

Sustained nuclear localization of p21/WAF-1 upon growth arrest induced by contact inhibition

Michelle G. Ritt^{a,1}, Jocelyne Mayor^{b,2}, John Wojcieszyn^{b,3}, Roger Smith III^b,
Claudia L. Barton^a, Jaime F. Modiano^{b,*}

^aDepartment of Small Animal Medicine and Surgery, Texas A&M University, College Station, TX 77843, USA

^bDepartment of Pathobiology, Texas A&M University, College Station, TX 77843, USA

Received 13 December 1999; received in revised form 27 March 2000; accepted 1 June 2000

Abstract

We assessed the expression and distribution of p21/Waf-1 in TLM1 melanoma cells that exhibit contact inhibition and require serum for growth. The growth stage of cells stimulated to enter the mitotic cell cycle synchronously and grow to confluence was characterized by distinct, yet consistent levels and patterns of distribution of p21/Waf-1. Significantly, sustained accumulation of p21/Waf-1 in the nuclear compartment was seen only after 4 days in culture when cell-to-cell contacts were established, leading to a diminished rate of cell growth. Overexpression of wild-type *waf-1* in melanoma cells reduced growth of subconfluent cells, decreased Cdk4 activity with a concomitant increase in hypophosphorylated Rb, and promoted cell death by apoptosis. The data support the premise that cell-to-cell contacts provide signals that mediate sustained nuclear localization of p21/Waf-1 leading to cell growth arrest; furthermore, an elevation in the activity of this protein can lead to apoptosis. © 2000 Elsevier Science Ireland Ltd. All rights reserved.

Keywords: Cell cycle; Contact inhibition; Cyclin-dependent kinase inhibitor; Melanoma

1. Introduction

The 21-kDa product of the *waf-1* gene, p21/Waf-1 is a prototypical cyclin-dependent kinase inhibitor (CDKI) that is important for cell growth regulation, particularly in cells of epithelial and neuroectodermal

origin [1–6]. This protein has multifactorial functions, including facilitating the assembly of cyclin-dependent kinase (Cdk) holoenzyme complexes [7], and modulating the activity of Cdks [8–11], stress-activated protein kinases [12], and the proliferating cell nuclear antigen, PCNA [13,14].

p21/Waf-1 expression is induced by p53 following stimuli that induce DNA damage [1], and it is also differentially regulated through the cell cycle, decreasing during the S phase [15,16]. This cell cycle-specific component of p21/Waf-1 expression is independent from regulation by p53 [16–18]. Moreover, the expression of p21/Waf-1 may be subject to additional mechanisms of control during the process of differentiation [19–21], and also may be modulated

* Corresponding author. AMC Cancer Research Center, 1600 Pierce Street, Denver, CO 80214, USA. Tel.: +1-303-239-3408; fax: +1-303-239-3560.

E-mail address: modianoj@amc.org (J.F. Modiano).

¹ Present address: Animal Hospital Center, 5640 County Line Place, Highlands Ranch, CO 80126, USA.

² Present address: Prodigene, Inc., 101 Gateway Boulevard, College Station, TX 77845, USA.

³ Present address: IHC Services, 3450 Highway 71 West, Smithville, TX 78957, USA.

by growth inhibitory signals such as those delivered by transforming growth factor β [22–25].

Despite the observations that p21/Waf-1 expression is subject to extensive regulation, these fluctuations in its expression do not always correlate with growth arrest. This suggests that the post-translational events such as the subcellular localization of the protein also could be relevant to its function. To further delineate the role of p21/Waf-1 in mammalian cell growth, we examined its expression and subcellular distribution in a melanoma cell line (TLM1). The data show that the expression of p21/Waf-1 varied as growth-arrested cells were stimulated to enter the mitotic cell cycle synchronously and grow to confluence. Sustained accumulation of p21/Waf-1 in the nuclear compartment was only seen following the establishment of cell-to-cell contacts. Our results also show that the overexpression of exogenous p21/Waf-1 was sufficient to inhibit growth and promote apoptosis of subconfluent melanoma cells. These results were recapitulated in a second, independent thymic epithelial cell line (Cf2Th). Taken together, the data indicate that targeting and maintaining p21/Waf-1 in the nuclear compartment may play a role in contact inhibition of cell growth, and support the possibility that functional loss of p21/Waf-1 may contribute to neoplastic transformation.

2. Materials and methods

2.1. Cell lines

The characteristics of the canine TLM1 melanoma cell line have been described previously [26,27]. These cells are diploid and retain normal properties of growth regulation, including contact inhibition and a serum requirement for growth. TLM1 cells synchronized in the G1 phase by serum-deprivation have a doubling time of 48–50 h following serum restimulation. The density of the TLM1 cells in culture at the initiation of each experiment was 1×10^5 cells/ml. Cf2Th canine thymic epithelial cells were obtained from the ATCC (Rockville, MD) and cultured under the same conditions as the TLM1 cells.

2.2. Reverse transcriptase–polymerase chain reaction (RT–PCR)

Total RNA was isolated as described [28]. The

expression of *waf-1* and β -actin mRNA was examined by RT–PCR. Degenerate primers for *waf-1* were designed from the conserved cyclin A-binding and Cdk2-binding domains of the human and murine *waf-1* cDNAs. The annealing temperature for amplification of the *waf-1* cDNA was 37°C for 5 cycles and 60°C for 25 cycles (1 min/cycle); for the β -actin cDNA it was 60°C for 30 1-min cycles. The oligonucleotide primers used for amplification of *waf-1* were 5'-CGNTGGAACCTNGACTTNGNC-3' (sense), and 5'-GAGTGNTAGAAATCTGTNANGCTGG-3' (antisense), which included positions 224–518 of the human cDNA and positions 218–509 of the murine cDNA, respectively, to produce an amplification product of 291–294 base pairs. Degenerate positions are indicated by the letter N. The product from these reactions was used to further amplify a 317 bp *waf-1* fragment using cDNA generated from a canine small intestine RNA pool, and also a clone from a canine cosmid library that may be syntenic to human chromosome 6. The PCR products were sequenced using a Perkin–Elmer Applied Biosystems model 377 automated sequencer (Perkin–Elmer, Norwalk, CT). The wild-type canine sequence for the primers is 5'-CGATGGAACCTT-GACTTCGTC-3' (sense), and 5'-GAGTGGTAGAA-ATCTGTAAGGCTGG-3' (antisense). The GenBank accession number for the partial cDNA sequence of canine *waf-1* (317 bp) is AF076469. The oligonucleotide primers used for amplification of β -actin were 5'-ATGTTTCGAGACGTTCAACACCCC-3' (sense) and 5'-GCCATCTCCTGCTCGAAGTCCAG-3' (antisense), based on the GenBank sequence for *C. familiaris* β -actin (AF021873) to produce an amplification product of 318 base pairs.

