Transcriptional upregulation of *p21/WAF/Cip1* in myeloid leukemic blasts expressing AML1-ETO

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ABSTRACT

An inducible model for conditional expression of AML1-ETO in myeloid U-937 cells was generated previously to determine cellular effects of AML1-ETO and to identify target genes. Induction of AML1-ETO expression in U-937 resulted in reduced cell growth, G1 arrest and apoptosis. Microarray analysis showed more genes up-regulated than down-regulated (180 vs. 69). Clustering of AML1-ETO-positive and -negative cell lines was possible based on these differentially expressed genes. *p21/WAF/Cip1* (*CDKN1A*) was up-regulated 4.6-fold upon induction of AML1-ETO which was confirmed in additional experiments. Knock-down of AML1-ETO by siRNA could reduce *p21/WAF/Cip1* expression in Kasumi-1 cells. mRNA expression analysis of *p21/WAF/Cip1* in a large cohort of acute myeloid leukemia patients demonstrated a significantly higher expression in AML1-ETO-positive leukemia. The increased expression of *p21/WAF/Cip1* in primary leukemic blasts suggests that elevated *p21/WAF/Cip1* levels may contribute to specific features observed in AML1-ETO positive leukemia.

Key words: cell cycle, acute myeloid leukemia, chromosomal translocation.

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Introduction

The t(8;21)(q22;q22) is a recurrent chromosomal translocation in acute myeloid leukemia (AML). It results in the expression of a chimeric gene product that fuses the DNA-binding domain of AML1 (RUNX1) with almost the entire ETO protein (RUNX1T1). AML1-ETO-positive leukemia has been modeled *in vitro* and *in vivo*. Ectopic expression of AML1-ETO in U-937 cells has been shown to block differentiation,[1] while suppression of AML1-ETO in t(8;21)-positive leukemic cells by small interfering RNAs (siRNA) supports it.[2] Inducible expression of AML1-ETO in a cell line model has been previously reported to induce growth arrest and apoptosis.[3-5] But AML1-ETO promotes hematopoietic stem/progenitor cell proliferation when expressed in CD34-positive cells.[6] As ETO and AML1-ETO interact with numerous co-repressors and histone deacetylases (HDACs), it has been hypothesized that AML1-ETO acts as a constitutive repressor of AML1-dependent genes.[7,8] This mechanism was suggested for the downregulation of genes involved in myeloid differentiation such as *C/EBPα*[9] and the tumor suppressor gene *p14ARF*.[10]

Thus far, only a limited number of studies have focused on AML1-ETO gains-of-function. We have previously generated an ecdysone-inducible AML1-ETO expression model in U-937 cells and showed that AML1-ETO can cause growth inhibition and induction of apoptosis.[4,5] We now used microarray analysis to identify additional potential regulators of the prominent cellular changes observed upon expression of AML1-ETO such as cell growth inhibition and subsequent apoptosis and tried to reconcile this observation with leukemogenesis by AML1-ETO. Strong upregulation was shown for *p21/WAF1/Cip1* (CDKN1A). Transcriptional upregulation, as demonstrated in a promoter-reporter assay, was independent of p53. Notably, we describe for the first time, that specific downregulation of AML1-ETO by siRNA led to repression of *p21/WAF1/Cip1* in Kasumi-1. Expression studies for *p21/WAF1/Cip1* in primary

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leukemic blasts from 47 patients with AML1-ETO-positive AML and 53 patients with normal karyotype AML demonstrated a significantly higher expression in AML1-ETO-positive samples. This observed upregulation of *p21/WAF1/Cip1* by AML1-ETO *in vitro* and *in vivo* might be responsible for some of the cellular effects of AML1-ETO.

