University of Münster
Department of Medicine
Hematology and Oncology
- Director: Univ.-Prof. Dr. W. E. Berdel -

Identification of Growth Suppressive Genes as Cyclin A1 Targets in Acute Myeloid Leukemia by Microarray Analysis

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Amber Yasmeen
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Dean: Univ.-Prof. Dr. Heribert Jürgens

1. Examiner: Univ.-Prof. Dr. Hubert Serve
2. Examiner: Prof. Dr. O. M. Koch

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University Hospital Münster
Clinic and Polyclinic for Inner Medicine A
-Director: Univ. Prof. Dr. W. E. Berdel-

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Summary

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Amber Yasmeen

Cyclin A1 is a tissue specific A-type cyclin, which is essential for spermatogenesis. Cyclin A1 is also highly expressed in blasts of acute myeloid leukemia. In a transgenic mouse model, cyclin A1 induced leukemia but its pathogenic role and the molecular mechanisms of its action remain unclear. In this study, we identified cyclin A1 induced genome wide changes in gene expression. RNA from U937 control vector transfected and cyclin A1 overexpressing U937 cells were analyzed at different serum conditions using high-density oligonucleotide arrays. A number of genes, which showed upregulation and downregulation by cyclin A1, were identified. WT1 and EGR1, two growth suppressor were downregulated by cyclin A1. This finding was confirmed by quantitative RT-PCR analysis. Furthermore, WT1 was shown to be downregulated at the protein level in U937 cells overexpressing cyclin A1 as compared to control cell lines. We also analyzed the expression of WT1 and EGR1 in different leukemia cell lines at the protein level. Expression of WT1 protein was low in Hutu, KCL22, ML1 and Cos cells. EGR1 protein was low in ML1, KCL22 and NB4. In cyclin $A1^{-/-}$ mice organs, WT1 and EGR1 were higher expressed in testis, bone marrow and ovary as compared to the wild type organs. Also, cyclin $A1^{-/-}$ murine embryonic fibroblasts (MEFs) expressed higher levels of EGR1 mRNA upon irradiation than irradiated wild type fibroblasts. WT1 and EGR1 protein levels were increased in cyclin $A1^{-/-}$ MEFs. Four different isoforms are known of WT1, which are characterized by the insertion or exclusion of two splice inserts (17aa and KTS) at two different positions. Interestingly, in U937 control cells the WT1-/- isoform was found predominantly. In U937 cells overexpressing cyclin A1, all isoforms WT1-/-, WT1+/+, WT1+/- and WT1-/+ could be found. The WT1-/+ isoform reduced colony formation and induced G1 cell cycle arrest in a myeloid cell line.

Taken together, cyclin A1 overexpression in U937 myeliod cells leads to significant changes in genome wide gene expression. WT1 and EGR1 are putative cyclin A1 target genes. These data provide evidence that cyclin A1 can lead to transcriptional suppression of tumor suppressor and antiproliferative genes.

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1. Introduction

1.1 Cyclins and Cell Cycle

The cell cycle is based on two key families of proteins. First, the family of cyclin-dependent protein kinases (cdks) activates downstream signaling by phosphorylating substrates at serine and threonine residues. Second, a family of specialized activating proteins, called cyclins, binds to cdk molecules and control their ability to phosphorylate appropriate target proteins.

The cyclic assembly, activation and disassembly of cyclin-cdk complexes are pivotal events driving the cell cycle. The name “cyclin” is based on the oscillating synthesis and degradation in each cell cycle division (101, 154) (126) (33). Cdks and their cyclin partners are positive regulators, which induce cell cycle progression. Cyclin dependent kinase inhibitors act as brakes to stop the cell cycle progression in response to regulatory signals. By direct association with cyclin-cdk complexes, cyclin dependent kinase inhibitors can downregulate cdk activity (73, 155).

The binding of two families of cyclin dependent kinase inhibitors modulates the activity of cdks. The first class includes the INK4 proteins, which specifically inhibit the catalytic subunits of cdk4 and cdk6. There are four such proteins named $p16^{INK4A}$, $p15^{INK4B}$, $p18^{INK4C}$ and $p19^{INK4D}$ (73, 156) consisting of multiple ankyrin repeats and which bind specifically to cdk4 and cdk6.

The second class (Cip/Kip) affects the activities of cyclin D, E and A dependent kinases. The Cip/Kip family includes $p21^{Cip1}$, $p27^{Kip1}$ and $p57^{Kip2}$, all of these contain characteristic motifs within their amino-terminus that enable them to bind both to cyclin and cdk subunits (47, 155).

Cyclins were first identified in marine invertebrates on the basis of their involvement in cell cycle periodicity during meiotic and early mitotic divisions (127, 162). There are 30 cyclin sequences in higher vertebrates named cyclins A-H, which are divided

into different categories. The cyclins A to H can be distinguished on the basis of conserved sequence motifs, by pattern of appearance as well as by different functional roles during specific phases of the cell cycle (reviewed in (125, 153) (156)).

The activity of cyclins usually requires physical association with cyclin dependent serine and threonine kinases (cdks). In mammalian cells, several cdks (cdk2, 3, 4, 5, 6 and 7) function at different stages of the cell cycle and their activities are regulated by cyclins, various co-factors and modifying enzymes. The negative regulators, so called cyclin-dependent kinase inhibitors (CKIs) bind to the cyclin and to cdk subunits inhibiting kinase activity and cell cycle progression (47, 135, 152, 156).

The cell cycle is comprised of different stages G1, S, G2, and M division. Cell cycle regulation depends on the balance between the presence of activated cyclins, activated cyclin dependent kinases and kinase inhibitors. Active cyclin-cdk complexes enable the cell to pass through specific phases of the cell cycle division (G1 and G2), DNA synthesis (S), and mitosis (M). Cyclin abundance is rate limiting for progression through the different stages of the cell cycle, e.g. progression through G1 depends upon cdk4 and cdk6 along with cyclin D regulatory subunits. D type cyclins are synthesized in early G1, bind to and activate cdk4 and cdk6 in response to mitogenic signals.

Subsequently, cyclin E interacts with cdk2 and regulates late G1 and early S Phase (25, 118) Cyclin A2 appears later in the cell cycle, interacting with cdk2 and cdk1, and regulating progression through S phase and early G2 phase. Cyclin B is active during mid-G2 and mitosis when it forms a kinase complex with cdk1 (Figure 1).

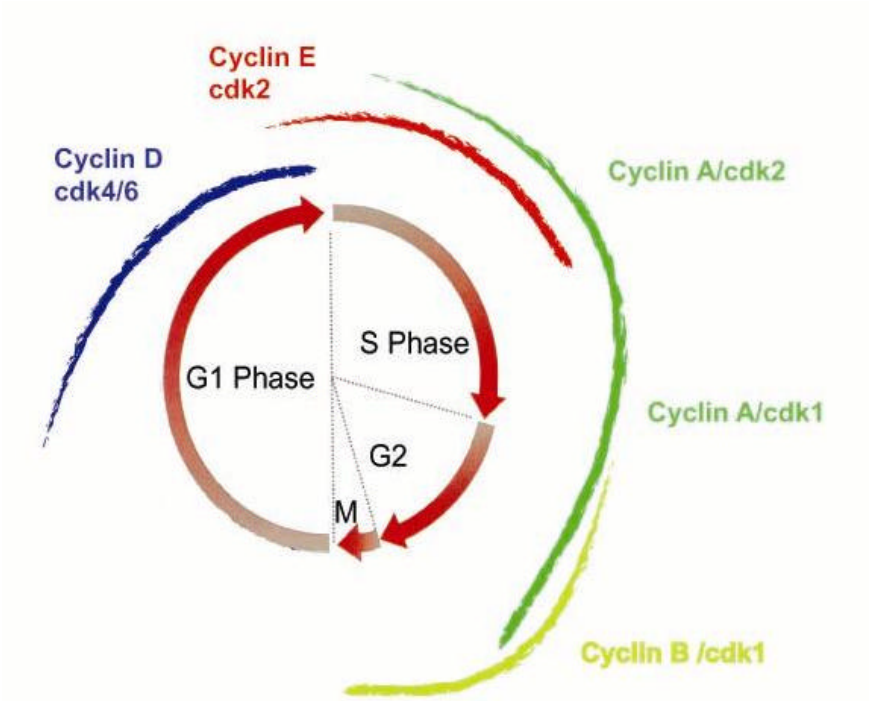

Figure 1: Expression of cyclins oscillates during cell cycle progression
Transcription of cyclin genes is induced in specific cell cycle phases. Also, co-ordinated cyclin degradation is crucial for cell cycle progression (122)

1.2 The Role of A-Type Cyclins in Cell Cycle

1.2.1 Cyclin A2

The A-type family of cyclins consists of two members: cyclin A1 and cyclin A2. Cyclin A2 (formerly known as cyclin A) can activate two different cyclin-dependent kinases. Cyclin A2 accumulates in the nucleus immediately after its synthesis and associates with both cdc2 (cdk1) and cdk2 (29, 40, 64, 120). In tissue culture systems, the activity and localization of cyclin A2 support the hypothesis that it binds to both cdk2 and cdc2 (cdk1) (55) (13). This leads to two distinct cyclin A2 associated kinase activities, one appearing in S phase, the other in G2. In addition to its role in S phase progression, cyclin A2 is also involved in G2/M transition and mitosis. Cyclin A2 has been reported to be expressed in meiotically arrested oocytes of several species, including fish (60) and frogs (49). In addition, cyclin A2 is a rate limiting component

for entry into mitosis (79, 117). Cyclin A2 deficient cells do not enter mitosis (75). Cyclin A2 acts earlier in the cell cycle than cyclin B (54, 63)

In Drosophila embryos, cyclin A2 mutations cause defects in neuronal progenitor cells (167). During mouse embryogenesis, cyclin A2 is present at high levels, (133, 161) and targeted mutagenesis of the coding region of murine cyclin A2 results in early embryonic lethality, apparently around the preimplantation stage (109). Somatic and germ cells of the ovary express cyclin A2 mRNA and protein, while cyclin A2 is not found in the oocyte and metaphase II arrested egg. Nonetheless, cyclin A2 is detected after fertilization and throughout the preimplantation development stages. Mouse cyclin A1 and A2 share 44% overall similarity at the amino acid level and (84%) amino acid identity within the highly conserved cyclin box (133, 161, 172).

Cyclin A2 regulation has been studied in different cell culture systems. Cyclin A2 is synthesized at the onset of DNA replication as well as during G2/M transition (151). Cyclin A2 expression is under negative transcriptional control during the cell cycle especially in G0 and early G1 phase while repression is not observed in later phases. Cyclin A2 expression is required for transcriptional derepression of its own gene. An inhibitory complex is found at the promoter *in vivo* and *in vitro* (53, 147, 180). The region responsible for repression has been named CDE (cell cycle dependent element) and CCRE (cell cycle responsive element). The cyclic association and dissociation of CDE/CCRE repressor complex is involved in the periodic transcription of cyclin A2. Another region in the cyclin A2 promoter named CHR (Cell cycle genes homology region) shows homology with sequences in the promoter of several other cell cycle regulated genes (181). This cell cycle gene homology region (CHR) is found in the promoters of cdc25C, B-myb, cdc2 and cyclin A2. In gel shift experiments, binding to the major DNA groove for the CDE/CCRE and to the minor groove of the CHR has been shown (53, 87, 88, 123). Mutation of either motif results in de-repression of the cyclin A2 promoter.

E2F plays critical roles in cell cycle progression by regulating the expression of genes involved in nucleotide synthesis, DNA replication, and cell cycle control. E2F is a heterodimeric transcriptional factor composed of the E2F and DP multi-gene families (105, 115). Transcriptional activation by E2F is modulated during the cell cycle by pocket proteins of the pRb family (116). E2F is repressed in G0 and early

G1, but during cell cycle progression cyclin dependent kinases effect the dissociation of the inhibitory E2F pocket protein complexes. This dissociation generates transcriptionally active free E2F and leads to the activation of E2F –regulated genes (72, 105, 115). E2F plays no significant role in the repression of cyclin A2 promoter in G0/G1 cells, but later in the cell cycle. E2F1 and E2F3 bind the CDE and displace a presumed repressor protein named CDF1 in late stage of G1/S phase (182) (86). Mutation in the CDE can abrogate the binding of E2F, and overexpression of E2F1 leads to an upregulation of the cyclin A2 gene (20, 21, 147). Cyclin A2 promoter activity is repressed by the CHR/CHR-binding factor (CHF) (123). The repressor elements are thought to interfere with the transactivation function of factors bound further upstream. Sp1, NF-Y, and CBP/cycA and the ATF/CREB/CREM family can all interact with upstream elements (20, 180, 183). The ATF/CREB/CREM family factors mediate regulation of the cyclin A2 promoter by cAMP (22). It was also reported that ATF/CREB/CREM family members show cell type specific binding to the cyclin A2 promoter (6). CBP/cycA is involved in adhesion-dependent cyclin A2 transcription (66).