2.3. Fractionation of cells for immunoblotting

Cell pellets were frozen and stored below -80°C until the end of each experiment. To make whole cell lysates that included cytosolic and nuclear proteins, cell pellets were disrupted in a high salt buffer containing 300 mM sodium chloride, 50 mM Tris (pH 7.6), 0.5% Triton X-100, 1 mM *N*-ethylmaleimide, 2 $\mu\text{g}/\text{ml}$ aprotinin, and 1 $\mu\text{g}/\text{ml}$ leupeptin. Insoluble material was removed by centrifugation, and protein concentrations of the cell lysates were determined using the bicinchoninic acid method (Pierce, Rockford, IL) as described [27]. To obtain cytosolic

and nuclear fractions, cell pellets were incubated at 4°C in a buffer containing 10 mM HEPES (pH 7.8), 10 mM potassium chloride, 0.16 mM magnesium chloride, 1 mM DTT, 1 mM PMSF, 2 µg/ml aprotinin, and 1 µg/ml leupeptin, and 0.5% NP-40, and monitored microscopically until significant swelling was evident without lysis. The supernatant obtained after centrifugation at 10 000 × *g* for 5 min (cytosolic extract) was preserved by addition of 20% (v/v) glycerol. The nuclear pellets were then incubated in incremental volumes of a buffer containing 20 mM HEPES (pH 7.8), 1 mM magnesium chloride, 0.5 M sodium chloride, 0.5 mM EDTA, 1 mM DTT, 0.1 mM PMSF, 2 µg/ml aprotinin, and 1 µg/ml leupeptin, and 1% NP-40 to eliminate the viscosity of the preparation. Nuclear proteins were extracted by three cycles of rapid freezing and thawing, followed by incubation at 4°C for 20 min and centrifugation at 10 000 × *g* for 15 min. The resultant supernatant (nuclear extract) was preserved by addition of 20% (v/v) glycerol. Protein concentrations in the cytosolic and nuclear extracts were determined in aliquots resuspended in phosphate-buffered saline (PBS) with 1% sodium dodecyl sulfate (SDS), using the bicinchoninic acid method.

2.4. Immunoblotting

Cellular proteins were separated by SDS–polyacrylamide gel electrophoresis (PAGE) (using equivalent amounts of protein in each lane) and transferred to nitrocellulose membranes (Hybond, Amersham, Arlington Heights, IL) as described [27]. The antibodies used were anti-p21/Waf-1 rabbit polyclonal antibody (C19; Santa Cruz Biotechnology, Santa Cruz, CA), anti-Cdk4 polyclonal antibody (C-22; Santa Cruz Biotechnology), anti-β-tubulin antibody (clone 5H1; PharMingen, San Diego, CA), or anti-Rb monoclonal antibodies (G3-245; PharMingen and IF8; Santa Cruz Biotechnology). The reactivity of the anti-p21/Waf-1 antibody to canine p21/Waf-1 was confirmed by competition studies using the immunizing peptide (Santa Cruz Biotechnology). Immunoblots were digitized using an IS-500 Image Analyzer (Alpha Innotech Corp., San Leandro, CA), and band densities were quantified with the NIH Image v.1.59 software package.

2.5. Transfections

Delivery of exogenous DNA (transfection) was performed using cationic liposomes with the Doper reagent (Boehringer Mannheim, Indianapolis, IN) following the manufacturer's recommendations. The full-length human *waf-1* gene encoding p21/Waf-1 [8] was subcloned into the multiple cloning site of the pBK-RSV eukaryotic expression vector (Stratagene, La Jolla, CA). The pGL3 expression vector (Promega) encoding the firefly luciferase gene under the control of a constitutive promoter was used to monitor transfection efficiencies. Three micrograms of the pBK-RSV expression vector encoding the *waf-1* gene under the control of the RSV promoter and 0.5 µg of pGL3 were mixed with 9 µg of the cationic liposome reagent in 100 µl of HEPES-buffered saline and incubated for 15 min at 25°C. The empty pBK-RSV vector was used as a negative control. These mixtures were added in 1 ml of OptiMEM serum-free medium (Gibco BRL) to TLM1 cells (3×10^5) that had been adhered to six-well plates by an overnight incubation. The cell–DNA–liposome mixtures were incubated for 6 h at 37°C, followed by the addition of 1 ml of complete media and incubation overnight at 37°C. Expression of p21/Waf-1 and cell recovery were measured 48 h after transfections. At this time, cells were allowed to adhere to 96-well plates and DNA synthesis was measured 24 h later. The successful delivery of the exogenous p21/Waf-1 also was determined by immunoblotting.

2.6. Immunocytochemistry

TLM1 cells or Cf2Th cells (5×10^4 in 100 µl) were grown in replicate double-chamber slides (Nalge Nunc) for 5 days and harvested at 1-day intervals. Immunostaining and analyses were performed as described [26,27]. The antigen retrieval steps were omitted when immunocytochemistry was performed on transfected cells. Images were acquired using a Vanox AHBS3 microscope (Olympus Corp., Lake Success, NY) with an Optronics DEI-750 digital camera (Goleta, CA), and the accumulation of nuclear p21/Waf-1 was quantified morphometrically using the NIH Image software.

2.7. Luciferase activity

Twenty-four hours after the transfections, aliquots of 1×10^5 cells were washed and prepared for luciferase activity assays using a commercial kit (Promega) following the manufacturer's instructions, with the following modifications. The cells were lysed in 100 μ l of cell lysis buffer and centrifuged to remove the non-extractable material. Triplicate samples of 30 μ l of lysate from each condition were transferred to opaque 96-well white plates. The luciferase activity was quantified using an ML3000 microplate luminometer with automatic injectors (Dynatech, Chantilly, VA) in the enhanced flash mode, and the data were confirmed for each experiment using the cycle mode. No light output was detectable from TLM1 cells cultured under the conditions described above that were not transfected with the pGL3 (luciferase) vector. The light output from the pGL3-transfectants was normalized to a recombinant firefly luciferase standard curve (0–100 ng/ml). Values are expressed in relative light units (RLU).

2.8. Cellular proliferation

Proliferation of transfected TLM1 cells was evaluated by enumerating viable cells in culture and by DNA synthesis as described [29].

2.9. Cyclin-dependent kinase 4 assays

Cyclin-dependent kinase 4 (Cdk4) activity was determined as described [30]. Briefly, active Cdk4 complexes were immunoprecipitated from TLM1 cells using the anti-Cdk4 polyclonal antibody C-22 (Santa Cruz Biotechnology), and immune complex kinase assays were performed using [γ - 32 P]ATP and p56/Rb (a truncated recombinant Rb protein) as a substrate. The reactions were terminated by addition of SDS sample buffer and separated by SDS-PAGE.

2.10. Apoptosis

The fraction of cells with sub-G1 DNA content (less than two copies of DNA) was used to quantify apoptotic cell death in control cells or TLM1 Cells transfected with the p21/Waf-1 expression vector. Cells were harvested after 48 h in culture, washed in 150 mM sodium chloride, and permeabilized with

70% ethanol overnight. The cells were then washed once in 150 mM sodium chloride, resuspended in PBS containing 0.5% Tween-20 and 25 μ g/ml RNase A (Boehringer Mannheim) at 37°C for 30 min and stained with propidium iodide (25 μ g/ml) for 30 min in the dark at room temperature. DNA content was evaluated by flow cytometry as described [26].

2.11. Software

Figures were compiled from digitized images using Adobe Photoshop and Claris Draw software programs.

