



Expression and regulation of G₁ cell-cycle inhibitors (p16^{INK4A}, p15^{INK4B}, p18^{INK4C}, p19^{INK4D}) in human acute myeloid leukemia and normal myeloid cells

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In hematological malignancies, structural alterations of genes for G₁-specific cyclin-dependent kinases inhibitors (CKIs) have been extensively investigated. G₁-CKIs might play an important role not only as tumor suppressor genes but also in cellular differentiation. We examined constitutive and differentiation-induced expression and regulation of the four members of the G₁-CKI family p16^{INK4A}, p15^{INK4B}, p18^{INK4C} and p19^{INK4D} in acute myeloid leukemia as well as their expression in normal granulocytes and monocytes. p18^{INK4C} and p19^{INK4D} mRNA were expressed constitutively at high levels in seven myeloid cell lines and 16 AML patient samples, whereas expression of p15^{INK4B} mRNA was very low and only detectable by nested RT-PCR analysis. During phorbol ester-induced monocytic differentiation of leukemic HL-60 cells expression of particular G₁-CKIs was disparately regulated. This process was associated with growth arrest of the majority of the cells (≥80%) in G₁/G₀, and in parallel p15^{INK4B} were upregulated whereas p18^{INK4C} and p19^{INK4D} expression was downregulated. In contrast, granulocytic differentiation induced by DMSO was accompanied by an increase of p18^{INK4C} and p19^{INK4D} expression only. PMA treatment of blast cells from two AML patients confirmed these cell line results. Disparate regulation of p15^{INK4B} and p18^{INK4C} mRNA was dependent on intermediary protein synthesis and occurred at the post-transcriptional level as shown by nuclear run-on analysis and mRNA half-life studies. In normal granulocytes and monocytes low constitutive p15^{INK4B} and p18^{INK4C} mRNA expression was detectable by RT-PCR only, but p19^{INK4D} transcripts were noted by Northern blotting in both cell types. Disparate expression of G₁-specific cell cycle inhibitors indicates complex and divergent roles of particular CKIs during normal and leukemic myeloid hematopoiesis.

Keywords: acute myeloid leukemia; cell cycle inhibitor; myeloid differentiation

Introduction

Progression through the cell cycle is coordinated by sequential formation, activation and inactivation of cyclin-dependent kinases (cdks).¹ Activation of cdks requires their binding of regulatory subunits, cyclins, and phosphorylation by cdk-activating kinases.² These kinases include cdk4 and cdk6 which act in G₁-phase, as well as cdk1 (cdc2) and cdk2 which are involved in G₂/M transition of the cycle. The active cdk-cyclin complex can be inhibited by phosphorylation and by binding to heterologous small regulatory proteins known as cyclin-dependent kinase inhibitors (CKIs).^{3,4} Two structurally defined classes of CKIs have been identified. The first class includes p21^{WAF1}, p27^{KIP1} and p57^{KIP2} which inhibits the activity of G₁- and S-phase and to a lesser extent the mitotic cdk-cyclin complexes.^{5–7} *In vitro* overexpression of p21^{WAF1}, p27^{KIP1} and p57^{KIP2} induces growth arrest which suggests their possible role as tumor suppressor genes. The second class of CKIs inhibit G₁-specific cyclin D-cdk4/6 kinase activity (*INK4*)

only. The most extensively characterized members are p15^{INK4B} and p16^{INK4A} whose genes are adjacent on chromosome 9p21.^{8,9} Mutations of the p15^{INK4B} and p16^{INK4A} genes have been found in a variety of primary solid tumors.¹⁰ In hematological malignancies, deletions of the p15^{INK4B} and p16^{INK4A} genes were frequently identified in acute lymphoblastic leukemia (ALL) and lymphoid transformation of chronic myeloid leukemia (CML) whereas point mutations were very rare.^{11–13} These findings and the early formation of spontaneous tumors in mice with targeted disruption of the *INK4A*-locus suggest that p15^{INK4B} and p16^{INK4A} are important tumor suppressor genes.¹⁴ It remains to be seen whether the recently characterized product of a second transcript from the p16^{INK4A}-locus using a different promoter (p19^{ARF}) also contributes to tumorigenesis.¹⁵

Two other members of the *INK4*-cell cycle inhibitor family, p18^{INK4C} and p19^{INK4D} inhibit the activity of cyclin D-bound cdks by binding to cdk4 or cdk6.^{16–18} The genes for p18^{INK4C} and p19^{INK4D} are located at 1p32 and 19p13, respectively.^{16,17} Although chromosome 1p32 is frequently rearranged in certain solid tumors, almost no tumors with alterations of the p18^{INK4C} gene have so far been identified with the rare exception of some childhood T cell lineage ALL.^{11,19,20} No rearrangements or deletions of the p19^{INK4D} gene were observed in pediatric ALL with translocations involving chromosome 19p13.²¹ We have found p19^{INK4D} to be altered in a minority of lymphomas and sarcomas (unpublished observation).

Cdk inhibitors might play an important role not only as tumor suppressor genes but also in development and cellular differentiation. Expression of p21^{WAF1} during embryogenesis in a subset of amitotic cells has indicated that it may function as an inducible growth inhibitor during development.²² We, as well as others, have shown that p53-independent upregulation of p21^{WAF1} expression occurs during induced terminal differentiation of hematopoietic cells.^{23,24} In contrast to the p21^{WAF1}/p27^{KIP1}/p57^{KIP2} class of CKIs, less is known about expression and regulation of the G₁-class of CKIs (p15^{INK4B}, p16^{INK4A}, p18^{INK4C}, p19^{INK4D}) during cellular differentiation. *In vitro* experiments revealed relatively constant levels of p16^{INK4A} throughout the cell cycle with a minor peak in S-phase, whereas p15^{INK4B} expression was upregulated in human keratinocytes by transforming growth factor β (TGF β 1).^{9,25} In normal tissue, constitutive expression of p16^{INK4A} was barely detectable or absent, whereas p15^{INK4B} expression was detected by RT-PCR in a wide array of different tissues (lung, prostate, brain, intestine).²⁶ p18^{INK4C} and p19^{INK4D} mRNA are constitutively expressed in different human tissues in highly variable levels which indicates a tissue-specific regulation and the possible involvement of p18^{INK4C} function in cellular differentiation and development.^{16–18}

We have shown previously, that in contrast to ALL, alterations of the p16^{INK4A} gene are rare in acute myeloid leukemia (AML), and transcripts for p16^{INK4A} are constitutively

expressed in the majority of cases.²⁷ To gain more insight into the role of G₁-CKIs in human myeloid hematopoiesis, we examined their constitutive expression in normal granulocytes and monocytes, myeloid leukemic cell lines as well as in cells from AML patients and their regulation during chemically induced myeloid differentiation. Our results suggest a divergent and complex role of G₁-specific cell cycle inhibitors during myeloid differentiation.

