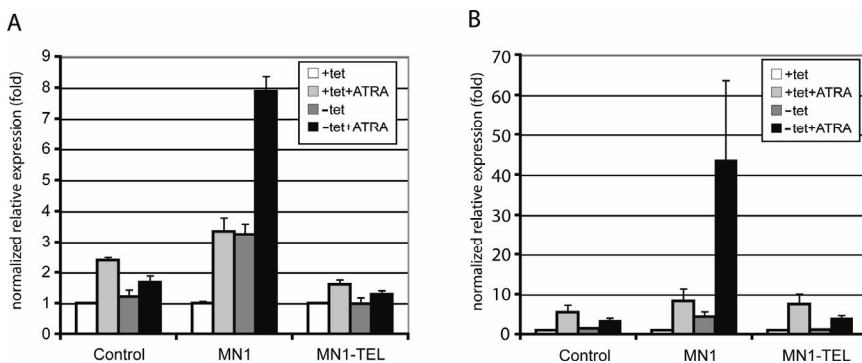
Figure 2. MN1-TEL does not synergize with RAR-RXR-mediated induction of *MEIS1* and *S100A8*. Expression of MN1 or MN1-TEL was induced by withdrawing tetracyclin (-tet) from stably transfected U937 cells in the absence or presence of ATRA. Subsequently, *MEIS1* (A) and *S100A8* (B) mRNA levels were determined by quantitative RT-PCR. ATRA and MN1 each induce *MEIS1* and *S100A8*, and together work synergistically. MN1-TEL, however, does not induce these RAR target genes and shows no synergy with ATRA.

MN1-TEL does not synergize with typical RAR-RXR coactivators

Stimulation of the MSV-LTR by MN1 is enhanced in a synergistic manner by p160 and p300, indicating that these coactivators mediate the synergy of MN1 and RAR-RXR.[2] To determine

whether MN1-TEL synergizes with p160 and p300, increasing amounts of RAC3 or p300 were co-transfected with either MN1 or MN1-TEL. MN1 stimulated transcription of the MSV-LTR, and a combination of MN1, ATRA and the p160 family member RAC3 markedly boosted the induction level (Figure 3A). An even stronger effect was observed when a combination of MN1, ATRA and p300 was used (Figure 3B). These effects were not recapitulated by a combination of MN1-TEL, ATRA and RAC3 (Figure 3C) or p300 (Figure 3D), and MN1-TEL had a repressing effect on the final induction level in the presence of RAC3 or p300. These results demonstrated synergy when RAC3 or p300 and MN1 were transfected, but a clear absence thereof when combinations of RAC3 or p300 and MN1-TEL were used.
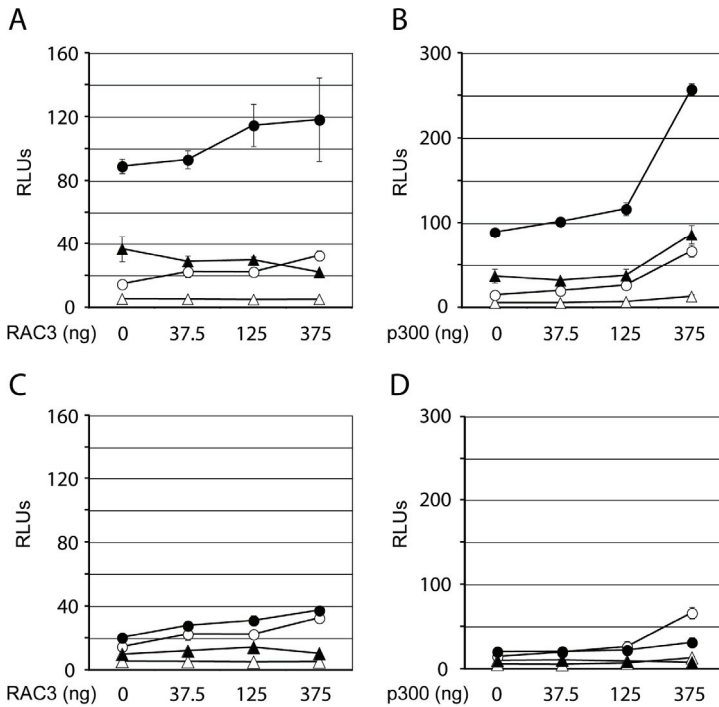
Figure 3. MN1-TEL does not synergize with RAC3 or p300. The MSV1 reporter and 125 ng of MN1 (A and B) or MN1-TEL (C and D) expression constructs were co-transfected with the indicated amounts of RAC3 or p300 expression constructs in the absence (open symbols) or presence (closed symbols) of ATRA. Triangles (△) represent the effects of RAC3 or p300 in the absence of MN1 or MN1-TEL, and circles (○) represent the effect in the presence of MN1 or MN1-TEL. MN1 shows clear synergy with RAC3 and p300. Under the same conditions, MN1-TEL does not show synergy with either coactivator.

The activation domains in MN1-TEL do not function properly

The MN1-TEL fusion protein contains nearly the complete MN1 sequence, including its transactivating domains that stimulate transcription through an interaction with p160 and p300.[2] Deletion of the MN1 transactivating domains from MN1-TEL abolishes activation of a TEL-responsive reporter.[13] Given that MN1-TEL does not synergize with p160 and p300, its transactivating domains may not function properly. We thus examined the functionality of the transactivating domains (TADs) in MN1-TEL with GAL4 DNA-binding domain (DBD) fusion
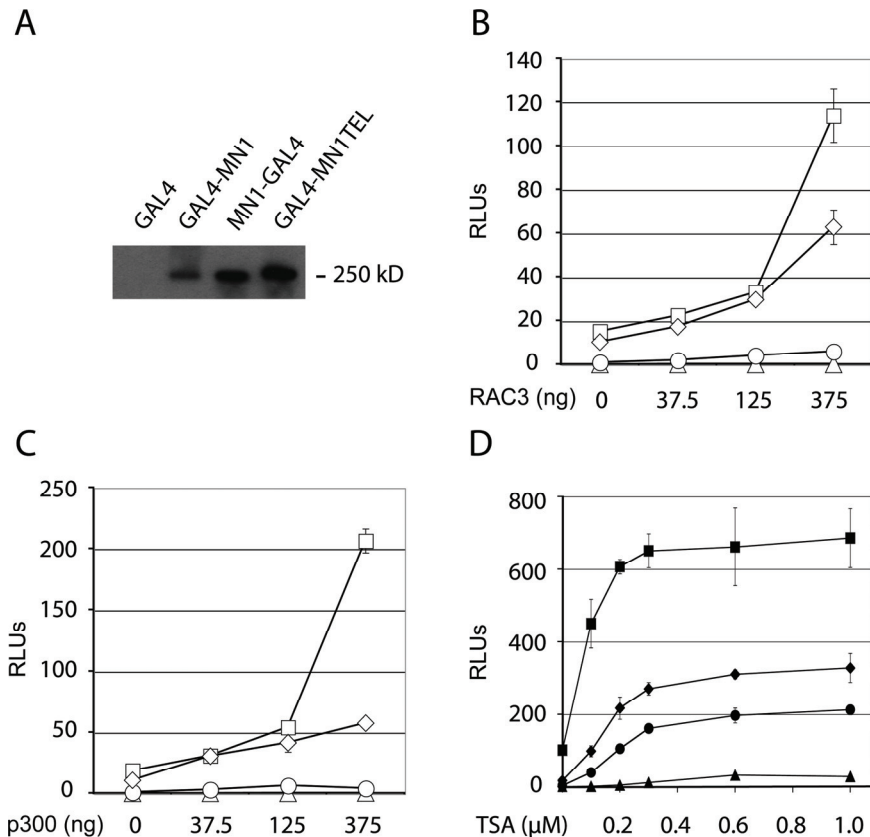
Figure 4. The transactivating domains in MN1-TEL do not respond to coactivators or histone deacetylase inhibitors. Expression constructs were transfected into Hep3B cells, and Western blot analysis using an antibody to MN1 showed comparable protein levels (A). Similar results were obtained using an antibody to GAL4 DBD (not shown). Luciferase reporters were co-transfected with fixed amounts of GAL4 fusion constructs and the indicated amounts of RAC3 or p300 constructs. GAL4-MN1 activates transcription in the presence of endogenous levels of coactivators. Whereas RAC3 (B) and p300 (C) enhance expression driven by GAL4-MN1 in a synergistic way (□). They stimulate MN1-GAL4 to a lesser degree (◇), and do not productively stimulate GAL4-MN1-TEL (○) hybrid protein or GAL4 (△) control. Luciferase reporters were co-transfected with 125 ng GAL4 fusion constructs and treated with increasing concentrations of TSA (D). Addition of histone deacetylase inhibitors restored GAL4-MN1-TEL (○) driven transcription only to levels well below those of GAL4-MN1 (□), and also below those of MN1-GAL4 (◇). The GAL4 (△) control plasmid did not activate transcription.

constructs and a reporter containing GAL4 upstream activator sequences. The nuclear localization of fusion proteins was verified using antibodies to GAL4 (not shown), and Western blots of lysates of transfected cells showed similar expression of the fusion proteins (Figure 4A). Fusions of MN1 and the GAL4-DBD showed a strong transactivation of the reporter, both when GAL4 was fused to the amino-terminus (GAL4-MN1) or the carboxy-terminus (MN1-GAL4). The transcription activation by GAL4-MN1, and to a lesser extent MN1-GAL4, could be further stimulated by RAC3 (Figure 4B) or p300 (Figure 4C). The fusion of MN1-TEL and the GAL4-DBD, however, activated transcription far less efficiently than GAL4-MN1 and MN1-GAL4. Additionally, GAL4-MN1-TEL driven transcription was stimulated ineffectively by RAC3 or p300. This indicates that, in contrast to MN1, MN1-TEL does not interact productively with p160 or p300.

Given that TEL represses transcription by recruiting a histone deacetylase complex,[22] it is conceivable that these deacetylases counteract coactivators bound to MN1-TEL. To test this hypothesis, GAL4-MN1-TEL and the reporter were transfected in the absence or presence of trichostatin A (TSA), a potent inhibitor of histone deacetylases.[26] Addition of TSA further stimulated activation by GAL4-MN1, and to a lesser degree by MN1-GAL4 (Figure 4D). Although GAL4-MN1-TEL was stimulated somewhat by TSA, its final activity was only one fifth of that of GAL4-MN1, and was lower than that of MN1-GAL4. TSA concentrations above 0.6 µM did not result in further activation of the reporter. These results show that histone deacetylases may inhibit MN1-TEL to some extent, but its inability to transactivate efficiently is the major reason for its modest transcription activation.

The MN1 carboxy-terminus contributes to the transcription activity of the protein
TEL fusion to the carboxy-terminus of MN1 eliminates its capacity to synergize with p160 and p300. Because GAL4 fusion to the MN1 carboxy-terminus also reduced the responsiveness towards coactivators, this may be a general phenomenon. To determine whether fusion of an unrelated protein domain could recapitulate this effect, we compared the transcription stimulation of the MSV-LTR by amino-terminal (GFP-MN1) and carboxy-terminal (MN1-GFP) fusions of MN1 with green fluorescent protein (GFP) (Figure 5). GFP alone was transfected as control. Western blots of lysates of transfected cells revealed that the fusions were expressed at a similar level (Figure 5A). Although both GFP-MN1 and MN1-GFP were able to stimulate the MSV-LTR and synergized with RAR-RXR, the carboxy-terminal fusion was far less active than the amino-terminal fusion (Figure 5B). Given that the carboxy-terminal fusion of GFP, GAL4 or TEL moieties all reduced MN1 performance, the carboxy-terminus contributes to its transcription activity. TEL fusion nonetheless appears to

have a more severe effect than the fusion of GAL4, as GAL4-MN1-TEL was less active than GAL4-MN1 and MN1-GAL4.
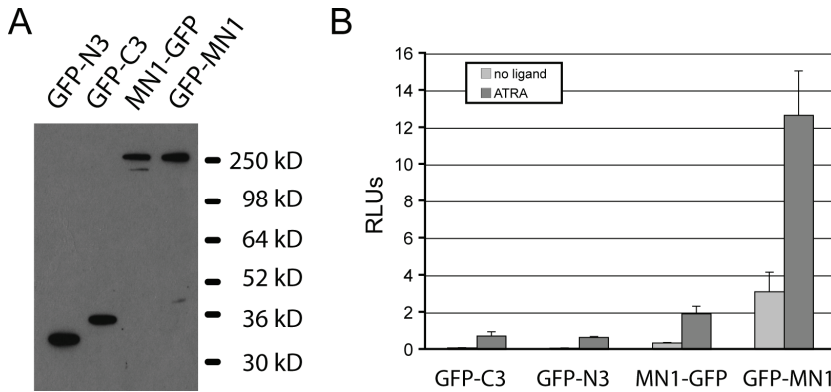
Figure 5. The MN1 carboxy-terminus contributes to its transcription-activating function. The MSV4 reporter was co-transfected with GFP alone or with GFP fused to the MN1 amino-terminus (GFP-MN1) or carboxy-terminus (MN1-GFP). Fusion constructs were transfected into Hep3B cells, and Western blot analysis of cell lysates with a GFP antibody showed comparable protein levels (A). Whereas GFP-MN1 efficiently stimulated transcription from the MSV-LTR, MN1-GFP was a markedly less potent activator (B). GFP alone (GFP-C3 and GFP-N3) did not stimulate transcription from the MSV-LTR.

MN1-TEL has a dual effect on transcription and acts as a dominant-negative mutant of MN1

Mutation of the TEL DBD abolishes the ability of MN1-TEL to activate the MSV-LTR, showing that this domain enables MN1-TEL to activate transcription through TEL-responsive elements.[13] On the other hand, MN1, RAR-RXR, p160 and p300 synergistically stimulate the MSV-LTR, whereas MN1-TEL does not. Therefore, the weak transactivation of the MSV-LTR by MN1-TEL compared to MN1 may be a combination of activation through TEL-responsive elements and repression through ATRA-responsive elements. To discriminate between these different modes of action, fully functional MN1-TEL was compared with a point mutant (MN1-TELDBDm) unable to bind TEL-responsive elements.[13] Whereas MN1-TEL may affect both TEL and RAR-responsive elements, MN1-TELDBDm is predicted to act only on RAR-responsive elements. The MSV-LTR was stimulated strongly by MN1, moderately by MN1-TEL, but was repressed by MN1-TELDBDm (Figure 6A). Hence, MN1-TEL has both a stimulatory and a repressing effect on transcription. TEL strongly repressed transcription. Because the DBD mutant of TEL locates to the cytoplasm instead of the nucleus,[13] it was not included in these experiments.
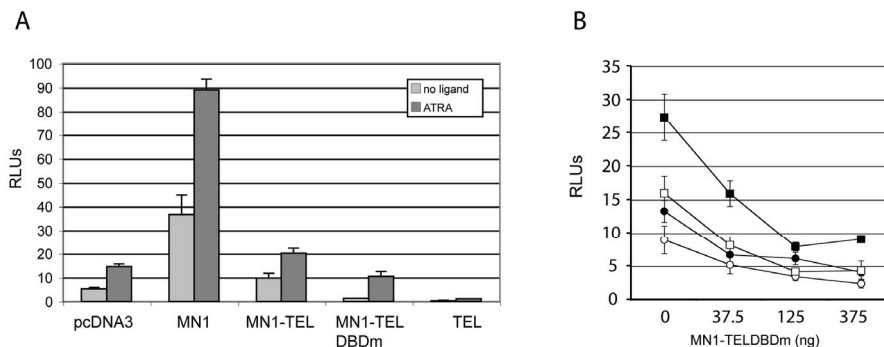
Figure 6. MN1-TEL has a dual effect on transcription from the MSV-LTR (A), and acts as a dominant-negative mutant (B). The MSV1 reporter was co-transfected with various expression constructs in the absence or presence of ATRA (A). Whereas MN1 and MN1-TEL stimulate reporter activity, MN1-TELDBDm and TEL inhibit transcription compared to the control. The MSV45 reporter was co-transfected with the indicated amounts of MN1-TELDBDm expression construct in the absence (○) or presence (□) of 125 ng MN1 expression construct and in the absence (open symbols) or presence (closed symbols) of ATRA (B). MN1-TELDBDm inhibits reporter activity in a dose-dependent manner under all conditions tested.

Several leukemia-associated fusion proteins affect the function of both partner proteins encoded by the remaining normal alleles.[6,7] To determine whether MN1-TEL interferes with MN1 function, a constant amount of MN1 construct was co-transfected with increasing amounts of MN1-TELDBDm, thus avoiding the stimulatory effect of MN1-TEL on TEL-responsive elements. In the absence of MN1, MN1-TELDBDm caused a dose-dependent repression of reporter activity (Figure 6B). MN1 and ATRA activated the MSV-LTR alone and in combination, but co-transfection of small amounts of MN1-TELDBDm efficiently repressed this activation. An equimolar ratio of MN1-TELDBDm and MN1 (125 ng), the situation that most likely mimics their expression in t(12;22)(p13;q11), activated the reporter only slightly above basal level and significantly diminished the stimulatory effect of ATRA. Higher amounts of MN1-TELDBDm did not further decrease activity. These results show that MN1-TELDBDm inhibits the normal activation of the MSV-LTR by MN1 and RAR-RXR, and indicate that MN1-TEL acts as a dominant-negative inhibitor of MN1 on promoters without TEL-responsive elements.