3. Results

3.1. Expression of p21/Waf-1 in synchronously growing TLM1 cells

We have previously described the growth characteristics of the TLM1 melanoma cell line [26]. After 3 days of culture in the absence of serum, approximately 90% of the TLM1 cells were in the G1 phase, as determined by DNA content analysis. When these serum-deprived cells were restimulated with serum, they entered the cell cycle rapidly (~40% of the cells were in the S, G2, or M phases of the cell cycle after 24 h). The cells proliferated for 4–5 days until they became confluent; by the sixth day of culture, 80–90% of the cells were in the G1 phase of the cell cycle. To assess the role of p21/Waf-1 in the control of TLM1 cell growth, we examined the expression of *waf-1* mRNA by RT-PCR and the expression of p21/Waf-1 protein by immunoblotting. The RT-PCR method used is not quantitative, thus the only interpretation that can be made is whether *waf-1* mRNA was present in the samples or not. The derivation of the PCR products from the *waf-1* message was confirmed by sequencing. There was 97% identity between this region of canine *waf-1* and human *waf-1*. Fig. 1 shows that *waf-1* mRNA was present in TLM1 cells throughout their growth cycle to confluence (days 1–5) as determined by amplification of a fragment of the predicted size by RT-PCR. A product of the same size was obtained from TLM1 cells that were transfected with human wild-type *waf-1* gene under the control of the RSV promoter

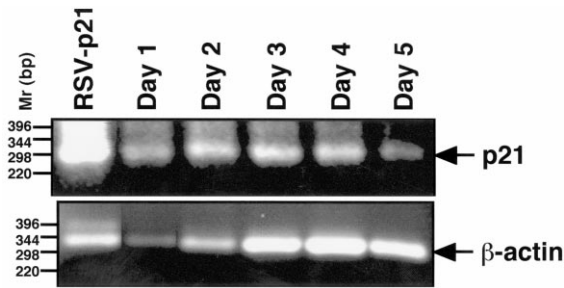


Fig. 1. Evaluation of *waf-1* mRNA expression by qualitative RT-PCR in growth-arrested TLM1 cells restimulated with serum. TLM1 cells were adhered to culture plates overnight in complete media and synchronized by serum deprivation for 3 days. Cells were restimulated with serum and harvested at 24-h intervals for RNA extraction. Asynchronously growing TLM1 cells were transfected with an expression vector encoding the human *waf-1* gene (RSV-p21). The expression of *waf-1* (p21) and β -actin mRNA was examined by RT-PCR. RT-PCR products were separated by electrophoresis in 1% agarose slab gels and visualized by staining with ethidium bromide. Oligonucleotide primers for *waf-1* included positions 224–518 of the human cDNA, and positions 218–509 of the murine cDNA, respectively to produce an amplification product of 291–294 base pairs. A PCR product of \sim 290 bp was amplified using the PCR primers for *waf-1* (top). A PCR product of the expected size (\sim 320 bp) was amplified using the β -actin primers (bottom).

(RSV-p21). The integrity of the TLM1 mRNA was confirmed by amplification of a β -actin fragment.

The steady-state levels of p21/Waf-1 protein accumulation were quantified by densitometric analysis of immunoblots. Lysates from TLM1 cells were prepared at 24-h intervals between days 0 and 5, normalized for protein content, and the proteins separated by one-dimensional electrophoresis. All the cellular proteins analyzed were markedly decreased in serum-starved cells. Fig. 2a shows that the levels of immunoreactive p21/Waf-1 increased during days 1 and 2 after serum restimulation. The band densities in the immunoblots were 5- to 8-fold greater on days 1 and 2 than on day 0 after normalization to the levels of β -tubulin, a structural protein expressed predominantly by cells of neuronal or neuroectodermal origin. However, p21/Waf-1 levels again dropped dramatically by day 3. The amount of p21/Waf-1 increased by \sim 5-fold again at day 4 (compared with days 0 and 3) as the cells became confluent, and remained elevated through day 5. To confirm that the immunoreactive 21-kDa protein was homologous to the p21/Waf-1 protein encoded by the *waf-1* gene, a human

waf-1 gene was introduced into TLM1 cells and the electrophoretic mobility of its gene product was compared to that of the endogenous canine p21/Waf-1 24 h after the transfection procedure. Fig. 2b shows that TLM1 cells transfected with a control plasmid encoding the bacterial β -galactosidase enzyme gene (Vector) had an immunoreactive 21-kDa protein

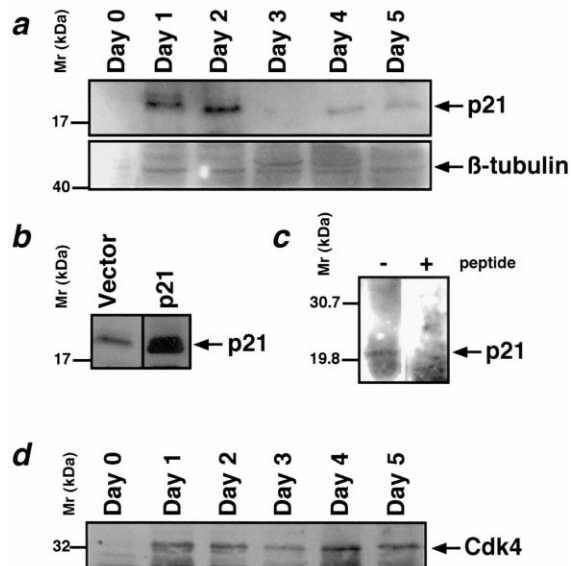


Fig. 2. Evaluation of p21/Waf-1 protein accumulation by immunoblotting in growth-arrested TLM1 cells restimulated with serum. (a) TLM1 cells were adhered to culture plates overnight in complete media and synchronized by serum deprivation for 72 h. Cells were restimulated with serum and harvested at 24-h intervals for protein extraction. Cells were detached, washed in serum-free PBS, and the cell pellets collected and frozen below -80°C . The accumulation of cellular p21/Waf-1 and β -tubulin proteins was determined by immunoblotting. Proteins for immunoblot analysis were separated by SDS-PAGE. The proteins that remained in the gel after completion of the transfer procedure were stained by Coomassie blue to ensure that the amounts of total protein present in each sample were approximately equivalent. The amount of protein in serum-starved cells (day 0) was substantially less than for days 1–5 in all the experiments performed. (b) Asynchronously growing TLM1 cells were transfected with 3 μg of plasmid DNA from an empty pBK-RSV vector (Vector), or the same pBK-RSV vector into which human *waf-1* was subcloned under the control of the RSV promoter (p21) using cationic liposomes for delivery of exogenous DNA. The accumulation of p21/Waf-1 was examined by immunoblotting 48 h after the transfections were performed. (c) Immunoblot of p21/Waf-1 from subconfluent TLM1 cells in the absence (–) or presence (+) of a 20-fold excess of the immunizing peptide consisting of the 19 C-terminal amino acids of the human p21/Waf-1 protein. (d) Immunoblot of Cdk4 in whole cell lysates prepared as in (a).

similar to that seen in the untransfected cells. The forced overexpression of the human *waf-1* gene in TLM1 cells resulted in a ~5- to 10-fold increase in the amount of an immunoreactive 21-kDa protein. Furthermore, pre-incubation of the anti-p21/Waf-1 antibody with 20-fold excess of the immunizing p21/Waf-1 peptide eliminated binding of the antibody to endogenous p21/Waf-1 from TLM1 cells in the immunoblot assays (Fig. 2c). Because p21/Waf-1 is a CDKI, we also evaluated the accumulation of Cdk4, one of its potential substrates that may be regulated in a cell cycle-specific manner [30,31]. Predictably, the levels of Cdk4 protein increased approximately ~3-fold upon serum restimulation of the TLM1 cells (Fig. 2d, compare days 0 and 1), and showed much less fluctuation than p21/Waf-1 thereafter.