Design and Methods

Cell culture

The human myeloid cell lines U-937 (monoblastic, AML1-ETO negative, p53-mutated), HL-60 (late myeloblastic, AML1-ETO negative, p53-deleted), KG-1 (early myeloblastic, AML1-ETO negative, p53-mutated) and Kasumi-1 (late myeloblastic, AML1-ETO positive, p53-mutated)[11-13] were obtained from DSMZ (Braunschweig, Germany) and cultured as recommended. SKNO-1 (late myeloblastic, AML1-ETO positive, p53-mutated; kindly provided by S. Nimer, MSKCC, New York) were grown in the presence of 10 ng/mL recombinant human GM-CSF (Cellgenix, Freiburg, Germany). HEK 293 (ATCC; CRL-1573) cells were maintained in Dulbecco's modified Eagles's medium (Invitrogen, Karlsruhe, Germany) supplemented with 10% fetal calf serum (Invitrogen) and 50 U/mL penicillin. The generation of the ecdysone-inducible AML1-ETO expression system in U-937 cells was described previously.[14] A single cell clone (9/14/18) was characterized in detail and used for all experiments.

Flow cytometry

To determine apoptosis rate, cells were resuspended in RPMI 1640 supplemented with 0.5% BSA and mixed with an equal volume of DiOC6 (3,3`dihexyloxacarbocyanine iodide)/PI double staining solution. Samples were incubated for 15 mins. at 37°C and analyzed on a Becton Dickinson FACSCalibur.

AML1-ETO knock-down

For AML1-ETO knock-down, siRNAs siAGF1, the mismatch control siAGF6, and unrelated controls were synthesized by Alnylam Europe AG (Kulmbach, Germany) and transfected into Kasumi-1 cells as previously described.[2]

Microarray analysis

RNA isolation was performed using the Qiagen RNeasy Kit (Qiagen, Hilden, Germany) and the AA Biotechnology RNA Mini Kit (AA Biotechnology, Gdansk, Poland) according to the manufacturers' recommendations. RNA was processed and hybridized with HG-U133A microarrays (Affymetrix, Santa Clara, CA, USA) as described.[15] After two independent AML1-ETO induction experiments in U-937 cells, gene expression in both induced samples was compared with both uninduced samples. Genes were considered up-regulated/down-regulated if they showed a >2-fold change in both experiments. Only probe sets with a signal intensity of >100 were considered as expressed. Microarray analyses from patient samples were performed at the Laboratory for Leukemia Diagnostics (Department of Internal Medicine III, Hospital Grosshadern, Munich, Germany) and the Munich Leukemia Laboratory, where samples were referred for routine cytomorphological and cytogenetic analyses. Patients' informed consent was obtained according to institutional standards.

Northern blot analysis

Northern Blot was performed as described (14). A 640-bp fragment from the 3'-region of full-length *AML1-ETO* cDNA was used as *AML1-ETO* probe. A full-length probe for *p21/WAF1/Cip1* was obtained from B. Vogelstein, The Johns Hopkins University School of Medicine, New York.

Quantitative real-time RT-PCR

For reverse transcriptase PCR (RT-PCR), RNA was extracted using a NucleoSpin RNA II Kit (Macherey-Nagel, Düren, Germany). First strand synthesis was done using 2 μg of RNA and Superscript II Reverse Transcriptase (Invitrogen). Real-time PCR was performed using LightCycler 480 SYBR Green I Master and a LightCycler 480 Real-Time PCR System (Roche Diagnostics-Applied Science, Mannheim, Germany). Primer sequences are available upon request.

Western blot analysis

Whole cell protein extracts were obtained from at least 5×10^6 cells with the Active Motif Nuclear Extract Kit (Active Motif Europe, Rixensart, Belgium). 25 μg protein were run on a 12% Novex Bis-Tris polyacrylamide gel (Invitrogen) using an Xcell SureLock Mini-Cell (Invitrogen) gel chamber and transferred to Hybond P membranes (GE Healthcare). After antibody hybridization, detection was performed with ECL Plus Western Blotting Detection Reagent (GE Healthcare). Gels and membranes were stained with Coomassie blue to confirm equal loading.

Promoter-reporter assays

The plasmid expression constructs WWp21-Luc (kindly provided by B. Vogelstein, The Johns Hopkins University School of Medicine, New York) and pcDNA3-AML1-ETO were transfected into HEK 293 cells with Fugene 6 transfection reagent (Roche, Nutley NJ, USA).[7] Measurements of transcriptional activity were conducted with at least four independent transfections.