The overexpression of viral proteins such as Adenovirus E1A, Simian virus 40, human herpesvirus-8 (HHV-8) and Polyoma T antigens or Papilloma E7 has been shown to increase cyclin A2 expression by binding to the CCRE/CDE-CHR. These findings further support to the notion that pocket proteins may modulate cyclin A2 transcription (27, 146, 178, 179).

The Retinoblastoma gene product (pRb) and the related p107 and p130 interact with cyclin A2. They are essential for repression of cyclin A2 in quiescent cells and play a role in early G1 phase and G1/S transition (124). The C-terminal regulatory domain of p53 contains a cyclin A2-cdk2 docking site, which suggests a role for cyclin A2 in p53 responses to DNA damage (89). Cyclin A2/cdk2 bind to E2F transcription factors (E2F-1, 2, 3A, 3B) *in vivo* and *in vitro* and modulates E2F activity by phosphorylation (147). In G1, CDC6 interacts specifically with the active cyclin A2/cdk2 complex *in vitro* and *in vivo*. Nuclear CDC6 relocalizes to the cytoplasm upon cyclin A2/cdk2 activation. This negative regulatory event could be implicated in the prevention of re-replication during S phase and G2 (16). Jun B and B-myb are also physiological targets for cyclin A2/cdk2. B-myb has been implicated in regulation of cell cycle

progression especially during S phase. Cyclin A2/cdk2 (141) enhances its transactivation capacity. Cell adhesion plays an essential role in the regulation of cellular proliferation. It was observed in both NRK and NIH3T3 fibroblasts that cyclin A2 is a target of adhesion dependent signals (20). Also, activation of human primary T lymphocytes via CD2 and CD28 adhesion molecules relieves Cyclin A2 gene transcriptional inhibition (128). Ectopic expression of c-myc causes deregulation of cyclin A2 levels and adhesion independent growth. Cyclin A2 is critically involved in chromosome ploidy, and mutations have been shown to be associated with endo-reduplication in certain cells, and silencing of cyclin A2 induces complete genome duplication (96). In mammalian somatic cells, cyclin A2 is also involved in the centrosome duplication. This requires phosphorylation of retinoblastoma protein (Rb), E2F transcription factors and cdk2 activity (95).

1.2.2 Cyclin A1

Cyclin A1 was isolated and characterized as a second human cyclin A gene (133, 161, 172, 175). Cyclin A1 has 48% identity with human cyclin A and is more related to the recently cloned murine cyclin A1 (84% identity). Cyclin A1 is specifically expressed in testis and brain. It is also highly expressed in several myeloid leukemia cell lines, including ML-1, U937, NB4, KG-1, and THP1. In contrast, very low expression at the mRNA level is observed in most nonleukemic, adherent cell lines (175).

There are different roles for the two A-type cyclins in the regulation of mitotic and meiotic cell cycles (133, 161). The mRNAs of the putative cdk partners of these cyclins, cdk1 and cdk2 are expressed in similar patterns during spermatogenesis (173). Murine cyclin A1 binds both cdk1 and cdk2 in testis (83, 84, 161). Cdk2 is highly expressed during all stages of spermatogenesis, especially in cells undergoing the meiotic reduction division. It was also reported that in testicular cells cdk1 is not the active partner of cyclin A1 (134) a fact confirmed by a yeast two-hybrid assay that showed human cyclin A1 does not interact with cdk1 (175).

The importance of cyclin A1 for spermatogenesis was demonstrated in a deletional mouse model (85). Male cyclin $A1^{-/-}$ mice are sterile due to a block of

spermatogenesis before the first meiotic division. This meiotic arrest is associated with increased germ cell apoptosis, synaptic abnormalities and reduction in cdc2 kinase activation at the end of the meiotic prophase. Besides defects in spermatogenesis, cyclin $A1^{-/-}$ mice develop normally, suggesting that cyclin A1 is not essential for embryonic and postnatal somatic cell division (109). Female cyclin $A1^{-/-}$ mice do not show noticeable defect during oogenesis, which indicates differences of regulatory mechanisms of meiosis between male and female.

Cyclin A1 expression is regulated at the transcriptional and translational level during cell cycle. The human cyclin A1 promoter initiates transcription from several sites and a construct containing a fragment from -190 to +145 shows the highest transcriptional activity. Promoter activity crucially depends on four GC boxes upstream of the transcriptional start sites. These GC boxes, which are binding site for Sp1 and Sp3 *in vitro* also regulate cell cycle regulated expression (103). Hypomethylation of the cyclin A1 promoter is important for the high transcriptional promoter activity in leukemia cell lines. Although tumor cells often express aberrant methyltransferase activity (61, 168), they have been shown to display hypomethylation of promoter regions of genes that promote growth and inhibit apoptosis (43). Methylation of the cyclin A1 promoter correlates with absence of endogenous cyclin A1 expression in solid cancer cell lines.

Functionally, cyclin A1 associates with the cell cycle regulatory proteins Rb and E2F1 *in vivo* and *in vitro*. Also, Rb family proteins and E2F1 are substrates for phosphorylation by cyclin A1 associated kinases (176). Cyclin A1 also interacts with B-myb. Cyclin A1/cdk2 posphorylates B-myb at functionally important serine and threonine residues in the C-terminus (106). Since B-myb is important for proliferation of normal hematopoietic and leukemia cells, cyclin A1 may be one of the co-activators that enable the transactivating ability of B-myb in these cells. C-myb is also involved in the transactivation of the human cyclin A1 promoter. This interaction shows a specific involvement of c-myb in the cell cycle of the hematopoietic cells. Thesed data provide evidence that cyclin A1 interacts with cell cycle regulatory proteins and all these findings suggest that Cyclin A1 has a distinctive role in the cell cycle of somatic cells (104).
However, most genes regulated by cyclin A1-cdk2 have not been identified, yet.

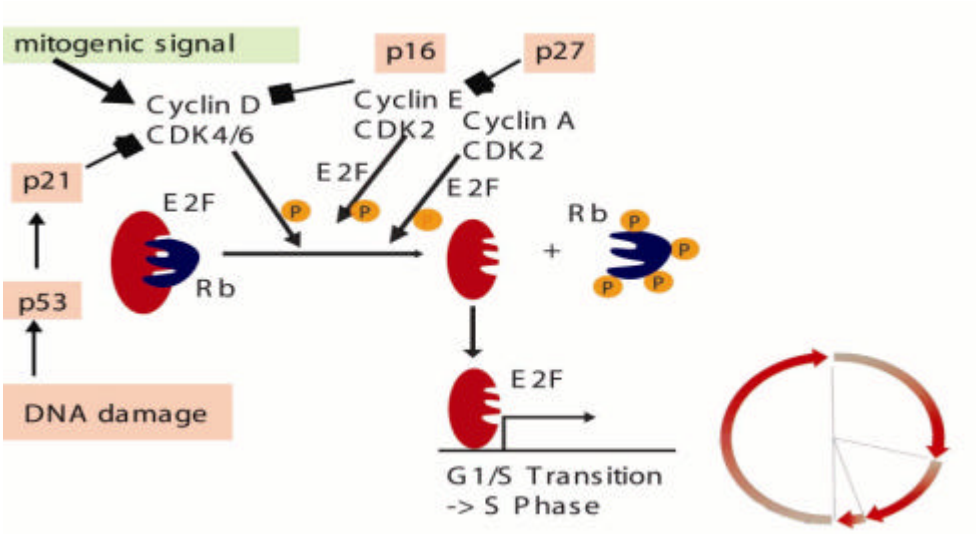

Figure 2: Regulation of Rb by cyclin/cdk complexes

Regulation of the Rb/E2F pathway by cyclin/cdk2 complexes is well established and is shown here to illustrate the principle of cyclin/cdk action. Mitogenic signals induce the synthesis of cyclin D and subsequent formation of cyclin D-cdk4/6 complexes. The cyclin D- cdk4/6 complexes then phosphorylate downstream targets, including Rb protein. Cyclin D-cdk4/6 activity leads to the activation of cyclin E-cdk2 complexes. Increased cyclin E-cdk2 kinase activity then results in enhanced Rb phosphorylation. Rb hyperphosphorylation releases E2F from transcriptionally repressive Rb/E2F complexes. E2F downstream genes lead to S phase initiation. Rb is also phosphorylated by cyclin A/cdk2. Cyclin/cdk activity is regulated by several cdk inhibitors and other regulatory factors e.g. cyclin activating kinase (CAK).

1.3 Acute Myeloid Leukemia

Acute myeloid leukemia (AML) is a malignant disease of the hematopoietic system. AML is characterized by an accumulation of granulocyte or monocyte precursors in the bone marrow and blood. Although leukemia originates from the bone marrow, it can spread to the blood, lymph nodes, spleen, liver, central nervous system and other organs. In Germany, the annual incidence of AML is about 2-5 per 100.000 humans and increases with increasing age on up to 12 per 100.000 humans at the age of > 65 years. In the past years, progress was made with investigating of characteristic genetic changes of AML blasts. Their association with clinical and prognostic characteristics and functional consequences were defined. Recent

findings led to the refinement and revision of the diagnostic criteria, influencing the new WHO classification of the disease (45).

1.3.1 Acute Myeloid leukemia- Diagnosis

The diagnosis of AML is based on the presence of more than 30% blasts in the bone marrow of the patient. AML is divided on the basis of morphological and cytochemical characteristics according to the French American British (FAB) - classification in sub-groups (see Table 1).

FAB-Classification in Acute Myeloid Leukemia

FAB	Name	Percentage	MPO	NBE	PAS	Associated Translocation	Involved Gene
M0	Undifferentiated	3%	-	-	-	inv(3q26), t(3;3) (1%)	EVI1
M1	Myeloblastic without maturation		+/-	-	-		
M2	Myeloblastic with maturation	25-30%	++	-	-	t(8;21) (40%)	AML1/ETO
						t(6:9) (1%)	DEK/CAN
M3	Acute Promyelocyctic leukemia	5-10%	+++	-	+/-	t(15;17) (98%)	PML/RAR?
						t(11;17 (1%)	PLZF/RAR?
						t(5;17) (1%)	NPM/RAR? ?
M4	Acute myelomonocytic Leukemia	20%	+++	++	-	11q23 (20%)	MLL
						inv(3q26), t(3;3) (3%)	EVI1
						t (6; 9) (1%)	DEK/CAN
M4eo	Myelomonocytic with bone marrow eosinophlia	5-10%	+++	++	-	inv(16), t(16;16) (80%)	CBFß/MYH 11
M5	Acute Monocyticleukemia	2-9%	+/-	+++	+/-	11q23 (20%)	MLL
						t(8;16) (2%)	MOZ/CBP
M6	Erythroleukemia	3-5%	-	-	+++		
M7	Acute Megakaryoblastic leukemia	3-12%	-	-	++	t(1;22) (5%)	GATA1 mutations

Table :1 FAB-classification of AML; MPO=Myeloperoxidase; NBE=N-Butylacetat-Esterase (unspecific); PAS=Periodic-acid Shift

Apart from this classification the international commission has developed new AML classification for World Health Organization (WHO) (45). Within each category, distinct diseases are defined according to a combination of morphology, immunophenotype, genetic features and clinical syndromes (See Table: 2).

WHO-classification of Acute Myeloid Leukemia:	
AML with recurrent cytogenetic translocations	
	AML with t (8; 21)(q22; q22), AML1/ETO
	Acute Promyelocytic leukemia (AML with t (15; 17)(q22; q11-22) and Variants, PML/RAR?)
	AML with abnormal bone marrow eosinophils (inv (16)(p13q22) or t (16; 16(p13; q11), CBFß/MYH11)
	AML with 11q23 (MLL)-Abnormalities
AML with multilineage dysplasia	
	With prior myelodysplastic syndrome
	Without prior myelodysplastic syndrome
AML and myelodysplastic syndromes, therapy related	
	Alkylating agent-related
	Epipodophyllotoxin-related (some may be lymphoid)
	Others type
AML not otherwise characterized	
	AML minimally differentiated
	AML without maturation
	AML with maturation
	Acute myelomonocytic Leukemia
	Acute monocytic Leukemia
	Acute erythroid Leukemia
	Acute megakaryocytic Leukemia
	Acute Basophilic leukemia
	Acute Panmyelosis with myelofibrosis
	Acute biphenotypic Leukemia

Table 2: WHO-classification of AML

In this classification, cytogenetic and pathogenetic criteria of the AML are taken into account, which determine prognosis and in the future also selection of therapy. The analysis of AML typical mutations and the identification of antigens on the surface of AML blasts and specific chromosome translocations have improved classification. The classification AML is facilitated by the powerful and well-developed techniques of cytogenetics, fluorescence *in situ* hybridization (FISH) and polymerase chain reaction (163). The cyctogenetics, FISH and PCR techniques enable the detection of quantitatively molecular changes or chromosomal translocations in leukemia cells and allow highly sensitive detection of remaining blasts in patients undergoing

remission. Progress in prognosis and treatment of childhood leukemia are considered as a remarkable success of modern medicine. Childhood leukemia, once was considered as a fatal disease, now exhibits overall cure rates ranging from 75% to 85% for acute lymphocyte leukemia and 40% to 50% for acute myelogenous leukemia (15).