3.2. Subcellular localization of p21/Waf-1 in TLM1 cells

Although the role of p21/Waf-1 and its expression throughout the cell cycle have been examined in several cell culture systems [5,32], to our knowledge, its subcellular distribution in growing cells has not been thoroughly examined. To this end, we used immunocytochemistry to evaluate the subcellular localization of p21/Waf-1 in synchronously growing TLM1 cells. Fig. 3 shows that there was a redistribution of p21/Waf-1 staining after induction of growth with serum. p21/Waf-1 was present in nucleoli of TLM1 cells throughout the culture period. However, this nucleolar staining was only seen in cells subjected to the antigen retrieval procedure (heating in a microwave for 6 min in a 0.1 M sodium citrate buffer, pH 6.0). This component of nucleolar p21/Waf-1 may be similar to that which can reportedly be sequestered within a detergent insoluble nuclear fraction [15]. Cytoplasmic p21/Waf-1 staining was seen in serum-starved cells with spindled to stellate morphology (Fig. 3a). Some cells with round morphology showed more intense staining, perhaps a reflection of the condensed cytoplasm in these cells. The significance of this morphological pleomorphism is not clear. The overall levels of p21/Waf-1 increased ~5-fold during the first 24 h following serum restimulation as determined by immunoblotting (Fig. 2), but there was no change in the subcellular localization of this protein (Fig. 3b). Cytoplasmic p21/Waf-1 and nucleolar

p21/Waf-1 were still detectable 2 days after serum restimulation of TLM1 cells; in addition, there was substantial translocation of p21/Waf-1 to the nuclear compartment (compare the pink-staining nuclei in Fig. 3c with the blue-staining nuclei in Fig. 3a,b). The nuclear p21/Waf-1 staining and much of the cytoplasmic p21/Waf-1 staining disappeared by day 3 (Fig. 3d; note the absence of red chromogen deposition in the cell nuclei). Other than the nucleolar p21/Waf-1, only cells that were undergoing mitosis stained positive for p21/Waf-1 at this time (Fig. 3h). On day 4 of culture, cytoplasmic p21/Waf-1 was again detectable, and the levels of nuclear p21/Waf-1 increased approximately 10-fold (Fig. 3e). This increase in nuclear p21/Waf-1 between days 3 and 4 was approximately twice as large as the increase in total p21/Waf-1 (~5-fold, see Fig. 2a). p21/Waf-1 also remained prominently associated with mitotic figures when these were present. The expression of both cytoplasmic and nuclear components of p21/Waf-1 was sustained with similar intensity into the fifth day of culture, when the monolayer was confluent and the TLM1 cells began to exhibit a decreased growth rate (Fig. 3f). In experiments extended into the sixth day of culture, the nuclear and cytoplasmic components of p21/Waf-1 expression continued to be sustained in these confluent TLM1 cells (see below). p21/Waf-1 staining was also evaluated in sections from three independent primary canine melanomas. Cells within these primary tumors would be predicted to exhibit asynchronous growth, and possibly have lost the constraints of contact inhibition. In each case, all the cells showed cytoplasmic staining for p21/Waf-1. One case showed prominent nuclear staining in >70% of the tumor cells; the second showed nuclear staining in approximately 10% of the cells and nucleolar staining in >90% of the cells; and the third showed no nuclear staining with sporadic nucleolar staining (data not shown).

The localization of p21/Waf-1 to the nucleus was specific, as determined by immunostaining for other nuclear and cytoplasmic markers in TLM1 cells (A. Koenig, B. Weeks, J. Modiano, unpublished results). Cells stained for the intermediate filament vimentin or for the isoform of the S100 calcium-binding protein found in melanoma cells (S100a) showed reactivity that was confined to the cytoplasm. Staining for the melanoma-specific antigen MelanA (also known as

MART-1) or for GD3 ganglioside (using antibody R24) showed reactivity that was confined to the cytoplasm and Golgi zone. Surface GD3 gangliosides also could be detected flow cytometrically [33]. The tumor suppressor protein p53 was confined to the cytoplasm in TLM1 cells throughout the culture period. The alternative possibilities that this may represent mutant p53, or a defect in other mechanisms that target p53 to the nucleus, have not been investigated. Finally, the

tumor suppressor protein Rb was shown to be predominantly confined to the nuclear or perinuclear compartment. None of these proteins showed nucleolar localization.

To establish that the differences in nuclear localization of p21/Waf-1 were related to changes in the subcellular localization of p21/Waf-1, cytosolic and nuclear fractions of TLM1 cells were evaluated for changes in p21/Waf-1 by immunoblotting. Fig. 4