DISCUSSION

We have studied the properties of the MN1-TEL fusion protein, the product of t(12;22,p13;q11) associated with AML. This fusion protein was shown to activate TEL-responsive transcription, whereas TEL itself is a repressor.[13,22] Now, we have compared the

properties of the MN1 and MN1-TEL with respect to RAR-RXR-mediated transcription, crucial for the proliferation and differentiation of blood cell lineages. Whereas MN1 functions as a coactivator for RAR-RXR, MN1-TEL inhibits RAR-RXR-mediated transcription. Furthermore, MN1-TEL seemed to act as a dominant-negative inhibitor of MN1, as it severely hampered the activation of RAR-RXR by MN1.

Using MN1-TELDBDm, a mutant that cannot bind DNA by itself, we have been able to distinguish between direct and indirect effects of MN1-TEL on promoters. MN1-TEL enhances transcription from TEL-responsive elements, by directly stimulating transcription, by competing with endogenous repressors such as TEL itself, or both. MN1-TEL may thus activate transcription of genes that are normally repressed by TEL. In our transfection experiments too, MN1-TEL stimulated transcription from the MSV-LTR. Unlike MN1, however, MN1-TEL did not synergize with RAR-RXR, p160 or p300, nor did it stimulate expression of the endogenous RAR-RXR target genes *MEIS1* and *S100A8*. MN1-TELDBDm, which only affects RAR-RXR function, strongly repressed transcription. These results show that MN1-TEL has at least two distinct and opposing effects on the MSV-LTR, a stimulatory activity dependent on direct recognition of responsive elements, and a repressive activity independent of direct DNA binding. MN1-TELDBDm not only repressed RAR-mediated transcription in the absence of MN1, but also diminished the stimulatory effect of ATRA when expressed at a concentration equimolar to that of MN1. This shows that MN1-TEL acts as a dominant-negative inhibitor of RAR and MN1 when eliminating the contribution of TEL-responsive elements.

ATRA did not prevent MN1-TEL from repressing RAR-RXR-mediated transcription. This suggests that MN1-TEL functions differently from the classical corepressor complexes containing NCoR and SMRT, which bind only in the absence of ATRA.[27] In t(15;17) acute promyelocytic leukemia, the PML-RARα fusion protein binds corepressor complexes more tightly than RAR alone. Corepressor complexes can be released from PML-RARα with pharmacological doses of ATRA, or can be counteracted by histone deacetylase inhibitors in the case of the PLZF-RAR fusion.[12] Because TEL is a transcriptional repressor that recruits corepressors and histone deacetylases,[22] MN1-TEL could recruit such corepressor complexes to RAR-RXR. Nonetheless, even pharmacological doses of ATRA only marginally relieved the repression in our transfection assays and experiments with histone deacetylase inhibitors showed that tight or irreversible binding of corepressor complexes is not the cause of MN1-TEL´s inhibitory activity. Instead, MN1-TEL appears to block RAR-RXR-mediated transcription by preventing the productive recruitment of coactivator

complexes. Accordingly, MN1-TEL inhibited the capacity of p160 and p300 to stimulate the MSV-LTR.

The fusion of amino-terminal TADs to the DBD of ETS family members is not restricted to leukemias. The EWS-FLI fusion protein that causes Ewing sarcoma bears very similar characteristics.[28] This fusion, too, may have both transcription-stimulating and transcription-repressing effects.[29] Future studies may thus reveal the importance of the disruption of gene expression by this type of fusions in a wide variety of tumors. Whereas MN1-TEL was shown to activate TEL-responsive elements,[13] we now reveal that the fusion protein is also a dominant-negative suppressor of MN1 and RAR-RXR. ATRA or histone deacetylase inhibitors are unable to relieve this effect. Thus, a possible treatment of t(12;22) leukemia will likely require a novel combined strategy, aiming to reduce the stimulation of TEL-responsive promoters and simultaneously overcome the inhibition of RAR-RXR.

ACKNOWLEDGEMENTS

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CHAPTER 5

MN1 affects expression of genes involved in hematopoiesis and can enhance as well as inhibit RAR/RXR-induced gene expression

Magda A. Meester-Smoor[1,5], Marjolein J.F.W. Janssen[1,5], Gerard C. Grosveld[2], Annelies de Klein[3], Wilfred F.J. van IJcken[4], Hannie Douben[3], Ellen C. Zwarthoff[1]

[1]Department of Pathology, Erasmus MC, Rotterdam, The Netherlands
[2]Department of Genetics and Tumor Cell Biology, St. Jude Children's Research Hospital, Memphis, TN, USA
[3]Department of Clinical Genetics, Erasmus MC, Rotterdam, The Netherlands
[4]Erasmus Center of Biomics, Erasmus MC, Rotterdam, The Netherlands
[5]Authors contributed equally to this work

ABSTRACT

The oncoprotein meningioma 1 (MN1) is overexpressed in several subtypes of acute myeloid leukemia (AML) and overexpression was associated with a poor reponse to chemotherapy. MN1 is a cofactor of retinoic acid receptor/retinoid-x-receptor (RAR/RXR)-mediated transcription and this study identified genes in the promonocytic cell line U937 that were regulated by MN1. We found that MN1 can both stimulate and inhibit transcription. Combining MN1 expression with all-*trans* retinoic acid (ATRA), the ligand of the RAR/RXR dimer, showed that MN1 could both enhance and repress ATRA effects. Many of the identified genes are key players in hematopoiesis and leukemogenesis (e.g. *MEIS1* and *BMI1*). Another interesting target is *DHRS9*. DHRS9 is involved in the synthesis of ATRA from vitamin A. MN1 inhibited *DHRS9* expression and completely abolished its induction by ATRA. *MN1* is also the target of a rare AML-causing translocation encoding the MN1-TEL protein. MN1-TEL induces expression of only a few genes and its most pronounced effect is inhibition of a large group of ATRA-induced genes including *DHRS9*. In conclusion, both MN1 and MN1TEL interfere with the ATRA pathway and this might explain the differentiation block in leukemias in which these genes are involved.

INTRODUCTION

Recently, *meningioma 1 (MN1)* was identified as an important player in myeloid leukemogenesis. Overexpression of *MN1* is observed in AML specified by the chromosomal aberration inv (16),[1,2] in some AMLs overexpressing the transcription factor ecotropic viral integration (*EVI1*),[2] and in some adult AMLs with normal karyotype.[3] A high level of *MN1* expression is a predictor of poor prognosis in these latter patients and recently it has been shown that low *MN1* expression can be used as a marker to predict sensitivity to all-*trans* retinoic acid (ATRA) treatment.[4] By retroviral insertional mutagenesis Slape *et al* have identified *Mn1* as an second hit that co-operates with the *NUP98-HOXD13* fusion in causing myelodysplastic syndrome (MDS) or AML in mice.[5]

However, the involvement of *MN1* in AML has already been known much longer. In 1995, we cloned the *MN1* gene[6] and soon thereafter it was identified as a target gene of the recurrent t (12;22) found in AML patients.[7] As a result, almost all of the coding sequence of *MN1* is fused to two-thirds of the coding sequence of *TEL (ETV6)*. MN1 appeared to act as a transcriptional coactivator of retinoic acid receptor/retinoid-x-receptor (RAR/RXR),[8] but recent research[4] and also this paper show that MN1 has a dual function. Depending on the type of RAR/RXR target gene, MN1 can either stimulate or repress transcription, with or without collaboration with ATRA. TEL, the other fusion partner of the MN1-TEL fusion, is a member of the E26 transformation-specific (ETS) family of transcription factors and represses transcription by binding to ETS elements in promoters.[9,10] MN1-TEL is thought to act as a novel transcription factor causing transcription deregulation of genes normally repressed by TEL.[11] Recently, we have shown that MN1-TEL has an additional characteristic important for its pathogenic properties. By the use of a point mutant that cannot bind to ETS elements, we have shown that MN1-TEL represses RAR/RXR-mediated transcription.[12]

Bone marrow (BM) transduction/transplantation experiments in mice have shown that both *MN1-TEL* and *MN1* are hematopoietic oncogenes. MN1-TEL induces AML within three months after BM transplantation.[13] MN1 overexpression in mouse BM causes myeloproliferative disease, a condition slightly different from AML. Mice died within 5-8 weeks after receiving transplants. Combined expression in mouse BM of MN1 and CBFb-MYH11, the product of inv (16), resulted in rapid development of AML strongly suggesting that overexpression of MN1 is indeed an important step in the development of inv (16) AML.[14] Heuser *et al* have also shown that *MN1* is a powerful oncogene in mouse BM and based on the rapid development of disease suggested that overexpression of MN1 alone

might be sufficient to cause AML in mice.[4] In agreement with the results of the *in vivo* studies, *Mn1* expression is found in the granulocytes-monocytes progenitor (GMP) fraction of hematopoietic cells in mice.[14] Wagner *et al* detected *MN1* in the slow dividing, primitive fraction of hematopoietic stem cells obtained from human umbilical cord blood .[15]

Although all these reports document the importance of MN1 in different forms of leukemia, little is known about the downstream targets of MN1 expression, which severely hampers our understanding of its involvement in leukemogenic transformation. This study aimed to identify genes regulated by MN1 and MN1-TEL by expression profiling in the promonocytic cell line U937[16] and the effect of expression of these genes in absence or presence of ATRA. The U937 cell line has a progenitor phenotype with features close to the granulocytes-monocytes progenitor (GMP) stage of hematopoiesis. The cell line has extensively been employed to study myeloid differentiation induced by agents such as ATRA and DMSO. We show that MN1, directly or indirectly, affects expression of many genes that play a role in hematopoiesis. Moreover, MN1 can both inhibit and stimulate expression of genes induced by RAR/RXR. MN1-TEL inhibits some genes that are also inhibited by MN1 but its greatest effect is in inhibiting RAR/RXR-induced genes.

MATERIALS AND METHODS

Origin of cell lines, cell culture, induction of gene expression and Western blotting
The cell lines were based on U937T[17] (derived from cell line U937[18]) and inducibly express *MN1* or *MN1-TEL* using the tet-off system. They were generated as described previously,[17] by selecting cells transfected with pUHD10S plasmid, containing complementary DNA (cDNA) of the gene of interest. The cell lines will be referred to as the MN1, MN1-TEL, and UHD cell line. The UHD cell line is transfected with empty expression plasmid. The cell lines were cultured in RPMI 1640 medium supplemented with 10% fetal calf serum, 1000 U/ml penicillin, 1 mg/l streptomycin, 1 ug/ml tetracyclin, 0.5 µg/ml puromycin, and 100 µg/ml hygromycin. Gene expression was induced by withdrawal of tetracyclin. Two separate induction experiments were performed a few weeks apart, to serve as biological duplicates. For each time point (0, 16, 20, 24, 48, 72h) 3×10^6 cells of each cell line were exposed to four different medium conditions: absence of tetracyclin (-tet: induction of target gene); presence of tetracyclin (+tet: no induction of target gene); both in absence or presence of 1 µM all-trans retinoic acid (ATRA, Sigma-Aldrich, Zwijdrecht, The Netherlands). The cells were harvested and washed in PBS. Dry cell pellets were stored at -80°C corresponding to a 1-ml portion (to be used later for RNA extraction) and two 100-µl portions of resuspended cells

(each to be used for Western blot analysis; enough for loading of 4 gels). The antibody against the N-terminal part of MN1 (2F2) has been described.[11] The antibody against BMI1 was F6 (Upstate, USA), for YES1 #610375 (BD Biosciences, Breda, the Netherlands) and ID1 SC-488 (Santa Cruz, CA, USA). The Western blots were incubated with horseradish peroxidase-labeled secondary antibodies (P044701, DAKO, Glostrup, Denmark) and IRDye 680 conjugated anti-mouse (LI-COR, Lincoln, NE, USA). The latter was used to scan and quantify the protein signals on an Odyssey infrared imaging system (LI-COR). Protein levels were calculated relative to the amount of β-tubulin present in the sample. The β-tubulin antibody was from hybridoma E7.

RNA extraction and amplification of RNA

Total RNA was extracted from the frozen cell pellets by TRIzol reagent (Invitrogen, Carlsbad, CA, USA) and further purified using RNeasy columns with on-column DNase treatment (Qiagen, Hilden, Germany). The concentration and purity of the RNA was measured on a NanoDrop ND-1000 UV-VIS spectrophotometer and the quality of the RNA was checked using the Agilent 2100 BioAnalyzer with an RNA 6000 Nano LabChip. Three microgram of total RNA was used for linear amplification using the Superscript III RNA Amplification kit (Invitrogen). The concentration and purity of the amplified RNA were measured on a NanoDrop instrument and the quality of the amplified RNA was checked by agarose gel electrophoresis.

Gene expression profiling with microarrays

Spotted oligo microarrays with the Operon V3.0 library (35K Human, http://omad.operon.com/humanV3) were obtained from the Netherlands Cancer Institute Central Microarray Facility (NKI-CMF). Protocols for sample preparation were taken from the NKI-CMF website (http://microarrays.nki.nl). In short, 1 μg of amplified RNA was labeled using the ULS[TM]-Cy3/5 aRNA fluorescent labeling kit (Kreatech, Amsterdam, The Netherlands), and was used for hybridization on the same day. The labeling efficiency was checked on a NanoDrop instrument. The labeled aRNA was fragmented (RNA Fragmentation Reagents, Ambion, Austen, TX, USA) and mixed with blocking solution containing Poly d(A), Cot-1 DNA, and yeast t-RNA (GE Healthcare, Zeist, the Netherlands and Roche, Basel, Switserland). The arrays were hybridized overnight at 42°C on a Tecan HS4800 hybridization station, according to the M016 protocol developed by the Erasmus Center for Biomics (http://www.biomics.nl). Samples were co-hybridized according to a multifactorial design scheme as shown in supplementary Figure 1, with each combination of

samples in a straight and dye-swap fashion for each biological duplicate. The hybridized arrays were scanned on a Perkin Elmer ScanArray Express HT instrument. The measured fluorescence intensities were determined using ImaGene software version 6.0 (Biodiscovery, El Segundo, CA, USA).

Microarray data analysis

The ImaGene data were uploaded into the CMF database (CMFdb, http://cmfdb.nki.nl) and normalized using the lowess subarray method (default settings, except for background correction). No background correction was applied, as overall background was very low and even (mean background 311±75 for Cy5 and 446±173 for Cy3, averaged over all arrays, versus a mean signal of 2376±592 for Cy5 and 2927±650 for Cy3, corresponding to 15%). Background correction would result in negative intensities for low-intensity spots, indicating that any background signal was not superimposed on the spots but rather present next to the spots. This was also indicated by the appearance of the blank spotting controls, which showed up as holes in the background. The normalized data were downloaded from the CMF database and further analyzed in R using limmaGUI.[19,20,21] The data were analyzed with each time point separately as well as combined for each condition. Linear parametrizations were created including each of the comparisons of interest. Each separate comparison at each time point is composed of four arrays (supplementary Figure 1). In addition, each biological duplicate was analyzed separately to assess the biological variability. Gene lists were ranked based on the B-statistic (log odds of differential expression); *P* values are also reported. Unsupervised hierarchical clustering was performed using Spotfire DecisionSite 9.0 (Tibco, Somerville, MA, USA) with default settings. In the cluster analysis, log2 ratios at the individual time points with *B* values <1 were set to 0 when the accompanying *B* value of the combined time points of a certain reporter was also <1.

Reverse transcription-polymerase chain reaction and real-time quantitative polymerase chain reaction

Total RNA was converted into cDNA with the M-MLV reverse transcriptase system (Invitrogen), using 3 µg of total RNA and 750 ng of random primer (Invitrogen). Real-time quantitative polymerase chain reaction (qPCR) was performed using the amount of cDNA equivalent to 25 ng total RNA in a total volume of 25 µl containing 330 nM of primers each and 12.5 µl Power SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA, USA). The primers used for qPCR for *MEIS1* and *YES1* were taken from the RTprimerDB database (http://medgen.ugent.be/rtprimerdb/)[22] (IDs indicated). The primer sets for *NDRG1*

and *POLR2A* were obtained from fellow researchers. Whenever possible, intron-spanning primers were used. The sequences are shown in supplementary Table 1. Expression levels were determined relative to a standard curve for which standards were prepared by making 4-fold serial dilutions of a pooled sample. For each biological duplicate, qPCR was performed in duplicate and thus the results are represented as the average and standard deviation of four measurements. PCR reactions and real-time fluorescence measurements were done on an ABI 7700 Sequence Detection System (Applied Biosystems; annealing: 60°C, measurement: 72°C). The qPCR results were normalized using the messenger RNA levels of *RNA polymerase 2A* (*POLR2A*). The results are presented relative to the expression levels in the presence of tetracyclin for each cell line.

Fluorescent in situ hybridization
The U937 cell line cultures were treated for 30 min with colcemid and fixed with ethanol/acetic acid (3:1). Nuclei were spotted onto slides and incubated with the nicktranslation-labelled plasmids (Bio-nicktranslation kit, Invitrogen) or BAC probes (Random Prime labellings system, Invitrogen). The amount of plasmid probes was 5 ng per slide and that of BAC probes 50-75 ng per slide. BAC clones were selected from the UCSC genome browser (UC Santa Cruz) and purchased from BACPAC Resources (Oakland, USA). FISH slides were analysed with an Axioplan 2 Imaging microscope (Zeiss, Göttingen, Germany) and images were captured using Isis software (MetaSystems, Altlussheim, Germany).