1.3.2 Acute Myeloid leukemia - prognosis factors

A set of clinical and molecular characteristics indicates both the probability of achieving complete remission and the duration of disease-free survival of AML patients.

According to another classification of AML (MRC) patients are divided into favourable, intermediate and unfavourable subtypes based on the prognosis (see Table 3).

Medical Research Council (MRC) classification of AML		
Subtype	**MRC**	**MRC and C/EBP? mutation status**
Favorable	**T(15; 17),Inv(16),t(8;21)**	**t (15,17), Inv (16) and t (8; 21), or C/EBP? mutation without FLT3-ITD or adverse cytogenetics**
Intermediate	**All others**	**C/EBP? mutation with FLT3-ITD or all others**
Unfavorable	**del (5q)/-5, -7,3q;complex karyotypic abnormalities**	**del (5q)/-5, -7,3q;complex karyotypic abnormalities**

Table 3: MRC-classification of AML; C/EBP? = CCAAT/enhancer binding protein-? ;ITD, internal tandem duplication. Modified from ref (41).

It has to be considered that the choice of the therapy is different among above-mentioned classification based on prognosis. The prognosis of the AML M3 with the translocation t (15; 17) is quite favourable upon treatment with conventional chemotherapy, the specific therapy with ATRA finally leads to excellent survival rates. Patients with the translocation t (8; 21) seem to have a more favourable prognosis upon the repeated application of high dose ARA C (10).
However, also the mortality of patients with favourable karyotype still reaches 40% to 50%. Secondary AML after MDS (myeloid dysplastic syndrome) and/or after the treatment with chemotherapeutic agents (i.e. Alkylation and Topoisomerase II-inhibitors) leads to a significantly worse prognosis than those of the average of the leukaemiapatients.

Many genes and signalling pathways are involved in development of AML. It was observed that high levels of cyclin A1 were found in AML cell lines and in AML samples from patients (175, 177). This observation suggests a possible role of cyclin A1 in AML and in the proliferation and differentiation of hematopoietic progenitors.

2. Materials & Methods

2.1 RNA extraction and hybridization

Total RNA was extracted from cells using TRIzol reagent (Invitrogen). 10 µg of total RNA were reverse transcribed into cDNA using an oligo-d (T)-T7-primer. Subsequently, T7 polymerase was used for *in vitro* transcription. During transcription, cRNA was labelled using biotinylated oligonucleotides. The resulting labelled cRNAs were fragmented and hybridized to U95A oligonucleotide microarrays, containing probes for approximately 12000 independent transcripts (Affymetrix, Santa Clara, CA). Arrays were scanned following staining with streptavidin-phycoerythrin, signal amplification with biotinylated anti-streptavidin antibodies and subsequent staining with streptavidin-phycoerythrin. Raw data were normalized and scaled to an average level of 2500.

2.2 Analyses of microarray data and bio informatics

Bioinformatical analyses were performed using Gene spring analysis software (Gene spring). A two-class procedure of the Significance Analysis of Microarrays software (Stanford) was used to identify differentially regulated genes (166). The delta value was set at 0.59. SPSS 10.0 was used to evaluate the statistical significance of gene expression differences.

For a gene to be classified as repressed, its expression had to be lower than 50% in each of the three different serum concentration samples as compared to U937-wildtype control and cyclin A1 transfected cells. Finally, gene candidates were excluded which were repressed more than 3-fold by U937 control cell *vs* cyclin A1 and with 1%, 5% and 10% FCS concentration. For a gene to be called induced, its expression had to be increased at least 3-fold in *each* sample compared to U937-

control vector vs overexpressing cyclin A1 with different FCS concentration samples had to be above 150 units (Affymetrix average intensity).

2.3 Quantitative real-time RT-PCR

Total RNA was isolated using TRIzol (Invitrogen) according to the manufacturers recommendations from U937 cell line. For RT-PCR, RNA was reverse transcribed using M-MLV (Moloney murine leukaemia virus) reverse transcriptase (Promega).
First, 1?g of RNA was incubated with 1?l RNAse out, in DEPC-H_2O (RNAse free water) at a total volume of 17,5?l for 5 minutes at 70°C. RNA samples were incubated for 5 minutes on ice and again for 10 minutes at room temperature. 1?l of M-MLV was added with 5?l of 5x buffer, 1,25?l of dNTP's and incubated for 1 hour at 42°C. After reverse transcription MMLV was inactivated by 70°C for 15 minutes. The cDNA samples were diluted to 100?l and 2.5?l of cDNA were used for each PCR reaction. PCR-amplification of the housekeeping gene glyceraldehydes-3-phosphate-dehydrogenase (GAPDH) was used to confirm the quality of cDNA and standardize the total amount of cDNA between different samples. The quantification of mRNA expression levels was carried out using a real-time fluorescence detection method based on TaqMan Instrument (PE Bio systems) (106).

The PCR reactions were prepared with 2x master mix (Applied biosystems) 12.5?l, forward and reverse primer (10?m) 0.125?l each, probe (5?m) 0.5?l and ddH_2O 6.75?l. Cycling conditions were 50°C for 5min, 95°C for 2min, 95°C for 2min, and 60°C for 2min. The steps 3 to 4 were repeated 40 times. The WT1 and EGR1 probes were labelled at the 5' end with the fluorescent dye FAM and the housekeeping gene (GAPDH) with VIC and at the 3' end with quencher TAMRA. The 5' nuclease activity of the Taq polymerase cleaved the probe and released the fluorescent dyes (FAM or VIC), which was excited by a laser, and the emitted flouresence was detected by a CCD camera. After the detection threshold was reached, the fluorescence signal was proportional to the amount of PCR product generated. Initial template concentration was calculated from the cycle number when the amount of PCR product passed a threshold set in the exponential phase of the PCR reaction. Relative gene expression levels were calculated using standard curves generated by serial dilutions of U937 cDNA. The relative amounts of gene expression were calculated by using the expression of GAPDH as an internal standard. At least three independent analyses

were performed for each sample and each gene. Primers and probes are listed in the 2.9 heading.

2.4 Cell Lines and Transfection

MEF (murine embryonic fibroblasts), Hela (human cervical carcinoma cells), Hutu 80 (human colon carcinoma cells), NIH3T3 (murine fibroblasts), KCL22 (human chronic myeloid leukemia), SW837 (adenocarcinoma cell line), NB4 (human acute promyelocyctic leukemia), K562 (human chronic myeloid leukemia in blast crisis), ML1 (human acute myelomonocytic leukemia), Cos- 7 (monkey kidney cells). In this study, two stable myeloid leukamia cell lines derived from U937 cells (human myeloid leukemia) cells were used (gift from Dr. Carmela Beger MHH Hannover). U937 cell were transfected with empty retroviral vector pLXSN as a control cell line or with pLXSN cyclin A1 mRNA(U937 overexpressing cyclin A1).

Suspension cell lines were grown in RPMI 1640 medium (Invitrogen), and adherent lines were maintained in Dulbecco's modified Eagle medium (DMEM, Invitrogen), each supplemented with 2 mM L-glutamine and 10% fetal calf serum (FBS, Biochrom KG), 100units/ml pencillin and 100?g/ml streptomycin (Biochrom KG), 2mM L-glutamine (Biochrom KG).

32D myeloid progenitor cells were cultured in RPMI-1640+ 20mM HEPES (Invitrogen) supplemented with 10% fetal bovine serum (FBS, Biochrom KG), 100-units/ml pencillin and 100?g/ml streptomycin (Biochrom KG), and 2mM L-glutamine (Biochrom KG). All mammalian cells were grown at 37°C and 5% CO_2. 32D myeloid progenitor cells were transfected following the Amaxa Kit (biosystems) protocol. A total of 5?g of plasmid DNA with 100?l of solution R per transfection was used. The cells were electroporated by Amaxa program T24, which was optimized for 32D cells.

2.5 Antibodies & Western blotting

Cells were lysed in Radioimmunoprecipitation buffer (RIPA) (150mM NaCl, 1.0% NP-40, 0.5% deoxycholic acid (DOC), 0.1% SDS, 50mM Tris–Cl (pH8.0) with complete EDTA-free protease inhibitor). The proteins were run on SDS-PAGE gradient gels (7-14% Bio-Rad). Proteins sample were electroblotted onto PVDF membranes

Immobilon-P (Millipore), probed with specific primary antibodies EGR-1 (588) and WT1 (F-6) from Santa Cruz Biotechnology, ?-Actin (Sigma) followed by IgG – conjugated peroxidase a coupled secondary anti-body against rabbit and mouse IgG (Jackson ImmunoResearch). Detection was performed with ECL plus (Amersham Biosciences) and exposure to a radiographic film.

2.6 Colony Assay

U937 control and overexpressing cyclin A1 or 32D cells were electroporated with 10ug of each pcDNA3, pCMV-EGR1 (pCMV EGR1 is a gift from Milbrandt Lab), pcDNA3-cyclin A1, pCMV-WT1+/-, pCMV-WT1-/+, pCMV-WT1-/- and pCMV-WT1+/+ expression vectors (WT1 isoforms were a gift from Dr Jerry Peteiller's Lab). After 16 hours cells were separated by gradient centrifuge and 1x 10^5 lived cells per 35mm dish were cultured with methylcellulose mix. The methylcellulose mix contained "Iscove modified Dulbecco medium" with 1% methylcellulose, 20% FCS, IL3 (1ng/ml) and in the presence of blastidin or neomycin (0.5-0.6mg/ml) as a selection marker, grown in triplicate in methylcellulose colony assays. Colonies were counted after 14 days of growth.

2.7 Flow cytometry

32D cells were transfected with GFP-Ras, pcDNA3, pcDNA3-cyclin A1, pCMV-EGR1, pCMV-WT1 +/-, pCMV-WT1-/+ expression vectors with Amaxa Kit (biosystems) protocol (described previously) and incubated for 24hours. For FACS-analysis, cells were washed with PBS and 0.1% BSA, fixed by resuspension in ice-cold 70% ethanol and incubated on ice for 1 hr. After extensive washing, cells were resuspended in PBS containing RNase A and propidium iodide and incubated at room temperature for 30 minutes up to 1 hour. FACS analyses were performed using FL-1 *vs* FL-2 channel (GFP *vs* PI) in order to get the cell cycle profiles of GFP positive (transfected) cells. Cell cycle distribution was calculated using the ModFitL T V2.0 (PMac) software.

2.8 Isoform Analysis

A forward primer labelled with fluorescent dye phosphoramidites FAM 5'CAG-ATG-AAC-TTA-GGA-GCC-ACC-TTA-A-3' and the unlabeled reverse primer 5'-TTG-GCC-ACC-GAC-AGC-TG-3' were used in the PCR. The cDNA from U937 control and U937 cyclin A1 cell line were prepared with MMLV reverse transcriptase (Promega) following manufacturers protocol (described previously). Each PCR mixture contained 50-100ng of cDNA, 2.5units of taq polymerase, 1?m of each primer, 10um each dNTP, 1.5mM $MgCl_2$, and PCR buffer at a 1X final concentration. (Applied Biosystem, CA, USA). Thirty cycles of PCR were performed in a DNA-thermal cycler (Master cycler personal, eppendorff). After initial denaturation at 95°C for 4 min, each cycle consisted of denaturing at 95°C for 4 min, 94°C for 20 seconds, annealing at 62°C for 20 seconds, and extension at 72°C for 30 seconds and cycle was repeated for 30-35 cycles. One microliter of PCR product was diluted in 20?l formamide containing 1IU internal size standard (Gene Scan ROX 1000, Applied Biosystems). Samples were denatured for 2 min at 90°C and run on a 3700 ABI Prism Genetic Analyzer (Applied Biosystem). The data were visualized on a histogram and analyzed by using ABI Gene Scan Software (Applied Biosystems).

2.9 Primers & Probes

<u>GAPDH Primers and Probes</u>

Human GAPDH-R

5'-GAA-GAT-GGT-GAT-GGG-ATT-TC-3'

Human GAPDH-F

5'-GAA-GGT-GAA-GGT-CGG-AGT-3'

Human GAPDH Probe

VIC -5'-CAA-GCT-TCC-CGT-TCT-CAG-CC-TAMRA-3'

<u>EGR1 Primers and Probes</u>

Human EGR1 Probe

FAM 5'-CCC-TAC-CAG-CAC-CTG-ACC-GCA-GA-TAMRA-3'

Human EGR1-F

5’-CGG-ACA-CGG-GCG-AGC-3’

Human EGR1-R

5’-TGT-TCA-GAG-AGA-TGT-CAG-GAA-AAG-A-3’

Mouse EGR1 Probe

FAM 5’ATC-CCA-GCC-AAA-CGA-CTC-GGT-TGC-TAMRA-3’

Mouse EGR1-F

5’-GGC-GAT-GGT-GGA-GAC-CAG-T-3’

Mouse EGR1-R

5’-CGG-CCA-GTA-TAG-GTG-ATG-GG-3’

WT1 Primers and Probes

Human WT1 Probe

FAM-5-’CCT-ACC-CAG-CCT-CGA-TTT-TTT-CAT-ATT-GCA-A-TAMRA-3’

Human WT1-F

5’- GAG-AAA-TCC-TCA-CTG-CCC-TGC-3’

Human WT1-R

5’-CGG-CCT-CGA-AGG-TGC-AT-3’

Mouse WT1 Probe

FAM-5’CGC-CCT-ACA-GCA-GTG-ACA-ATT-TAT-ACC-AAA-TG-TAMRA-3’

Mouse WT1-F

5’-CCA-GGC-CCT-GCT-CCT-GA-3’

Mouse WT1-R

5’-GTC-ATG-CAT-TCA-AGC-TGG-GA-3’

3. Results

3.1 Identification of targets gene by Microarray analysis

High-density oligonucleotide microarrays were used to identify genes that were transcriptionally regulated by cyclin A1 in U937 cells. As an additional control, U937-PML-RAR? expressing cells were used. These cells have been shown to increase cyclin A1 expression upon Zinc-induction of the fusion protein. Since cyclin overexpression might relieve serum dependence, which was usually necessary for S-phase entry, the microarray analyses were performed in the presence of 1%, 5% or 10% of fetal calf serum. Total RNA was extracted from the cell lines and hybridized to Affymetrix U95A arrays. These arrays contain a total of ~12.500 independent genes with 40 oligonucleotides ascribed to each gene. Twenty of these oligonucleotides contain point mutations and serve as controls for specificity. Following hybridization, arrays were laser scanned. Primary bioinformatics analyses were carried out using the Affymetrix Microarray Suite 4.0 software. Data were tested for reliability and were normalized with regard to signal intensity.