Results and Discussion

Forced AML1-ETO expression results in strong upregulation of multiple genes involved in cell cycle regulation including p21/WAF1/CIP1 (CDKN1A)

To detect changes in the transcriptome mediated by AML1-ETO, RNA from two independent experiments was obtained from clone 9/14/18 after 48 hrs. incubation with or without Ponasterone A. RNA was hybridized to Affymetrix HG U133A oligonucleotide arrays. A total of 249 probe sets were identified as differentially expressed. Surprisingly, 180 genes were found up-regulated and only 69 down-regulated (Figure 1A and *Online Supplementary Table S1*). The differentially expressed probe sets can distinguish AML1-ETO positive cell lines from AML1-ETO

negative cell lines in a principal components analysis (Figure 1B). Among the genes with a known function in cell cycle regulation and apoptosis, *p21/WAF1/CIP1* showed a particularly strong upregulation with a 4.6-fold increase (Figure 1A and *Online Supplementary Table S2*).

Induction of p21/WAF1/Cip1 upon conditional AML1-ETO expression is accompanied by cell growth arrest and subsequent apoptosis

When 9/14/18 cells were induced to express AML1-ETO for up to ten days, a dramatic reduction in cell number was observed beginning three days after addition of Ponasterone A, with a nearly complete loss of proliferation observed by day 6. An inducible LacZ control clone was unaffected by the hormone treatment (Figure 2A). As we previously demonstrated, the observed effects of AML1-ETO on cell growth are attended by a partial G1 arrest followed by apoptosis.[4,5] Cell cycle analyses with flow cytometry of propidium iodide-stained (PI) nuclei showed a transient increase of cells in G1 by 6% after 24 hrs. of PonA treatment which was reversed at 48 hrs. (*data not shown*). After three days, an increase of cells with hypodiploid DNA content (sub-G1) by a median of 12 % occurred. Control cells treated just with ethanol did not exhibit the increase of cells in G1 and sub-G1 (*data not shown*). Apoptosis was confirmed using a FACS assay for early apoptotic events based on $DiOC_6$/PI staining (Figure 2B). Hormone treatment of the clone 9/14/18 for 48 hrs. caused a decrease in viability by 10.8±3.6% SEM; p=0.048 by t-test) in 5 independent experiments and the proportion of apoptotic cells increased by 4.1±1.4% SEM; p=0.02 by t-test). The observed maximum decreases in viability and increases in apoptosis were 24% and 9% respectively. U-937 treated with Ponasterone A showed no decrease in viability (-0.2±0.4% SEM) and no increase in apoptosis (-0.1±0.12%). No effect was observed in a control clone expressing LacZ.

AML1-ETO is linked to increased p21/WAF1/Cip1 expression in vitro and in vivo

Based on the microarray data and the cellular effects observed after AML1-ETO induction in U-937 cells, we hypothesized that AML1-ETO expression might regulate *p21/WAF1/Cip1* expression. After confirmation by Northern Blot (*Online Supplementary Figure S1*), we analyzed the kinetics of AML1-ETO induction and *p21/WAF1/Cip1* expression in 9/14/18 cells. Quantitative PCR analysis revealed that the increase in *p21/WAF1/Cip1* expression first observed after eight hours was clearly preceded by the increase in AML1-ETO expression, which was already detectable after two hours (Figure 3A). Western Blot confirmed an increase of *p21/WAF1/Cip1* on the protein level (*Online Supplementary Figure S2*). In order to examine whether AML1-ETO positivity is linked to higher *p21/WAF1/Cip1* transcription, its expression level was quantified both in AML1-ETO-positive (Kasumi-1 and SKNO-1) and -negative (HL-60 and U-937) leukemic cell lines by Northern Blot. Notably, both AML1-ETO-positive cell lines displayed detectable levels of *p21/WAF1/Cip1* compared to HL-60 and U-937 (Figure 3 B), thus supporting a link between AML1-ETO status and *p21/WAF1/Cip1* expression. To confirm the observed reg-