Materials and methods

Reagents

Phorbol-12-myristate-13-acetate (PMA) (Sigma, St Louis, MO, USA) was dissolved in acetone to a stock solution of 1 mM and stored at -20°C. All-*trans* retinoic acid (Sigma) was dissolved in 95% ethanol to stock concentrations of 1 mM and stored at -20°C. Actinomycin D (Fluka, Buchs, Switzerland) was kept as stock solution (2 mM) dissolved in dimethyl sulfoxide (DMSO; Merck, Darmstadt, Germany). Final concentrations of DMSO never exceeded 0.5% (vol/vol). Recombinant human transforming growth factor β 1 (TGF β 1) was purchased from R&D Systems (Minneapolis, MN, USA).

Cell samples

The following human myeloid leukemia cell lines were cultured in McCoy's medium (Gibco, Grand Island, NY, USA) supplemented with 10% fetal bovine serum (FBS; Gibco, Grand Island, NY, USA) in a humidified atmosphere containing 5% CO₂ at 37°C: HL-60 (promyelocytic), KG-1 (myeloblastic) and KCL-22 (myeloblastic, Ph⁺), U-937 (monoblastic), HEL and K-562 (erythroid/myeloblastic, Ph⁺). All cell lines were obtained from the American Type Culture Collection (ATCC; Rockville, MD, USA). In addition, normal diploid human lung fibroblasts (WI-38) and immortalized human keratinocytes (HaCat; Dr NE Fusenig, German Cancer Research Center, Heidelberg, Germany) were used. Differentiation of HL-60 cells was assessed by morphology, esterase staining and by flow-cytometric analysis of expression of lineage-specific surface antigens (CD11b/CD11c). After exposure to PMA, RA or DMSO, >85% of HL-60 cells displayed monocytic or granulocytic features, respectively. Fresh bone marrow or peripheral blood cells were obtained at initial diagnosis from 16 AML (diagnosis established according to the French-American-British FAB classification and by immunophenotyping). Mononuclear cells from leukemic samples were separated by Ficol-Hypaque density gradients (1.077 g/l; Nyegaard, Oslo, Norway) and cryopreserved in liquid nitrogen until use. All leukemic mononuclear cell samples were composed of >95% blast cells as shown by morphology and specific esterase staining on cytopsin preparations. Neutrophils and monocytes were isolated from buffy coats of donor blood provided by the Swiss Central Laboratory Blood Transfusion Service, SRK, according to established methods.²⁸

RNA analysis

Northern blot analysis was performed as described.²³ Total RNA samples (10 μ g) were size-separated by an agarose-formaldehyde gel (1% wt/vol) and transferred to nylon membrane (Hybond-N; Amersham, Amersham, UK). Hybridization with random-primer ³²P-labeled probes (1-

2 \times 10⁶ c.p.m./ml hybridization solution) was performed for 16 to 24 h at 42°C. Filters were washed to a final stringency of 0.25% SSC/55°C to 0.1% SSC/65°C and exposed for 6 to 96 h at -70°C to X-ray films (3M; Trimax, Ferrara, Italy). Purified inserts were used as human cDNA probes: p21^{WAF1} (2.1 kb; BamHI/HindIII) from pCEP,⁵ p15^{INK4B} (0.5 kb, EcoRI) from pBS,⁹ p16^{INK4A} (0.9 kb; EcoRI) from pBS,⁸ p18^{INK4C} (0.5 kb; BamHI) from pUC118,¹⁶ p19^{INK4D} (1.3 kb; EcoRI) from pSP72,¹⁷ p24^{CD11} (0.8 kb; EcoRI) from p7B18-0,²⁹ p27^{KIP1} (1.3 kb; EcoRI) from pBS,⁶ myeloperoxidase (MPO; PstI) from pUC8,²³ and interleukin-8 (IL-8; EcoRI) from pBR322.²³ Normal human diploid fibroblasts (WI-38) and transformed human keratinocytes (HaCat) were used as controls where indicated. In the half-life experiments, autoradiograms were quantified by optical scanning using the ImageQuant software (version 3.3, Molecular Dynamics, Sunnyvale, CA, USA) on a personal computer.

RT-PCR analysis was performed using the Gene Amp RNA-PCR Kit (Perkin Elmer, Branchburg, NJ, USA). Briefly, 1 μ g of total RNA was reverse transcribed by MuMoLV reverse transcriptase (2.5 U) using random hexamers (2.5 μ M) in a final volume of 20 μ l. cDNA samples were 10-fold diluted and amplified in a nested PCR approach in a volume of 30 μ l containing 100 ng of DNA template, 50 mM KCl, 10 mM Tris-HCl (pH 8.3), 1.5 mM MgCl₂, 1 μ M of each primer, 100 μ M of each nucleotide, and 0.8 U Taq DNA Polymerase using the p15^{INK4B}-specific primers: first round: p15-s1 (exon 1-pos 263; 5'-gag tgt cgt taa gtt tac gg-3') and p15-as1 (exon 2-pos 791; 5'-ggg taa gaa aat aaa gtc gt-3') spanning a 528 bp fragment; and second round: p15-s2 (exon 1-pos 324; 5'-gga atg cgc gag aac aag ggc atg-3') and p15-as2 (exon 2-pos 789; 5'-gt aag aaa ata aag tcg ttg-3') spanning a 465 bp fragment.³⁰ PCR conditions: first round: 35 cycles at 94°C for 1 min, 50-58°C for 1 min, and 72°C for 1 min; 1 μ l of the product was then amplified in the second round: 40 cycles at 94°C for 1 min, 52°C for 1 min, and 72°C for 1 min, each round with a final elongation step for 5 min at 72°C.