Finally, all the data were scaled to an average of 2500 Affymetrix units. These data were imported into the Excel-Software (Microsoft) and further analyzed using the Statistical Analysis of Microarray (SAM) –Software (Stanford). This software package calculates the likelihood of differences in expression based on the variability of each gene´s expression variability compared to expression variability of the entire dataset. A delta value of =2.50 or of ≠2.50 was regarded as significant. Overall, 726 genes were significantly regulated as indicated by these analyses. To increase the likelihood that only the most significant genes were identified and to exclude false positives, the number of genes was further reduced by the following criteria: To be regarded as induced, the overall induction in U937-cyclin A1 cells had to be greater than 3-fold and in each of the pairs at 1%, 5% and 10% expression of U937-cyclin A1 had to be greater than for U937-control cells.

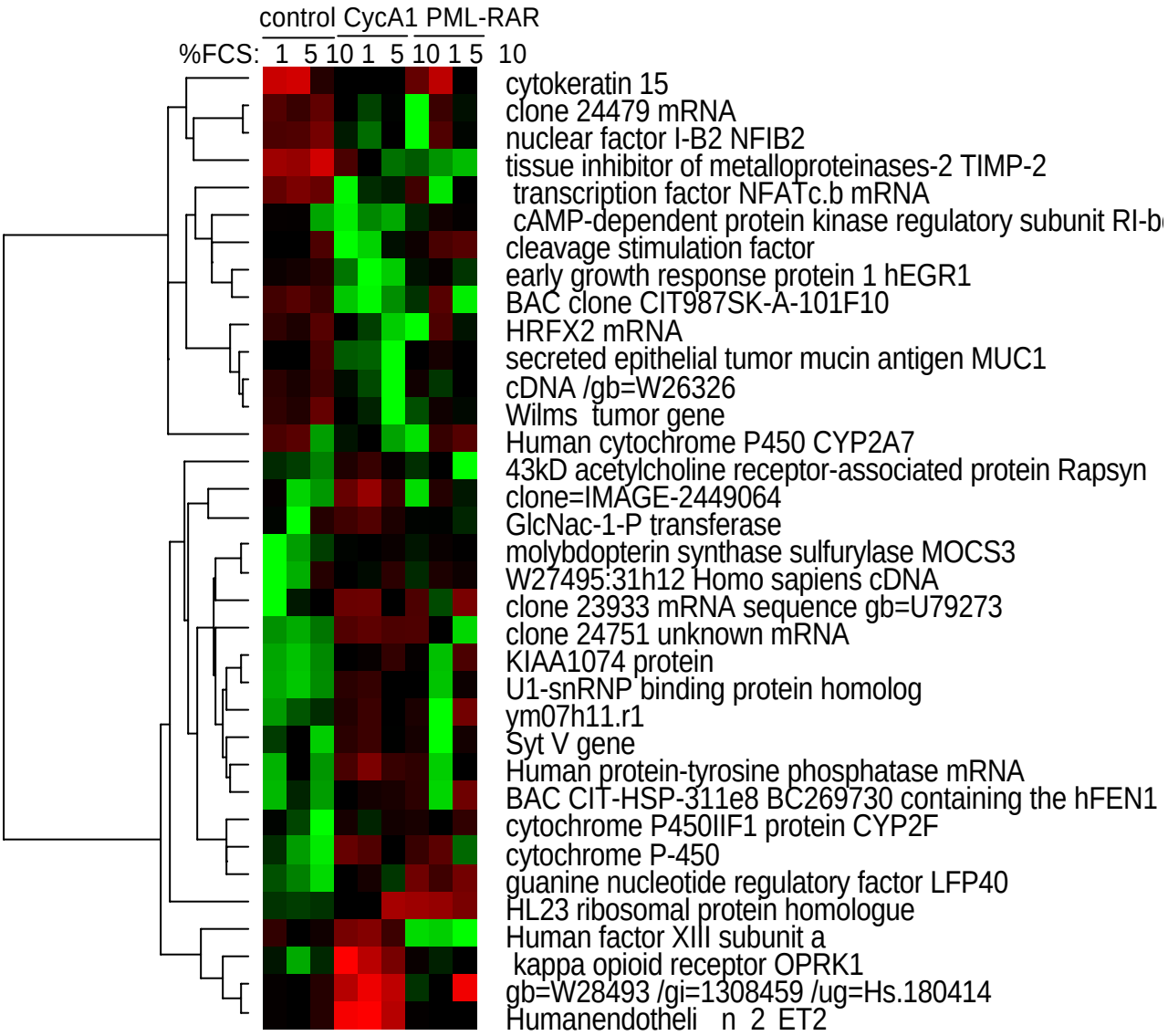

Fig. 3: <u>Hierarchical cluster analysis of cyclin A1 regulated genes.</u>

Oligonucleotide microarray analyses were performed using U95A arrays that contain about 12,500 independent genes. Genes that were differentially expressed between U937-vector control and U937-Cyclin A1 cells were identified using SAM-Analysis and selection criteria based on the strength of induction. For details see text. Hierarchical cluster analysis was performed and visualized using the Cluster and Tree software (Stanford).

For a gene to be regarded as decreased, overall repression of the gene in U937-cyclin A1 cells had to be greater than 3-fold (induction below 0.33) and in each of the pairs at 1%, 5% and 10% expression of U937-cyclin A1 had to be lower compared to U937-control cells. These relatively strict criteria might limit the number of false positives. Using these criteria, 35 genes were regarded as significant with 21 being

induced and 14 being repressed upon cyclin A1 induction. The data were hierarchically clustered and the diagram is presented in Fig. 1

Also, It was analyzed which genes were regulated by cyclin A1 and PML-RAR?, since these genes might be PML-RAR? regulated *via* cyclin A1 (Table 4).

Gene	**Cyclin A1**		**PML-RAR?**	
	SAM	**Induction**	**SAM**	**Induction**
Cluster Incl. W27495: 31h12 Homo sapiens cDNA	4.85	3.71	5.48	3.42
cytochrome P-450	2.74	7.57	3.21	9.88
molybdopterin synthase sulfurylase (MOCS3)	3.25	3.89	2.88	4.30
HL23 ribosomal protein homologue	20.84	3.25	2.53	5.11
Tissue inhibitor of metalloproteinases-2 (TIMP-2) /len=1069	-3.84	.08	-3.46	.02

Table 4: Genes that are simultaneously regulated by Cyclin A1 and PML-RAR? .

Genes identified to be regulated by cyclin A1 were analyzed for being regulated by PML-RAR?. Only genes that fulfilled the criteria to be regulated in the SAM (Significance Analysis of Microarrays) analysis (>=+2.5 or <=-2,5) and to be induced or repressed more than 3-fold were regarded as significant. Four genes were induced by PML-RAR? and one gene (TIMP-2) was repressed by PML-RAR?

In the current study, two genes were identified, which were repressed, and also were known to be involved in growth regulation and tumor pathogenesis, the Wilms' tumor gene and EGR1. The Wilms tumor gene was a known tumor suppressor that was also overexpressed in many leukemias. The Early growth reponse gene (Egr-1) was another growth suppressive gene. Both proteins function as transcription factors and the proteins bind to the similiar DNA binding sites. Finally, both are connected to the functions of the p53 tumor suppressor protein. For these reasons, WT-1 and Egr-1 were selected to be further investigated in this study.

3.2 Cyclin A1 regulates WT1 and EGR1 at mRNA level

In microarray analysis, it was found that U937 cells overexpressing cyclin A1 expressed lower levels of EGR1 and WT1. In order to verify the microarray results,

U937 cyclin A1 overexpressing and control cells were used to confirm EGR1 and WT1 mRNA downregulation by quantitative real-time RT-PCR.

Therefore, quantitative RT-PCR was performed with U937 control and cyclin A1 overexpressing cells cultured in the presence of 10% FCS. The expression of WT1 in cyclin A1 overexpressing U937 cells was reduced by 70% as compared to expression in U937 control cells (Fig. 4A). EGR1 expression was reduced by 15% in the cyclin A1 overexpressing cells relative to the control cells (Fig. 4B). Therefore, the expression of WT1 and EGR1 was downregulated, and confirmed that cyclin A1 was involved in the suppression of these two genes at the mRNA level.

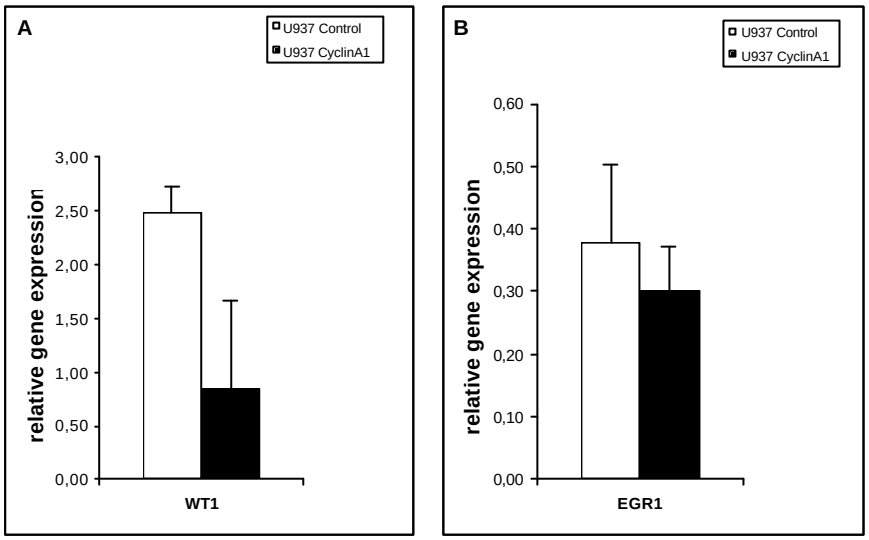

Figure 4: WT1(A) and EGR1(B) are downregulated in U937 overexpressing cyclin A1 cells as compared to U937 control cells. Total RNA of U937 control and overexpressing cyclin A1 cells was used in quantative RT-PCR analysis to determine WT1 and EGR1 expression relative to their GAPDH expression.

Since the microarray expression results were confirmed by quantitative RT-PCR, we checked if the expression of WT1 and EGR1 was upregulated in the absence of cyclin A1 in murine embryonic fibroblasts (MEFs). It was observed previously that UV irradiation induced cyclin A1 expression *via* a p53-dependent mechanism (Müller-Tidow et al, unpublished data). It was also reported previously that ionizing irradiation induces EGR1 expression (19, 52), (18). Therefore, it was interesting to examine the expression of WT1 and EGR1 after UV irradiation. MEFs were treated with UV

irradiation and collected at different time points to monitor the alteration of these two genes in the presence and absence of cyclin A1.

Cyclin A1$^{-/-}$ MEFs showed a 2-fold increase in the EGR1 level at 24hrs after UV irradiation, but the increase in EGR1 expression decreased over time (Fig. 5B). However, there was decreased WT1 expression after UV irradiation in cyclin A$^{-/-}$ during the time course (Fig. 5A). Cyclin A1$^{+/+}$ MEFs showed an increase in WT1 expression at 24hrs, which decreased over time.