RESULTS

Induction of expression of MN1 and MN1-TEL in U937T cells
Expression of *MN1* and *MN1-TEL* was induced in U937T cells by tetracyclin (tet) withdrawal for 16, 20, 24, 40, 48 and 72h. A Western blot visualizing the induction of MN1 or MN1-TEL protein is shown in Figure 1A. In the presence of tetracyclin (0h) there was no detectable expression of either protein but expression gradually increased from 16h onward, as indicated by the appearance of single bands of the correct molecular weight. At induction times exceeding 24h, additional bands appeared, possibly representing breakdown products of MN1 and MN1-TEL. The blots were stained with Coomassie blue to verify equal loading of the lanes (not shown). In parallel, the messenger RNA expression levels were examined by qPCR analysis as shown in Figure 1B. Both cell lines contained a low, but clearly detectable level of *MN1* or *MN1-TEL* transcripts even in the presence of tetracyclin, whereas

these products were not detected in the other inducible cell lines or the control cell line UHD. Tetracyclin release resulted in a 10-fold increase in transcript levels within 16-24h, increasing further with longer induction times. Interestingly, the presence of ATRA during tetracyclin release led to elevated expression levels in both cases (25-30 fold for MN1 and 30-40 fold for MN1-TEL at 16-24h). In pilot experiments, a similar effect of ATRA on induction of MN1 and MN1-TEL protein was observed (data not shown).
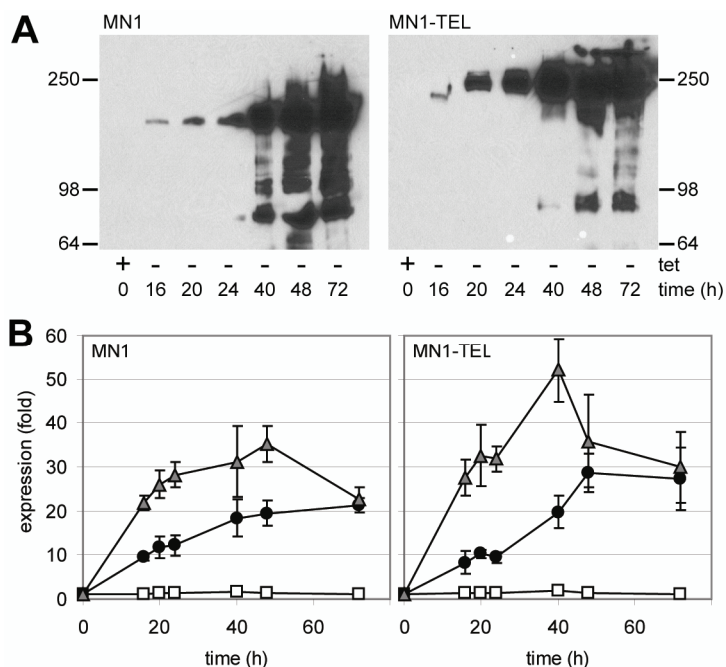
Figure 1. Induction of MN1 and MN1-TEL expression in U937T cells. The expression of MN1 and MN1-TEL was induced by withdrawal of tetracyclin for 16, 20, 24, 40, 48, and 72h, after which samples were collected for Western blotting and real-time qPCR. The cells collected at 0h were grown in the presence of tetracyclin and without the addition of ATRA. (A) Assessment of the expression of MN1 or MN1-TEL by Western blot, using an antibody raised against the N-terminal part of MN1 (2F2). (B) Assessment of the levels of *MN1* and *MN1-TEL* messenger RNA expression by qPCR. The qPCR results were normalized, using the messenger RNA levels of POLR2A, and are presented relative to those in the presence of only the tet-repressor. (white squares (□): +tet and +ATRA; black circles (●): -tet, no ATRA; grey triangles (▲): -tet and +ATRA).

Alterations in gene expression mediated by MN1 and MN1-TEL and ATRA in U937 cells

To examine the effects of MN1 and MN1-TEL on the expression of genes and the influence of ATRA on these effects, oligonucleotide microarray analysis was performed following the scheme presented in supplementary Figure 1. Only samples taken after 16, 20, and 24h of induction were used for microarray analysis because there is a clear expression of the

genes at these time points and we want to detect early effects and not focus on possible secondary effects of expression of MN1 and MN1-TEL. For assessment of the effect of ATRA, only samples induced for 16 and 20h were used. Each combination of samples was hybridized in a straight and dye-swap fashion for two biological duplicates, adding up to four arrays per combination. Both raw and normalized data have been deposited in NCBI's Gene Expression Omnibus[23] and are accessible through GEO Series accession number GSE11441 (http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE11441).

On average, 29129±2699 unflagged spots were quantified per array (out of 37632 on each of the 100 arrays in total). We identified 4468 spots showing statistically significant differences (*B* values of 1 or higher when all the time points (16, 20, 24h) were combined for analysis) in mean expression levels under the conditions selected for supplementary Figure 3. The unsupervised hierarchical clustering (supplementary Figure 3) of these signals shows that time points of each different treatment cluster together, and that all the ATRA effects cluster together. It is obvious that addition of ATRA results in higher expression levels than those observed with MN1 or MN1-TEL expression alone. When analyzing the expression data, we realized that *MN1*, although present on the array, did not show up as one of the differentially expressed genes. A closer inspection of the signal showed that it was consistently low for both Cy5 and Cy3 in all arrays (514±114 and 516±115, respectively), suggesting that the oligo for *MN1* most probably did not hybridize very well with the labeled amplified RNA.

To assess the reproducibility between biological duplicates for the genes affected by MN1, MN1-TEL and ATRA, correlation plots for the resulting reporter sets are shown in supplementary Figure 2. For these, the microarrays were analyzed for each biological replicate separately. Correlation coefficients (R^2) were obtained by linear regression of the log2 ratios in each biological duplicate plotted against each other. This resulted in correlation coefficients between the log2 ratios in each biological duplicate of 0.900 for genes affected by MN1, 0.843 for genes affected by MN1-TEL and 0.938 for genes affected by ATRA alone (all time points combined), indicating very similar behavior in both experiments. Even though most of the log2 ratios found were relatively low, just above 0.2 (absolute value), with maximal values up to 1.0 for MN1 and up to 0.6 for MN1-TEL, these high correlation coefficients give us confidence that even low log2 ratios are meaningful.

Genes affected by MN1, MN1-TEL and ATRA
A list was assembled consisting of all genes influenced by MN1 or MN1-TEL expression with a cutoff of log2 ratios >0.2 or <-0.2 (*n*=85) supplemented with 60 genes whose expression
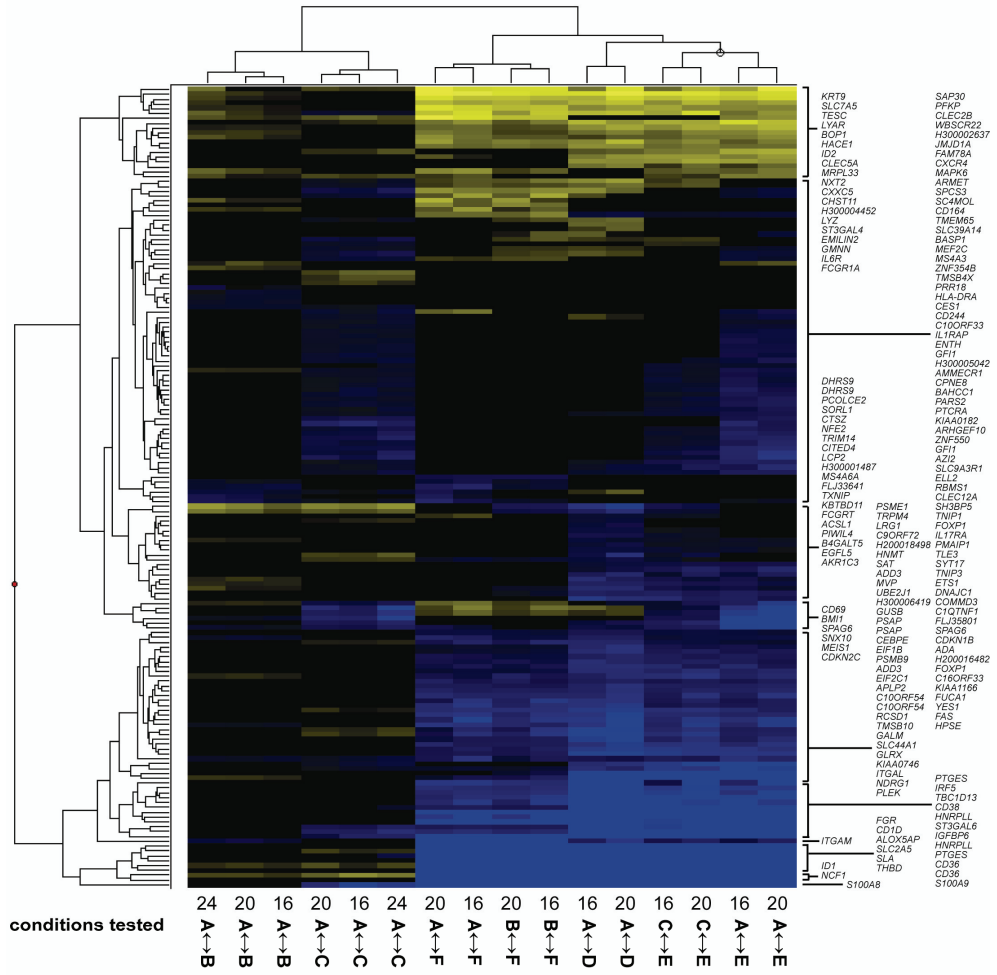
Figure 2. A selected set of 165 reporters cluster together for subsequent time points of MN1 or MN1-TEL expression and/or ATRA treatment in U937 cells. The log2 ratios of changes in expression between the conditions indicated were subjected to unsupervised hierarchical clustering. The conditions tested on the different arrays are indicated at the bottom with numbers (16, 20 or 24) and letter codes A-F. Numbers correspond to hours after induction of MN1 or MN1-TEL. The table underneath the figure explains the letter codes (+: presence of MN1 or MN1-TEL or ATRA; -: absence of MN1 or MN1-TEL or ATRA). For instance, A ↔ B means that a sample with induced MN1-TEL expression is hybridized against a sample without MN1-TEL expression. Blue color represents stimulation and yellow repression.

P-value	log2 ratio	Gene	log2 ratio	P-value	Gene Name	Genbank
1,6E-14	-0,49	*DHRS9*	-0,54	5,2E-19	**NADP-dependent retinol dehydrogenase/reductase**	**AF240698**
1,5E-13	-0,37	*DHRS9*	-0,35	1,4E-14	**NADP-dependent retinol dehydrogenase/reductase**	**AF529288**
1,9E-03	-0,25	*ID1*	-0,49	6,8E-13	**DNA-Binding inhibitor ID-1**	**BC012420**
1,9E-03	-0,25	*ID2*	-0,33	1,8E-06	DNA-binding protein inhibitor ID-2	M97796
7,3E-03	-0,19	*SLA*	-0,24	4,8E-05	SRC-like adapter	BC007042
2,9E-03	-0,17	*FCGR1A*	-0,26	2,0E-08	High affinity immunoglobulin gamma Fc receptor I precursor	L03418
	0	*KRT9*	-0,24	7,7E-03	Keratin, type I cytoskeletal 9	S69510
	0	*TMSB4X*	-0,36	3,8E-03	Thymosin beta-4 (FX)	M26759
	0	*MS4A6A*	-0,29	2,3E-06	Membrane-spanning 4-domains, subfamily A, member 6A	AF261136
	0	*LYZ*	-0,22	4,6E-04	lysozyme (renal amyloidosis)	BC004147
	0	*PRR18*	-0,27	6,4E-03	proline rich region 18	BC034775
	0	*C10ORF54*	-0,22	2,3E-06	Chromosome 10 open reading frame 54	AK024449
	0	*FLJ33641*	-0,22	7,1E-10	cDNA FLJ33641 fis, clone BRAMY2023719	AK090960
	0	*CD1D*	-0,20	8,5E-03	T-cell surface glycoprotein CD1D precursor	AF142668
	0	*HLA-DRA*	-0,21	9,5E-03	MHC class II, DR beta 3	Z84814
2,6E-07	-0,29	*IL6R*	0		Interleukin-6 receptor alpha chain (CD126 antigen)	S72848
1,6E-06	-0,22	*CXXC5*	0		CXXC finger 5	BC002490
1,7E-03	-0,22	*ZNF354B*	0		Zinc finger protein 354B	AK057737
4,7E-03	-0,20	*H300002637*	0		unknown protein, oligo ID H300002637	
8,2E-07	-0,21	*FAM78A*	0		family with sequence similarity 78, member A	BC029924
1,4E-04	-0,20	*NXT2*	0		NTF2-related export protein 2	AL031387
1,7E-03	-0,20	*MS4A3*	0		membrane-spanning 4-domains, subfamily A, member 3 (hematopoietic cell-specific)	BC008487
3,8E-03	-0,21	*HACE1*	0,22	9,8E-04	HECT domain and ankyrin repeat containing, E3 ubiquitin protein ligase 1	AB037741
5,4E-04	-0,16	*SLC9A3R1*	0,22	1,5E-08	Solute carrier family 9, isoform 3 regulatory factor 1	BC011777
	0	*SH3BP5*	0,23	4,2E-07	SH3 domain-binding protein 5	BC010123
	0	*CPNE8*	0,22	1,8E-06	copine VIII	AK098593
	0	*CLEC12A*	0,23	8,1E-11	C-type lectin protein CLL-1	AF247788
	0	*AMMECR1*	0,21	2,1E-07	AMMECR1 protein	AJ012226
	0	*TLE3*	0,20	2,3E-06	Transducin-like enhancer protein 3	BC015729
	0	*H200016482*	0,21	1,0E-02	unknown protein, oligo ID H300016482	
	0	*KIAA0182*	0,25	2,7E-06	KIAA0182, genetic suppressor element 1, GSE1	D80004
	0	*PFKP*	0,21	1,0E-05	6-Phosphofructokinase, platelet type	M64784
	0	*ARHGEF10*	0,25	9,8E-06	Rho guanine nucleotide exchange factor (GEF) 10	AB002292
	0	*PMAIP1*	0,20	1,1E-05	Phorbol-12-myristate-13-acetate-induced protein 1	D90070
	0	*KIAA0746*	0,28	1,4E-05	unknown protein	AB018289
	0	*PARS2*	0,23	6,4E-07	prolyl-tRNA synthetase 2, mitochondrial	AL117473
	0	*FOXP1*	0,23	3,4E-06	Forkhead box protein P1	AF151049
	0	*BAHCC1*	0,21	2,9E-05	BAH domain and coiled-coil containing 1	AB040880
	0	*ELL2*	0,20	1,8E-09	elongation factor, RNA polymerase II, 2	U88629
	0	*TMEM65*	0,23	3,8E-17	Transmembrane protein 65	BC017881
	0	*PTCRA*	0,22	6,8E-03	Pre-T-cell receptor alpha	AF084941
	0	*SLC39A14*	0,22	4,0E-09	solute carrier family 39 (zinc transporter), member 14	BC015770
	0	*IL17RA*	0,28	7,0E-10	interleukin 17 receptor A	U58917
	0	*ZNF550*	0,30	1,6E-00	zinc finger protein 550	DC034610
	0	*H300005042*	0,24	8,7E-13	unknown protein, oligo ID H300005042	
	0	*TNIP1*	0,30	1,1E-13	NEF-associated factor 1 (NAF1) (HIV-1 NEF interacting protein)	U83844
1,2E-03	-0,16	*CD69*	0,42	3,0E-20	Early activation antigen CD69	BC007037
	0	*WBSCR22*	0,31	2,3E-09	Putative methyltransferase hussy-3	BC011696
	0	*FOXP1*	0,27	2,0E-10	Forkhead box protein P1	AF151049
	0	*MEF2C*	0,33	4,7E-12	Myocyte-specific enhancer factor 2C	S57212
	0	*GFI1*	0,29	9,5E-12	Growth factor independence-1 (zinc finger protein)	U67369
	0	*GFI1*	0,27	6,2E-06	Growth factor independence-1 (zinc finger protein)	U67369
	0	*ETS1*	0,37	1,1E-17	C-ETS-1 protein (P54)	P14921
	0	*BASP1*	0,41	2,1E-17	Brain acid soluble protein 1	AF039656
	0	*C1QTNF1*	0,37	1,4E-11	Complement-C1q tumor necrosis factor-related protein 1 precursor	Q9BXJ1
	0	*FLJ35801*	0,41	1,9E-20	Hypothetical protein FLJ35801	AK098685
	0	*CD164*	0,39	1,4E-20	Putative mucin core protein 24 precursor (CD164 antigen)	D14043
	0	*SYT17*	0,47	7,5E-13	synaptotagmin XVII	BC004518
	0	*CLEC2B*	0,45	4,3E-19	C-type lectin superfamily member 2	AB016628
	0	*DNAJC1*	0,42	3,0E-17	DNAJ homolog subfamily C member 1	AK027263
	0	*SPAG6*	0,43	6,9E-21	Sperm associated antigen 6 isoform 1	AL080136
	0	*S100A9*	0,58	7,4E-22	Calgranulin B	A12032
	0	*COMMD3*	0,49	6,7E-26	BUP protein	BC022898
	0	*CD36*	0,56	2,1E-15	Platelet glycoprotein IV (CD36 antigen)	AY095373
	0	*CDKN1B*	0,54	2,6E-21	Cyclin-dependent kinase inhibitor 1B	BC001971
	0	*MEIS1*	**0,58**	**4,1E-37**	**Homeobox protein MEIS1**	**U85707**
	0	*TNIP3*	0,67	1,5E-32	TNFAIP3-interacting protein 3	AJ320534
	0	*CD36*	0,66	6,1E-25	Platelet glycoprotein IV (CD36 antigen)	AY095373
	0	*SPAG6*	0,65	1,0E-32	Sperm associated antigen 6 isoform 1	BC030585
	0	*S100A8*	**0,89**	**1,2E-29**	**Calgranulin A**	**BC009528**
	0	*BMI1*	**0,76**	**1,5E-27**	**Polycomb complex protein BMI-1**	**S62198**
7,5E-03	0,17	*SNX10*	0,77	1,0E-32	Sorting nexin 10	BC031050
1,2E-05	0,20	*CDKN2C*	0,50	4,0E-25	Cyclin-dependent kinase 6 inhibitor, P18-INK6	BC016173
3,7E-03	0,20	*CES1*	0		carboxylesterase 1 (monocyte/macrophage serine esterase 1)	M65261
1,1E-06	0,21	*CD244*	0		CD244 natural killer cell receptor 2B4	AJ245376
3,6E-03	0,21	*C10ORF33*	0		chromosome 10 open reading frame 33	AK075265
2,1E-04	0,23	*IL1RAP*	0		Interleukin 1 receptor accessory protein	AF167343
5,9E-08	0,24	*TRPM4*	0		Transient receptor potential cation channel, subfamily M, member 4	AF497623
2,1E-11	0,32	*FUCA1*	0		Alpha-fucosidase I	AK092914
4,1E-06	0,36	*ITGAM*	**0**		**Integrin alpha-M precursor**	**M84477**
4,6E-17	0,37	*FAS*	0		Apoptosis-mediating suface antigen FAS (CD95)	BC012479
1,1E-13	0,34	*YES1*	0,15	1,0E-03	**Proto-oncogene tyrosine-protein kinase YES**	**M15990**
1,2E-21	0,42	*HPSE*	0,11	7,0E-03	Heparanase	AF152376
2,3E-04	0,21	*GLRX*	0,26	7,2E-07	glutaredoxin (thioltransferase)	BC010965
1,1E-06	0,24	*ENTH*	0,19	4,3E-05	Enthoprotin	AF434813