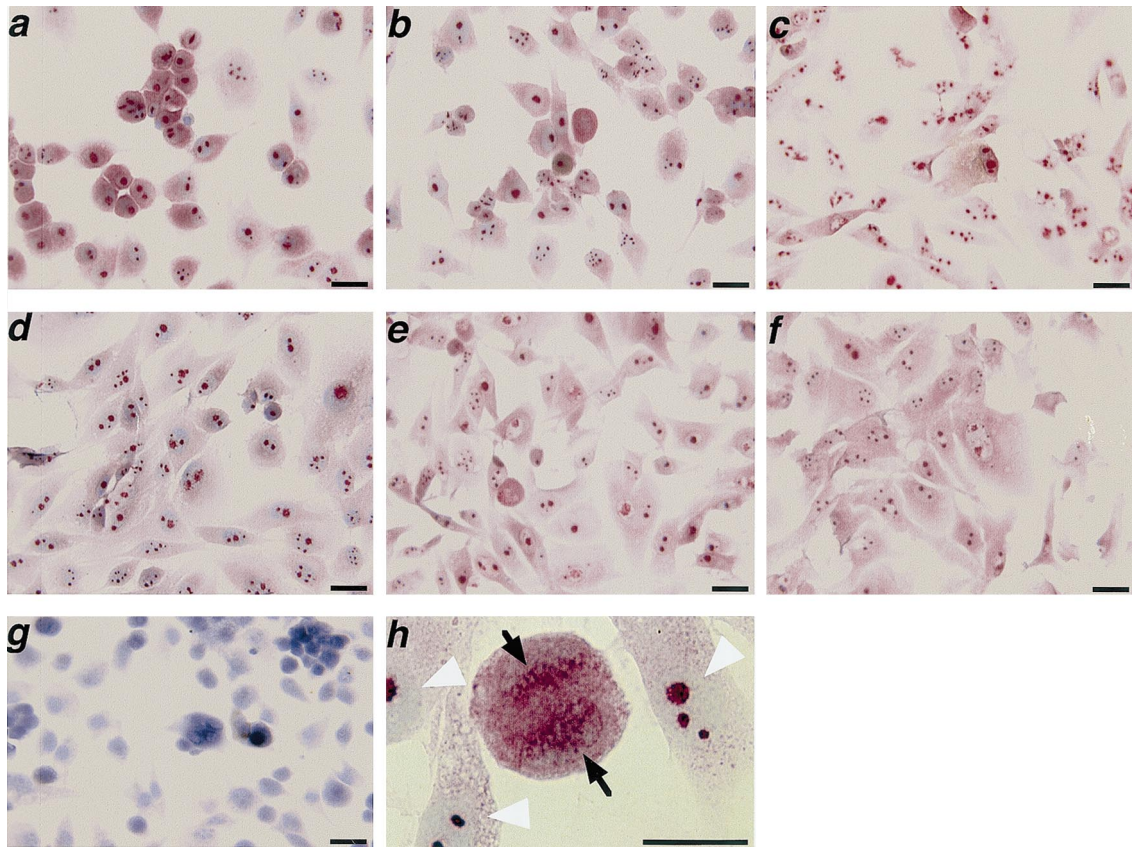


Fig. 3. Distribution of p21/Waf-1 in growth-arrested TLM1 cells restimulated with serum. Photomicrographs of cultured TLM1 cells harvested at 1-day intervals, and stained for p21/Waf-1 by immunocytochemistry using a streptavidin-biotin-alkaline phosphatase complex reaction. The distribution of p21/Waf-1 is demonstrated by the pink to red staining. (a) Cells harvested at day 0 (synchronized in the G1 phase by 3 days of serum withdrawal, immediately prior to the addition of serum). (b) Cells harvested 1 day following stimulation. (c) Cells harvested 2 days following stimulation. (d) Cells harvested 3 days following stimulation. (e) Cells harvested 4 days following stimulation. (f) Cells harvested 5 days following stimulation. Note that the monolayers were partially disrupted by the antigen unmasking procedure. (g) Negative control using rabbit IgG in place of the anti-p21/Waf-1 primary antibody to stain cells harvested 2 days after stimulation. (h) Higher magnification photomicrograph of TLM1 cells after 3 days in culture showing several non-dividing cells, and one cell in anaphase. p21/Waf-1 in the non-dividing cells is seen only in the cytoplasm and nucleoli (compare with (d)); nuclear p21/Waf-1 is undetectable (white arrowheads). The anaphase cell, lacking a discernible nuclear membrane, shows diffuse staining distributed throughout the cytoplasm and prominent staining associated with the mitotic plate (black arrows). The relative changes in nuclear staining for p21/Waf-1 in (a–f) were quantified by analysis of deconvoluted digital images of 100 nuclei from each sample using NIH Image. Bars: 25 μ m.

shows that immunoreactive p21/Waf-1 was detectable in cytoplasmic extracts of TLM1 cells between days 0 and 6 of culture. The levels of cytosolic p21/Waf-1 were maximal at day 2 of culture, declined at day 3, and remained at approximately the same levels for the duration of the culture period. In contrast, p21/Waf-1 was undetectable in the nucleus during days 0 and 1. Marginal amounts of immunoreactive p21/Waf-1 were seen in TLM1 cells after 2 days of culture; they disappeared at day 3, and they were again detectable after 4 days of culture. The levels of p21/Waf-1 in the nuclear compartment remained elevated during the fifth and sixth days of culture.

These findings were not unique to TLM1 cells. We examined the subcellular localization of p21/Waf-1 in another canine cell line derived from fetal thymic epithelium (Cf2Th) that also exhibits contact inhibition. Cf2Th cells were released from contact inhibition and cultured for 5 days to obtain a confluent monolayer. As was seen in the TLM1 cells, there was a transient localization of p21/Waf-1 to the nucleus of Cf2Th cells after 2 days in culture. The nuclear component of p21/Waf-1 was markedly decreased or absent on day 3 of culture; however, it was restored on day 4 and sustained into days 5 and 6 when the Cf2Th cell monolayer was confluent (data not shown).

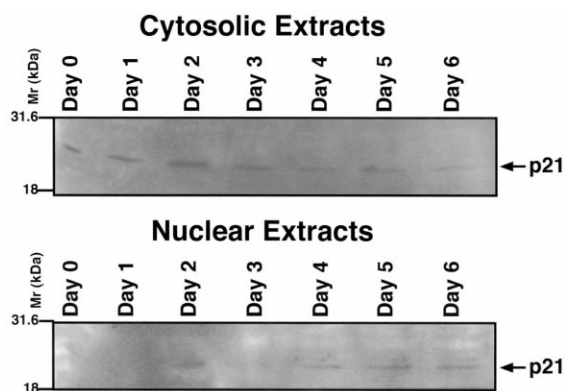


Fig. 4. Subcellular distribution of p21/Waf-1 in growth-arrested TLM1 cells restimulated with serum. Cytoplasmic extracts and nuclear extracts of TLM1 cells harvested at 24-h intervals following serum restimulation were prepared as described in Section 2. The accumulation of cytosolic and nuclear p21/Waf-1 was determined by immunoblotting. Proteins remaining in the gel after completion of the transfer procedure were stained by Coomassie blue to ensure that the amounts of total protein present in each sample were approximately equivalent.

3.3. Overexpression of p21/Waf-1 impairs proliferation and promotes apoptosis of subconfluent TLM1 cells

As noted previously, subconfluent TLM1 cells were not growth-inhibited although p21/Waf-1 was detected transiently in the nucleus. We examined whether p21/Waf-1 overexpression would impair the proliferation of TLM1 cells. Cells were transfected with the vector encoding human *waf-1* (RSV-p21) or the empty vector as a control (RSV-neo) and were cultured for 48 h. Fig. 5 shows that the transfection procedure did not appreciably affect the proliferation of TLM1 cells, as untransfected cells and cells transfected with the control vector exhibited similar increases in cell numbers (Fig. 5a) and DNA synthesis (Fig. 5b). In contrast, TLM1 cells transfected with human *waf-1* that contained approximately 5- to 10-fold higher levels of p21/Waf-1 (Fig. 2b) showed a 33% ($\pm 5\%$) reduction in cell numbers (Fig. 5a) and a 27% ($\pm 6\%$) decrease in cellular proliferation as measured by DNA synthesis (Fig. 5b). There was no statistical difference in viable cell numbers between untransfected cells and cells transfected with the control vector ($P = 0.2$), but the cell numbers in both of these conditions were significantly different than those in cells transfected with human *waf-1* ($P < 0.01$). Similarly, the proliferation observed in TLM1 cells transfected with *waf-1* was significantly lower ($P = 0.02$) than that of untransfected or control-transfected TLM1 cells. The DNA content and cell cycle distribution of the untransfected cells and the cells transfected with the control vector at 48 h were similar. Approximately 55–60% of the cells had two copies of DNA (G1 phase); 15–20% had more than two and less than four copies of DNA (S phase), and 22–26% had four copies of DNA (G2/M phases). Less than 6% of the cells had less than one copy of DNA (sub-G1, or apoptotic cells). In contrast, distinct peaks reflecting cell cycle distribution were not evident in cells transfected with human *waf-1*, and approximately 50% of the cells had less than one copy of DNA (apoptotic). This suggests that the decrease in TLM1 cell numbers and proliferation was due, at least partly, to increased apoptosis of transfected cells [26].