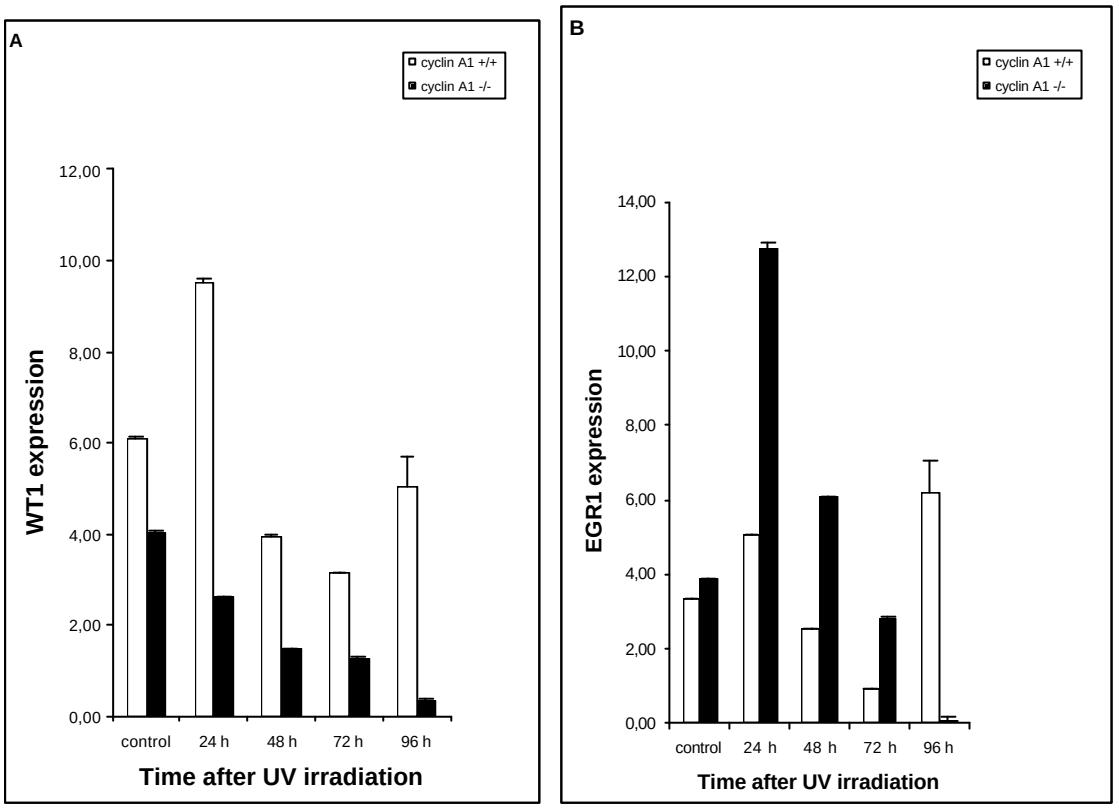

Figure 5: <u>WT1(A) and EGR1 (B) expression in cyclin A1 $^{+/+}$ and cyclin A1 $^{-/-}$ murine embryonic fibroblast cells upon UV irradiation.</u> Total RNA of cyclin A1 $^{+/+}$ and cyclin A1 $^{-/-}$ MEFs was prepared at different time points after UV irradiation and then used in quantitative RT- PCR analysis or WT1 and EGR1 expression.

These data showed that cyclin A1 was possibly involved in the regulation of WT1 and EGR1. The U937 cell model hinted to a putative function of cyclin A1 in the regulation of WT1 and EGR1 expression, but in MEFs, the relation between WT1 and cyclin A1 was not clarified. However, EGR1 expression was induced after 24 hrs UV irradiation and cyclin A1 $^{-/-}$ cells showed a clear increase in EGR1 expression.

Since WT1 and EGR1 expression was so far determined only in cultured cell lines, it was interesting to examine the expression of WT1 and EGR1 in different tissues of cyclin A1$^{+/+}$ and cyclin A1 $^{-/-}$ mice.

Interestingly, bone marrow of cyclin A1 $^{-/-}$ mice expressed higher levels of WT1 (Fig. 6A) and EGR1 (Fig. 6B). Moreover, in testis, liver, and ovary the expression of WT1 and EGR1 was also increased in absence of cyclin A1 (Fig. 6C and 6D). However, in lung there were decreased levels of WT1 and EGR1 in cyclin A1 $^{-/-}$, while EGR1 expression in brain and spleen was also lower in cyclin A1 $^{-/-}$.

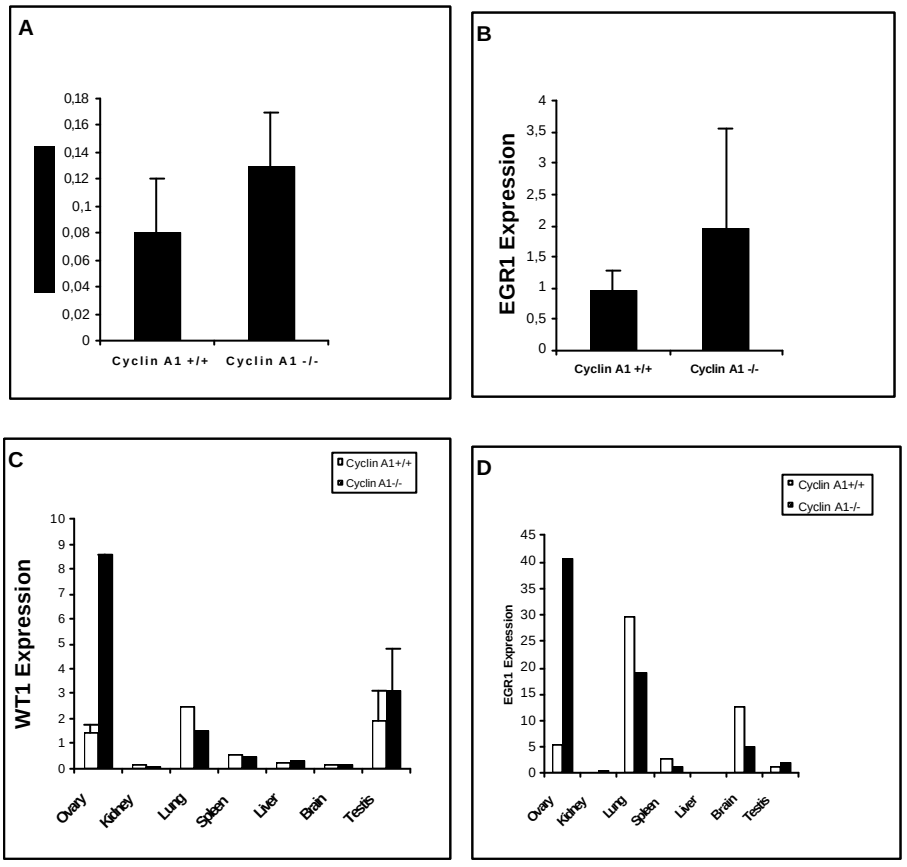

Figure 6: mRNA expression levels of WT1(A+C) and EGR1 (B+D) in bone marrow (A+B) and different organs (C+D) of cyclin A1 $^{+/+}$ and cyclin A1 $^{-/-}$ mice.

Expression levels of the WT1 and EGR1 were determined by quantitative RT-PCR.

The expression levels of WT1 and EGR1 genes were standardized using expression of GAPDH as a standard. Expression levels were determined in triplicates and were shown as the mean +SE.

In summary, these experiment showed that cyclin A1 could lead to transcriptional suppression of WT1 and EGR1 under different conditions. This further substantiates the assumption that WT1 and EGR1 might be downstream targets of cyclin A1 and that cyclin A1 is involved in the regulation of tumor suppressor genes.

3.3 WT1 and EGR1 protein expression in different cell lines

At the mRNA level WT1 and EGR1 were found to be downregulated in U937 cells overexpressing cyclin A1. To investigate the effect of cyclin A1 at the protein level, we performed immunoblot analyses in U937 cell lines. Cyclin A1 protein was expressed at higher levels in U937 cells overexpressing cyclin A1 as compared to control cell line (Fig. 7 upper blot). WT1 and EGR1 expression was examined in the same lysates of U937 control and cyclin A1 overexpressing cells. While WT1 was downregulated and EGR1 was not (Fig. 7 middle blot and lower blot).

The two bands detected at 54-52 kDa and 42 kDa appeared to be isoform of WT1. It has been reported in other studies that WT1 imunoblots showed different isoforms in some cell lines (58, 157, 158).

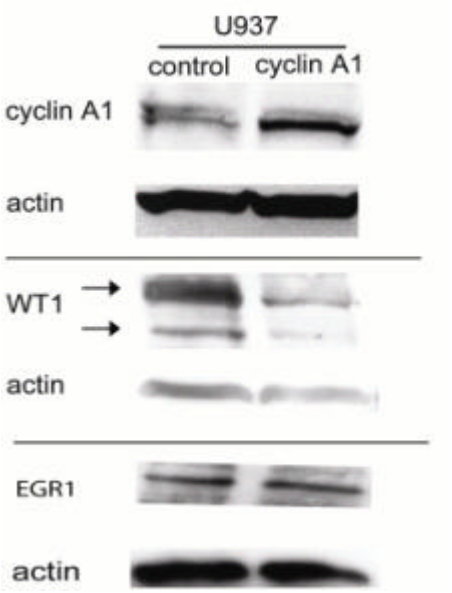

Figure 7: WT1, but not EGR1 was downregulated at the protein level in U937 cells overexpressing cyclin A1. The level of cyclin A1 protein was also shown in this immunblot for comparison. The blots were probed with antibodies for cyclin A1, WT1, and EGR1 respectively and subsequently for actin. The WT1 antibody detected

bands 54-52 kDa and 42 kDa, EGR1 antibody at 80 kDa and the actin antibody at 42 kDa.

No clear difference was observed for EGR1 at protein level in U937 cells overexpressing cyclin A1 (Fig. 7 lower blot). At mRNA level, the EGR1 showed only 15% difference in U937 cyclin A1 overexpressing cells.

Expression of WT1 and EGR1 protein was also examined in different leukemic and solid tumor cell lines (Fig. 8A & 8B). In NB4 and KCL22 cell line, the 42-kDa isoform of WT1 was clearly observed same as in U937 control and overexpressing cyclin A1. In the myeloid cell line ML1, the expression of WT1 was lower compared to Hela (human cervix carcinoma cells) and two lung cancer cell lines Hutu and SW837 (Fig. 8A). Expression of EGR1 was observed at low levels in KCL22 (human chronic myeloid leukaemia cells), NB4 (human acute promyelocytic cells), ML1 (human myeloid cells) and Cos-7 (monkey kidney cells) (Fig. 8B).

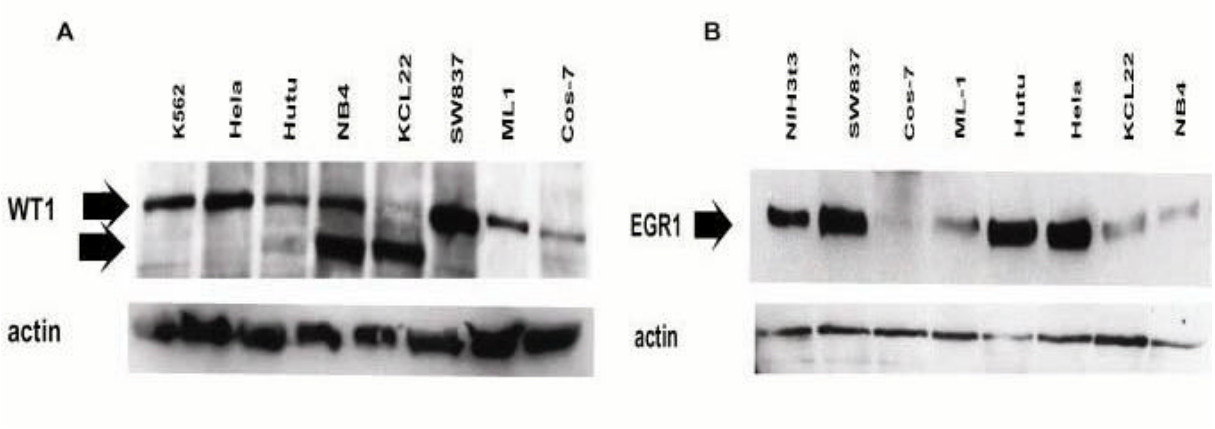

Figure 8: Expression of WT1 and EGR1 protein in different leukemic and solid tumor cell lines. K562 and NIH 3t3 cell lines were used as a positive control for WT1 and EGR1. The blots were probed with antibodies for WT1 or EGR1, and subsequently for actin.

WT1 and EGR1 expression was also determined in cyclin $A1^{+/+}$and cyclin $A1^{-/-}$ MEFs at protein level. EGR1 and WT1 protein expression was significantly higher in cyclin $A1^{-/-}$ MEFs (Fig. 9A and 9B). Interestingly, it was observed that at protein level there was an increase in WT1 expression, which was not detected at mRNA level (Fig. 5A).

The lane 1 and 3 lysates from EGR1 was used to detect for WT1 expression (Fig. 9B).

