Abstract. Cyclin A1 is a cell cycle protein that is expressed in testes, brain and CD34-positive hematopoietic progenitor cells. Cyclin A1 is overexpressed in a variety of myeloid leukemia cell lines and in myeloid leukemic blasts. Transgenic cyclin A1 overexpressing mice develop acute myeloid leukemia with low frequency. In this study, we looked for putative target genes of cyclin A1 in hematopoietic cells. Microarray analysis of U937 myeloid cells overexpressing cyclin A1 versus control cells detected 35 differential expressed genes, 21 induced and 14 repressed ones upon cyclin A1 overexpression. Among the differentially expressed genes WT1 was chosen for further analysis. Repression of WT1 expression was confirmed on the mRNA and protein level. In addition, WT1 expression was higher in bone marrow, liver and ovary of cyclin A1-/- mice. Isoform analysis showed a profound change of the WT1 isoform ratio in U937 cyclin A1-overexpressing versus control cells. Functional analysis revealed an inhibition of colony growth when WT1 isoforms were transfected into U937 cells, which was not affected by the overexpression of cyclin A1. In addition, overexpression of the WT1-/- isoform induced a G1 cell cycle arrest which was abrogated upon cotransfection with cyclin A1. This study identified WT1 as a repressed target of cyclin A1 and suggests that the suppression of WT1 in cyclin A1-overexpressing leukemias might play a role in the growth and suppression of apoptosis in these leukemic cells.

Introduction

Cyclin A1 is a cell cycle protein whose high level expression in human normal tissues is restricted to testes and brain (1,2). Like other cyclins, cyclin A1 shows an oscillating expression during the cell cycle and interacts with cyclin-dependent kinases (CDK’s). In case of cyclin A1, it shows a predominant expression in the S and G2 phase of the cell cycle and interacts with CDK1 and 2 (1,2). As a result, downstream targets of the cyclin A1/CDK complexes are activated, including the retinoblastoma (Rb) protein, E2F transcription factors (3) and b-myb (4), resulting in cell cycle progression through the S to G2 checkpoint. Male cyclin A1 -/- mice display a defect in spermatogenesis before the first meiotic cell division (5), suggesting a role for cyclin A1 in spermatogenesis. Apart from this defect, both male and female cyclin A1 -/- mice develop normally.

Additional to testis and brain, cyclin A1 expression can be detected in CD34-positive hematopoietic progenitor cells, and cyclin A1 is overexpressed in a variety of myeloid leukemia cell lines and acute myeloid leukemic blasts of patients (1,6). In addition, transgenic mice overexpressing cyclin A1 display abnormal myelopoiesis and develop acute myeloid leukemia at a low frequency (7). These results suggest a possible role of the overexpression of cyclin A1 in acute myeloid leukemia, but most of the putative target genes of cyclin A1 are still unknown. Herein, we analyzed miRNA expression profiles of leukemic cells overexpressing cyclin A1.

Materials and methods

RNA extraction and hybridization. Total RNA was extracted from cells using TRIzol reagent (Invitrogen). Total RNA (10 μg) was reverse transcribed into cDNA using an oligo-dT-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 ~12000 independent transcripts (Affymetrix, Santa Clara, CA).
Arrays were scanned following staining with streptavidin-phycocerythrin, signal amplification with biotinylated anti-streptavidin antibodies and subsequent staining with streptavidin-phycocerythrin. Raw data were normalized and scaled to an average level of 2500.

**Analyses of microarray data and bioinformatics.** 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 (8). The delta value was set at 0.59, SPSS (SPSS Inc.) was used to evaluate the statistical significance of gene expression differences.

A gene was classified induced or repressed only if its expression level was increased at least 3-fold or decreased at least 2-fold respectively, in each sample in U937 cells overexpressing cyclin A1 compared to U937-control vector transfected cells with all 3 different FCS concentration samples. In addition, its absolute expression value had to be >150 units (Affymetrix average intensity).

**Quantitative real-time RT-PCR.** Total RNA was isolated using TRIzol (Invitrogen) according to the manufacturer’s recommendations from U937 cell line. For RT-PCR, RNA was reverse transcribed using M-MLV (Moloney murine leukemia virus) reverse transcriptase (Promega).