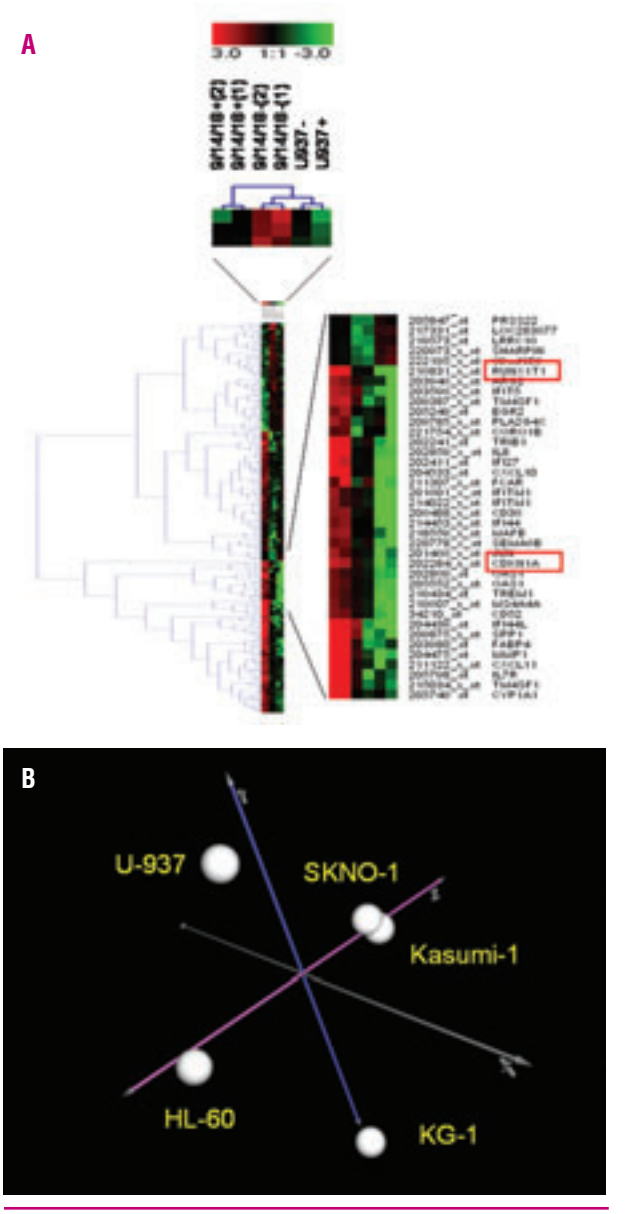

Figure 1. Identification of AML1-ETO regulated genes. DNA microarray analysis of wild type U-937 cells and and a single cell clone of U-937 cells with inducible expression of AML1-ETO (clone n. 9/14/18) was performed using Affymetrix HG-U133A microarrays in 2 independent experiments. 9/14/18 and wild type U-937 cells were cultured in the presence or absence of 5 µM Ponasterone for 48 hrs. Primary image analysis was performed by using Microarray Suite 5.0. To allow comparison between different array hybridizations, images were scaled to an average hybridization intensity of 500. Differentially expressed genes were identified based on the criteria described in Design and Methods. Cluster analysis, principal component analysis, and data visualization was performed with the Genesis software package.[27] (A) Cluster analysis (Manhattan distance, complete linkage clustering) of the different U-937 samples was performed by using the identified differentially expressed genes as data points. The positions of probe sets corresponding to *p21/WAF1/Cip1* and ETO (RUNX1T1) detecting the fusion transcript are indicated. The probe set for ETO (detecting the fusion transcript) was among the most strongly induced transcript. Ponasterone A had no clearly detectable effect upon overall gene expression in wild type U-937. *p21/WAF1/Cip1* (CDKN1A) expression was absent in wild type U-937 cells treated with PonA (absence call on microarray). (B) Principal component analysis with AML1-ETO positive (SKNO-1, Kasumi-1) and negative cells lines. The same probe sets as in panel A were used. AML1-ETO positive cell lines cluster together whereas the other cell lines are clearly separated.