Nuclear run-on assays were performed as described.²³ A total of 6 \times 10⁷ HL-60 cell nuclei, suspended in 100 μ l buffer consisting of 50 mM Tris-HCl, pH 8.3, 40% glycerol, 5 mM MgCl₂ and 0.1 mM EDTA were added to 100 μ l transcription buffer (20% glycerol, 100 mM KCl, 5 mM MgCl₂, 1 mM each of ATP, GTP, and CTP, 5 mM dithiothreitol, 100 μ Ci α -³²P-UTP (400 Ci/mmol) and 100 units RNasin). The elongated labelled transcripts were extracted and purified by the acid guanidinium thiocyanate phenol-chloroform procedure. RNA was dissolved in 2 ml hybridization buffer and hybridized to denatured plasmid cDNAs (p15^{INK4B}, p16^{INK4A}, p18^{INK4C}, p21^{WAF1}, p27^{KIP1}, MPO, IL-8 and pcDNA1 (internal control) immobilized on nitrocellulose membranes (5 μ g per dot) at 42°C for 72 h. Membranes were washed for 20 min at 65°C in 0.2 \times SSC/0.1% SDS and in 0.1 \times SSC/0.1% SDS. Following treatment with 10 μ g/ml RNase A at 37°C for 1 h, filters were washed in 2 \times SSC for 20 min at room temperature and then exposed to X-ray film at -70°C. Quantification of the ³²P-labelled transcripts hybridized to plasmid DNAs was performed by analysis with a Molecular Dynamics PhosphorImager using the ImageQuant software (ImageQuant (version 3.3) Molecular Dynamics, Sunnyvale, CA, USA).

Western blot analysis

Laemmli buffer-solubilized total cellular protein (50 μ g) was separated on a 18% SDS-polyacrylamide gel. Equal loading

was checked by Coomassie staining of the gel and BSA-standard calibration. After transfer to nitrocellulose, p15^{INK4B} and p18^{INK4C} proteins were detected by enhanced chemoluminescence following the manufacturer's instructions (ECL; Amersham). Nonspecific binding was blocked overnight with dried fat milk in TBS-T (tris-buffered saline, Tween 20) according to the manufacturer's protocol (Santa Cruz, Santa Cruz, CA, USA). Two polyclonal, rabbit anti-human p18^{INK4C} (N-20) specific antibodies and anti-human p15^{INK4B} (K-18) (Santa Cruz) were used at a dilution of 1:200–1:500, respectively.

Cell cycle analysis

Percentage of cells in G₀/G₁ stage of the cycle was determined by cell cycle analysis using the Cycle Test Plus DNA reagent kit (Becton Dickinson Immunocytometry Systems, San Jose, CA, USA) according to the manufacturer's instructions on a FACScan flow cytometer (Becton Dickinson). Cell cycle distribution was then determined using the MultiCycle software (Phoenix Flow Software Products, San Diego, CA, USA) on a personal computer.

Results

Constitutive mRNA expression of G₁ cell cycle inhibitors in human normal and malignant myeloid cells

We and others have shown that in contrast to ALL, genomic alterations of p16^{INK4A} are very rare events in AML and low levels of p16^{INK4A} mRNA are constitutively expressed in most cases.^{27,31} We now compared constitutive expression of the other members of the G₁-CKIs family (p15^{INK4B}, p18^{INK4C}, p19^{INK4D}) mRNA in normal granulocytes and monocytes, seven myeloid cell lines (HL-60, K-562, KG-1, KCL-22, U-937, HEL, DAMI) and in 16 fresh cell samples (>95% blastic cells) from AML-patients (Table 1). No p15^{INK4B} transcripts were found in any of these cell samples as assessed by Northern blot analysis. Yet, by nested RT-PCR analysis, low levels of transcripts for p15^{INK4B} were detected in four of seven cell

Table 1 Constitutive expression of G₁ CKIs (p15^{INK4B}, p18^{INK4C}, p19^{INK4D}) mRNA in human myeloid cells

Myeloid cells	p15 ^{INK4B}		p18 ^{INK4C} Northern blot	p19 ^{INK4D} Northern blot
	Northern blot	RT-PCR		
Granulocytes	–	+	– ^a	+
Monocytes	–	+	– ^a	+
HL-60	–	+	+	+
HEL	–	–	+	+
KG-1	–	+	+	+
K-562	–	–	+	+
U-937	–	+	+	+
KCL22	–	+	+	+
DAMI	–	–	+	+
AML (n = 16)	0/16	12/16	16/16	16/16

^aRT-PCR positive.

lines known to contain a normal p15^{INK4B} gene.²⁷ In contrast high levels of constitutively expressed p18^{INK4C} and p19^{INK4D} mRNA was found in all seven cell lines by Northern blotting. Similarly, constitutive expression of p15^{INK4B} transcripts was detected in 12 of 16 AML cases only by RT-PCR analysis. All patients samples showed higher levels of expression of p18^{INK4C} and p19^{INK4D} mRNA as determined by Northern blotting. No aberrant transcripts were seen and there was no correlation with morphological AML subtypes. In normal granulocytes and monocytes p15^{INK4B} and p18^{INK4C} mRNA expression were detectable by RT-PCR only (Figure 1, Table 1). However, in both cell types transcripts for p19^{INK4D} were easily detectable by Northern blotting. p21^{WAF1} mRNA was noted in normal monocytes in Northern blots, but not in granulocytes where RT-PCR experiments yielded positive results (not shown).