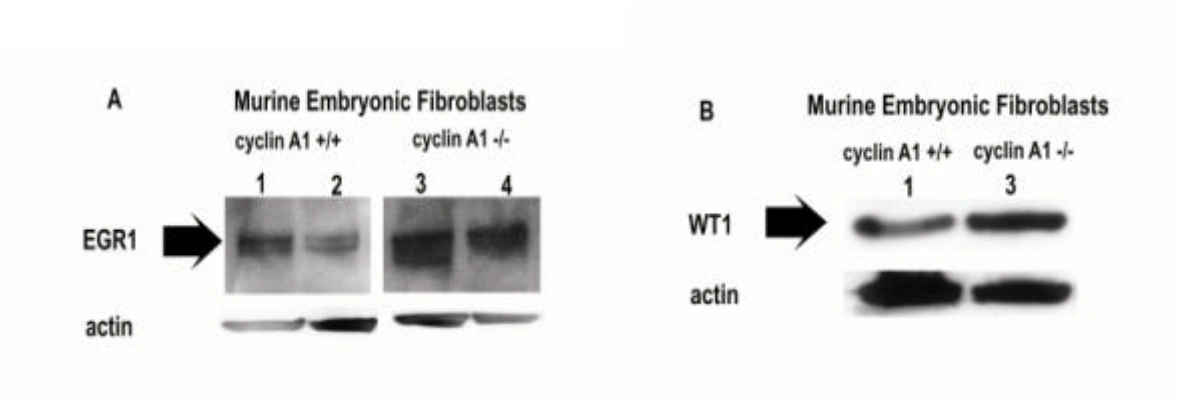

Figure 9: (A) Induction of EGR1 and (B) WT1 protein in cyclin$^{-/-}$ murine embryonic fibroblasts. Extracts was prepared from two different samples of murine embryonic fibroblasts of cyclin A1$^{+/+}$ and cyclin A1$^{-/-}$ in EGR1 immunoblot. The same lysate from lane 1 and 3 was used for WT1 immunoblot. The blots were probed with EGR1 (80kDa) or WT1 (54-52kDa), and subsequently for actin.

To summarise, it was observed that there was an increase in the protein expression of EGR1 in MEFs (Fig. 9A). Moreover, it was confirmed at mRNA level that EGR1 expression was high in MEFs without UV irradiation (see control Fig. 5B). When the MEFs were UV irradiated, the EGR1 expression was induced 8 fold (see Fig. 5B). No conclusive result was obtained concerning the WT1 expression at mRNA level, although an increase in WT1 expression was detected in MEFs at protein levels (Fig. 9B).

3.4 WT1 isoforms inhibit colony growth

Furthermore, it was interesting to see whether the WT1 and EGR1 showed any significant effects on colony growth in myeloid cell lines. Normal function of WT1 correlated with the expression of WT1 with or without a certain amino acid insertion.
The WT1 primary transcript has been described to undergo two alternatives splicing events, giving rise to four isoforms of mRNA (42). Alternate splicing of exon 5 removes 17 amino acids from the middle of the protein. The other alternate splicing (KTS) removes 3 amino acids between the third and fourth zinc of the finger domains

(Fig. 13). WT1+/-, WT1-/+ isoforms, and EGR1 respectively were transfected with and without cyclin A1 into 32D cells to investigate differences in colony formation.

The transfection of WT1-/+ isoform reduced the colony growth up to 75% (Fig. 10A). The co-transfection of WT1 isoforms with cyclin A1 did not change colony growth (Fig. 10A). EGR1 was transfected with or without cyclin A1 in 32D cell line and it did not lead to suppression in colony growth (Fig. 10B).

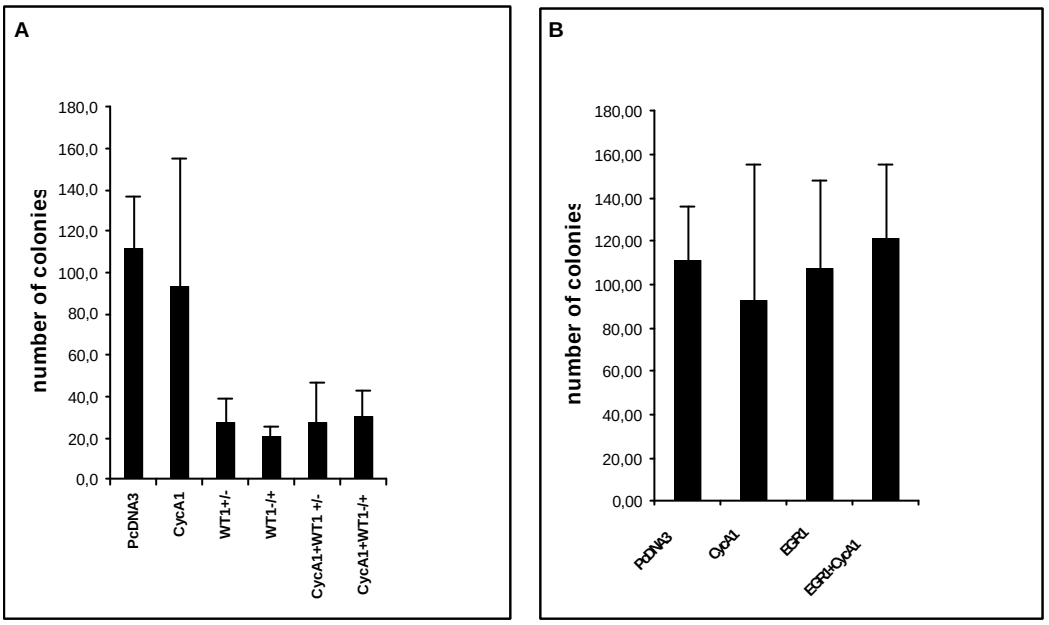

Figure 10: The effect of WT1 isoforms and EGR1 with and without cyclin A1 on colony growth. 32D cells were transfected with pcDNA3, cyclin A1, WT1-/+, WT1 +/- (Fig. 10A) and EGR1 (Fig. 10B). The following day, cells were seeded in triplicate in methylcellulose colony assays in the presence of neomycin as selection marker. Colonies were counted after 14 days. The result was observed in at least three independent experiments and is shown as the mean +SE.

In this study, the U937 cells overexpressing cyclin A1 and control were used as a model system. U937 cells overexpressing cyclin A1 and control were used in the microarray analyze to detect the genes, which were regulated by cyclin A1.

Further, it was interesting to analyze the endogenous effect of cyclin A1 on colony growth. Two additional isoforms of WT1 -/- and WT1 +/+ were included in the next experiment to determine their effect on colony growth.

No significant difference was found in colony formation of U937 overexpressing cyclin A1 and control cells with WT1 +/-, WT1 +/+, WT1 -/- isoforms, but the WT1-/+ isoform

induced 50% reduction of colony growth in U937 cells overexpressing cyclin A1. It was also observed that there was a general reduction in colony formation compared to control vector (Fig. 11).

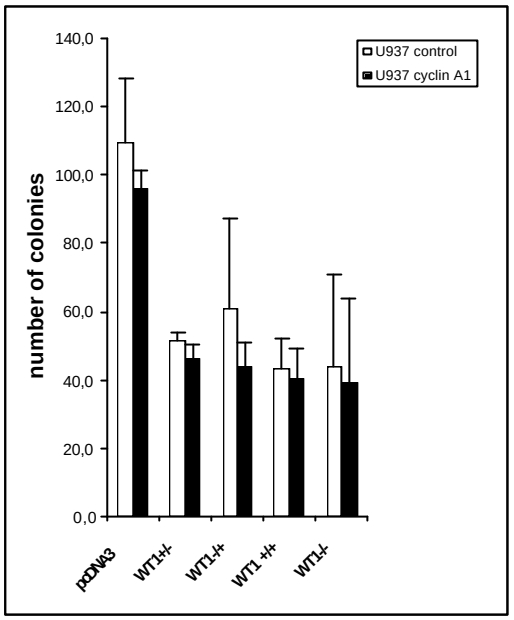

Figure 11: <u>Colony assay in U937 control and overexpressing cyclin A1 with different WT1 isoforms.</u> U937 cells were transfected with pcDNA3, WT1-/+, WT1 +/- WT1+/+ and WT1-/-. The following day, cells were seeded in triplicate in methylcellulose colony assays in the presence of neomycin as a selection marker. Colonies were counted after 14 days. The result was observed in at least three independent experiments and is shown as the mean +SE.

To summarize, these data showed that WT1 isoforms reduced colony growth formation as compared to the control vector. The addition of cyclin A1 did not further affect colony formation.

3.5 WT1 isoform induces G1 arrest

Cyclin A1 was assumed to be involved in the G1/S transition in cell cycle progression. Therefore, it was tested if expression of WT1-/+, WT1+/-, and EGR1 either with or without cyclin A1 showed any change in cell cycle progression in 32 D cells.

For cell cycle analysis, WT1-/+, WT1+/-, EGR1, and cyclin A1 were transfected in 32D cells and stained after 24 hours with propidium iodide for cell cycle analysis by flow cytometry.

The expression of the WT1-/+ lead to a cell cycle block. This block occurred in G1 to S phase progression in 32D cells (Fig. 12A). WT1 induced G1 cell cycle arrest. When

cyclin A1 was co-transfected with the WT1 isoforms, cyclin A1 abrogated the WT1 growth arrest (Fig. 12A). Either with or without cyclin A1 co-transfection, EGR1 did not show any change in cell cycle progression (Fig. 12B).

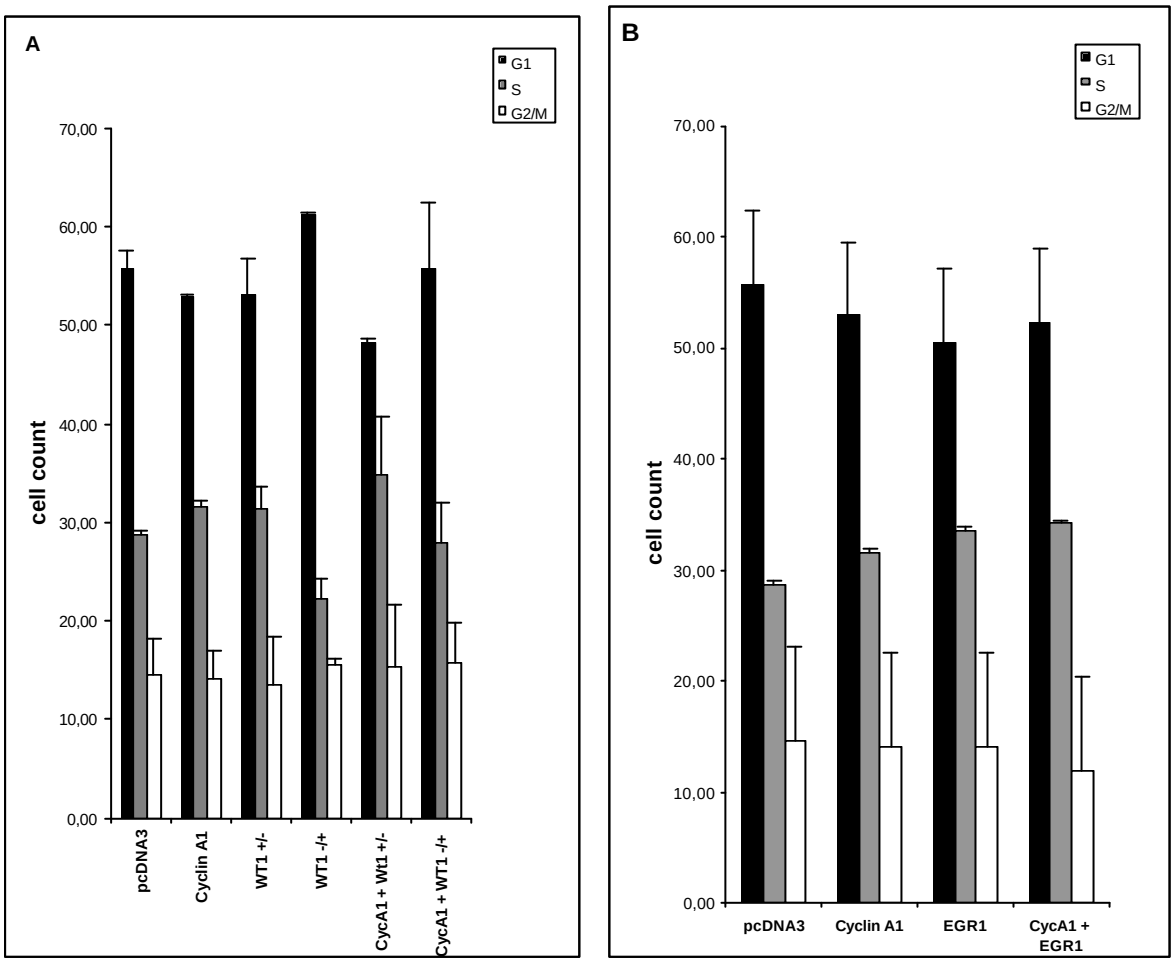

Figure 12: Effects of WT1(A) isoforms and EGR1 (B) on cell cycle regulation by FACS . The pcDNA3, cyclin A1, WT1+/-, WT1-/+, EGR1 were transfected with and without cyclin A1 in 32D cells. After 24 hours the cells were stained with propidium iodide, sorted by FACS, and cell cycle distribution was calculated using the ModFitL T V2.0 (PMac) software. The number of cells transfected in different phases was compared to the cells transfected with control vector. The result presented was the mean of three independent experiments.

The percentage of cells distributed in the different phases of cell cycle is shown in Table 5.