ulation in primary t(8;21)-positive leukemia, mRNA expression levels of *p21/WAF1/Cip1* in 43 cases of newly diagnosed AML with t(8;21) were compared to 57 cases of AML M2 with normal karyotype using microarray data. The average signal strength from the microarray for *p21/WAF1/Cip1* was 43% higher in samples from patients with t(8;21) than in samples from patients without t(8;21) (Figure 3C). Analyzed by Wilcoxon two-sample test, this difference was statistically significant (p=0.0005). This differential expression was validated further by analyzing published datasets (*Online Supplementary Table S3).*

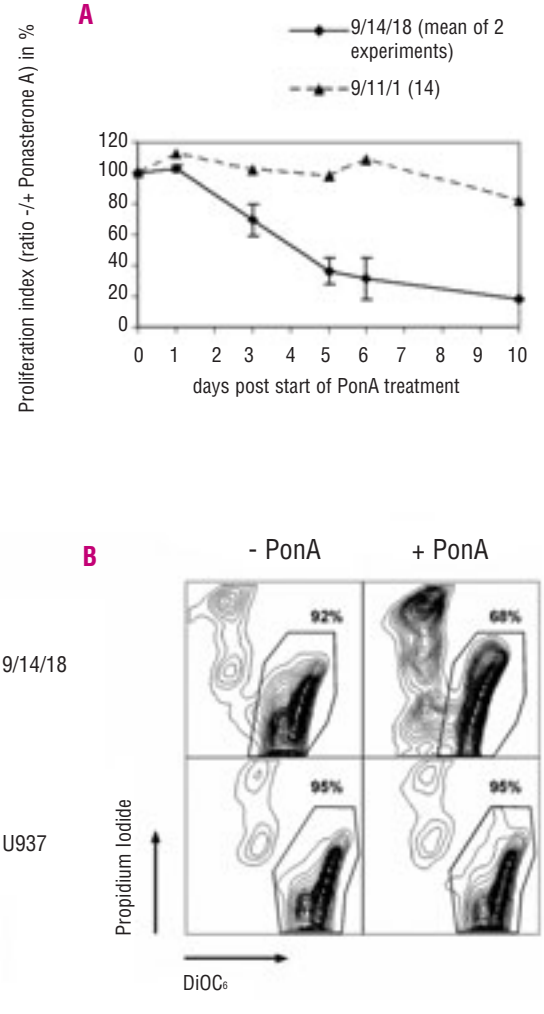

Figure 2. Inducible expression of AML1-ETO in U-937 cells results in growth arrest and apoptosis. Inducible AML1-ETO expression in U-937 cells was accomplished based on an ecdysone-inducible system. AML1-ETO was expressed after treatment with 5 μM Ponasterone A. (A) Growth inhibition of U-937 after induction of AML1-ETO expression. Cell growth was determined by counting viable cells using trypan blue exclusion. The ratio of viable cells in the presence or the absence of Ponasterone A is shown for each cell line in percent. 9/14/18 represents a clone of U-937 with inducible expression of AML1-ETO. 9/11/1 (with inducible expression of LacZ) is shown as control. Almost complete loss of cell growth was observed upon expression of AML1-ETO, but not upon expression of LacZ. (B) Apoptosis analysis by flow cytometry using $DiOC_6$/PI double staining after induction of AML1-ETO expression. Treatment with Ponasterone A (PonA) led to an increase in apoptosis in the inducible system, but not in wild type U-937 cells starting at 48 hrs. of treatment. The number of viable cells is given as percent value.