RNA (1 μg) was incubated with 1 μl RNase out, in DEPC-H2O (RNase free water) at a total volume of 17.5 μl for 5 min at 70°C. RNA samples were incubated for 5 min on ice and again for 10 min at room temperature. M-MLV (1 μl) was added with 5 μl of 5X buffer, 1.25 μl of dNTP’s and incubated for 1 h at 42°C. After reverse transcription MMLV was inactivated by 70˚C for 15 min. The cDNA samples were diluted to 100 μl and 2.5 μl of cDNA were used for each PCR reaction. PCR-amplification of the housekeeping gene glyceraldehydes-3-phosphate-dehydrogenase (GAPDH) was carried out using TaqMan Instrument of mRNA expression levels was carried out using a real-time PCR reaction. PCR-amplification of the housekeeping gene glyceraldehydes-3-phosphate-dehydrogenase (GAPDH) was optimised for 32D cells.

### Cell lines and transfection.

32D cells were purchased from DSMZ (Braunschweig, Germany). In this study, 2 stable myeloid leukemia cell lines derived from U937 cells (human myeloid leukemia) were used. U937 cells 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’s medium (DMEM, Invitrogen), each supplemented with 2 mM L-glutamine and 10% FCS (Biochrom KG), and 100 U/ml penicillin and 100 μg/ml streptomycin (Biochrom KG), and 2 mM L-glutamine (Biochrom KG). For 32D cells, the RPMI-1640 medium was additionally supplemented with 20 mM HEPES (Invitrogen). The mammalian cells were grown at 37°C and 5% CO2. The U937 and 32D cells were transfected following the Amaxa kit (Biosystems) protocol. A total of 5 μg of plasmid DNA with 100 μl of solution R per transfection was used. The cells were electroporated by Amaxa program T24, which was optimized for 32D cells.

**Antibodies and Western blotting.** Cells were lysed in radioimmunoprecipitation buffer (RIPA) (150 mM NaCl, 1.0% NP-40, 0.5% deoxycholic acid (DOC), 0.1% SDS, 50 mM Tris-Cl (pH 8.0) with complete EDTA-free protease inhibitor). The proteins were run on SDS-PAGE gradient gels (7-14% Bio-Rad). Proteins sample were electrotblotted onto PVDF membranes Immobilon-P (Millipore), probed with specific primary antibodies WT1 (F-6) from Santa Cruz Biotechnology and ß-actin (Sigma) followed by a secondary antibody against rabbit and mouse IgG antibody conjugated with peroxidase (Jackson ImmunoResearch). Detection was performed with