The predisposition of p21/Waf-1 over-expressing cells to undergo apoptosis precluded definitive assessment of the distribution of this protein by immunocy-

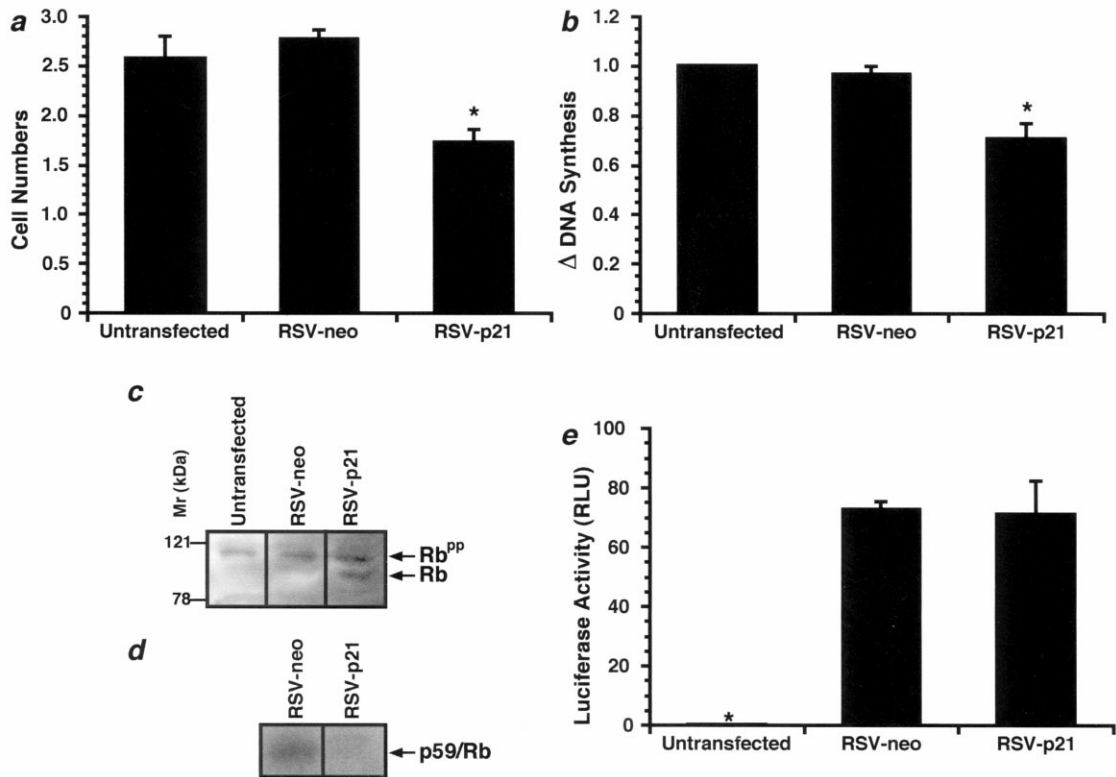


Fig. 5. Overexpression of p21/Waf-1 impairs TLM1 cell proliferation and Rb phosphorylation. Asynchronously growing TLM1 cells were allowed to remain untransfected, or transfected with the pGL3 vector encoding the firefly luciferase gene along with an empty pBK-RSV vector (RSV-neo), or along with the same pBK-RSV vector into which human *waf-1* was subcloned under the control of the RSV promoter (RSV-p21). Bars that represent values that are significantly different ($P < 0.05$) are marked by an asterisk. The data represent the mean \pm SEM of viable cells after 48 h in culture (normalized to a starting number of 1) from six independent experiments, each performed in duplicate (a,b), a representative experiment of two done (c,d), or mean \pm SEM from three independent experiments expressed in relative light units (RLU) (e). (a) Number of viable cells recovered from untransfected TLM1 cells, TLM1 cells transfected with the empty vector, and TLM1 cells transfected with *waf-1*. (b) TLM1 cells recovered 48 h after the transfection procedures were transferred to 96-well culture plates, and proliferation was evaluated 24 h later by incorporation of [3 H]thymidine into DNA. Data are shown as the ratio of the DNA synthesis in counts/min from each experimental condition divided by the DNA synthesis in counts/min from the untransfected cells. (c) TLM1 cells recovered 24 h after the transfection procedures were assessed for phosphorylation and content of Rb protein by immunoblot analysis. The hypophosphorylated form of Rb (Rb) can be resolved as a distinct band with faster electrophoretic migration than the hyperphosphorylated form of Rb (Rb^{pp}). (d) In vitro Cdk4 kinase activity measured using an immune complex assay in TLM1 cell extracts 24 h after the transfection procedure. Phosphorylation of the truncated form of Rb (p59/Rb) used as a substrate was resolved by separating the reaction products using SDS-PAGE followed by autoradiography (2-h exposure). (e) Cells harvested 24 h after the transfection procedures were assayed for luciferase activity in triplicate using an enhanced flash luminometer. Untransfected cells had no detectable luciferase activity (0.014 ± 0.003 RLU). The light output from each experiment was normalized using a recombinant firefly luciferase standard curve.

tochemistry. Nevertheless, there was a concomitant reduction in the levels of hyperphosphorylated Rb protein (Fig. 5c) and a substantial reduction in Cdk4 activity in the *waf-1* transfectants at 24 h (Fig. 5d) that was not seen in the control transfectants. This suggests

that the transfected p21/Waf-1 reduced Cdk activity in the TLM1 cells proportionately. These changes were not due to differences in the transfection efficiency between the two conditions, since there were no differences (at 24 h) in the activity obtained from a

luciferase reporter gene that was co-transfected with either the control or the *waf-1* vectors (Fig. 5e). Luciferase activity was undetectable in untransfected cells.

4. Discussion

We examined the relationship between the expression and localization of the *waf-1* gene product, p21/Waf-1, and its possible influence on cellular proliferation in a canine melanoma cell line. Our results suggest that cell-to-cell contacts promote nuclear translocation of p21/Waf-1, and also that the action of this protein to induce cellular growth arrest following contact inhibition could be related to its sustained nuclear localization. It is noteworthy that the transient localization of endogenous p21/Waf-1 to the nucleus did not stop cell cycle progression in subconfluent TLM1 cells. However, overexpression of p21/Waf-1 reduced proliferation and increased apoptosis in these cells.