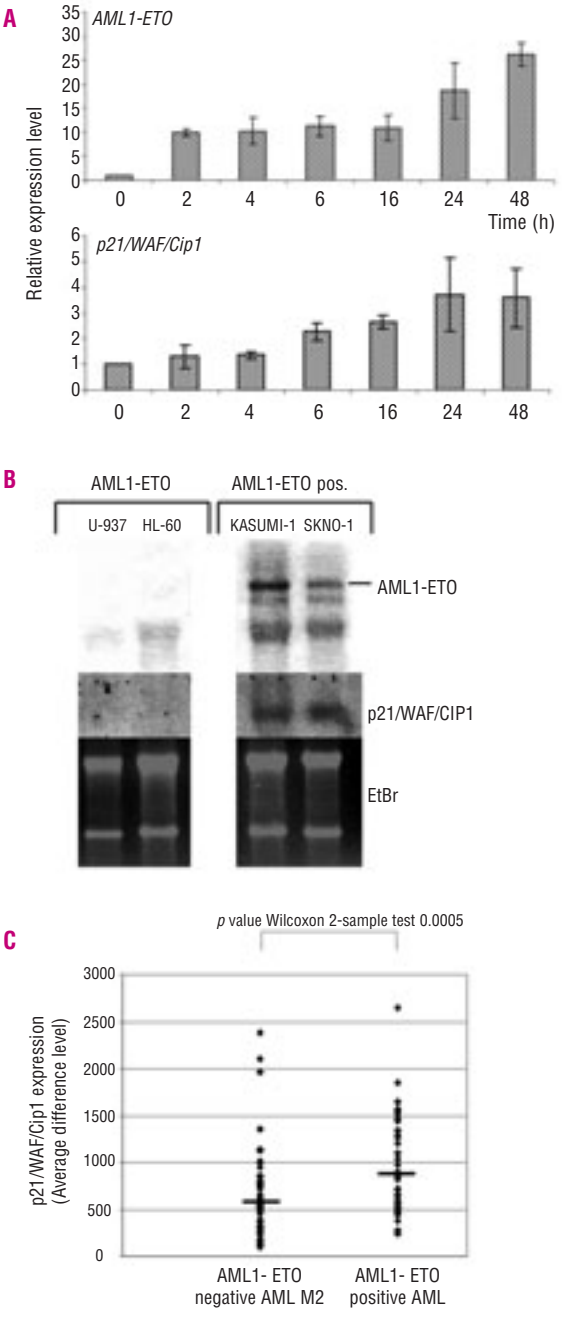

Figure 3. Increased expression of p21/WAF1/Cip1 in an inducible AML1-ETO model system and in primary AML samples with t(8;21). (A) Time course analysis of *p21/WAF1/Cip1* and *AML1-ETO* expression analyzed by real-time quantitative RT-PCR in the inducible U-937 cell line demonstrates that expression of p21/WAF1/Cip1 increases in response to increased AML1-ETO expression levels. Expression data was normalized using β-glucuronidase expression. The expression level is shown as relative expression compared to the expression at 0 hours (B) *p21/WAF1/Cip1* and *AML1-ETO* expression is readily detectable in two AML1-ETO positive cell lines (right) but undetectable in two AML1-ETO negative cell lines (left) as assessed by Northern Blot. Ethidium bromide stained gels are shown as loading control. Two week exposure time was required for detection of *p21/WAF1/Cip1*. (C) Expression of p21/WAF/Cip1 in primary AML samples from 100 patients with acute myeloid leukemia and either t(8;21) or normal karyotype. Comparison of scaled signal intensities of p21/WAF/Cip1 expression from microarray analyses (Affymetrix) of 43 patients with t(8;21) and of 57 control patients with AML of FAB subtype M2 with normal karyotype. The average signal strength from the microarray for p21/WAF/Cip1 was 43% higher in samples from patients with t(8;21) than in samples from patients without t(8;21). The *p*-value was calculated by a Wilcoxon 2-sample test.

AML1-ETO activates the p21/WAF1/Cip1 promoter and "knock-down" in Kasumi-1 cells by siRNA decreases p21/WAF1/Cip1 expression

Activation of the *p21/WAF1/Cip1* promoter by AML1-ETO was studied in promoter-reporter experiments. AML1-ETO caused a dose-dependent activation of the *p21/WAF1/Cip1* promoter (Figure 4A). No accumulation of p53 upon induction of AML1-ETO was noted which is in accordance with published data, as U-937 does not carry functional p53 (*data not shown*). Thus, the observed upregulation of *p21/WAF1/Cip1* is p53-independent.