16 20 24   16 20 24

MN1-TEL expression   MN1 expression

Figure 3. Genes regulated by MN1 and MN1-TEL. The clustering analysis was performed with expression data from the three different time points (16, 20 and 24 hours after induction of MN1 or MN1-TEL). All genes listed show a significant change in expression (log2 ratio ≤0.2 or ≥0.2) for all three separate time points. The listed log2 ratios together with the *P* value are calculated from combined time points. Genes indicated in bold are used for qPCR analyses (Fig 5 and 6 and supplementary Figure 5). Blue represents stimulation and yellow repression.

was most significantly affected by ATRA treatment. A cutoff value of 0.2 corresponds to a 15% difference in expression between conditions. The list was supplemented with a set of genes that do not meet the above criteria, but of which the expression is influenced by ATRA treatment in combination with MN1 or MN1-TEL (n=28). Genes were removed from the lists if a similar significant change in expression was observed by tetracyclin release of the control cell line as that invoked by MN1 or MN1-TEL expression. The final list, consisting of 165 reporters comprising 155 different genes, was used for cluster analysis. The results are visualized in Figure 2 and it is clear that there is clustering of the different time points of each different treatment and all the ATRA effects. Supplementary Table 2 contains all data including the extended gene names, Genbank accession numbers, combined log2 ratios, B values and P values. Figure 3 displays the genes that are regulated by MN1 or MN1-TEL (n=85). The different time points are represented by cluster analysis and accompanied by the combined log2 ratios and P values. It is obvious that MN1 expression affects many more genes than MN1-TEL expression. About 50% of the genes that are repressed by MN1 are also repressed by MN1-TEL (yellow), whereas MN1-TEL has no effect on most genes that are induced by MN1 (blue). This suggests that the transcription-stimulating properties of MN1 are lost in the MN1-TEL protein. In addition, we identified a small set of genes that is influenced by MN1-TEL and that does not respond to MN1.

Figure 4 summarizes those genes that are differentially affected in the combination of MN1 or MN1-TEL with ATRA when compared with either treatment alone. Clearly the differentials in expression in the presence of ATRA are in general larger than those of MN1 and MN1-TEL. An explanation for this striking difference might be that, in contrast to the effect of ATRA, which impacts all cells, only part of the cells are effectively expressing the gene of interest when released from tetracyclin inhibition. Limitation of expression to a sub population of the culture is not uncommon in the expression system used in this study (Dr. Judith Boer, Leiden University Medical Center, personal communication, 2006). Synergistic induction or repression of transcription is indicated in Figure 4 with a black or grey background, respectively. We concluded there was synergy when the observed effect of ATRA in combination with MN1 or MN1-TEL was higher or lower than that expected from the sum of the observed separate effects (cutoff value log2 > 0.2). Figure 4A lists genes induced by ATRA. A small subset of ATRA-stimulated genes is synergistically stimulated by MN1 (indicated with a black box). These include the leukemogenic oncogene *MEIS1*. MN1-TEL either has no effect on these genes or inhibits the ATRA effect. Figure 4A also shows that expression of MN1 results in a synergistic inhibition of gene expression of about half of the ATRA-stimulated genes (indicated by the grey background) and an even larger group of
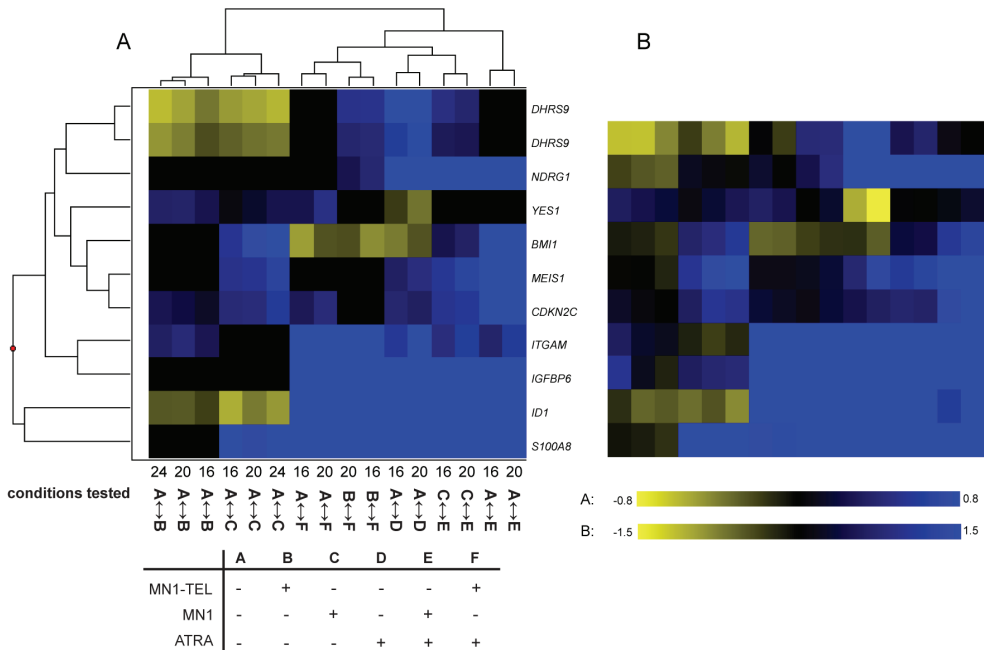
genes is repressed by MN1-TEL. For instance, the induction of *DHRS9*, an enzyme involved in ATRA synthesis,[24] by ATRA is completely annulled by both MN1 and MN1-TEL. The integrin family member *ITGAM* is the only gene whose expression is synergistically induced by MN1-TEL and ATRA. Figure 4B shows that MN1 or MN1-TEL can sometimes revert gene inhibition by ATRA. Most notable is the high induction of *BMI1* by MN1, which is even further stimulated in the presence of ATRA. Figure 4C shows a selection of genes of which expression is not affected by ATRA alone, but that are affected by the combination of MN1 or MN1-TEL and ATRA. Of special interest are the genes *SPAG6, DNAJC1* and *COMMD3* that, together with *BMI1* (Figure 4B), are similarly regulated by ATRA and MN1. These genes are all located on chromosome 10p12.31. None of these genes is upregulated in the control cell line or in the MN1-TEL cell line. *BMI1* is a known common viral integration site in for B-cell lymphomas in mice[25] and the possibility existed that integration of the *MN1* expression plasmid in this region of the genome might have caused an artificial upregulation of the entire group of genes. We therefore performed fluorescent insitu hybridisation on metaphase chromosomes using a plasmid containing the *MN1* cDNA and a 10p12.31 specific BAC clone as probes. No integrations of the plasmid were detected in the 10p12.31 region of the genome (Supplementary Figure 4). Thus, we conclude that the stimulation of these genes by MN1 is genuine. In conclusion, Figure 4 shows that both MN1 and MN1-TEL have considerable effects on RAR/RXR-mediated transcription.

Validation and extension of microarray results by qPCR analysis

To validate the microarray results, we selected a number of genes for qPCR evaluation. Included were genes exhibiting interesting expression differences between MN1 and MN1-TEL or upon ATRA addition. The results were compared to the corresponding microarray data. This comparison is visually represented by heat maps in Figure 5. This shows that the qPCR results are very similar to those obtained by the microarray analysis, as indicated by the overall similarity in patterning of the heat maps. Notably, there is a 2-fold difference in magnitude in the color scale, which runs from –0.8 to 0.8 for the microarray data and from –1.5 to 1.5 for the qPCR data. This indicates that the microarray data give an ~2-fold underestimation of the change in gene expression. RNA samples were not only collected for the time points used for microarray analysis, but also at 0h and at longer induction times of 40, 48, and 72h. Therefore, we were able to follow the behavior of interesting candidate genes beyond the limited 24 hour interval of the microarray analysis, and generally, the magnitude of the observed effects continued to increase. Some of the results for *S100A8*, *NDRG1*, *DHRS9*, *BMI1*, *YES1* and *ID1* are highlighted in Figure 6, and the full data set for

Figure 4. ATRA-regulated genes and the influence of MN1 and MN1-TEL on their expression. Log2 ratios of changes in expression are indicated for ATRA treatment, MN1 or MN1-TEL induction, and the combination of MN1 or MN1-TEL induction and ATRA treatment. Black or grey background represents a synergistic induction or repression of ATRA effects (cut off value log2 ratio 0.2). (A) ATRA-stimulated genes. (B) ATRA-repressed genes. (C) Genes not influenced by ATRA treatment alone, but affected by the combination of ATRA and MN1 or MN1-TEL. Gene names and Genbank number are shown in supplementary Table 2 (available at http://carcin.oxfordjournals.org). Genes indicated in bold are used for qPCR analyses (Figures 5 and 6 and supplementary Figure 5).

Figure 5. Comparison of microarray and qPCR results. The RNA samples used for microarray analysis were also used for qPCR analysis. Expression levels were determined relative to a standard curve and used without further normalization. The results were compared to the corresponding microarray data by expressing the qPCR data as mean log2 ratios. (A) Unsupervised hierarchical clustering of the microarray data (log2 ratios) for the genes selected for qPCR validation. The conditions tested on the different arrays are indicated at the bottom with numbers (16, 20 or 24) and letter codes A-F. A detailed explanation is given in legend of Figure 2. (B) Heat map of the qPCR data in the order corresponding to the unsupervised hierarchical clustering of the microarray data. Blue color represents stimulation and yellow repression.

these genes and for *MEIS1*, *CDKN2C*, *ITGAM*, *ID1*, and *IGFBP6* is shown in supplementary Figure 5. Figure 6A shows that *S100A8* expression after 72h is 100-fold increased by ATRA, 600-fold by MN1 and 1800-fold by the combination of the two. On the contrary, MN1-TEL expression seems to negatively influence the ATRA effect (supplementary Figure 5B). ATRA also stimulates *NDRG1* expression that is completely abolished by MN1-TEL (Figure 6B). Both MN1 and MN1-TEL abolish the 8 to 10-fold induction by ATRA of *DHRS9* expression (Figures 6C and 6D). Figure 6E shows that MN1 expression causes a considerable increase in *BMI1* levels, regardless of the presence of ATRA. *YES1* is a gene that is repressed by the addition of ATRA (supplementary Figure 5I). Upon MN1 and MN1-TEL expression there is an increase in *YES1* expression that is similar in absence or presence of ATRA. The increase is most pronounced upon MN1-TEL expression at time points beyond 24h (Figure 6F). *ID1* expression is decreased upon MN1 induction, whereas the presence of ATRA stimulates (Figure 6G). Figure 6H shows the validation of the observed effects on the protein

level for BMI1, ID1 and YES1. Together, the qPCR data and Western blots confirm our microarray observations and show that many of the effects continue after the 24h time point that was the end point for the microarray experiments.
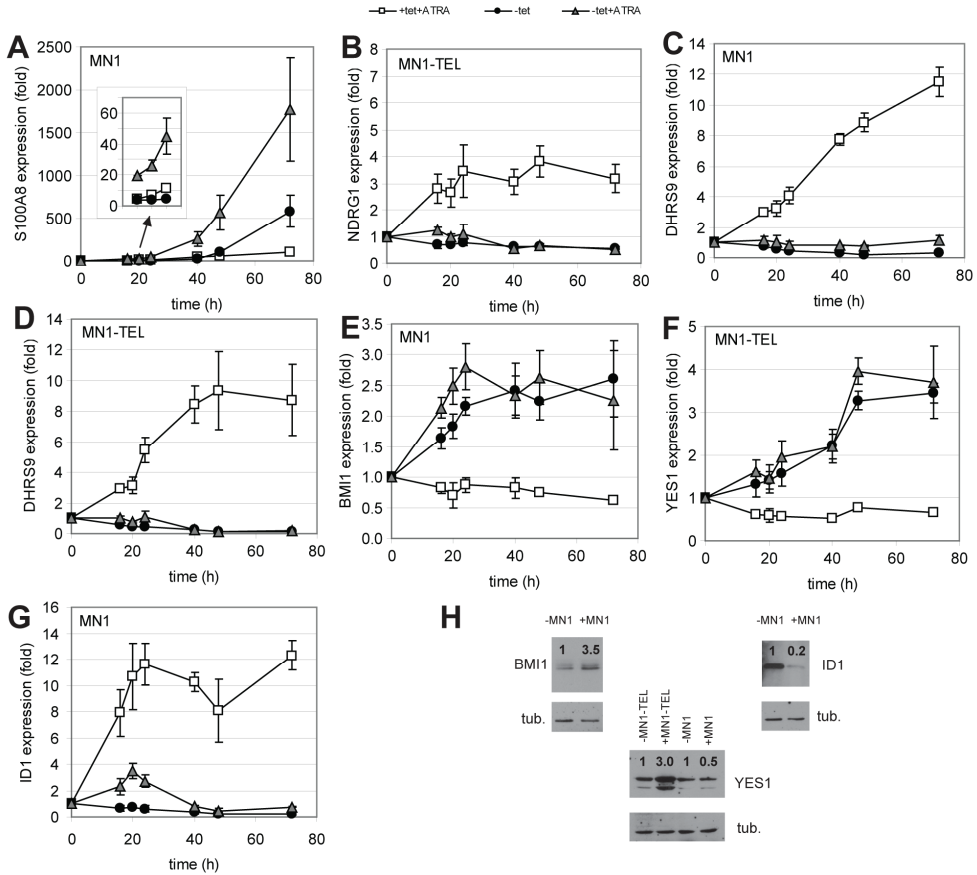
Figure 6. Time dependence of effects of MN1 and MN1-TEL on expression levels and validation of qPCR results by Western blot. MN1 and MN1-TEL expression were induced by tetracyclin release in the presence or absence of ATRA. In parallel with the RNA samples used for the microarray hybridizations (16, 20, and 24h), samples were also collected and analyzed with qPCR for time point 0h and longer incubation times of 40, 48, and 72h. The qPCR results were normalized, using the messengerRNA levels of *POLR2A*, and are presented relative to those in the presence of only the tet-repressor: white squares (□): +tet & +ATRA; black circles (●): -tet, no ATRA; grey triangles (▲): -tet and +ATRA. The results are shown only for the cell line indicated in the upper left corner of each graph. (A) *S100A8* (MN1 cell line). (B) *NDRG1* (MN1-TEL cell line). (C) *DHRS9* (MN1 cell line). (D) *DHRS9* (MN1-TEL cell line). (E) *BMI1* (MN1 cell line). (F) *YES1* (MN1-TEL cell line). Three of the target genes (*BMI1*, *ID1* and *YES1*) were analysed on the protein level. Time points after induction of MN1 or MN1-TEL expression and treatments were chosen (*BMI1*, 24 h; *YES1*, 48 h and *ID1* 72 h) and protein levels detected and quantified. Protein expression levels (indicated with a ratio above the signal) were calculated relative to the amount of β-tubulin present in the sample and the not-induced sample. tub, tubulin.