![Figure 1. Heatmap of of cyclin A1 overexpression associated transcriptional changes. 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 with differentially expressed genes and clusters were visualized using the Cluster and Tree software (Stanford).](image-url)
were prepared with M-MLV reverse transcriptase (Promega) The cDNA from U937 control and U937 cyclin A1 cell line were used in the PCR. GCC-ACC-TTA-A-3' and the unlabeled reverse primer 5'-dye phosphoramidites FAM 5'CAG-ATG-AAC-TTA-GGA-isoform analysis was calculated using the ModFitLT V2.0 (PMac) software. of GFP positive (transfected) cells. Cell cycle distribution up to 1 h. FACS analyses were performed using FL-1 vs. FL-2 channel (GFP vs. PI) in order to get the cell cycle profiles incubated at room temperature for 30 min after 16 h, cells were separated by gradient centrifuge and 1x10^5 living cells per WT1-/+ expression vectors with Amaxa kit (Biosystems) after retroviral transduction were analyzed for differences in mRNA expression by high density oligonucleotide arrays. Differentially expressed genes were determined as described in Materials and methods. With this stringent criteria, 35 genes were regarded as differentially expressed with 21 being induced and 14 being repressed this stringent criteria, 35 genes were regarded as differentially expressed with 21 being induced and 14 being repressed upon cyclin A1 induction (Fig. 1). Out of these genes the Wilms tumor gene (WT1) was selected for further analysis since this gene is known to be involved in growth regulation and tumor pathogenesis. Expression of WT1 is reduced in cyclin A1-overexpressing U937 cells. 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 U937 control cells. Quantitative RT-PCR was performed with U937 overexpressing cyclin A1 cells as compared to U937 control cells. Total RNA of U937 control and overexpressing cyclin A1 cells was used in quantitative RT-PCR analysis to determine WT1 expression relative to their GAPDH expression. Colony assay. U937 control and overexpressing cyclin A1 or 32D cells were electroporated with 10 μg of each pcDNA3, pcDNA3-cyclin A1, pCMV-WT1++, pCMV-WT1+, pCMV-WT1+ and pCMV-WT1+++ expression vectors (WT1 isoforms were a gift from Dr Jerry Peteiller). After 16 h, cells were separated by gradient centrifuge and 1x10^6 living cells per 35-mm dish were cultured with methylecellulose mix. The methylecellulose mix contained Iscove’s modified Dulbecco’s medium (IMDM, Invitrogen) with 1% methylecellulose, 20% FCS, IL3 (1 ng/ml) and blastidin or neomycin (0.5-0.6 mg/ml) as a selection marker. Cells were grown in triplicate in methylecellulose colony assays. Colonies were counted after 14 days of growth. Flow cytometry. 32D cells were transfected with GFP-Ras, pcDNA3, pcDNA3-cyclin A1, pCMV-WT1++, pCMV-WT1+ and pCMV-WT1+++ expression vectors with Amaxa kit (Biosystems) protocol (as described in Materials and methods) and incubated for 24 h. 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 h. After extensive washing, cells were resuspended in PBS containing RNase A and propidium iiodide and incubated at room temperature for 30 min up to 1 h. 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 ModFitLT V2.0 (PMac) software. 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 M-MLV reverse transcriptase (Promega) following the manufacturer’s protocol (described previously). Each PCR mixture contained 50-100 ng of cDNA, 2.5 units of taq polymerase, 1 μM of each primer, 10 μM each dNTP, 1.5 mM MgCl2, and PCR buffer at a 1X final concentration. (Applied Biosystems). Thirty cycles of PCR were performed in a DNA-thermal cycler (Master cycler personal, Eppendorf). Initial denaturation at 95°C for 4 min was followed by 30-35 cycles of denaturing at 94°C for 20 sec, annealing at 62°C for 20 sec, and extension at 72°C for 30 sec, followed by a terminal extension step at 72°C for 10 min. PCR product (1 μl) was diluted in 20 μl formamide containing 1 IU 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 Biosystems). The data were visualized on a histogram and analyzed by using ABI Gene Scan Software (Applied Biosystems).


Results

Identification of target genes by microarray analysis in cyclin A1 overexpressing U937 cells. U937 cells overexpressing cyclin A1 after retroviral transduction were analyzed for differences in mRNA expression by high density oligonucleotide arrays. Differentially expressed genes were determined as described in Materials and methods. With this stringent criteria, 35 genes were regarded as differentially expressed with 21 being induced and 14 being repressed upon cyclin A1 induction (Fig. 1). Out of these genes the Wilms tumor gene (WT1) was selected for further analysis since this gene is known to be involved in growth regulation and tumor pathogenesis. Expression of WT1 is reduced in cyclin A1-overexpressing U937 cells. 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 U937 control cells (Fig. 2). The reduction of WT1 expression in cyclin A1-overexpressing U937 cells was confirmed on the protein level by Western blot analysis (Fig. 3). The two bands detected at 54-52 kDa and 42 kDa appeared to be different isoforms of WT1, as previously reported (10-12).

WT1 expression in cyclin A1-/+ mice tissues. Bone marrow of cyclin A1-/+ mice expressed higher levels of WT1 mRNA

![Figure 2. WT1 is 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 quantitative RT-PCR analysis to determine WT1 expression relative to their GAPDH expression.](image-url)
Moreover, in testis, liver, and ovary the expression of WT1 was also increased in the absence of cyclin A1 (Fig. 4B). This is in accordance with the findings in the leukemia cell lines. However, in lung tissue WT1 expression was decreased in cyclin A1-/- mice (Fig. 4B).