We next asked whether downregulation of AML1-ETO in a t(8;21)-positive cell line results in decreased expression of *p21/WAF1/Cip1*. Kasumi-1 cells were electroporated with siRNA targeting AML1-ETO (siAGF1). As controls, electroporations without siRNA (mock) or with the mismatch control siRNA siAGF6 were performed. For AML1-ETO, a 75% reduction was achieved with two sequential electroporations. Knock-down of AML1-ETO decreased *p21/WAF1/Cip1* mRNA 4.9-fold and protein respectively (*Online Supplementary Figure S3*; Figure 4B). This adds further evidence that the observed upregulation of *p21/WAF1/Cip1* is a direct effect of AML1-ETO.

Although a number of potential AML1-ETO target genes in myeloid cells have been determined by differential gene expression analyses,[14,16-18] it is as yet unclear which downstream targets are involved in AML1-ETO-mediated leukemogenesis and its cell cycle effects. Based on gene ontology information we identified cell cycle- and apoptosis-related genes that were regulated.[19] *p21/WAF1/Cip1* was found up-regulated by this approach. It belongs to the family of cyclin-dependent kinase inhibitors and is a known Runx1-target gene.[20]

We noted that cell cycle delay and subsequent apoptosis were induced when AML1-ETO was conditionally expressed in U-937, consistent with previous studies showing apoptosis induction by AML1-ETO.[3] It is tempting to speculate that a higher apoptosis rate in AML1-ETO positive AML may be functionally related to the higher response rate observed in this leukemia subgroup as AML with a higher apoptosis rate has been shown to have a better outcome.[21] However, experiments in which AML1-ETO was transduced into normal hematopoietic stem cells suggest that the anti-proliferative and proapoptotic effects of AML1-ETO occur primarily in more committed hematopoietic progenitors.[6]

Upregulation of *p21/WAF1/Cip1* mRNA was preceded by AML1-ETO expression. The hypothesis that *p21/WAF1/Cip1* is transcriptionally induced by AML1-ETO was further supported by activation of the *p21/WAF1/Cip1* promoter in a promoter-reporter assay. An interesting and novel finding of the present study was that *p21/WAF1/Cip1* expression is reduced by knock-down of AML1-ETO. Enhanced expression of *p21/WAF1/Cip1* upon expression of AML1-ETO *in vitro* is consistent with other reports in which AML1-ETO was transduced into K562 cells.[22,23] In these reports, AML1-ETO has to bind to DNA to cause its effect and occupancy of the *p21/WAF1/Cip1* promoter by AML1-ETO was demonstrated by chromatin immunoprecipitation.[23] It was also demonstrated that both full-length and a strongly leukemogenic truncated variant of AML1-ETO cause