DISCUSSION

In this study, we have identified genes regulated by the oncoprotein MN1 and the fusion protein MN1-TEL in U937T cells. During the past year, *MN1* and *MN1-TEL* have been identified as potent leukemogenic oncogenes in mice. In humans, MN1 is overexpressed in different subgroups of AML, especially in inv (16) AML, while its overexpression in AML with a normal karyotype has been associated with a poor response to ATRA treatment. In the past we and others showed that MN1 influences RAR/RXR-regulated gene expression and therefore we also studied the effect of MN1 expression on the activation of endogenously expressed retinoid receptors in the presence of ATRA.

The expression profiling resulted in a list of genes up or downregulated by ATRA, MN1 or by the combination of MN1 and ATRA. We focussed on direct effects by choosing the time points following induction of MN1 expression when, and shortly after, expression was visible. We, however, cannot rule out the possibility that some genes are indirectly affected by MN1 expression. For some genes such as *MEIS1, CDKN2C, S100A8* and *9*, the combination of MN1 and ATRA synergistically induced expression (Figure 4A). This regulation pattern is identical to the one we observed in transient transfection experiments using a mouse sarcoma virus long terminal repeat-driven reporter gene.[8] In previous studies, we showed that the transactivating N-terminal domain of MN1, that binds coactivators such as p300 and RAC3, is responsible for this type of regulation. Here, we also show that MN1 can inhibit gene expression induced by ATRA, which is concordant with the data presented by Heuser *et al*[4] who described that MN1 opposes the effects of ATRA and even conferred ATRA resistance to cells. At present, we do not know how MN1 exerts these effects. Besides the transactivating domain, not much is known about functional domains in the rest of this large protein. Finally, there are genes whose expression is minimally affected by ATRA alone but whose expression is induced to a higher level by the combination of MN1 and ATRA than by MN1 alone. Among these are *BMI1, SPAG6, DNAJC1* and *COMMD3,* four genes that are located within 0.5 Mb of each other on chromsome 10p12. The joint regulation of these genes suggests that MN1 might be able to extend its influence over a larger genomic area. Interestingly, *BMI1* and *SPAG6* are also coordinately and highly expressed in inv (16) leukemias in which high expression of MN1 is thought to be obligatory.[2]

It is obvious that many of the genes affected by MN1 play a role in hematopoiesis and leukemia. *BMI1* is a member of the polycomb repression complex 1 and is required for the maintenance of adult hematopoietic stem cells. It functions by repressing genes that

promote lineage specification and cell-cycle arrest, such as p16/Arf.[26,27,28] *SPAG6* was recently identified as a marker for minimal residual disease in AML patients[29] in cases where expression of *SPAG6* is high. In case of a remission, the expression of *SPAG6* drops, whereas a relapse is characterized by rising *SPAG6* expression levels. The same is true for *S100A8* and *S100A9*. The expression of these two calcium-binding proteins is upregulated by MN1 and they were recently described as genes upregulated in AML.[30] The genes are downregulated in response to treatment with methotrexate. The *Meis1* locus is a common retroviral integration site and was shown to be important for the pathogenesis of AML in mice.[31,32]

Overexpression of MN1 in mouse BM produces highly proliferating immortalized cell lines and mice receiving transplants with MN1 retrovirus-transduced cells rapidly develop a lethal myeloproliferative disease/myeloid leukaemia.[4,14] Heuser *et al*[4] showed that p21, p27 and PU.1 were repressed in these cells. We see no effect of MN1 and/or ATRA on p21 and PU.1 expression in U937 cells. However, we do see upregulation of the cyclin dependent kinase inhibitors *CDKN1B* (p27) and *CDKN2C* (p18). A counterintuitive effect is that upregulation of these genes is expected to lead to cell cycle arrest. On the other hand, it has been shown that *CDKN2C* is abundantly expressed in hematopoietic progenitors, AML, and cell lines and its expression is downregulated along with myeloid differentiation.[33,34]

The MN1-TEL fusion gene contains most of the coding sequence of MN1 and TEL (ETV6), thereby combining the transcription-activating domains of MN1 with the DNA-binding domain of TEL. The fusion protein is thought to act as a deregulated transcription factor possibly disturbing the function of both MN1 and TEL. We therefore expected a much more extensive list of genes whose expression would be affected by MN1-TEL. However, only few genes are induced or inhibited by MN1-TEL. Perhaps its most profound effects reside in the fact that it is unable to stimulate a large set of genes that are induced by MN1 (Figure 3). The other most noticeable activity of the fusion protein is its inhibition of the stimulatory effects of ATRA on a large set of genes (Figure 4A). Disturbing the function of TEL is most likely caused by the binding of MN1-TEL to ETS regulatory sequences using TEL's DNA-binding domain. Further scrutiny of genes that are induced by MN1-TEL identified two genes, *ITGAM* and *HPSE*, that are indeed regulated by the ETS factors PU.1 and GABP.[35,36,37] It is not known whether TEL also regulates these genes, but it has been described that ETS family members can bind to different ETS elements making it possible that MN1-TEL binds the ETS responsive elements in these genes. In agreement with our q-PCR and array data there are retinoic acid-responsive elements in the promoter region of

the *ITGAM* gene,[37] explaining the increase in *ITGAM* expression in response to ATRA treatment.

The most interesting tumorigenesis-related genes that are influenced by MN1-TEL are *NDRG1, ITGAM* and *DHRS9*. The expression of *NDRG1* is not directly influenced by the presence of MN1-TEL, but in combination with ATRA treatment it inhibits the ATRA effects on NDRG1 expression. *NDRG1* is a metastasis suppressor, frequently downregulated in more advanced and poorly differentiated tumors.[38] ITGAM, also known as CD11b or MAC-1, belongs to the family of integrins. CD11b/MAC-1 is expressed on mature monocytes and macrophages and is present on AML cells with a myelomonocytic differentiation. In fact, antibodies against CD11b/MAC-1 are widely used to sort and define the differentiation status of leukemic cells. High CD11b/MAC-1 expression was also seen in leukemias induced by expression of MN1-TEL in mouse bone marrow cells.[14] Overexpression of CD11b/MAC-1 has also been associated with a unfavorable prognosis in AML.[39,40]

Finally, the effect of MN1 and MN1-TEL on *DHRS9* is interesting in relation to the differentiation block observed in most leukemias. The gene encodes an enzyme that is essential for the synthesis of ATRA from vitamin A. We show that ATRA stimulates expression of this gene and may thus enhance its own synthesis in mammals. Both MN1 and MN1-TEL inhibit *DHRS9* expression and completely abolish its induction by ATRA. Thus, this might inhibit the ATRA-induced differentiation in AML in humans and of MN1 and MN1-TEL-induced leukemia in mice. One could hypothesize that AML with high MN1 expression would perhaps benefit from adjuvant treatment with ATRA. However, the contrary appears to be the case. AML patients that were treated with ATRA only benefited from this treatment when MN1 expression was low. High MN1 expression apparently conferred ATRA resistance to the leukemic cells.[4] These findings suggest that perhaps other MN1 target genes play a more important role in defining the leukemia phenotype.

In conclusion, we have shown that MN1 and MN1-TEL, directly or indirectly, regulate expression of many genes that have been implicated in leukemogenesis. Both proteins are able to enhance or to inhibit ATRA effects on gene expression. Further research is necessary to fully understand the pleiotropic effects executed by MN1 and MN1-TEL on gene expression.

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SUPPLEMENTARY MATERIALS

Supplementary Table 1. Primersets used for qPCR

gene	forward primer	reverse primer
BMI1	5'-ACTTCATTGATGCCACAACC-3'	5'-CAGAAGGATGAGCTGCATAA-3'
CDKN2C	5'-ATGGATTTGGAAGGACTGC-3'	5'-GTGTGCTTCACCAGGAACTC-3'
DHRS9	5'-TCAGAGAGACTTCGTACTGTGC-3'	5'-TGTAGTCCTCTAGTGTCAGCCA-3'
ID1	5'-TCCAGCACGTCATCGACTAC-3'	5'-TCAGCGACACAAGATGCG-3'
IGFBP6	5'-CATGCCGTAGACATCTGGAC-3'	5'-AGGAGCTTCCATTGCCATC-3'
ITGAM	5'-GTGAAGCCAATAACGCAGC-3'	5'-TCTCCATCCGTGATGACAAC-3'
MEIS1 (ID 3185)	5'-ATGTGACAATTTCTGCCACCG-3'	5'-CCTGAACGAGTAGATGCCGTG-3'
MN1	5'-TCGCTGATGGCAGAGCACAG-3'	5'-GTCATTCAAGTTAGGGCAG-3'
MN1TEL	5'-TCGCTGATGGCAGAGCACAG-3'	5'-CTCCGGCTGTGTGTGTATAG-3'
NDRG1	5'-GACCCAACAAAGACCACTCTC-3'	5'-TGCCATCCAGAGAAGTGACG-3'
POLR2A	5'-CGGATGAACTGAAGCGAATG-3'	5'-GAGTCCACAAGCAGTTTGG-3'
S100A8	5'-ATGTTGACCGAGCTGGAGAA-3'	5'-CAGAATGAGGAACTCCTGGAAG-3'
YES1 (ID 441).	5'-CAGCAAGACAAGGTGCAAAA-3'	5'-TGCAGAATTCCAAATGACCA-3'

All newly designed primersets are deposited in the primer database.

Supplementary Table 2 is available at http://carcin.oxfordjournals.org
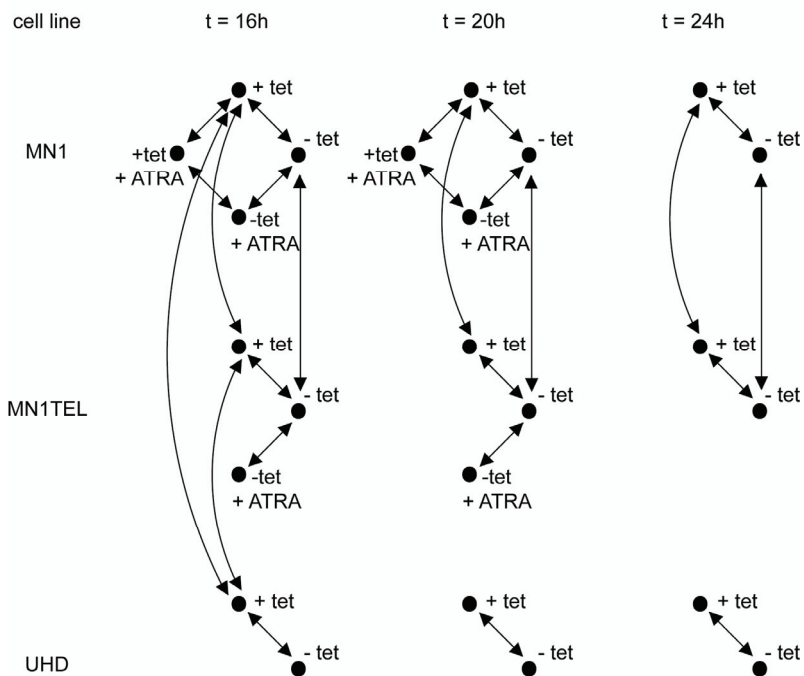
Supplementary Figure 1. Experimental design of the microarray study. Expression of MN1 or MN1-TEL using the tet-off system was induced by withdrawal of tetracyclin from stably transfected U937T cells, in presence or absence of ATRA. Each dot represents the samples of the corresponding cell line (U937T-MN1, U937T-MN1-TEL, or U937T-UHD as control) after incubation for the indicated time under the condition indicated, that were used for hybridization. The presence (+) or absence (-) of tetracyclin or ATRA is indicated. Each double-headed arrow represents 4 arrays, consisting of two biological duplicates that were each hybridized in a straight and dye-swap fashion. A total of 100 arrays were performed. Conditions with ATRA were compared to their untreated counterparts at time points 16 and 20h only. The cell lines without expression of the target gene (+tet, no tet release) were compared to each other at time point 16h only.
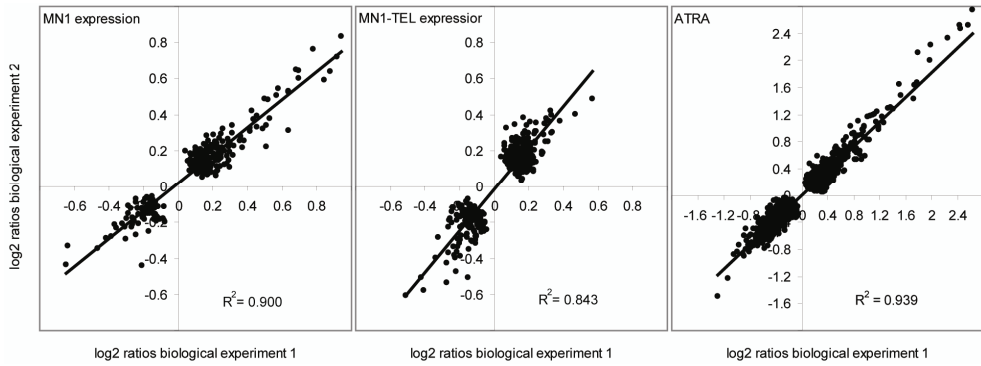
Supplementary Figure 2. Reproducibility of microarray results between biological duplicates. The microarrays were analyzed according to the design in Fig S1, but now for each biological experiment separately, with each arrow in Fig S1 representing only two arrays for one biological replicate, hybridized in a straight and dye-swap fashion. Correlation plots included only reporters with a B value ≥1 in the combined analysis of all time points and both biological replicates together. The mean log2 ratios obtained for biological experiment 2 were plotted against the mean log2 ratios obtained for biological experiment 1. The correlation coefficients (R^2) indicated in the graphs were obtained by linear regression analysis. (A) Correlation plot of the 297 reporters affected upon MN1 expression. (B) Correlation plot of the 420 reporters affected upon MN1-TEL expression. (C) Correlation plot of the 3162 reporters affected upon ATRA treatment.