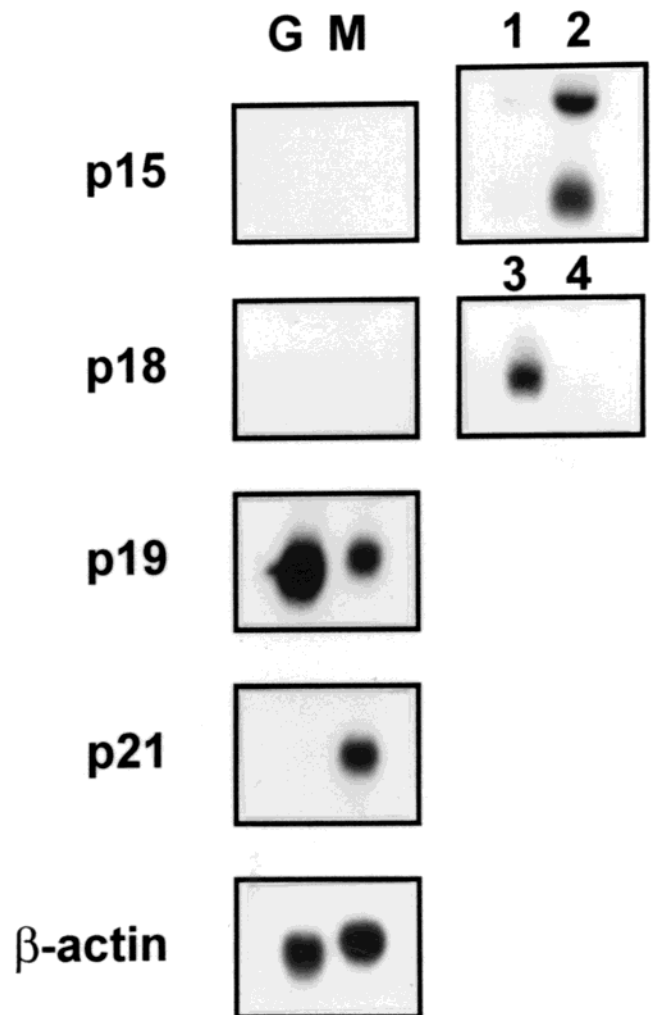


Figure 1 Constitutive expression of human CKI mRNA (p15^{INK4B}, p18^{INK4C}, p19^{INK4D} and p21^{WAF1}) expression in normal human myeloid cells. Northern blot analysis using total RNA (10 μg) of freshly separated granulocytes (G) and monocytes (M). TGF-β1, (2 ng/ml, 24 h) treated human keratinocytes (HaCat) served as positive control for p15^{INK4B} expression; HL-60 cells treated with PMA (60 ng/ml, 24 h) were used as control for p18^{INK4C} expression (see also Figure 2). 1 = HaCat uninduced; 2 = HaCat, TGFβ induced; 3 = HL-60 cells uninduced; 4 = HL-60, PMA induced.

Disparate expression of G₁ cell cycle inhibitors during leukemic myeloid differentiation

We examined in the HL-60 cell line model whether the G₁ cell cycle inhibitors p15^{INK4B}, p16^{INK4A}, p18^{INK4C} and p19^{INK4D} might be regulated during myeloid cell differentiation. HL-60 cells used in this study do not contain a deletion in the p15^{INK4B} and p16^{INK4A} genes.^{27,31,32} HL-60 cells were induced to differentiate toward monocytic-like cells by treatment with phorbol ester (PMA; 60 ng/ml). The differentiation process was linked to growth arrest as shown by cell cycle analysis. A significant fraction of the cells was growth-arrested in G₁/G₀ stage of the cycle after 24 h (G₁: 82.7% (vs uninduced control cells: 67%); G₂/M: 17.0% (vs 5.3%); S: 0.3% (vs 27.7%)) and remained growth-arrested at 96 h of PMA treatment. In parallel to the increased fraction of growth-arrested cells, p15^{INK4B} mRNA expression was induced, reaching detectable levels after 12 h (not shown) which peaked after 24 to 48 h and slowly declined again after 72 to 96 h (Figure 2). A temporary two-fold increase of p16^{INK4A} transcript level was confined to 24 and 48 h of PMA treatment. In contrast, a marked decrease of both p18^{INK4C} transcripts (1.9 kb and 1.1 kb) was seen after 24 h of PMA treatment with very low levels after 96 h. An additional transcript of 1.5 kb was transiently detected after 24 h of PMA treatment. Similarly, transcripts for p19^{INK4D} (1.3 kb) were downregulated after 24 h and remained low up to 96 h.

We also compared the expression pattern of G₁-CKIs with that of other human CKIs during monocytic differentiation of HL-60 cells by rehybridization of the Northern blots with cDNA probes encoding human p21^{WAF1}, p27^{KIP1} and p24^{CD11} (Figure 2). Expression of p24^{CD11} (another small cell-cycle inhibitory protein which was initially characterized to delay G₁ progression by dephosphorylation of cdk2) was markedly downregulated.²⁹ In contrast, expression of p21^{WAF1} was considerably induced as shown previously.²³ No marked changes of mRNA levels for p27^{KIP1} were seen. As control for the induced myeloid differentiation process, the blot was rehybridized with a myeloperoxidase (MPO) cDNA which showed the expected marked decrease of MPO mRNA expression (not shown).

To assess whether disparate regulation of p15^{INK4B} and p18^{INK4C} monocytic differentiation of HL-60 cells might occur at the protein level, Western blot analyses were performed. As seen in Figure 3, protein levels for p15^{INK4B} showed an increase upon treatment with PMA while p18^{INK4C} protein decreased and became undetectable after 24 h. Taken together, these data show a disparate expression pattern of various cell-cycle inhibitors at the RNA and protein levels during induced monocytic differentiation in HL-60 cells.

Differentiation of HL-60 cells towards granulocyte-like cells by DMSO (1.25%, 0–96 h) was also associated with a G₁-arrest of the cycle (24 h: G₁: 88.2% (vs uninduced control cells: 54.8%); G₂/M: 3.1% (vs 10.3); S: 8.7% (vs 29.9%)). This process was paralleled by an approximately five- to 10-fold increase of p18^{INK4C} and p19^{INK4D} mRNA levels after 16 to 96 h of DMSO exposure, whereas levels for p16^{INK4A} mRNA remained low (Figure 4) and no transcripts for p15^{INK4B} were found at any time points. Similar results were found when the cells were induced by all-trans retinoic acid. In order to exclude whether the observed changes simply represent 'side products' of pleiotropic effects of differentiation agents such as PMA rather than genuine biological phenomena during cellular differentiation, we studied three myeloid cell lines other than HL-60 (K-562, HEL, DAMI) with different or no

differentiation responses to either PMA or DMSO. In K-562 cells which contain a homozygous deletion of the p15/16 locus,²⁷ downregulation of p18^{INK4C} mRNA was seen after 24 h of PMA exposure (60 ng/ml) whereas in contrast to HL-60 cells p19^{INK4D} transcript levels remained unchanged. Similar results were obtained with the megakaryoblastic DAMI cell line. In HEL cells that also display a deleted INK4 locus²⁷ PMA exposure resulted in a temporary decrease of p18^{INK4C} expression. Similarly, exposure of these cells to TGF-β1 (2 ng/ml, 24 h) resulted in a marked decrease of p18^{INK4C} mRNA levels. Treatment of K-562 and HEL cells with DMSO (1.25%) which does not induce differentiation in these particular cells showed no effect on p18^{INK4C} and p15^{INK4B} expression. These results indicate that the observed changes in HL-60 cells cannot simply be attributed to nonspecific PMA or DMSO effects.