Previous studies have shown that the levels of p21/Waf-1 expression are variable as cells progress through the cell cycle [16,25] leading to distinct functional consequences. Changes in the abundance of p21/Waf-1 during cell growth and differentiation have been shown in many cell types, including epithelial cells [20,21,24] and carcinomas [11,25,34]; mesenchymal cells such as differentiating myoblasts [19,24] and hematopoietic cells [17,35]; and neuroectodermally-derived cells such as human melanocytes [4]. However, the fluctuations in expression of p21/Waf-1 do not always correlate with growth arrest, suggesting that the subcellular localization of the protein, rather than the amount present, may be relevant to its function. This possibility was addressed in an elegant study using W138 human diploid fibroblasts that showed p21/Waf-1 co-localized with PCNA to the nuclear compartment following DNA damage induced by ultraviolet irradiation [15]. In parallel experiments, fibroblasts with a deficient DNA excision and repair mechanism failed to undergo growth arrest upon DNA damage. In the cells with defective DNA repair mechanisms, the function of p21/Waf-1 may have been impaired by sequestration within a detergent-insoluble compartment of the nucleus [15]. Additional evidence supporting the importance of subcellular localization

of p21/Waf-1 in mediating growth arrest was seen in MCF-7 mammary adenocarcinoma cells exposed to TGF β [25]. TGF β exerts an antiproliferative effect in MCF-7 cells that leads to nuclear translocation of p21/Waf-1 and inhibition of Cdk2 activity. The importance of these effects was highlighted by the observations that nuclear translocation of p21/Waf-1 and inhibition of Cdk2 were not seen following treatment with TGF β in an MCF-7 subline that is refractory to the antiproliferative effects of this cytokine [25].

The role of p21/Waf-1 in mediating contact-induced growth arrest remains less well understood. Expression of p21/Waf-1 was shown to increase in Caco-2 intestinal epithelial cells following confluence, but the subcellular localization of the protein was not documented in these experiments [20]. Similarly, the levels of p27/Kip-1, a closely related Cdk inhibitor, increase substantially following E-cadherin-dependent cell-to-cell contact in mammary adenocarcinoma cells [36], and p27/Kip-1 also undergoes nuclear translocation in rat fibroblasts cultured at high density [37]. Our present results show that the accumulation and subcellular distribution of p21/Waf-1 varied over the course of TLM1 cell growth in culture. p21/Waf-1 mRNA was detectable throughout the 5-day culture period, but the RT-PCR technique used to assess the presence of p21/Waf-1 mRNA was not quantitative. The levels of p21/Waf-1 protein varied substantially over the 5-day culture period, suggesting that post-transcriptional mechanisms may contribute to the regulation of p21/Waf-1 protein accumulation. Maximal p21/Waf-1 accumulation with nuclear localization was seen prior to the height of DNA synthesis for the first replicative cell cycle. A remarkable decrease in p21/Waf-1 with localization away from the nucleus coincided with the time of maximal DNA synthesis, and preceded re-entry into the G1 phase based on the doubling time of this cell line [26]. Thus, nuclear localization of p21/Waf-1 in subconfluent cells may be required in preparation for initiation of the subsequent cell cycle. Our data also demonstrate that the sustained localization of p21/Waf-1 to the nucleus coincided with the TLM1 cells becoming confluent. It is noteworthy that in one case, TLM1 cells carried beyond 30 passages became refractory to contact inhibition. This TLM1 subline was not preserved, but a posteriori analysis

showed that these cells had spontaneously lost expression of p21/Waf-1. Finally, experiments to address the role of p21/Waf-1 using mouse embryo fibroblasts derived from animals with a targeted deletion of *waf-1* showed that absence of p21/Waf-1 did not lead to anchorage independent growth, but the cells showed a slightly reduced capacity for contact inhibition [38]. This suggests that contact-induced growth arrest is not uniquely mediated by p21/Waf-1 and that the TLM1 cells that were refractory to contact inhibition likely harbored additional mutations. Together, these results support the concept that nuclear p21/Waf-1 may contribute to slowing or stopping cell cycle progression once cell-to-cell contacts are established. However, additional factors are necessary for contact-induced cell growth arrest.

The inability of nuclear p21/Waf-1 to arrest cell growth of subconfluent cells after 2 days in culture may be a quantitative phenomenon. We and others have shown that the overexpression of p21/Waf-1 in asynchronously growing melanoma cells or mammary epithelial cells reduced proliferation and increased apoptosis [26,39], indicating that growth in subconfluent conditions could be overridden, at least partially, by exaggerated levels of p21/Waf-1. These supraphysiologic levels of p21/Waf-1 may act stoichiometrically by abrogating Cdk activity and preventing Rb inactivation that are necessary for cell cycle progression and cellular survival. Similar effects may account for the increased apoptotic tendency of TLM1 cells maintained in culture as a confluent monolayer, even in the presence of serum [26]. In these cells, sustained nuclear accumulation and activity of p21/Waf-1 may interfere with events required for cellular survival. Additional investigations to delineate the precise role of p21/Waf-1 to constrain cell growth and prevent transformation are warranted.

Acknowledgements

The authors gratefully acknowledge Dr David Beach for providing the plasmid encoding wild-type human *waf-1*. We also wish to thank Cheryl Morales and Maren Fuentes for technical assistance, John B. Roths for assistance with image analysis, and Drs Stuart Helfand, Gheorge Stoica, Tom Welsh, Jennifer

Thomas, Carol Chitko-McKown, and Peter Nowell for reading the manuscript and for helpful discussions. This work was supported in part by NIH Clinical Investigator Development Award K08-HL03130, Grant 1626 from the Canine Health Foundation of the American Kennel Club and Grant 98-CA36 from Morris Animal Foundation to J.F.M.

References

- [1] W.S. El-Deiry, T. Tokino, V.E. Velculescu, D.B. Levy, R. Parsons, J.M. Trent, D. Lin, W.E. Mercer, K.W. Kinzler, B. Vogelstein, WAF1, a potential mediator of p53 tumor suppression, *Cell* 75 (1993) 817–825.
- [2] W.S. El-Deiry, T. Tokino, T. Waldman, J.D. Oliner, V.E. Velculescu, M. Burrell, D.E. Hill, E. Healy, J.L. Rees, S.R. Hamilton, K.W. Kinzler, B. Vogelstein, Topological control of p21/waf1/cip1 expression in normal and neoplastic tissues, *Cancer Res.* 55 (1995) 2910–2919.
- [3] M.E. Miele, G. Robertson, J.H. Lee, A. Coleman, C.T. McGary, P.B. Fisher, T.G. Lugo, D.R. Welch, Metastasis suppressed, but tumorigenicity and local invasiveness unaffected, in the human melanoma cell line MelJuSo after introduction of human chromosomes 1 or 6, *Mol. Carcinog.* 15 (1996) 284–299.
- [4] H. Jiang, J. Lin, Z.Z. Su, M. Herlyn, R.S. Kerbel, B.E. Weissman, D.R. Welch, P.B. Fisher, The melanoma differentiation-associated gene *mda-6*, which encodes the cyclin-dependent kinase inhibitor p21, is differentially expressed during growth, differentiation and progression in human melanoma cells, *Oncogene* 10 (1995) 1855–1864.
- [5] A.L. Gartel, M.S. Serfas, A.L. Tyner, p21 – negative regulator of the cell cycle, *Proc. Soc. Exp. Biol. Med.* 213 (1996) 138–149.
- [6] C. Missero, F. Di Cunto, H. Kiyokawa, A. Koff, G.P. Dotto, The absence of p21Cip1/WAF1 alters keratinocyte growth and differentiation and promotes ras-tumor progression, *Genes Dev.* 10 (1996) 3065–3075.
- [7] J. LaBaer, M.D. Garrett, L.F. Stevenson, J.M. Slingerland, C. Sandhu, H.S. Chou, A. Fattaey, E. Harlow, New functional activities for the p21 family of CDK inhibitors, *Genes Dev.* 11 (1997) 847–862.
- [8] Y. Xiong, G.J. Hannon, H. Zhang, D. Casso, R. Kobayashi, D. Beach, p21 is a universal inhibitor of cyclin kinases, *Nature* 366 (1993) 701–704.
- [9] J.W. Harper, G.R. Adami, N. Wei, K. Keyomarsi, S.J. Elledge, The p21 Cdk-interacting protein Cip1 is a potent inhibitor of G1 cyclin-dependent kinases, *Cell* 75 (1993) 805–816.
- [10] J.W. Harper, S.J. Elledge, K. Keyomarsi, B. Dynlacht, L.H. Tsai, P. Zhang, S. Dobrowolski, C. Bai, L. Connell-Crowley, E. Swindell, M.P. Fox, N. Wei, Inhibition of cyclin-dependent kinases by p21, *Mol. Biol. Cell* 6 (1995) 387–400.
- [11] N. Barboulet, P. Chadebecq, V. Baldin, S. Vidal, A. Valette, Involvement of p21 in mitotic exit after paclitaxel treatment in