**Expression of WT1 isoforms in U937 cyclin A1 overexpressing and control cells.** The WT1 primary transcript has been described to undergo two alternatives splicing events, giving rise to four isoforms of mRNA (13). Alternative 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 the third and fourth zinc finger domain (see Fig. 5). The 4 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 an imbalance in WT1 isoforms can lead to tumor development (14,15).

In U937 control cells only the WT1-/- isoform was expressed (Fig. 6, upper histogram). In contrast, in U937 cells overexpressing cyclin A1 all isoforms were present (Fig. 6, lower histogram) with the following relative expression values, as determined by histogram: WT1-/- (482 bp, 12%); WT1-/+ (489 bp, 24%); WT1+/- (528 bp, 26%); WT1+/+ (535 bp, 36%).

**WT1 isoforms inhibit colony growth.** When transfected into either U937 control cells, each WT1-/-, WT1-/+ and WT1+/+ isoform, reduced the colony formation by ~50% compared to a transfection with the empty vector (Fig. 7). This reduction was not affected by the overexpression of cyclin A1 in cyclin A1 overexpressing U937 cells (Fig. 7). Cyclin A1 has been reported to enhance cell cycle progression and proliferation in leukemic cells. Suppression of the growth inhibitory WT1 would fit into the picture of the cell cycle enhancing features of overexpressed cyclin A1.

**WT1-/- isoform induces G1 arrest.** Cyclin A1 is known to be involved in the G1/S transition in cell cycle progression. Therefore, we analyzed whether expression of WT1-/- or WT1+/+ isoforms in the presence or absence of cyclin A1 would alter cell cycle progression in 32 D cells.
The expression of WT1 -/+ led to a cell cycle arrest. This block occurred in G1 to S phase progression in 32D cells (Fig. 8). WT1 -/+ induced G1 cell cycle arrest. When cyclin A1 was co-transfected with the WT1 isoforms, cyclin A1 abrogated the WT1 +/−-induced cell cycle arrest (Fig. 8). The percentage of cells distributed in the different phases of cell cycle is shown in Table I.

### Discussion

In this study, we identified genes with a differential expression upon overexpression of cyclin A1. None of these genes was previously known to be associated with cyclin A1. The repression of the WT1 gene was verified both on the mRNA and on the protein level. The WT1 gene is a tumor suppressor gene which encodes a member of the Cys2-His2 zinc finger family of transcription factors. Some data suggest that WT1 plays a role in hematopoietic differentiation: Expression of the WT1 gene has been detected in several myeloid cell lines and CD34+ hematopoietic precursors (16) and its expression disappears during differentiation (17,18). In addition, WT1 overexpression was found to interfere with the induced differentiation in these cells (19,20) and leukemic cells treated with wt1 siRNA showed a loss in proliferation and increase in apoptosis (21).

A possible role of WT1 in AML is supported by the finding that high levels of WT1 expression have adverse prognostic significance in AML (22). Heterozygous WT1 mutations are

### Table I. Cell cycle distribution of WT1 in 32D cells.

<table>
<thead>
<tr>
<th>Transfected constructs</th>
<th>Cell cycle distribution (%)</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>G1</td>
<td>S</td>
</tr>
<tr>
<td>pcDNA3</td>
<td>55.68</td>
<td>28.73</td>
</tr>
<tr>
<td>Cyclin A1</td>
<td>52.95</td>
<td>31.53</td>
</tr>
<tr>
<td>WT1 +/−</td>
<td>53.08</td>
<td>31.43</td>
</tr>
<tr>
<td>WT1 −/+</td>
<td>61.25</td>
<td>22.35</td>
</tr>
<tr>
<td>Cyclin A1 and WT1 +/−</td>
<td>48.30</td>
<td>34.79</td>
</tr>
<tr>
<td>Cyclin A1 and WT1 −/+</td>
<td>55.77</td>
<td>27.89</td>
</tr>
</tbody>
</table>

pcDNA3, WT1 +/−, WT1 −/+ 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.
Figure 7. Colony assay in U937 control and overexpressing cyclin A1 with different WT1 isoforms. 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.