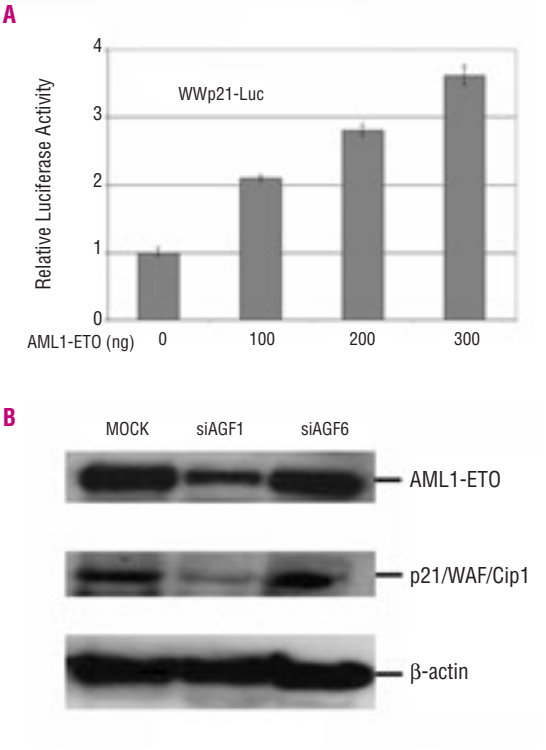

Figure 4. **Promoter-reporter assay demonstrating *p21/WAF1/Cip1* upregulation by AML1-ETO and downregulation of *p21/WAF1/Cip1* in Kasumi-1 by siRNA-mediated repression of AML1-ETO. (A) p21/WAF1/Cip1 expression is upregulated by activation of the p21/WAF1/Cip1 promoter as demonstrated by promoter-reporter assay for *p21/WAF1/Cip1* expression. 5 µg WWp21-Luciferase (containing a 2.4 kb *p21/WAF1/Cip1* promoter fragment) was transfected together with increasing amounts (given in ng) of an AML1-ETO-expression vector into HEK293 cells with total DNA kept at 500 ng. A β-galactosidase vector was cotransfected as control. Cells were harvested 24 hrs. after transfection. Transcriptional activity was determined using a luciferase assay (Promega, Madison WI, USA) and a tube luminometer (Berthold, Dortmund, Germany). Luciferase values were normalized against β-galactosidase activity and expressed as relative light units (RLU) of the normalized luciferase activity. Luciferase activity is given in arbitrary units relative to baseline (without AML1-ETO). AML1-ETO increases the expression from the *p21/WAF1/Cip1* promoter in a dose-dependent manner.(B) *p21/WAF1/Cip1* and *AML1-ETO* mRNA and protein expression in Kasumi-1 after double-transfection with siRNAs (siAGF1=active siRNA against AML1-ETO, siAGF6=mismatch control siRNA, mock=mock siRNA). Results from a double transfection experiment are shown. Protein from these experiments was obtained in Urea lysis buffer (9 M urea, 4% (w/w) 3-[(3-Cholamidopropyl)-dimethylammonio]-propansulfonat (CHAPS), 1% w/w Dithiotheitol) with 2.5 U/mL Benzonase (Merck, Darmstadt, Germany). Western blot analysis confirms that siAGF1 can reduce AML1-ETO and *p21/WAF1/Cip1* expression but not MOCK and siAGF6. β-actin expression is shown as loading control.**

p21/WAF1/Cip1 upregulation. Recently, AML1-ETO has been shown to activate and sensitize towards a p53 response.[24] However, our results suggest that *p21/WAF1/Cip1* upregulation occurs independently of p53. We suggest that an interaction between AML1-ETO and specific, as yet unidentified regulatory cofactors are required in order to increase the expression of *p21/WAF1/Cip1* which is consistent with the recently described cofactor exchange model of AML1-ETO mechanism.[25] Results of a murine model imply that disruption of *p21/WAF1/Cip1* can enhance leukemogenicity of full-length AML1-ETO resulting in leukemia development in

part of the *p21/WAF1/Cip1* animals.[23] But *p21/WAF1/Cip1* deficiency has not been described in AML1-ETO-positive AML and in the present paper we demonstrate that overexpression of *p21/WAF1/Cip1* is not only found in cell line models, but also in primary leukemic blasts from AML patients. The functional role of *p21/WAF1/Cip1* for leukemogenesis of AML1-ETO positive leukemia remains to be determined. As *p21/WAF1/Cip1* plays a role in maintaining an intact stem cell pool in normal hematopoiesis allowing continuous proliferation in the setting of sequential transplantation,[26] it may have an important function for early hematopoietic cells together with other genes up-regulated by AML1-ETO. Therefore, it is tempting to speculate that among other factors, *p21/WAF1/Cip1* expression might support the maintenance of the leukemic stem cell pool.

Authorship and Disclosures

TB and MF: experimentation, manuscript preparation, JAB flow cytometry experiments, MSS and SB: processing and analysis of microarrays, SL: promoter/reporter analysis, NS and OH: siRNA experiments, TH: microarray data of patient samples, MHW: promoter/reporter analysis; design and interpretation of experiments; manuscript preparation; ML: design and interpretation of experiments; manuscript preparation. The authors reported no potential conflicts of interest.

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