To assess further that the observed disparate regulation of p15^{INK4B} and p18^{INK4C} expression during PMA-induced differentiation is not an HL-60 cell-specific or a mere cell line phenomenon, we examined short-time cultured blast cells from two AML patients (1, AML M₂; and 2, AML M₁) neither of which contained any alterations of the 9p21 locus. Similar to HL-60 cells, PMA treatment induced differentiation of the cells associated by morphologic changes (lammellopodieae formation, cell adhesion) and growth arrest. As shown in Figure 5, p18^{INK4C} mRNA expression markedly decreased upon treatment with PMA for 24 and 48 h in both patients, as detected by Northern blotting. Transcripts for p15^{INK4B} in cells from patient 2 were slightly upregulated, while those of patient 1 were only detected by RT-PCR. p21^{WAF1} mRNA was markedly upregulated in both cell samples. These data show that similar to the HL-60 cell line model, two G₁ cell cycle inhibitors are disparately expressed in blastic cells from myeloid leukemia patients upon phorbol ester-induced myeloid differentiation.

Regulation of p15^{INK4B} and p18^{INK4C} mRNA expression during differentiation of HL-60 cells

To examine whether changes of p15^{INK4B} and p18^{INK4C} mRNA expression might depend on *de novo* protein synthesis, experiments were performed with cycloheximide (CHX). In HL-60 cells, CHX at 20 μg/ml blocks >95% of protein synthesis as determined by ³⁵S-methionine incorporation. Cells were exposed to PMA (60 ng/ml) or CHX alone, or were preincubated for 1 h with CHX and then treated with PMA for 6, 15, and 24 h. As shown in Figure 6, upregulation of p15^{INK4B} and downregulation of the 1.9 kb transcript of p18^{INK4C} were both dependent on *de novo* protein synthesis. The smaller p18^{INK4C} transcript (1.1 kb) which is barely expressed in uninduced cells was superinduced by CHX alone and by CHX in combination with PMA. Western blot analysis showed a decrease of p18^{INK4C} expression which suggests that the formed protein is encoded by the 1.9 kb transcript (not shown). In contrast to p18^{INK4C}, both p15^{INK4B} transcripts were regulated in parallel and no superinduction was seen in the absence of ongoing protein synthesis.

To determine whether alteration of the transcription rate might be responsible for the increase of p15^{INK4B} and the decrease of p18^{INK4C} mRNA expression after PMA treatment, nuclear run-on analysis was performed. Although HL-60 cells express p18^{INK4C} constitutively at easily detectable levels, its transcription rate was very low and no profound decrease was observed after treatment with either PMA or DMSO for 48 h (Figure 7). Similarly no

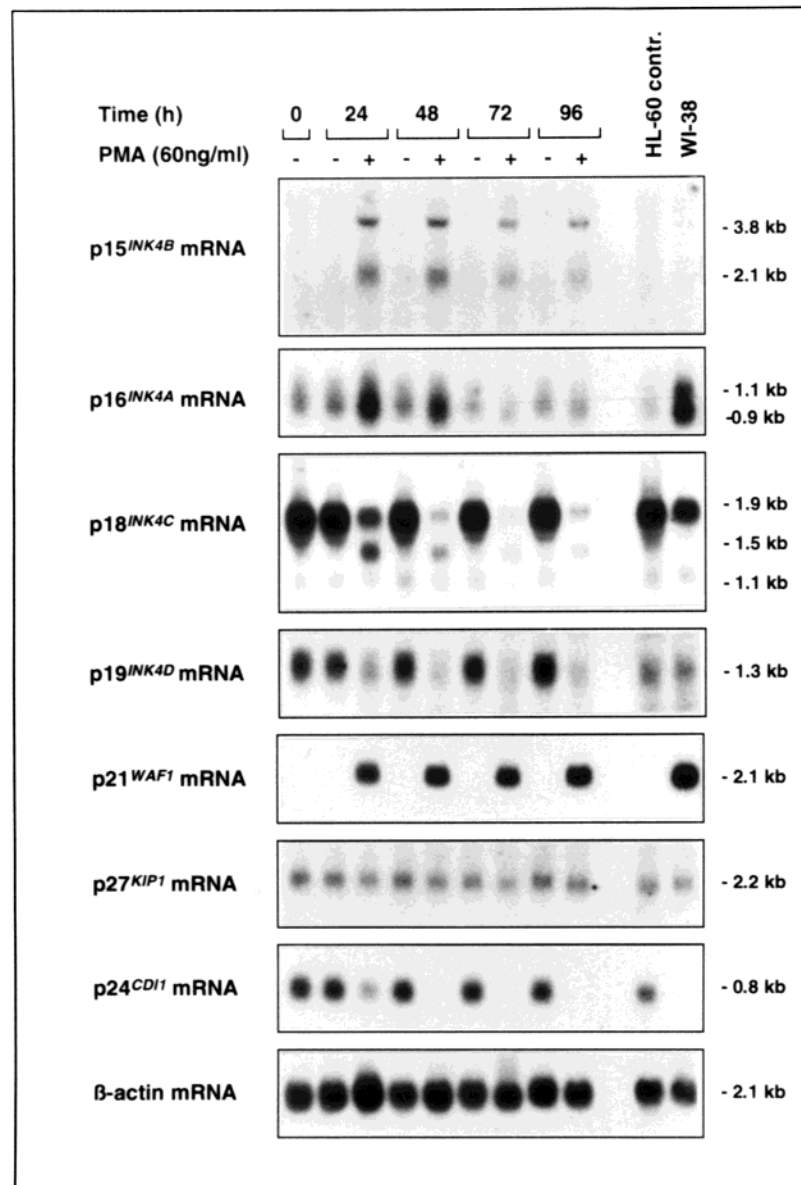


Figure 2 Comparative analysis of human CKIs (p15^{INK4B}, p16^{INK4A}, p18^{INK4C} and p19^{INK4D}, vs p21^{WAF1}, p27^{KIP1} and p24^{CDI1}) mRNA expression during differentiation of HL-60 cells towards the monocytic lineage when induced by PMA. Northern blot analysis of RNA levels for CKIs in HL-60 cells after treatment with PMA (60 ng/ml) for 0 to 96 h as indicated. Analysis was performed by blotting 10 μg total RNA per lane and sequential hybridization with specific cDNA probes. Rehybridization of the blot with a β-actin cDNA demonstrates integrity and equivalent RNA-loading per lane.²³

changes in the rate of transcription were seen for p15^{INK4B} in uninduced and induced cells. MPO and IL-8 used as internal controls were markedly down- and upregulated after PMA or DMSO treatment, respectively, as described previously.²³