Figure 8. Effects of WT1 isoforms on cell cycle regulation by FACS. Empty vector, cyclin A1, WT1\(^{+/-}\), WT1\(^{-/-}\) were transfected with and without cyclin A1 in 32D cells. After 24 h the cells were stained with propidium iodide, sorted by FACS, and cell cycle distribution was calculated using the ModFitLT 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 ± SE of three independent experiments.
Furthermore, the WT1 +/+ (+17aa) and WT1 -/+ (-17aa) ratio is mutation interfering with the ratio of WT1 (+KTS) and WT1 at different ratios albeit at much lower overall levels. A cyclin A1 in these cells led to the expression of all isoforms WT1 isoform, compared to all other published results with identified a cell line with predominant expression of a sole the U937 control samples. This observation for the first time investigated using capillary electropherosis of fluorescent essential for normal functioning. The different isoforms were investigated by further studies.

In mammals, exons 5 and 9 of WT1 are alternatively spliced (13,25). Splice variant 1 results in the insertion of 17 amino acids upstream of the zinc finger domain and enhances the transcriptional activity of protein (26). Splice variant 2 occurs within the zinc finger domain, inserting three amino acids (Lys-Thr-Ser; KTS) between the third and the fourth zinc finger. This splice variant alters the DNA binding specificity of the protein (27,28). Alternative splicing of WT1 gives rise to 4 different splice isoforms (25), termed WT1+/- (both splice variants 1 and 2 are present), WT1+/+ (both splice variants 1 and 2 are present), and WT1+-/- (neither splice variant present). These 4 isoforms display molecular masses in the range of 52-54 kDa (29).

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 samples. This observation for the first time identified a cell line with predominant expression of a sole WT1 isoform, compared to all other published results with respect to isoform distribution. In contrast, overexpression of cyclin A1 in these cells led to the expression of all isoforms at different ratios albeit at much lower overall levels. A mutation interfering with the ratio of WT1 (+KTS) and WT1 (-KTS) protein reportedly leads to the Frazier syndrome (30). Furthermore, the WT1+/- (+17aa) and WT1+/- (-17aa) ratio is also important, and absence of WT1+/- showed poor postnatal survival and glomerular abnormalities in mice (31,32).

In concordance with previous results, the transfection of WT isoforms into myeloid cell showed a pronounced reduction in colony growth (33). In previous studies it was reported that WT1 (-KTS) and WT1 (+KTS) differently affect the growth suppression of primary hematopoietic cells (33,34). However, in this study the growth reduction by WT1 was equal in every isoform. In addition, the growth inhibition by WT1 isoforms was not affected by the overexpression of cyclin A1. WT1+/- was shown to induce G1 arrest in 32D cells, which further suggest that this isoform was involved in different functions of myeloid cells. Different reports indicate that WT1 has relevance for the induction of growth arrest and apoptosis (35-37). Overexpression of WT1 blocked the G1-S transition of NIH3t3 and induced apoptosis in F9 embryonal carcinoma cells and in an osteosarcoma cell line (36,38,39). The M1 murine myeloblastic cell line expressing no endogenous WT1 reacts very differently to ectopic expression. Constitutive expression of the WT1 (+KTS) form induces monocyctic differentiation independent of external stimuli. In contrast, M1 cells stably expressing the WT1 (-KTS) isoform could not be established due to the induction of G1 arrest and/or apoptosis (11,35). 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 (38). In this study, the co-transfection of cyclin A1 released the G1 arrest. In conclusion, cyclin A1 might interact with WT1 directly or indirectly during the cell cycle.

Collectively, this study identified several potential target genes of cyclin A1 and identified WT1 as a repressed target gene of cyclin A1. Furthermore, this study suggests that the suppression of WT1 in cyclin A1-overexpressing leukemias might play a role in the growth and suppression of apoptosis in these leukemic cells.

Acknowledgements

This study was supported by the Deutsche Forschungsgemeinschaft, the Deutsche Krebshilfe and the IZKF at the University of Münster.

References