|	A	B	C	D	E	F
MN1-TEL	-	+	-	-	-	+
MN1	-	-	+	-	+	-
ATRA	-	-	-	+	+	+
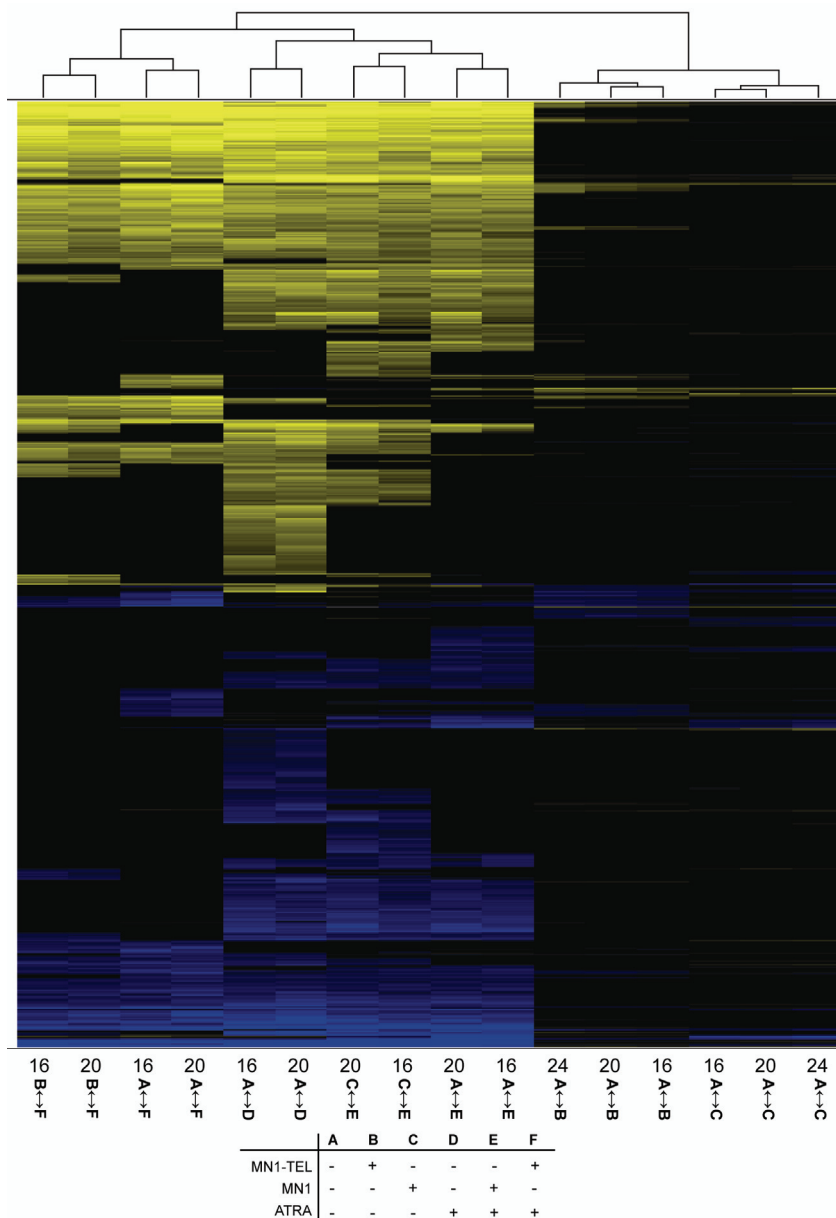
Supplementary Figure 3. Unsupervised hierarchical clustering of 4468 spots that showed statistically significant differences in mean expression levels under any of the conditions shown. The conditions tested on the different arrays are indicated at the bottom with numbers (16, 20 or 24) and letter codes A to F. Numbers correspond to hours after induction of MN1 or MN1-TEL. The table underneath the figure explains the letter codes (+: presence of MN1 or MN1-TEL or ATRA; -: absence of MN1 or MN1-TEL or ATRA). For instance: A ↔ B means that a sample with induced MN1-TEL expression is hybridized against a sample without MN1-TEL expression. Blue color represents stimulation and yellow repression.
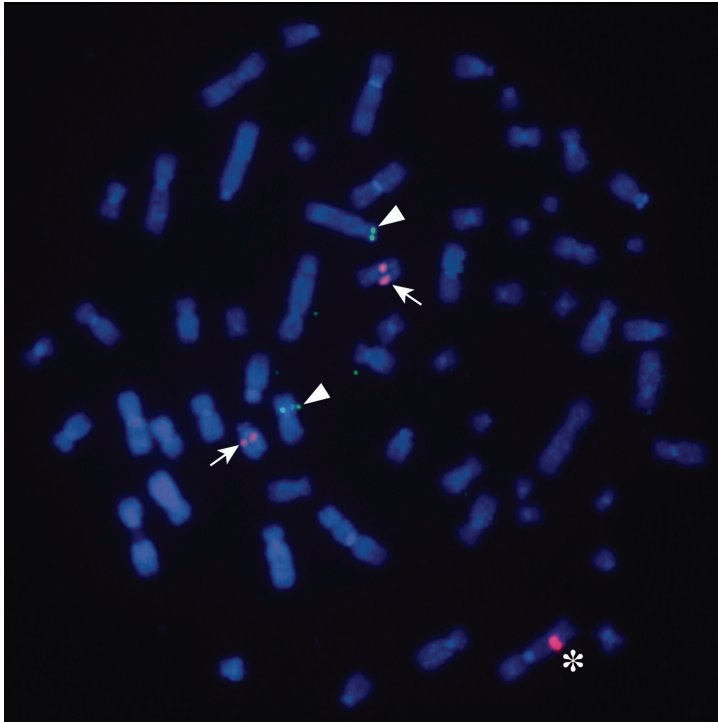
Supplementary Figure 4. Fluorescent in-situ hybridisation in cell line U937 MN1. Metaphase chromosomes were hybridized with plasmid pMN50, containing the cDNA of MN1 (red), and the BAC clone RP11-293I13 (green). Note that the pMN50 plasmid will detect all integrated plasmids because of homologous vector sequences. Signal of the RP11-293I13 BAC is observed on the two chromosome 10 derivatives (arrowhead/green) and plasmid pMN50 detects 3 plasmid integration sites (red) in this cell line. The big dot on the M5 marker chromosome,[41,42] indicated by the asterisk, was present in all three different cell lines and thus derived from the tetracyclin-repressor plasmid. The other signals, indicated by arrows, located on two smaller marker chromosomes, are derived from the pUDH10S plasmid containing the MN1 cDNA or the co-transfected hygromycin selection plasmid.
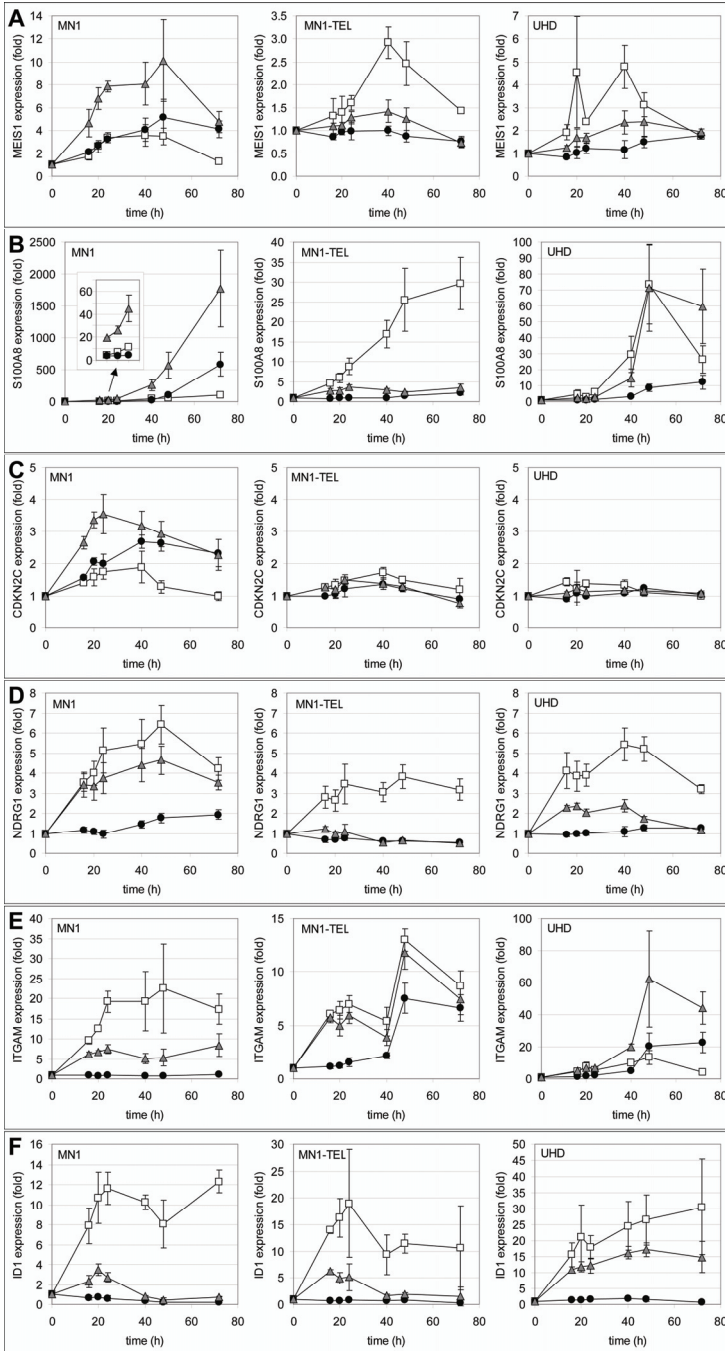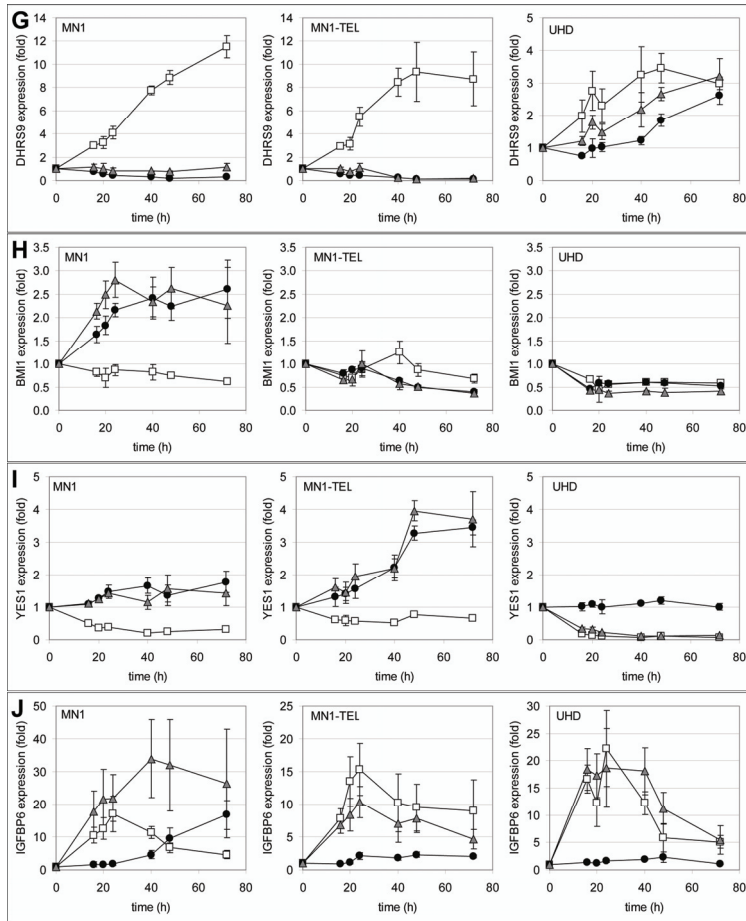
Supplementary Figure 5. Effects of MN1 and MN1-TEL on expression levels of several genes. MN1 and MN1-TEL expression were induced by tetracyclin release in the presence and absence of ATRA. Time points used for the qPCR analyses are 0, 16, 20, 24, 40, 48, and 72h. The qPCR results were normalized, using the mRNA levels of *POLR2A*, and are presented relative to those in the presence of only the tet-repressor (white squares (□): +tet & +ATRA; black circles (●): -tet, no ATRA; grey triangles (▲): -tet & +ATRA). Left graphs: effects in MN1 cell line; middle graphs: effects in MN1-TEL cell line; right graphs: effects in UHD cell line. (A) *MEIS1*. (B) *S100A8*. (C) *CDKN2C*. (D) *NDRG.* (E) *ITGAM.* (F) *ID1*. (G) *DHRS9*. (H) *BMI1*. (I) *YES1.* (J) *IGFBP6*. Please note the different ranges between y-axis of the different cell lines for *MEIS1, S100A8, ITGAM, ID1 and DHRS9*.

CHAPTER 6

General discussion

GENERAL DISCUSSION

Since the *MN1* gene was cloned in 1995, subsequent research has shown that the MN1 protein is implicated in the development of various tissues within the body, both during early development and in adult life. In the developing mouse embryo, expression of *Mn1* has been detected mainly in the developing facial structures and in the brain. *Mn1* knockout mice have shown that Mn1 is crucial for proper craniofacial bone development during embryogenesis. In adult mice, analysis of the bone marrow has detected high *Mn1* levels in progenitors of the granulocyte and macrophage lineage and possibly in more primitive hematopoietic stem cells. In humans, overexpression of *MN1* has been detected in bone marrow derived from patients with distinct subtypes of leukemia. Moreover, MN1 has been shown to present an independent marker for poor treatment outcome and shorter overall survival. Studies in mice have confirmed that induced *MN1* expression in the bone marrow contributes to a leukemic phenotype. *In vitro* studies using cell lines that overexpress MN1 have shown that MN1 promotes proliferation. Based on these observations in humans, mice and in cell lines, MN1 is thought to maintain an undifferentiated state of cells and possibly promote self-renewal. Although *MN1* expression has been detected in other tissues, for example in skeletal muscle, these areas have not been investigated yet. *In vitro* studies have provided a basis to unravel the molecular mechanisms involved: MN1 has been shown to be a transcriptional cofactor for the vitamin A and D nuclear receptors. Expression profiling of the *MN1*-inducible leukemic cell line U937 has shown that MN1's activity is not restricted to nuclear receptors, because MN1 also inhibits and induces transcription without the involvement of vitamin A. For example, expression of *IGFBP5* is regulated by MN1 through activation of the *IGFBP5* promoter and a transcription factor that cooperates with MN1 has been shown to bind a CACCC-rich element within this promoter. The role of MN1 in leukemia is reviewed by G. Grosveld.[1]

This chapter will discuss recent papers in relation to the chapters presented in this thesis. Furthermore, some important issues and leads for future research on MN1 are discussed.

THE CRUCIAL ROLE OF MN1 IN EARLY MAMMALIAN DEVELOPMENT

Defects detected in *Mn1* knockout mice clearly point to an important role for Mn1 in the development of cranial bone structures. In addition to the observed cranial bone defects, these mice suffer from a cleft palate, causing the mice to die shortly after birth. We

hypothesized that the palatal defects are most likely an additional effect to the malformations in the bone structures of the head, although an intrinsic role of Mn1 in palatal development was not ruled out. As mentioned in the introduction, Liu *et al* have investigated Mn1's role in palatal development in more detail. *Mn1* expresssion was detected within distinct regions of the developing palate. In palatal structures of *Mn1* knockout mice *Tbx22* expression was greatly reduced. Reporter studies using the *Tbx22* promoter have demonstrated that indeed Mn1 is capable of upregulating *Tbx22* levels. Altogether, this showed that Mn1 and Tbx22, function in a novel molecular pathway regulating mammalian palatal development.[2] The palatal defect as detected in the *Mn1* knockout mice is thus, at least partially, accounted for by a primary defect in palatal growth.

MacDonald *et al* have investigated the *Mn1* knockout mice and have focused on the role of Mn1 in bone formation. Calvarial osteoblasts derived from *Mn1* knockout mice displayed reduced differentiation, impaired proliferation and produced less mineralized matrix.[3] Additionally, it was shown that Mn1 is able to stimulate RANKL, an important osteoclast-inhiting factor. Altogether, these results show that Mn1 is important for appropriate osteoblast proliferation and differentiation and regulates osteoclast function through stimulation of *RANKL* gene transcription. However, it does not explain why the lack of Mn1 only creates cranial bone malformation; osteoblasts and osteoclasts are involved in bone formation throughout the skeleton and thus a more widespread effect would be expected. Most of the bones affected in the *Mn1* knockout mice are formed by intramembranous bone formation. However, as mentioned before, also several bones that are formed by endochondral ossification processes are affected. Another point of view towards this issue focusses on the embryonic origin of the cells that form the skeleton. Cells that form the vertebrate skeleton are derived from three distinct embryonic regions. The craniofacial bone structures originate from cranial neural crest cells, a layer of cells located between the neural tube and the epidermis whereas the remainder of the skeleton is formed from paraxial and lateral plate mesoderm. The agenesis or improper development of craniofacial bones could therefore also be caused by defects in neural crest cell migration and/or differentiation.

MN1's role in leukemias, either by overexpression or by a translocation, indicates additional functions for MN1 in the adult hematopoietic system. The perinatal death of the *Mn1* knockout newborns prevents us to study the role of MN1 in adult hematopoiesis directly, but transplantation of hematopoietic cells derived from livers of *Mn1* newborn knockout mice into irradiated adult recipients is possible. Hematopoietic cells from the *Mn1* knockout mice will home in the bone marrow of recipient mice and this enables studies on

aberrant hematopoiesis caused by Mn1 deficiency. These experiments are currently being performed and will hopefully shed light on Mn1's role in murine hematopoiesis (personal communication G. Grosveld). Whether Mn1 also plays an important role during embryonic hematopoesis is unknown and has not been investigated yet.

The role of Mn1 in postnatal skeletal dynamics and other physiological processes in, for instance, the brain where *Mn1* expression is detected in normal mice, is impossible because of the perinatal death of *Mn1* knockout mice. To address these questions, conditional knockout models that survive postnatally need to be created.

MN1 STIMULATES AND INHIBITS GENE TRANSCRIPTION

We have shown that MN1 stimulates the *Insulin Like Growth Factor Binding Protein 5* (*IGFBP5)* gene through a CACCC-rich element in its proximal promoter. IGFBP5 belongs to a protein family with six members, which are expressed and secreted in a tissue and developmental stage-specific manner throughout the body. The proteins bind the most important regulators of mammalian growth and development, IGF-I and IGF-II. IGFBP5 expression has also been observed in bone. IGFBP5's specific role in bone is interesting in relation to Mn1's effect on bone formation: does a disturbed regulation of *IGFBP5* by Mn1 contribute to the observed bone defects? The role of IGFBP5 in bone formation, however, is highly contradictory. Knocking out *IGFBP5* in mice had minimal impact on bone[4] and overexpression caused impaired mineralization.[5,6] In contrast, administration of IGFBP5 and IGF-I led to increased bone formation and enhanced bone mineral density, both in cortical and calvarial bones.[7] Studies in cultured osteoblasts and bone cell lines have also shown these opposing results.[8,9,10]

Expression profiling of our *MN1*-inducible cell line U937 has revealed a large list of genes that are regulated by MN1. The data have also shown that the effects of MN1 are more widespread: MN1 not only functions as a cofactor for retinoic acid (RA)-mediated transcription, but it can also stimulate and inhibit transcription independent of RA. Interestingly, within the list of genes that are upregulated by MN1 are four genes that are localized in a small region of chromosome 10. This combined upregulation of neighboring genes might possibly point to a long distance effect of MN1 on transcription. Also two closely related genes, namely S100A8 and S100A9, both localized close to each other on chromosome 1, are regulated in a similar way by MN1. Within the genome several clusters of genes are shown to be upregulated simultaneously. Enhancer sequences can be separated from the genes often by thousands of base pairs or can even be located on

separate chromosomes.[11] Simultaneously upregulated, clustered genes are usually closely related with regard to their function. An well studied example is the β-globin locus.[12] Functional relations between the genes that are simultaneously upregulated by MN1 on chromosome 10 is however not recognized.

Expression profiling of *MN1*-inducible cell lines has provides important data about which genes are switched on of off after induction of MN1 expression, but it does not give clues on which sequences within promoters of genes are crucial for MN1. Earlier studies have identified different DNA response elements that are important for MN1 function. These include CACCC sequences, DR1 and DR5 repeats and vitamin D responsive elements. We have searched for these response elements in promoters of identified genes, but they were not recognizable. Chromatin immune precipitations (ChIPs) enable the identification of the exact regions within a promoter where a transcription factor or cofactor binds. Activation or downregulation of a gene is accompanied by specific histone modifications and the recruitment of specific protein complexes. Combining the MN1-inducible cell lines with ChIP technology enables to monitor these changes in histone modifications and protein complexes in response to MN1 induction. A combination of ChIP with high-throughput sequencing technology, so called ChIP-seq, enables the analysis of protein-DNA interaction sites on a genome-wide scale. We are currently setting up these techniques in order to perform conventional ChIPs and ChIP-seq analyses on *MN1*-induced cell lines in the near future. A more direct approach is achieved by cloning the upstream regions of genes regulated by MN1. Subsequent transient transfection assays and deletion of these promoter regions will most likely help us to gain insight into transcription regulation by MN1.