To determine whether the stability of p15^{INK4B} and p18^{INK4C} transcripts might change after PMA stimulation of the cells, half-life ($t_{1/2}$) studies were performed by blocking overall transcription with actinomycin D (10 μg/ml). In the absence of PMA, no p15^{INK4B} transcripts could be detected even after long exposure of the autoradiograph, and thus no estimation of the half-life was possible. After 12 h of PMA treatment, the

estimated half-life of p15^{INK4B} transcripts was approximately 1 h and markedly increased after 21 and 48 h to approximately 3.5 h and 4.7 h, respectively (Figure 8a). Estimated half-life of constitutively expressed p18^{INK4C} mRNA was approximately 3 h. Exposure to PMA for 12 h did not change $t_{1/2}$ of p18^{INK4C} mRNA, but exposure for 21 h to PMA decreased the half-life from 3 h to 1.8 h (Figure 8b). Taken together, these data indicate that regulation of p15^{INK4B} and p18^{INK4C} mRNA expression during PMA-induced differentiation of HL-60 cells is dependent on *de novo* protein synthesis and is due to altered mRNA stability.

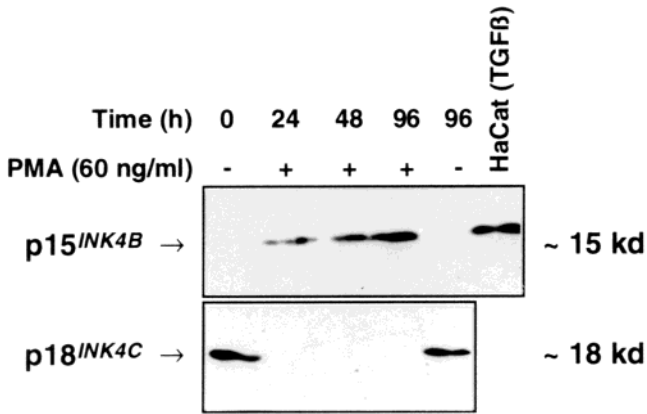


Figure 3 Assessment of p15^{INK4B} and p18^{INK4C} protein levels during PMA-induced differentiation of HL-60 cells. Total cellular protein (50 μg) solubilized in Laemmli-buffer of HL-60 cells treated with PMA (60 ng/ml) for 0 to 96 h was separated on a 18% SDS-polyacrylamide gel and transferred to a nitrocellulose filter. TGF-β-treated HaCat cells served as positive control.

Discussion

We show that members of the G₁-CKI family (p15^{INK4B}, p18^{INK4C}, p19^{INK4D}) are constitutively expressed in the majority of myeloid leukemic cell lines as well as in AML patients' cell samples at variable levels.^{11,27,31} Whereas transcripts levels of p15^{INK4B} were only detectable by RT-PCR, higher constitutive expression of p18^{INK4C} and p19^{INK4D} mRNA was seen in all specimens. This suggests that, at least in AML, these molecules do not play a role as tumor suppressor genes but might exert additional functions in proliferating myeloid cells.

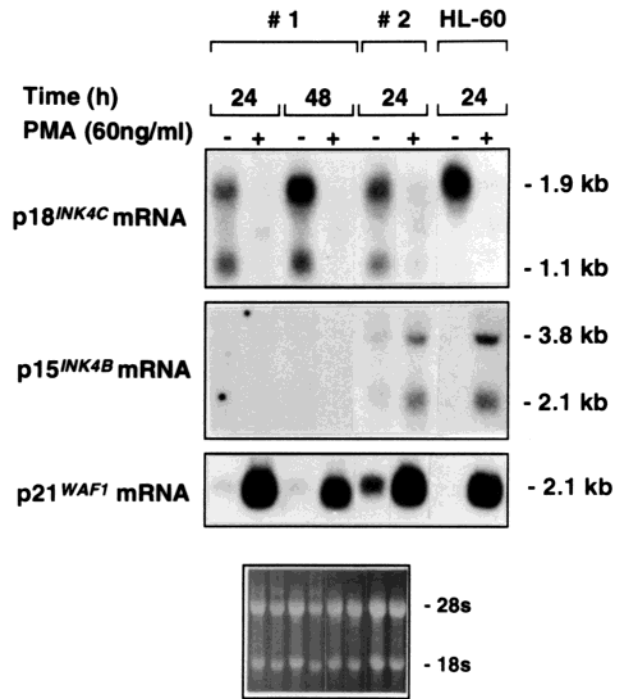


Figure 5 Expression of p15^{INK4B} and p18^{INK4C} (vs p21^{WAF1}) mRNA in PMA-induced differentiation of blastic cells from two AML patients. Northern blot analysis of RNA levels for p15^{INK4B}, p18^{INK4C} and p21^{WAF1} in myeloid leukemic cells from two patients (1 and 2) after treatment with PMA (60 ng/ml) for 0 to 48 h as indicated. PMA-treated HL-60 cells served as controls.

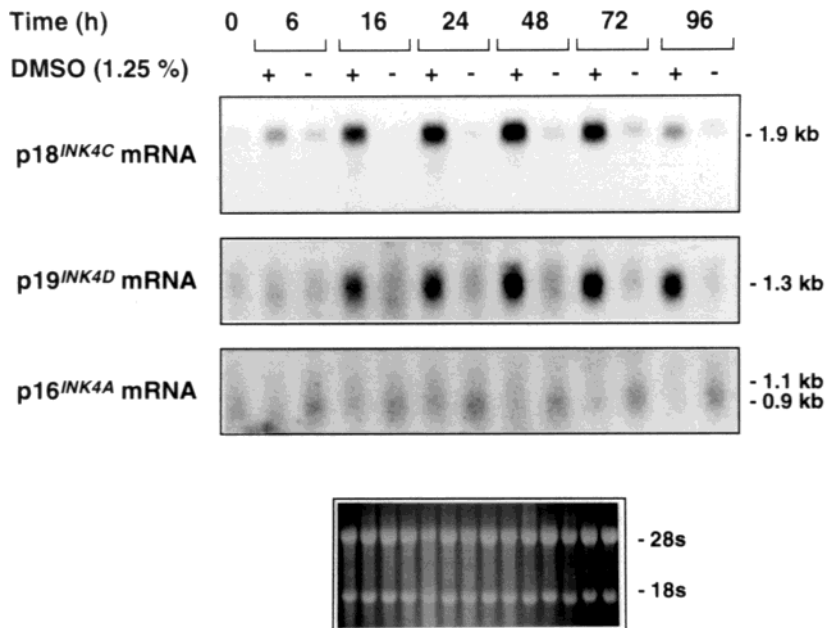


Figure 4 Comparative analysis of G₁-CKIs (p16^{INK4A}, p18^{INK4C}, and p19^{INK4D}) mRNA expression during differentiation of HL-60 cells towards the granulocytic lineage when induced by DMSO. Northern blot analysis of RNA levels for p16^{INK4A}, p18^{INK4C} and p19^{INK4D} in HL-60 cells after treatment with DMSO (1.25% v/v) for 0 to 96 h as indicated. Analysis was performed by blotting 10 μg total RNA per lane and sequential hybridization with the respective CKI cDNA probes.