Transfected constructs	cell cycle distribution (%)		
	G1	S	G2/M
pcDNA3	55,68 (1,9)	28,73 (0,4)	14,59 (3,7)
Cyclin A1	52,92 (0,1)	31,53 (0,7)	14,06 (2,9)
EGR1	50,40 (3,7)	33,52 (0,7)	14,09 (5,8)
WT1 +/-	53,08 (0,1)	31,43 (2,2)	13,49 (4,9)
WT1 -/+	61,25 (0,4)	22,35 (1,9)	15,59 (0,6)
Cyclin A1& EGR1	52,31 (6,6)	34,22 (0,3)	11,98 (8,5)
Cyclin A1&WT1 +/-	48,30 (1,8)	34,79 (6,0)	15,41 (6,2)
Cyclin A1&WT1 -/+	55,77 (7,4)	27,89 (4,1)	15,84 (4,0)

Table 5: Cell cycle distribution of EGR1 and WT1 in 32D cells. pcDNA3, WT1 +/-, WT1-/+, EGR1 were transfected with or without cyclin A1 in 32D cells. Cell cycle analysis was performed using propidium iodide staining. Figures indicate the percentage of cells in the different phases of the cell cycle, and the ratio of cells in G1 to cells in S and G2/M phase. Numbers in parentheses represent the +SE.

To summarize, in cell cycle analysis, the WT1-/+ isoform induced G1 cell cycle arrest even though the difference between cells with or without control vector was only 7%. Furthermore, co-transfection of cyclin A1 was observed to result in the release of G1 arrest.

3.6 WT-/- isoform predominantly expressed in U937 control

The WT1 primary transcript has been described to undergo two alternatives splicing events, giving rise to four isoforms of mRNA (42). Alternate splicing of exon 5 removes 17 amino acids from the middle of the protein. The other alternate splicing event (KTS) removes 3 amino acids between third and fourth zinc of the finger domains (see Fig. 13). The four isoforms are produced in a constant ratio that is conserved throughout in different species suggesting that they have non-overlapping functions. It was also reported that there was an imbalance in WT1 isoforms, leading

to tumor development (4, 62). In order to further analyze the function of WT1 isoforms, the ratio of the WT1 isoforms in the U937 control and in U937 overexpressing cyclin A1 cells was examined.

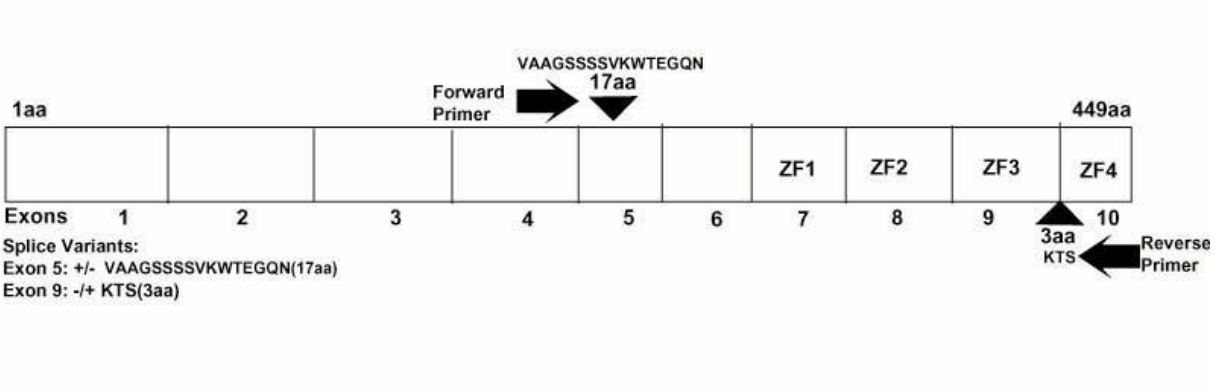

Figure 13:Scheme of the WT1 gene and the position of primers. The insertion and exclusion of the two splice inserts (17aa and KTS) generates the four isoforms (WT1-/-, WT1+/+, WT1+/- and WT1-/+). The forward primer (upstream of the17aa) was labelled with FAM (Flourescent dye phosphoramidites), the reverse primer (downstream of the KTS region) remained unlabelled.

The original histogram showed the different isoforms of WT1 (Fig. 14). The product size was calculated according to an internal standard (Rox 1000, Applied Biosystems), which was run with each sample. WT1 isoforms were calculated as WT1-/- (482bp), WT1-/+(489bp), WT1+/-(528) and WT1+/+(535bp).

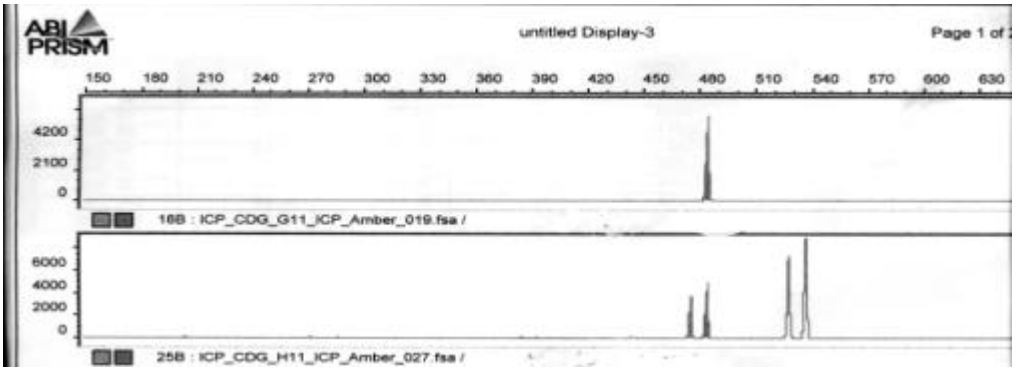

Figure 14: WT-/- isoform predominantly expressed in U937 control (upper histogram). The PCR product was run along with internal size standard (Gene Scan Rox 1000) on a 3700 ABI Prism Genetic Analyzer. The data were visualized in a histogram and analyzed using ABI Gene Scan Software (Applied Biosystems).

Interestingly, WT1-/- was found in 100% of U937 control cells, whereas the other isoforms were absent (upper histogram). In contrast, in U937 cells overexpressing cyclin A1 all isoforms were present, and measured at different ratios (lower

histogram). The WT1-/- isoform was present in 12 % and the WT1-/+ (24%), WT1+/- (26%), WT1+/+(36%) isoforms were expressed.

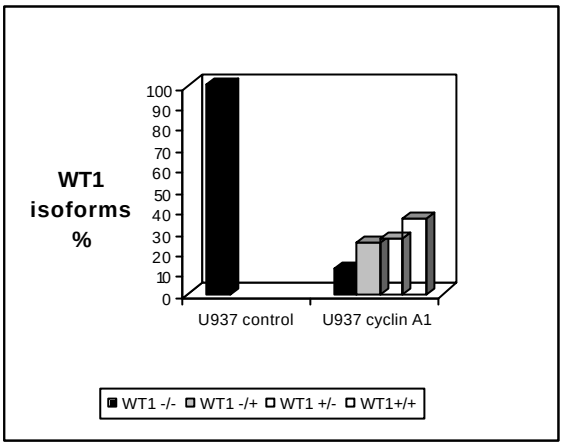

Figure 15: Distribution of WT1 isoforms in U937 control and cyclin A1 overexpressing cells. The 100 percent value represents the sum of all WT1 isoforms detected in one cell line.

In summary, these results show that the WT1 isoforms were present in different ratios in U937 control and overexpressing cyclin A1 cells. (26) (136).

Discussion

The deregulation of cell cycle is a main feature of all cancers (73). Many studies on cancer have focused on molecules that control cell cycle progression e.g. cyclins, cyclin dependent kinases (Cdks) and kinase inhibitors (CKIs). In many cancers, A-type cyclin genes are overexpressed, and protein concentrations and related kinase activities are often altered (9, 28, 107).

Cyclin A1 is highly expressed in the majority of myeloid and undifferentiated haematological malignancies as well as in normal hematopoietic progenitor cells.
It was observed that high levels of human cyclin A1 are present in acute myeloid leukemia cell lines (175). One potential explanation for cyclin A1 overexpression in AML leukemias could be that the leukemia cells that express high levels of cyclin A1 may be arrested at the stage of differentiation when cyclin A1 is normally expressed (177). However, cyclin A1 is highly expressed in leukemia and its overexpression contributes to enhanced leukemogenesis in mice (80).

In the current study, microarray analysis was used for the first time to identify the putative target genes, which are down- or upregulated by cyclin A1. Their functional relevance to AML was also analyzed.

Identification of relevant target genes in the microarray data set was performed using a two-step procedure. In a first step, 726 genes were identified as potential targets for cyclin A1. The high number of genes demanded further selection. In a second step, only those genes were selected that were 3 fold induced or repressed in all pairs of the 1%, 5% and 10% of U937-overexpressing cyclin A1 cells compared to control cells. These criteria limited the number of identified significant genes to 35.

Within this group of significant genes, there were 14 genes repressed and 21 induced in U937 cells overexpressing cyclin A1. They were involved in DNA repair system, growth regulation and also in differentiation e.g. Fen1, BRCA1, EGR1 and WT1 (48) (57, 90) (52).

In this study, two genes were selected which were downregulated by cyclin A1 and involved in acute myeloid leukemia (AML): EGR1 and WT1. The main interest was to identify and examine target genes of cyclin A1 and to analyze the common pathways of these genes with reference to cellular growth and differentiation

EGR1 (also known as NGFI-A, TIS8, Krox-24, and Zif268) was selected as the first gene. It is a member of the immediate early gene family (148) (97), (76, 82) (14).
The EGR1 gene encodes a nuclear protein, which contains three zinc fingers of the C_2H_2 subtype (38). Structure- function analysis of the EGR1 protein suggests that it contains an extended transcriptional activation domain on the N-terminus and a DNA-binding domain, consisting of three zinc finger motifs (164). EGR1 is involved in the regulation of cell growth and differentiation in response to signals such as mitogens, growth factors, and stress stimuli (52, 83). When EGR1 is induced, often displaces Sp1 or Sp3, other 3-zinc finger gene transcription factors from common or overlapping GC-rich binding sites, where Sp1 and Sp3 support basal expression of genes (17, 36). The GC-rich DNA-binding element for EGR1 is present in a large number of gene regulatory regions, including growth factors, signal transduction genes, transcription factors and oncogenes. Cyclin A and the thymidine kinase gene were shown to be regulated by EGR1 during the G0 to G1 transition (100, 103). However, EGR1 is induced very early in the apoptotic process and mediates the activation of downstream regulatory genes such as p53, and the tumor necrosis factor ? (1, 110, 112). EGR1 is also reported to interact with the transcription factor c-Jun and to modulate its activity (78). EGR1 is involved in p21Cip1 expression by directly interacting with a specific sequence on its gene promoter (131) and EGR1 is implicated as a major regulator of cell senescence (69).

EGR1 is also involved in the development, growth control, and differentiation of hematopoietic cells. Ectopic EGR1 expression in normal hematopoietic progenitors stimulated development along the macrophage lineage at the expense of development along granulocyte or erythroid lineages (67). Further, EGR1 target genes play an important role in development of progenitors from myeloid lineages toward macrophage differentiation (67, 81).

Several genes were identified which are involved in both cellular attachment to the extracellular matrix and in cell –cell interactions as potential targets for EGR1.The intracellular adhesion molecule 1 and CD44 were found to be a target for EGR1 in B cells (92) (34). The CD31 promoter was shown to contain Sp1 and EGR1 elements that conferred phorbol myristate acetate inducibility of a reporter gene to myeloid cells (2). EGR1 was not only involved in the development of myeloid progenitor along the macrophage lineage but also accelerated this process by suppressing the proliferative phase of the macrophage growth to differentiation developmental program (67). EGR1 was also involved in the growth inhibition and suppression of transformation of many cell types of both hematopoietic and nonhematopoietic origins (83). The ectopic expression of EGR1 impaired the leukemogenicity of M1 myeloblastic leukemia cells in vivo (68). Mice lacking the EGR1 gene were not impaired in macrophage differentiation (74). It was suggested that other members of the EGR1 family of transcription factors were capable of compensating for the function of EGR1 in macrophage developments.

This study showed that in U937 cells overexpressing cyclin A1, EGR1 expression was downregulated at the mRNA level. Possibly, EGR1 and cyclin A1 were regulating the cellular pathway through common genes, particularly those encoding multifunctional transcription factors such as c-jun, c-myc, c-myb and Sp1 which were involved in regulation of hematopoietic cell differentiation and proliferation (12, 36, 103, 104, 150). When cyclin A1 expression was low, EGR1 expression in leukemia cells was induced. Cyclin A1 was involved in regulation of multifunctional transcription factors, which also regulated EGR1. Bone marrow, testis, liver, and ovary showed high expression of EGR1 in cyclin $A1^{-/-}$ mice and further confirmed that cyclin A1 was involved in EGR1 regulation.

Many human tumor and leukemia cell lines express little or no EGR1 in contrast to their normal counterparts (51, 84). At protein level, it was found that EGR1 did not show any difference between U937 cyclin A1 overexpressing and control cells. It could be assumed that the EGR1 protein was stable and was not degraded by cyclin A1.