OVEREXPRESSION OF *MN1* IN LEUKEMIA

The overexpression of *MN1* in various leukemia subgroups has been detected by the use of microarray platforms. In a subset of patients, these data have been verified by reverse transcriptase (RT)-PCRs. Comparison of microarray and RT-PCR results has shown that microarray results greatly underestimate the level of *MN1* overexpression in inv(16) patients.[13] Presumably, small increases in *MN1* expression levels are present in other subclasses of leukemia that are undetectable in a microarray setup. It would therefore be interesting to analyze expression levels of *MN1* in well-defined subsets of leukemia by RT-PCR. Of special interest are the CBF-related leukemias. CBF is a heterodimeric transcription factor and both its subunits, AML1 and CBFβ, are subject to translocations. Overexpression of *MN1* seems to be restricted to the translocation that involves the

regulatory subunit CBFβ, the inv(16)-created CBFβ-SMMHC. The importance of MN1 in the development of leukemia by the CBFβ-SMMHC fusion protein has been confirmed by mice studies. It would be interesting to investigate whether fusion genes of the AML1 subunit are similarly promoted by MN1 in their leukemic potential. Five different translocations have been described that involve the AML1 subunit of CBF of which AML1-ETO is most frequent. It is found in up to 12% of AML patients and, as CBFβ-SMMHC, is associated with AML. Several mouse models have been created in order to study the leukemogenic properties of *AML1-ETO.* These have shown that expression of AML1-ETO in the bone marrow alone is not sufficient to induce leukemia in mice.[14,15,16,17,18] These mice were healthy and only after treatment with *N*-ethyl-*N*-nitrosourea (ENU), a powerful mutagen, cooperating mutations were acquired that were necessary to develop AML. Indications whether *MN1* overexpression in humans is detectable in AML1-ETO patients is deducible from expression profiling data that is stored in public databases. Analysis of the data in the Oncomine database[19] (www.oncomine.org) reveals four independent microarray experiments that have analysed RNA of AML1-ETO patient material. None of these experiments, however, report *MN1* as up or down regulated in AML1-ETO patients. A RT-PCR-based approach to analyse AML1-ETO patient samples will elucidate if even a mild overexpression of *MN1* is detectable in AML1-ETO patient samples. Notably, expression profiling of leukemic cells of patients that are diagnosed with *CBFβ* and *AML1* fusions have shown that these translocations are associated with distinct expression profiles.[20,21] This suggests that, although the two proteins CBFβ and AML1 function together as a heterodimer, the fusion proteins that are formed with either of these two subunits each have their specific function and pathways involved in leukemogenesis.

Although various studies have identified overexpression of MN1 in bone marrow of AML patients, no studies have been conducted so far that have investigated the basis behind this upregulation of *MN1*. In general, upregulation of a gene in cancer can be attributed to several different mechanisms: (1) amplification of a gene, (2) upregulation of proteins that upregulate the expression of the gene, (3) down regulation of a protein or microRNA (miRNA) that normally downregulates the expression of the gene, (4) mutations in regulatory sequences of a gene, either leading to less inhibition or increased stimulation, (5) placement of transcription-stimulating, regulatory elements derived from another locus near the gene by a translocation, (6) the increase of the mRNA stability of the gene by mutations in the untranslated regions or (7) demethylation of a promoter region resulting in initiation of the expression of the gene. It is unknown which option is responsible for the detected overexpression of *MN1* in leukemias. Translocations near the *MN1* gene possibly

causing an upregulation of *MN1* are not described in leukemia, making this option less likely. Two inv(16) patients with high *MN1* levels were shown to have a diploid copy number of the *MN1* locus, ruling out the possibility of amplification of the *MN1* gene as a upregulatory mechanism in these two patients.[13] Which of the other options is valid for the overexpression of MN1 that is detected in leukemias is hard to say. Future research will hopefully provide clues on the regulation of the *MN1* gene. This infomation, combined with array-based comparative genomic hybridization (CGH) or large-scale single nucleotide polymorphism (SNP) analysis and expression profiling data of inv(16) patients should be able to identify the mechanisms behind the upregulation of MN1. It is unknown if any miRNAs target the *MN1* mRNA. Searching the miRNA database (http://microrna.sanger.ac.uk/) revealed two miRNAs, namely hsa-miR-512-5p and hsa-miR-31, that possibly target the *MN1* mRNA. Importantly, target genes of the majority of miRNAs are not well mapped to date, which is also evident for the postulated *MN1*-regulating miRNAs: hsa-miR-512-5p and hsa-miR-31 have 924 and 810 possible target genes listed, respectively. Additionally, a literature search on miRNA expression profiles has not revealed differential expression for either of these two miRNAs in leukemia.[22,23,24,25,26]

Various mouse studies have provided additional evidence for the important role of MN1 overexpression in the pathogenesis of certain subtypes of leukemias. The CBFβ-SMMHC fusion protein only causes leukemia in adult mice when it is accompanied by MN1 overexpression. Additionally, two fusion proteins, CALM-AF10 and NUP98-HOXD13, are able to induce leukemia in mice, whereby integration of a retrovirus near the *MN1* gene leading to overexpression of *MN1,* has been shown to shorten the onset dramatically. Analyses of other integration sites have revealed that integrations near the *MN1* locus are frequently accompanied by integrations near the *zinc finger E-box binding homeobox 2* (*Zeb2*) gene. *CALM-AF10* transgenic mice develop leukemias in the myeloid lineage, whereas integrations near the *Zeb2* gene result in B-cell ALL, suggesting that expression of *Zeb2* strongly influences the phenotype of the *CALM-AF10* mice. It is unknown if overexpression of *MN1* in these mice contributes to the *Zeb2*-mediated B-cell ALL. In humans, ALL has been shown to be associated with downregulation of *MN1* by methylation of the *MN1* promoter region. This could represent a species-related difference in pathways involved in hematopoiesis.

Zeb2, also known as Smad-interacting protein (SIP1), is a transcriptional repressor that binds to DNA and interacts with SMADs, the downstream signal transducers of the transforming growth factor (TGF) family. In several epithelial cell lines, upregulation of *MN1* has been reported in response to TGFβ.[27,28] These two observations, namely *Mn1*

overexpression in *CALM-AF10* mice that is frequently accompanied by *Zeb2* integrations and upregulation of *Mn1* by TGFβ, make the TGFβ pathway an interesting candidate for future studies on MN1. The TGFβ pathway is complex and has been implicated in the development and maintenance of various organs. In hematopoiesis, TGFβ has been shown to have inhibitory signals on the proliferation of hematopoietic progenitors.[29] This seems to be in contrast to the role of MN1 during hematopoiesis: it maintains the progenitor state of cells and promotes proliferation. Expression of MN1, however, is not limited to the hematopoietic system. Expression is found in the developing brain, bone and in adult skeletal muscle. An interplay between TGFβ signaling and MN1 is thus still plausible and future studies will hopefully unravel if this interplay really exists.

THE MN1-TEL FUSION PROTEIN

The MN1-TEL fusion protein has been shown to influence both the normal function of TEL and MN1. MN1-TEL is able to bind to ETS-responsive elements and subsequently it uses the transcription stimulating domains of the MN1 moiety to stimulate promoters that are normally repressed by TEL. In addition, MN1-TEL functions as a dominant negative mutant of MN1 on RAR-RXR-mediated transcription. In contrast to MN1, the fusion protein is unable to cooperate with p300 and RAC3 and prevents MN1 to perform its normal function. Expression profiling of the leukemic cell line U937 with inducible expression of *MN1-TEL* has provided additional evidence for these mechanisms. After induction of MN1-TEL, inhibition of RA-induced transcription has been detected for many genes. Additionally, a small set of genes that are normally repressed by TEL is stimulated by MN1-TEL. Both mechanisms, namely the inhibition of RA effects and the stimulation of TEL-regulated genes, will contribute to leukemogenesis by MN1-TEL. Mice studies have shown however that MN1-TEL expression in bone marrow is not sufficient to cause AML.[30,31] Additional mutations or activation of other genes, such as *HOXA9*, are required to develop a leukemic phenotype. HOXA9 overexpression in mice bone marrow induces AML, but MN1-TEL is able to accelerate the onset dramatically. The upregulation of *N-Myc* in these mice implicates a role for *N-Myc* in the leukemogenic pathways by MN1-TEL.

Molecular mechanisms involved in the leukemogenic properties of MN1-TEL rely, at least partially, on the fact that the DBD domain of TEL is maintained in the fusion. A number of other translocations of *TEL* have been described in which, as for MN1-TEL, the DNA binding domain (DBD) domain of TEL contributes to the fusion. These include *TTL-TEL*,[32] *CHIC2-TEL*,[33,34] *PAX5-TEL*,[35] *EVI1-TEL*[36,37] and *HLXB9-TEL*.[38,39] The molecular

mechanisms for the fusions involving *TTL*, *CHIC2* and *EVI* genes with *TEL* are yet unknown. Notably, patients with an *EVI1-TEL* fusion also express the reciprocal fusion. The *EVI1*, *PAX5* and *HLXB9* genes encode transcription factors and their fusions with *TEL* possibly generate deregulated transcription factors. The fusion of the *HLXB9* and *TEL* genes leads to an in-frame fusion in about half of the patients with the t(7;12)(q26;p13). Additionally, it has been shown that all patients carrying this translocation have overexpression of the non-translocated allele of *HLXB9*, which might contribute to leukemogenesis.[40] It is unknown if patients carrying a *MN1-TEL* fusion overexpress the non-translocated *MN1* allele. It is, however, unlikely that the above mentioned mechanism plays a major role for the *MN1-TEL* fusion: molecular studies have shown that both MN1 and TEL segments of the fusion protein are important for oncogenesis by MN1-TEL and, moreover, *MN1* expression was not detectable in UCSD/AML1, a cell line that harbors a MN1-TEL fusion.[41]

Like *MN1-TEL*, the *PAX5-TEL* fusion has been shown to create a deregulated transcription factor.[42] The PAX5 protein binds DNA with its DBD and is both an activator and repressor of transcription. The fusion, unlike MN1-TEL, retains the DBD of PAX5 and activator/inhibitory domains of PAX5 are lost. The breakpoint within the *TEL* gene is exactly the same as for the *MN1-TEL* fusion, thereby contributing the DBD to the fusion protein. Molecular studies have indicated that PAX5-TEL acts as a repressor through the recruitment of the mSin3A co-repressor, which subsequently will attract histone deacetylases (HDACs). Our results on MN1-TEL has indicated that the repressive effect of MN1-TEL on RA-mediated transcription is not caused by recruitment of HDACs but rather the inability of the transcription stimulating domains of MN1 to cooperate with coactivators like p300 and RAC3. Accordingly, fusion of *MN1* to *TEL* is different compared to the other fusions involving *TEL*: MN1-TEL functions as both an activator and repressor of transcription and this relies on both MN1 and TEL sequences.

CONCLUSION

Over the last years, it has become clear that MN1 is an important oncogene for leukemogenesis. Inv(16) leukemias are associated with upregulation of *MN1* and in AMLs without cytogenetically detectable aberrations upregulation of *MN1* is linked to poor survival. Future research will bring to light if MN1 is also involved in other subtypes of leukemia.

Basic research, as described in this thesis, aims to unravel the function of MN1. We have shown, amongst others, the important interplay between RA and MN1. RA is used as therapy for AML patients and upregulation of *MN1* was shown to predict a poor response to

RA therapy. Future studies to characterize the cofactor function of MN1 should focus on MN1 itself, its binding partners, and its target genes. These studies are crucial to create hypotheses as to how to treat patients that present with high *MN1* expression. Judging from the size of the protein it is expected that MN1 is a multifunctional protein and if these different functions are linked to distinct regions, it might be possible to block the oncogenic function of MN1.

Studies aiming to unravel the regulation of the *MN1* gene might present ideas for future therapy in relation to the upregulation of MN1. It is well possible that miRNAs will be identified that regulate the expression of *MN1* and these or modified forms of these miRNAs might represent important tools for future therapy.

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CHAPTER 7

Appendices

SUMMARY

Leukemia is a cancer of the blood and bone marrow and is characterized by an abnormal proliferation of poorly differentiated blood cells. The overgrowth of normal blood cells by leukemic cells leads to failure of the blood to perform its normal functions, ultimately resulting in symptoms, such as fatigue, easy bleeding and a failure to fight infections. The main subgroups of leukemia are classified according to the cell lineage involved (myeloid or lymphoid) and the development of the disease (acute or chronic).

Leukemias are frequently associated with specific chromosomal translocations and inversions. These genomic abnormalities lead to fusion proteins that, contributed by other mutations, disturb normal hematopoiesis. Some of these aberrations, such as the inversion (inv) of chromosome 16, have been detected in up to 12% of acute myeloid leukemia (AML) patients, whereas others have been found in only a small number of patients. An example of the latter type of translocations is the t(12;22) that fuses the *MN1* and *TEL* genes. The *MN1-TEL* fusion gene is one of topics of this thesis.

MN1's involvement in leukemias is not restricted to the fusion gene *MN1-TEL*. Expression profiling of inv(16) patient samples has shown that the *MN1* gene is upregulated in these patients. Up or downregulated genes associated with a specific subgroup of leukemias are likely to represent genes involved in the molecular mechanisms behind these leukemias. Mice studies have indeed confirmed that upregulation of MN1 contributes to leukemogenesis: the fusion protein generated by the inv(16) only leads to leukemia in mice when it is combined with overexpression of MN1. In addition, *MN1* was shown to be upregulated in a subset of cytogenetically normal AML patients and within this patient group, *MN1* overexpression is an independent prognostic marker for poor treatment outcome and worse overall survival.

Retinoic acid (RA) is a crucial stimulator of differentiation for hematopoietic cells, and AML patients benefit from treatment with RA in addition to standard chemotherapy. A retrospective study has shown that high *MN1* expression levels correlate with resistance to RA treatment in AML patients, whereby only patients with low levels of *MN1* benefit from RA treatment. The association between *MN1* expression levels and RA treatment is explained by the fact that MN1 functions as a cofactor, both stimulatory and inhibitory, for the vitamin A receptor. During differentiation of hematopoietic cells, MN1's cofactor activity is mainly inhibitory and high MN1 levels thus result in failure to respond to RA treatment.

In order to unravel the function of the *MN1* oncogene and its leukemogenic fusion gene *MN1-TEL,* a whole range of different research lines have been conducted over the last years in our laboratory and these form the basis of this thesis. We have studied MN1

function during embryogenesis in mice and molecular studies have focused on the transcriptional regulation of genes by MN1 and MN1-TEL.

By homologous recombination, an *Mn1* null mice strain has been generated allowing us to study the effects of omitting the *MN1* gene and in **chapter 2** the phenotype of these mice is described. The knockout mice are characterized by a palatal defect and severe malformations in the cranial/facial bone structures pointing to a role for Mn1 in the formation of these bone structures. In mice, palatal defects are not compatible with life, resulting in perinatal death of the newborn mice. In heterozygous mice, hypoplastic cranial bones and incomplete penetrance of the cleft palate have been observed. In later life, no higher tumor incidence or other pathology was observed in heterozygous mice. Two research groups have extended these studies focussing on the developing palate and the bone formation within the skull. Liu and coworkers have shown differential expression of *Mn1* in the developing palate and molecular studies have shown that Mn1 regulates the expression of Tbx22, a protein important for proper palatal development. MacDonald and coworkers have isolated calvarial osteoblasts from *Mn1* knockout mice. The osteoblasts were shown to have a reduced growth rate and produced less mineralized bone matrix. The function of Mn1 is not restricted to the stimulation of osteoblasts. Mn1 also regulates osteoclast function through the stimulation of RANKL, a gene involved in regulation of osteoclast function.

Shortly after cloning of the *MN1* gene in 1995, it was realized that MN1 is able to activate transcription. The amino acid sequence, however, did not reveal a DNA binding domain. In order to identify DNA elements in promoters binding to MN1 directly or indirectly, i.e. via other transcription factors, we have performed an oligo selection assay. Amongst others, a CACCC-rich element has been identified as a MN1-responsive element. Previous northern blots on meningioma samples with probes against *MN1* and *IGFBP5* have shown the coordinated expression of *MN1* and *IGFBP5* in this type of cancer. The presence of CACCC elements in the promoter region of the *IGFBP5* gene has prompted us to study the possible regulation of *IGFBP5* by MN1. **Chapter 3** describes that MN1 indeed upregulated the *IGFBP5* gene. Deletion constructs of the promoter lacking the CACCC elements, located 140 bp upstream of the transcription start site, failed to be stimulated by MN1. We have shown that MN1 does not bind to this sequence directly but most likely interacts with other, yet unknown, DNA-binding transcriptional regulators. The CACCC element has also been shown to be important for the induction of the *IGFBP5* promoter by RA. Induction of *IGFBP5* by RA depends on co-expressed MN1 in the Hep3B liver carcinoma cell line that we used in this study. MN1-TEL, the leukemogenic fusion protein, is capable of stimulating the *IGFBP5* promoter, but is unable to cooperate with RA in Hep3B cells, suggesting that effects of RA can be negatively affected in leukemias caused by MN1-TEL.