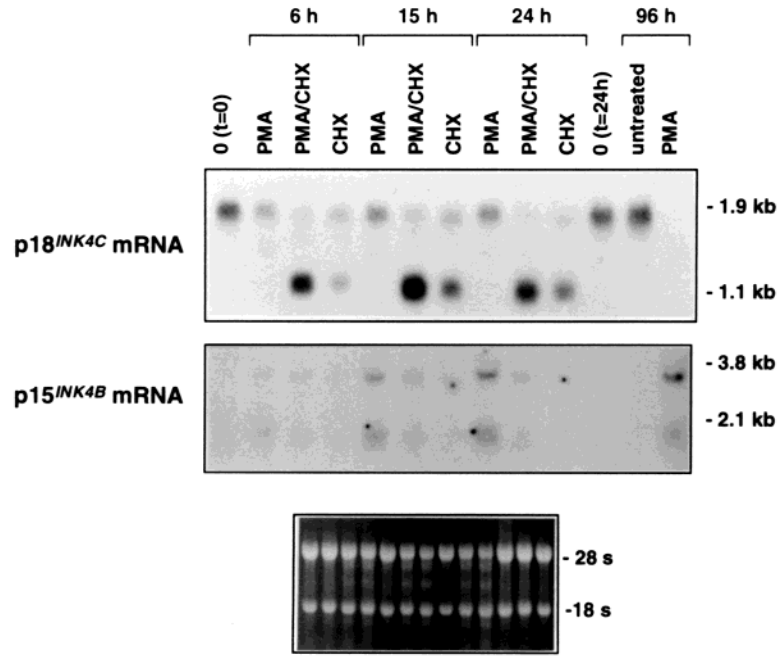


Figure 6 Influence of ongoing protein synthesis on the PMA-induced expression of p15^{INK4B} and p18^{INK4C} mRNA. Northern blot analysis in HL-60 cells which were pretreated for 1 h with the protein synthesis inhibitor cycloheximide (CHX; 20 μg/ml) and then with PMA (60 ng/ml) as indicated. Analysis was performed by blotting 10 μg total RNA per lane and sequential hybridization with specific CKI cDNA probes.

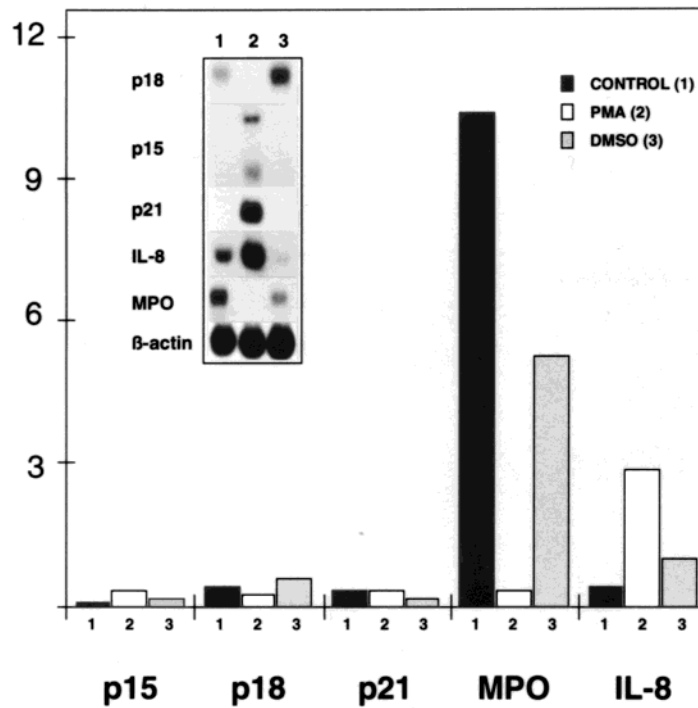


Figure 7 Rate of p15^{INK4B} and p18^{INK4C} (vs p21^{WAF1}) transcription in HL-60 cells exposed to PMA. After culturing HL-60 cells for 15 h with or without addition of PMA (60 ng/ml), nuclear run-on experiments were performed. ³²P-labeled RNA transcripts were hybridized to p15^{INK4B}, p18^{INK4C}, p21^{WAF1}, MPO, and IL-8 cDNAs immobilized on nylon filters. The bars are showing the relative levels of labelled transcripts as determined by Phosphorimager analysis. Lanes: 1: untreated cells; 2: PMA (48 h), and 3: DMSO (48 h). Plasmid vector DNA (pUC) served as control for nonspecific binding of radiolabeled mRNA (not shown). The small insert represents a Northern blot analysis of separate aliquots of the cells used in the nuclear run-on experiment showing changes of mRNA levels for p15^{INK4B}, p18^{INK4C} and p21^{WAF1}.