Different studies showed that certain types of cancer expressed high levels of EGR1, whereas others expressed low levels (77) (165). The current study reveals that EGR1 expression was low in myeloid and MEF cells but its expression was high in colon and cervical carcinoma cell line. It was suggested that expression of EGR1 was varied in different tumor cell lines.

In addition, UV irradiation caused an increase in expression of EGR1 in MEF cells thus confirming previous reports that EGR1 was involved in the DNA repair system (19, 130). EGR1 induced pro-apoptotic function after irradiation and was directly mediated by the target genes p53 and Rb (18). Cyclin A1 was involved in a DNA repair system, and irradiation induced apoptosis (Müller-Tidow et al, unpublished data) and mediated its action via a p53 dependent pathway. It could be speculated that enhanced levels of EGR1 protein may be needed to regulate genes involved in DNA repair, cell survival, and apoptosis.

In the 32D cell line, the constitutive expression of EGR1 did not show any difference in colony formation. It was reported in different studies that EGR1 is involved to promote cell growth in prostate cancer cells (169) and rat kidney tumor cells (144). However, in breast cancer, fibrosarcoma and glioblastoma, EGR1 functioned as a growth suppressor (12, 51). Previous studies did also show a clear role of EGR1 as a growth suppressor or enhancer (12, 51, 169). It was suggested that constitutive EGR1 expression was affected in cell growth differently in different cell lines, what might suggest a tissue specific role of EGR1.

It was also observed that ectopic expression of EGR1 in 32D myeloid cells did not show any change in cell cycle distribution. It was reported that EGR1 was involved in cell cycle regulation during G_0 to G_1 transition of certain genes e.g. cyclin A, thymidine kinase (100, 103, 174). In a human melanoma cell line (A375-C6), EGR1 impedes the Interleukin-1 inducible G_0-G_1 growth arrest (150). In this experiment, cells were used from mixed population and this could be a reason that EGR1 did not show any effect in cell cycle. It was also suggested that EGR1 was not directly involved in cell cycle regulation and this could be another reason why ectopic expression of EGR1 did not show any effect in cell cycle analysis.

The second gene examined in the current study was WT1, which was repressed by cyclin A1. Wilms tumor gene (WT1) is known to be a tumor suppressor gene, which encodes a member of the Cys2-His2 zinc finger family of transcription factors. The WT1 gene has been reported to be expressed in normal and malignant tissues as expressed in urogenital tissue, kidneys, gonads, uterus, brain, spleen and hematopoietic precursors (145). WT1 expression has also been described in malignant tissues e.g. Wilms' tumors, ovarian cancer, severe types of adult leukemia, many types of lung, thyroid, breast, testicular carcinoma and in melanoma. (3, 7, 11, 98, 99, 121, 129, 138)

The WT1 gene was first identified in 1990 by genetic analysis of the region of the chromosome band 11p13 in which constitutional heterozygous deletions correlate with hereditary predisposition to Wilms' tumor (11, 50). In mammals, exons 5 and 9 of WT1 are alternatively spliced, giving rise to four different splice isoforms (39). Splice variant 1 results in the insertion of 17 amino acids upstream of the zinc finger domain and enhances the transcriptional activity of protein (140). Splice variant II occurs within the zinc finger domain, inserting three amino acids (Lys-Thr-Ser; KTS) between third and fourth zinc fingers and changes the DNA binding specificity of the protein (RauscherIII, FJ 1990, Bicjmore, WA et al 1992). The four predominant WT1 isoforms generated *in vivo* are termed WT1 +/+ (both splices 1 and 2 are present), WT1+/-(splice 1 present only), WT1-/+(splice 2 present only), and WT1-/- (neither splice present). These four isoforms display molecular masses in the range of 52-54 kDa (102). In addition to above mentioned four isoforms of WT1, there are eight WT1 protein isoforms generated through translation initiation at an in-frame CUG codon upstream of the initiator AUG resulting in WT1 protein isoforms with molecular masses of 60-62 kDa (8). Internal translation initiation at an in-frame AUG127 codon downstream of the initiator AUG generates smaller WT1 isoforms with molecular masses of 36-38 kDa (142). WT1 isoforms with splice1 and 2 differences have distinct DNA-binding and transcriptional activities (23, 140, 171).

The WT1 zinc finger domain binds to the characteristic GC-rich EGR1 DNA-binding element, although with approximately 40-fold less affinity than EGR1 itself (24, 132). WT1 protein binds to the EGR1 DNA-binding element, which present in various

growth factor gene promoters such as the platelet-derived growth factor A chain (PDGF-A) promoter (170), p21, E-cadherin, insulin like growth factor II (IGF-II) promoter, and the murine colony-stimulating factor, 1(CSF-1) promoter ((23, 44). WT1 binding to the PDGF-A promoter region results in downregulation of this growth factor gene *in vivo.* Interestingly, WT1 can exert a dual transcriptional function. WT1 acts as a transcriptional repressor of the EGR1 gene in the presence of wild type p53, and it acts as a transcriptional activator in the absence of p53 (91). WT1 isoform with and without KTS play a role in sexual development by interacting with steroidogenic factors 1 (SF1) and Dax1 (111). In addition, WT1 binds to multiple sites of its own promoter region resulting in a feedback downregulation of the WT1 gene (140). It has been suggested by previous studies that WT1 is involved in cellular proliferation, differentiation, apoptosis and sex determination.

Normally, WT1 is expressed in CD34+ hematopoietic progenitor cells and disappears during differentiation (35, 98). WT1 is overexpressed in leukemic cells in the majority of human acute leukemias and in the blasts crisis of chronic myeloid leukemia. Most commonly, heterozygous WT1 mutations are found in approximately 15% of AML cases and are associated with poor response to chemotherapy (37, 119). WT1 is overexpressed in most adult and pediatric acute leukemias, CML and advanced MDS. High levels of WT1 expression have adverse prognostic significance in AML (both adult and pediatric) and pediatric ALL (139).

WT1 has been considered to be a prognostic marker in acute leukemia (5, 56). Immunization with WT1 peptides can raise a cytotoxic response to chemotherapy (37, 119). Induction of differentiation in several myeloid cell lines is associated with rapid decrease in WT1, and overexpression was found to interfere with the induced differentiation in these cells (149, 159). Thus, it is likely that WT1 has a role in hematopoietic differentiation.

In this study, WT1 was induced both at mRNA and protein levels when cyclin A1 was expressed at low level. It was suggested that cyclin A1 was involved indirectly in the phosphorylation of genes, which bind to WT1, followed by inhibition of WT1 expression. It was also reported that WT1 is involved in the transcriptional regulation of c-myc, c-myb, p21, p53 and RAR-? (32, 46, 93) (143), which alter the

transcriptional regulation properties of WT1. Functionally, cyclin A1 associates with the cell cycle regulatory proteins Rb and E2F1 *in vivo,* and (59) it also interacts with B-myb. Cyclin A1/cdk2 posphorylates B-myb at functionally important serine and threonine residues at the C-terminus (106). C-myb is also involved in the transactivation of the human cyclin A1 promoter.

Additionally, UV irradiation did not effect WT1 expression in MEF cells. Morever, it has been reported that treatment of HEK293 cells with UV light strongly enhanced transcriptional activation by the WT1 (+17aa) construct, correlating with an induction of endogenous Par4 expression (137). Other studies suggested that different WT1 isoforms have overlapping function. The experimental results of this study do not rule out this possibility.

Transfection of WT isoforms into myeloid cell lines inhibited colony formation in methylcellulose medium. WT1-/+ isoform was observed to have more effect in 32D and U937 overexpressing cyclin A1 cell lines. It was examined that transfection of different WT1 isoforms showed pronounced reduction in colony growth. Different studies showed that WT1 was involved in growth suppression and differentiation in hematopoietic cells. The different isoforms of WT1 behaved differently in growth suppression and differentiation of hematopoietic cells. It was reported that WT1 (-KTS) and WT1 (+KTS) affect differently growth suppression of primary hematopoietic cells (30, 160).

WT1 -/+ was shown to induce G1 arrest in 32D cells, which further confirmed that this isoform was involved in different functions of myeloid cells. Different reports mentioned that WT1 has relevance for the induction of growth arrest and apoptosis (31, 94, 108). Overexpression of WT1 blocked the G_1-S transition of NIH3t3 and induced apoptosis in F9 embryonal carcinoma cells and in an osteosarcoma cell line (31, 70, 71). The M1 murine myeloblastic cell line expressing no endogenous WT1 reacts very differently to ectopic expression. Constitutive expression of the WT1 (+KTS) form induces monocytic differentiation independent of external stimuli. Whereas M1 cells stably expressing the WT1 (-KTS) isoform cannot be established because they induce G1 arrest and/or apoptosis (108, 158). These findings also indicate that the effects of WT1 isoforms are different with respect to the various cell

backgrounds. In the current study, WT1 (+KTS) was involved in G1 arrest, which was contradictory to previous results. A possible explanation for this could be the specific background of cells. In a previous study, it was observed that overexpression of cyclin E/cdk2 or cyclin D1/cdk4 induced cell cycle progression into S phase, which is blocked by WT1 (70). In this study, the co-transfection of cyclin A1 was also observed to release the G1 arrest. It was suggested that cyclin A1 might interact with WT1 directly or indirectly during cell cycle.

The WT1 splice variants are conserved in all mammals and it seems that the ratio between different isoforms remains constant throughout development. The combined expression of all splice variants might be essential for normal functioning. The different isoforms were investigated using capillary electropherosis of fluorescent labelled PCR products. Only the WT1 -/- isoform was found in the U937 control sample compared to U937 overexpressing cyclin A1 sample. It was further observed that U937 overexpressing cyclin A1 cells showed all isoforms at different ratios. A mutation interfering with the ratio of WT1 (+KTS) and WT1 (-KTS) protein reportedly leads to the Frazier syndrome (65). Furthermore, the WT1+/+ (+17aa) and WT1-/+(-17aa) ratio is also important, and absence of WT1 (-17aa and –KTS) showed poor postnatal survival and glomerular abnormalities in mice (113, 114). The current study is in accordance with previous findings that the isoform ratio could be depending upon the type and stage of cells.

Taken together, several lines of evidence indicate interaction between cyclin A1 and the two newly identified target genes. Cyclin A1 was involved in regulating WT1 and EGR1 through multifunctional transcription factors and in interfering their pathways. The current study has set the first step to identify two cyclin A1 target genes by microarray analysis and examine their co-relation.

WT1 and EGR1 are involved in cellular growth and differentiation in the hematopoietic system, and their deregulation plays a role in AML. However, much remains to be learned about their role in the hematopoietic system and AML.
Apart from this, the exact role of cyclin A1 itself in the hematopoietic cell cycle and in the pathogenesis of acute myeloid leukemia demands further analysis.

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Amber Yasmeen

Contact Address

Potstiege 3
Münster 48161,
Germany.
Tel: +49-251-2035775
Mobil: 0179-4751000
E-mail: ambyas2002@yahoo.com

Permanent Address

E-91,Cantt Bazar Area,
Malir Cantt, Karachi-75070,
Pakistan

Personal Information

Gender	Female
Family name	Yasmeen
Name	Amber
Date of Birth	March 28, 1973
Nationality	Pakistani
Marital Status	Single

Education

1996-1998 **Master of Philosophy in Biotechnology**
NIBGE / Quaid-i-Azam University, Islamabad.
Thesis: Enhanced production of glucoamylase by cultures of *Aspergillus niger.*
Percentage: 79%

1994-1995 **Master Degree in Human Physiology**
University of Karachi, Karachi.
Specialization in Molecular Endocrinology
Percentage: 64%

1991-1994 **B.Sc. (Honors)**
University of Karachi, Karachi
Main subjects: Human Physiology, Chemistry, and Microbiology
Percentage: 66%

Work Experience

July 2001- to date Working as a researcher at the University Hospital Münster, Hematology/Oncology, Münster-48149, Germany.

Jan 2000-June 2001 Research Officer at the Pharma and Fine Chemical Division of The Pakistan Council for Scientific and Industrial Research (PCSIR) Lab Complex, Karachi, Pakistan.

Feb 1999- Nov 1999 Medical Research Officer at the Cirin Pharmaceuticals Ltd.

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Languages

Urdu (Mother Tongue)

	written	spoken	understanding
English (Language of Studies)	Fair	Good	Good
German	Fair	Fair	Good

Publications

1- Rajoka, M.I., Shahid, R. and Yasmin, A. (2000) Gamma ray-induced mutagenesis of *Aspergillus niger* for hyperproduction of glucoamylase in solid state fermentation. Ibid.
2- Amber Yasmeen, Wolfgang E. Berdel, Hubert Serve and Carsten Müller-Tidow. E-type and A-type cyclins as markers for cancer diagnosis and prognosis. Expert Rev. Mol. Diagn. 3 (5), 617-633, 2003.
3- Hanif,A., A. Yasmin, and *M.I. Rajoka* (2003). Production, repression and derepression of ß-cellobiosidase synthesis in *Aspergillus niger*.*Biores. Technol.* (Accepted).

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