The *MN1-TEL* fusion gene formed by the t(12;22) fuses the 5' part of the *MN1* gene with the 3' part of the *TEL* gene. TEL is a sequence specific transcriptional repressor and member of a large family of transcription factors with highly homologous domains. We have previously shown that MN1 is a cofactor for the vitamin A receptor. The fusion protein harbors large portions of both proteins including the transcription stimulating domains of MN1 and the DNA binding domains of TEL, thus creating a deregulated transcription factor. In the study presented in **chapter 4** we have studied the characteristics of the MN1-TEL fusion protein with respect to MN1 on a viral promoter. We have shown that cofactor MN1 is highly stimulated by the coactivators p300 and RAC3, whereas MN1-TEL is not. Using a mutant of MN1-TEL that can not bind DNA, we have shown that MN1-TEL functions as a dominant-negative mutant of MN1 and blocks vitamin A-mediated transcription. This blockage is caused by dysfunctional MN1-derived activation domains rather than by recruitment of corepressors by the TEL moiety of the fusion protein.

Several studies have shown the importance of MN1 expression levels in leukemias. Identification of genes regulated by MN1 provides tools to further study the pathways underlying these MN1-involved leukemic processes. In **chapter 5,** we have conducted a gene expression profiling study using a leukemic cell line with inducible expression of *MN1*. In the same setup, we have also studied the expression profile resulting from *MN1-TEL* induction. We have shown that MN1 can both stimulate and inhibit transcription. Combining MN1 expression with RA showed that MN1 can both enhance and repress RA effects. Many of the identified genes are key players in hematopoiesis and leukemogenesis (e.g. *MEIS1* and *BMI1*). Another interesting target is the *DHRS9* gene, which encodes the DHRS9 protein, an enzyme involved in the synthesis of RA. The strong stimulation of this gene by RA is completely abolished by MN1. MN1-TEL induces expression of only a few genes and its most profound effect is inhibition of a large group of RA-stimulated genes, including *DHRS9*. In conclusion, both MN1 and MN1-TEL interfere with the RA pathway and this might explain the differentiation block in leukemias in which MN1 and MN1-TEL are involved.

SAMENVATTING

Leukemie is een kanker van het bloed en het beenmerg die gekenmerkt wordt door een hoge proliferatie, gecombineerd met een slechte differentiatie van bloedcellen. De abnormale bloedcellen overgroeien de normale bloedcelpopulatie waardoor deze de normale functies niet meer kunnen uitvoeren. Dit leidt uiteindelijk tot de symptomen van de ziekte, zoals moeheid, slechte bloedstolling en een verminderd vermogen om infecties te bestrijden. Leukemieën worden geclassificeerd op grond van het beloop van de ziekte (acuut of chronisch) en de betrokken cellen (myeloïde of lymfoïde).

Leukemieën zijn vaak geassocieerd met specifieke chromosomale translocaties en inversies. Deze creëren fusiegenen welke, tezamen met andere mutaties in het genoom, uiteindelijk leiden tot een verstoorde bloedcelvorming. Ongeveer 15 translocaties vormen samen de belangrijkste afwijkingen die bekend zijn in leukemie patiënten. Daarentegen is er een grote groep translocaties die zeldzaam zijn en maar in een kleine groepen patiënten gevonden worden. Een voorbeeld hiervan is de translocatie van chromosoom 12 en 22, die leidt tot een fusie tussen de *MN1* en *TEL* genen. Het fusiegen *MN1-TEL* is een van de onderwerpen van dit proefschrift.

De betrokkenheid van *MN1* bij leukemieën is niet beperkt tot het fusiegen *MN1-TEL*. Genexpressie profielen van inv(16) leukemie patiënten hebben laten zien dat *MN1* hoog tot expressie komt in de leukemische cellen. Genen waarvan het expressiepatroon geassocieerd is met een bepaalde vorm van leukemie, zouden betrokken kunnen zijn bij de moleculaire mechanismen die ten grondslag liggen aan het ontstaan van deze leukemie. Met behulp van muismodellen is inderdaad aangetoond dat een verhoogde expressie van MN1 een belangrijke bijdrage levert aan het ontstaan van leukemie door het inv(16) fusiegen.

Daarnaast is ook aangetoond dat het *MN1* gen tot overexpressie komt in een gedeelte van de patiënten waarbij in de leukemische cellen geen chromosomale afwijkingen gevonden worden, de zogenaamde cytogenetisch normale leukemie patiënten. In deze patiëntengroep is gevonden dat het *MN1* expressie niveau een onafhankelijke prognostische factor is. Een hoge expressie van *MN1* voorspelt zowel een slechte reactie op chemotherapie als een slechtere overlevingskans voor de patiënt.

Vitamine A vervult een belangrijke rol bij de differentiatie van bloedcellen. Een verstoorde differentiatie van bloedcelpopulaties is een belangrijk kenmerk van leukemie en het is dan ook gebleken dat leukemie patiënten baat hebben bij een behandeling met vitamine A naast conventionele chemotherapie. Een retrospectieve studie toonde dat een

hoog expressieniveau van *MN1* in patiënten gecorreleerd is met een resistentie voor vitamine A behandeling. Alleen patiënten met een lage expressie van het *MN1* gen hebben daarom baat bij een adjuvante behandeling met vitamine A. De associatie van MN1 met de vitamine A behandeling is te verklaren met het feit dat MN1 een cofactor is van de vitamine A receptor. MN1 kan zowel stimulerend als remmend werken op de transcriptie die door de vitamine A receptor gereguleerd wordt. In het proces van bloedcelvorming werkt MN1 voornamelijk remmend samen met de vitamine A receptor, waardoor een hoog expressie niveau van *MN1* een situatie bewerkstelligt waarbij patiënten niet reageren op een behandeling met vitamine A.

De afgelopen jaren zijn er veel verschillende onderzoekslijnen gevolgd om de functie van het *MN1* gen en het leukemie veroorzakende fusie gen *MN1-TEL* te bestuderen. Deze vormden de basis voor dit proefschrift. Er is onderzocht wat de functie van Mn1 gedurende de embryogenese in muizen is en moleculaire studies hadden tot doel de transcriptie regulatie door MN1 en MN1-TEL te onderzoeken.

Met behulp van homologe recombinatie is een muizenlijn gecreëerd die het *Mn1* gen niet tot expressie brengt; een zogenaamde *Mn1* knockout muizenlijn. Het fenotype van deze muizen is beschreven in **hoofdstuk 2**. De muizen lijden aan een gespleten verhemelte en vertonen ernstige afwijkingen aan de botten van het aangezicht en het schedeldak. Dit duidt op een belangrijke rol voor Mn1 in het ontstaan van deze structuren. Defecten aan het verhemelte zijn voor muizen lethaal, waardoor alle *Mn1* knockout embryo's direct na de geboorte overlijden. Het overgrote deel van de heterozygote muizen, die dus nog maar 1 kopie van het *Mn1* gen bezitten, blijven na de geboorte in leven en hebben een normale levensverwachting. De botten van het gelaat en het schedeldak zijn aangelegd maar zijn dunner in vergelijking met de normale muizen. Het deel van de heterozygote muizen die na geboorte overlijden bleken een gespleten verhemelte te hebben.

Twee onafhankelijke onderzoeksgroepen hebben de *Mn1* knockout muizenlijn gebruikt voor vervolgstudies. Liu en medewerkers hebben de ontwikkeling van het verhemelte bestudeerd. In normale muizen bleek *Mn1* in verschillende regionen van het verhemelte tot expressie te komen. In de *Mn1* knockout muizen werd aangetoond dat Mn1 de expressie van Tbx22, een belangrijk eiwit voor de ontwikkeling van het verhemelte, reguleert. MacDonald en medewerkers hebben de botvorming van de schedel in de *Mn1* knockout muizen nader onderzocht. Osteoblasten afkomstig van *Mn1* knockout muizen vertoonden een lage groei snelheid en produceerden minder box matrix. De studie heeft laten zien dat de rol van Mn1 in het proces van botvorming zich niet beperkt tot het stimuleren van osteoblasten. Ook de functie van osteoclasten wordt gereguleerd door Mn1.

Na het kloneren van het *MN1* gen in 1995, bleek al snel dat MN1 betrokken is bij transcriptieregulatie. De voorspelde aminozuur sequentie gaf echter geen indicaties voor het bestaan van een DNA bindend domein in het eiwit. Met behulp van een zogenaamd oligoselectie experiment is getracht de DNA sequenties te identificeren die belangrijk zijn voor het functioneren van MN1. MN1 zou deze sequenties direct of indirect, dat wil zeggen via andere transcriptiefactoren, kunnen binden. Er werd o.a. een CACCC-rijk element geïsoleerd wat belangrijk zou kunnen zijn voor de functie van MN1. Eerder was er onderzoek gedaan aan meningiomen, een type goedaardige hersentumoren. Studies die betrekking hadden op RNA expressie niveaus van verschillende transcripten lieten zien dat de expressie niveaus van *MN1* en *IGFBP5* sterk variëren in meningiomen. Opvallend daarbij was dat de expressie van deze twee transcripten aan elkaar gecorreleerd is. De aanwezigheid van CACCC elementen in de regulerende sequenties van het *IGFBP5* gen heeft er toe geleid dat onderzocht is of MN1 de expressie van het *IGFBP5* gen kan reguleren. **Hoofdstuk 3** beschrijft dat MN1 inderdaad het *IGFBP5* gen kan stimuleren. De CACCC elementen liggen ongeveer 140 baseparen voor de positie waar de transcriptie gestart wordt en constructen die deze elementen missen worden nauwelijks door MN1 gestimuleerd. Dit laat zien dat de CACCC elementen in de regulerende sequenties van het *IGFBP5* gen betrokken zijn bij de stimulatie door MN1. Diezelfde elementen zijn ook betrokken bij de stimulatie van *IGFBP5* door o.a. vitamine A. We hebben aangetoond dat deze stimulatie in de gebruikte cellijn afhankelijk is van de expressie van MN1. Het fusie eiwit MN1-TEL is in staat om het *IGFBP5* gen te stimuleren, maar kan hierbij niet samenwerken met vitamine A zoals MN1 dit wel kan. Dit suggereert dat het niet kunnen samenwerken van MN1-TEL met vitamine A op vele andere genen een rol zou kunnen spelen in het ontstaan van leukemie door MN1-TEL.

Het *MN1-TEL* fusie gen, dat gevormd wordt bij de translocatie tussen chromosoom 12 en 22, fuseert het voorste deel van het *MN1* gen met het achterste deel van het *TEL* gen. TEL is een DNA bindende repressor van transcriptie en behoort tot een grote familie van transcriptiefactoren die een aantal goed gedefinieerde, homologe domeinen hebben. MN1 kan o.a. als cofactor de transcriptie beïnvloeden die door de vitamine A receptor gereguleerd wordt. Het fusiegen bevat van beide genen een groot gedeelte waardoor het transcriptie stimulerende deel van *MN1* en het DNA bindende domein van *TEL* gecombineerd worden. Het fusiegen codeert dus voor een transcriptiefactor met unieke nieuwe eigenschappen. In **hoofdstuk 4** is de functie van het MN1-TEL fusie-eiwit onderzocht in relatie tot het MN1 eiwit. De activiteit van MN1 als cofactor voor de vitamine A receptor wordt gestimuleerd door de cofactoren p300/CBP en RAC3. Het fusie-eiwit MN1-

TEL daarentegen niet. Met behulp van een mutant van MN1-TEL die niet meer aan DNA kan binden hebben we laten zien dat MN1-TEL een dominant-negatief effect heeft op MN1 en transcriptie die door vitamine A gestimuleerd wordt kan remmen. Deze remming wordt niet veroorzaakt door het aantrekken van corepressoren met het TEL gedeelte van het fusie eiwit, maar door het niet goed functioneren van de stimulerende domeinen uit het MN1 deel van het fusie eiwit.

Een aantal studies heeft laten zien dat *MN1* expressie niveaus belangrijk zijn in leukemieën. Het identificeren van genen die gereguleerd worden door MN1 levert een bijdrage voor het verder bestuderen van de mechanismen die betrokken zijn bij het ontstaan van leukemieën waarbij MN1 betrokken is. In **hoofdstuk 5** wordt een studie beschreven met een leukemische cellijn waarin MN1 tot expressie gebracht kan worden. Er is een genexpressie profiel bepaald van deze cellijn onder verschillende condities: in de aan- en afwezigheid van MN1 zonder of in combinatie met vitamine A. Op dezelfde manier is ook een cellijn gebruikt waarin het *MN1-TEL* fusie gen tot expressie gebracht kan worden. We hebben laten zien dat MN1 zowel transcriptie kan stimuleren als remmen, zowel in aan- als afwezigheid van vitamine A. Veel van de geïdentificeerde genen spelen een belangrijke rol bij het ontstaan van leukemie in het algemeen. Voorbeelden hiervan zijn *MEIS1* en *BMI1*. Een ander interessant gen dat door MN1 gereguleerd wordt is *DHRS9*. Het gen codeert voor een enzym dat betrokken is bij het metabolisme van vitamine A. *DHRS9* wordt zelf ook gestimuleerd door vitamine A en MN1 neutraliseert deze stimulatie volledig. MN1-TEL stimuleert de expressie van maar een selecte groep genen en het belangrijkste effect van het fusie-eiwit is de remming van een grote groep genen die gestimuleerd worden door vitamine A, waaronder *DHRS9*. Concluderend kan gezegd worden dat zowel MN1 als MN1-TEL de vitamine A gereguleerde processen beïnvloeden. Daar vitamine A een cruciale rol speelt bij de differentiatie van bloedcellen tijdens de bloedcel vorming zou dit een verklaring kunnen zijn voor de blokkering van de bloedcel differentiatie die gezien wordt in leukemieën waarbij MN1 en MN1-TEL een rol spelen.

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PHD PORTFOLIO

Name PhD student:	Magda Meester-Smoor	
Erasmus MC department:	Pathology	
Research Schools:	Molecular Medicine (Molmed)	
	Medical Genetic Center (MGC)	
PhD period:	August 1998 - May 2009	
Promoter:	Prof.dr. E.C. Zwarthoff	

1. PhD training

	Year	Workload
General academic skills		
- Laboratory animal science, article 9	1999	3.0 ECTS
- Biomedical English writing and communication	2006	2.0 ECTS
In-depth courses		
- Oncogenesis and tumor biology	1998	1.0 ECTS
Presentations		
- Oral presentations at the Josephine Nefkens Institute (JNI)	2002	0.3 ECTS
Scientific Lab meetings, Erasmus MC Rotterdam	2006	0.3 ECTS
	2007	0.3 ECTS
	2008	0.3 ECTS
- Oral presentations at the Study group Nucleic Acids	2006	0.3 ECTS
NWO/CW meeting in Lunteren	2007	0.3 ECTS
- Oral presentation at the Tumor Cell Biology meeting of the	2007	0.3 ECTS
Dutch Cancer Society in Lunteren		
- Poster presentation at American Association for Cancer	2008	0.3 ECTS
Research (AACR), San Diego, USA		
- Poster presentations at Molecular Medicine Day	2005	0.3 ECTS
in Rotterdam	2007	0.3 ECTS
	2008	0.3 ECTS
	2009	0.3 ECTS
- Poster presentation at the Study group Nucleic Acids	2008	0.3 ECTS
NWO/CW meeting in Veldhoven		
International conferences		
- Federation of European Biochemical Societies (FEBS)	2006	0.8 ECTS
Istanbul, Turkey		
- American Association for Cancer Research (AACR)	2008	1.0 ECTS
San Diego, USA		

Seminars and workshops

-	JNI oncology lectures	1998-2009	1.0 ECTS
-	Annual Molecular Medicine Day in Rotterdam (total of 8 visits)	1999-2008	2.5 ECTS
-	Tumor Cell Biology meeting of Dutch Cancer Society in Lunteren (total of 3 visits)	2002-2007	1.8 ECTS
-	Study section Nucleic Acids NWO/CW meeting in Lunteren or Veldhoven (total of 5 visits)	2000-2008	3.0 ECTS
-	MGC day in Noordwijkerhout or Rotterdam (total of 4 visits)	2002-2008	1.2 ECTS

2. Teaching activities

Internships of students: supervising practical work and writing

-	Francine van Loon from Avans Hogeschool, Breda April 2002 - September 2002	2002	4.0 ECTS
-	Merel Boogaard from Avans Hogeschool, Breda November 2002 - June 2003	2002-2003	4.0 ECTS
-	Diny van Dalsum-Verbiest from Avans Hogeschool, Breda April 2004 - October 2004	2004	4.0 ECTS
-	Gertine van Oord from Avans Hogeschool, Breda Febuary 2007 - September 2007	2007	4.0 ECTS
-	Gabriëlle van Tilburg from Avans Hogeschool, Breda November 2007 - June 2008	2007-2008	4.0 ECTS
-	Anton van der Stoep and Yvonne Muizer from Calvijn, Rotterdam (1 week, Junior Science project)	2006	1.0 ECTS
-	Gerdie de Jong and Kirsten Geneugelijk from Wartburg College, Rotterdam (1 week, Junior Science project)	2007	1.0 ECTS
-	Dominique Berger and Cho-land Yuan from Walburg College, Zwijndrecht (1 week, Junior Science project)	2008	1.0 ECTS

Other

-	Assistant for first-year medical students at VO	2005	0.4 ECTS
	'Verschillen tussen kleincellig en niet kleincellig	2006	0.4 ECTS
	longcarcinoom'	2007	0.4 ECTS
		2008	0.4 ECTS

One ECTS (European Credit Transfer System) equals a study workload of approximately 28 hours.

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