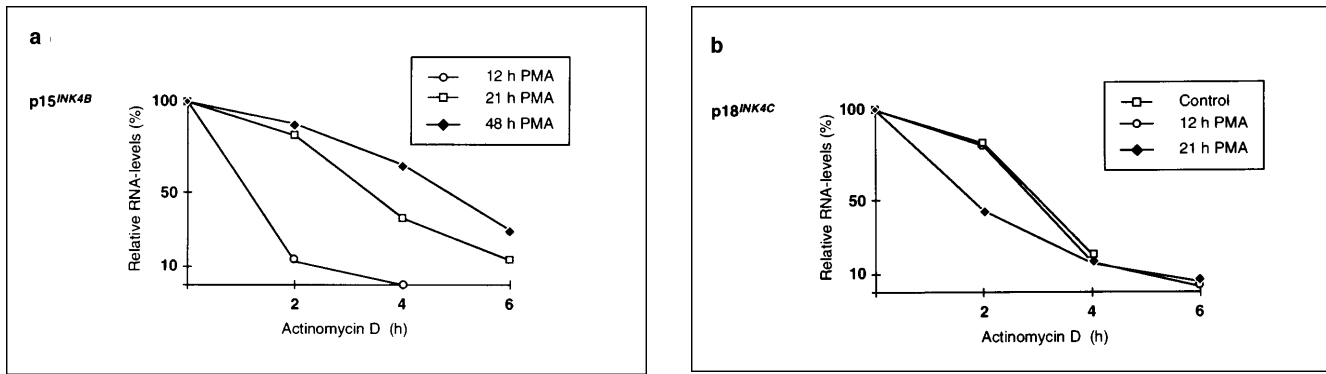


Figure 8 (a) p15^{INK4B} and (b) p18^{INK4C} mRNA stability during PMA-induced differentiation of HL-60 cells. HL-60 cells were induced for 12, 21 and 48 h with PMA (60 ng/ml) and then actinomycin D (10 μg/ml) was added for up to 6 h. Analysis was performed by blotting of 10 μg total RNA per lane and hybridization with specific CKI cDNA probe. Relative RNA levels were estimated after optical scanning of the autoradiograms using the ImageQuant software.

Induced terminal differentiation of transformed hematopoietic cells involves both the cessation of DNA synthesis and the expression of genes crucial for the differentiated phenotype. The decision as to whether a cell continues to divide or withdraws from the cell cycle and differentiates, occurs during the G₁-phase.³³ Induction of growth arrest of various cell types in G₁-phase of the cycle by several factors (eg TGF-β₁, phorbol esters) is linked to upregulation of p21^{WAF1}, p27^{KIP1} and p15^{INK4B}, all of them blocking cdk-cyclin complex activity in G₁.^{9,23,24} In our experiments, HL-60 cells induced to differentiate towards monocytic-like cells accumulated in the G₁/G₀ phase.³⁴ Growth arrest was accompanied by upregulation of p15^{INK4B} and to a much lesser extent of p16^{INK4A} whereas p18^{INK4C} and p19^{INK4D} were downregulated. Disparate expression patterns of CKIs during myeloid differentiation do not seem to be restricted to HL-60 cells. Downregulation of p18^{INK4C} was also observed during TGFβ₁-induced erythroid differentiation of K-562 cells and PMA-induced HEL cells. Since K-562 and HEL do not express p15^{INK4B} and p16^{INK4A} because of homozygous deletion in the 9p21 locus, this finding indicates that disparate regulation of p15^{INK4B} and p18^{INK4C} are independent events. The observed disparate regulation seems not to be cell line-restricted since a similar expression pattern of G₁-CKIs was seen upon PMA-induced differentiation of cultured blasts from two AML patients. These findings indicate that disparate regulation of G₁-CKIs is a common event during differentiation of myeloid leukemic cells. However, as recently reported by Liu *et al*,³⁵ induction of U-937 cells by 1,25 dihydroxyvitamin D₃ to differentiate into monocyte/macrophages was associated with an increase of p21^{WAF1}, p27^{KIP1}, p15^{INK4B}, p16^{INK4A} and p18^{INK4C} expression. This suggests that the recruitment of G₁-CKIs upon treatment with a differentiation-inducing agent might be primarily dependent on the inducing factor and the signaling pathway activated by this agent. In addition, it also suggests a cooperation between various CKIs to induce cell cycle arrest as demonstrated for mink lung epithelial cells and human keratinocytes.³⁶

Given the fact that overexpression of G₁-CKIs is able to inhibit cellular growth by blocking the cycle in the G₁/G₀ stage, their disparate expression pattern during myeloid differentiation is somewhat surprising. It might reflect their expression pattern during the normal cell cycle. Whereas a peak of p18^{INK4C} and p19^{INK4D} mRNA expression was

observed at the G₁/S boundary, maximum p21^{WAF1} expression was detected in an earlier G₁ stage of the cycle.^{17,18,37} Thus this suggests that some of the human CKIs (eg p18^{INK4C}, p19^{INK4D}) might play an important role in controlling the G₁-S transition of rapidly proliferating cells whereas others (eg p21^{WAF1}, p27^{KIP1}, p15^{INK4B} and presumably p16^{INK4A}) might be responsible for G₁ arrest enabling the cells to further differentiate. The differentiation-associated CKI expression pattern we observed seems not to be limited to hematopoietic cells since a recent study demonstrated an increase of p15^{INK4B} and p16^{INK4A} mRNA and protein in association with terminal neuronal differentiation of NT2 teratocarcinoma cells.³⁰ p15^{INK4B} might be unique among human G₁-CKIs since it was only found to be expressed at high levels in growth-arrested cells.^{26,30}

As opposed to monocytic differentiation, granulocytic differentiation of HL-60 cells was associated with an increase of p18^{INK4C} and p19^{INK4D} expression at growth arrest, while no induction of p15^{INK4B} transcripts occurred and p16^{INK4A} expression remained at low levels. These findings suggest a certain lineage specificity of CKI expression during HL-60 differentiation which has also been reported for other genes involved in cell cycle regulation. The central kinases (cdc2, cdk2, cdk4) were downregulated during induced differentiation of HL-60 cells towards both monocytes or granulocytes, while their regulatory cyclins were differently regulated. For example during monocytic differentiation the expression of cyclin D1 was highly upregulated. In contrast, cyclin D1 expression levels remained unchanged or became downregulated during granulocytic differentiation.^{38,39}

Our experiments further revealed that p15^{INK4B} and p18^{INK4C} were regulated during differentiation of HL-60 cells at the post-transcriptional level similar to p21^{WAF1}.²³ Rapid stabilization of p21^{WAF1} mRNA occurred within 90 min of PMA exposure, whereas the observed regulation of p15^{INK4B} and p18^{INK4C} mRNA appeared to be a late effect (24–48 h) and was inhibited by protein synthesis inhibitors. However, these findings also indicate that the modified expression of p15^{INK4B} and p18^{INK4C} genes might also be a consequence of an already established different cell phenotype and may not be related to the differentiation process itself. On the other hand, and in contrast to p21^{WAF1} and p27^{KIP1}, which act as primary 'immediate-early' growth brakes directing the cells towards cell cycle exit, G₁-CKIs might be involved in later events dur-

ing differentiation.^{23,35} In conclusion, disparate expression and regulation of the functionally and structurally highly related G₁-CKIs, family (p15^{INK4B}, p18^{INK4C}, p19^{INK4D}) indicates different roles of these genes during myeloid differentiation which might also depend on the resulting cellular phenotype as well as on the agent used to initiate the process.

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