- MCF-7 breast adenocarcinoma cell line, *Oncogene* 15 (1997) 2867–2875.
- [12] J. Shim, H. Lee, J. Park, H. Kim, E.J. Choi, A non-enzymatic p21 protein inhibitor of stress-activated protein kinases, *Nature* 381 (1996) 804–806.
- [13] J. Chen, P.K. Jackson, M.W. Kirschner, A. Dutta, Separate domains of p21 involved in the inhibition of Cdk kinase and PCNA, *Nature* 374 (1995) 386–388.
- [14] S. Waga, G.J. Hannon, D. Beach, B. Stillman, The p21 inhibitor of cyclin-dependent kinases controls DNA replication by interaction with PCNA, *Nature* 369 (1994) 574–578.
- [15] R. Li, G.J. Hannon, D. Beach, B. Stillman, Subcellular distribution of p21 and PCNA in normal and repair-deficient cells following DNA damage, *Curr. Biol.* 6 (1996) 189–199.
- [16] Y. Li, C.W. Jenkins, M.A. Nichols, Y. Xiong, Cell cycle expression and p53 regulation of the cyclin-dependent kinase inhibitor p21, *Oncogene* 9 (1994) 2261–2268.
- [17] K.F. Macleod, N. Sherry, G. Hannon, D. Beach, T. Tokino, K. Kinzler, B. Vogelstein, T. Jacks, p53-Dependent and independent expression of p21 during cell growth, differentiation, and DNA damage, *Genes Dev.* 9 (1995) 935–944.
- [18] W. Zhang, L. Grasso, C.D. McClain, A.M. Gambel, Y. Cha, S. Travali, A.B. Deisseroth, W.E. Mercer, p53-Independent induction of WAF1/CIP1 in human leukemia cells is correlated with growth arrest accompanying monocyte/macrophage differentiation, *Cancer Res.* 55 (1995) 668–674.
- [19] K. Guo, J. Wang, V. Andres, R.C. Smith, K. Walsh, MyoD-induced expression of p21 inhibits cyclin-dependent kinase activity upon myocyte terminal differentiation, *Mol. Cell Biol.* 15 (1995) 3823–3829.
- [20] B.M. Evers, T.C. Ko, J. Li, E.A. Thompson, Cell cycle protein suppression and p21 induction in differentiating Caco-2 cells, *Am. J. Physiol.* 271 (1996) G722–G727.
- [21] W.S. El-Deiry, T. Tokino, T. Waldman, J.D. Oliner, V.E. Velculescu, M. Burrell, D.E. Hill, E. Healy, J.L. Rees, S.R. Hamilton, K.W. Kinzler, B. Vogelstein, Topological control of p21WAF1/CIP1 expression in normal and neoplastic tissues, *Cancer Res.* 55 (1995) 2910–2919.
- [22] M.B. Datto, Y. Li, J.F. Panus, D.J. Howe, Y. Xiong, X.F. Wang, Transforming growth factor beta induces the cyclin-dependent kinase inhibitor p21 through a p53-independent mechanism, *Proc. Natl. Acad. Sci. USA* 92 (1995) 5545–5549.
- [23] A. Elbendary, A. Berchuck, P. Davis, L. Havrilesky, R.C. Bast Jr., J.D. Iglehart, J.R. Marks, Transforming growth factor beta 1 can induce CIP1/WAF1 expression independent of the p53 pathway in ovarian cancer cells, *Cell Growth Differ.* 5 (1994) 1301–1307.
- [24] S.B. Parker, G. Eichele, P. Zhang, A. Rawls, A.T. Sands, A. Bradley, E.N. Olson, J.W. Harper, S.J. Elledge, p53-Independent expression of p21Cip1 in muscle and other terminally differentiating cells, *Science* 267 (1995) 1024–1027.
- [25] P. Mazars, N. Barboule, V. Baldin, S. Vidal, B. Ducommun, A. Valette, Effects of TGF-beta 1 (transforming growth factor-beta 1) on the cell cycle regulation of human breast adenocarcinoma (MCF-7) cells, *FEBS Lett.* 362 (1995) 295–300.
- [26] J.F. Modiano, M.G. Ritt, J. Wojcieszyn, R. Smith III, Growth arrest of melanoma cells is differentially regulated by contact inhibition and serum deprivation, *DNA Cell Biol.* 18 (1999) 357–367.
- [27] M.G. Ritt, J. Wojcieszyn, J.F. Modiano, Functional loss of p21/Waf-1 in a case of benign canine multicentric melanoma, *Vet. Pathol.* 35 (1998) 94–101.
- [28] J.F. Modiano, D.A. Amran, G. Lack, K. Bradley, C. Ball, J. Domenico, E.W. Gelfand, Post-transcriptional regulation of T cell IL-2 production by human pooled immunoglobulin, *Clin. Immunol. Immunopathol.* 83 (1997) 77–85.
- [29] J.F. Modiano, Y. Kokai, D.B. Weiner, M.J. Pykett, P.C. Nowell, C.R. Lyttle, Progesterone augments proliferation induced by epidermal growth factor in a feline mammary adenocarcinoma cell line, *J. Cell. Biochem.* 45 (1991) 196–206.
- [30] J.F. Modiano, J. Domenico, A. Szepesi, J.J. Lucas, E.W. Gelfand, Differential requirements for interleukin-2 distinguish the expression and activity of the cyclin-dependent kinases Cdk4 and Cdk2 in human T cells, *J. Biol. Chem.* 269 (1994) 32972–32978.
- [31] D.O. Morgan, Principles of CDK regulation, *Nature* 374 (1995) 131–134.
- [32] V. Chiarugi, L. Magnelli, M. Cinelli, G. Basi, Apoptosis and the cell cycle, *Cell. Mol. Biol. Res.* 40 (1994) 603–612.
- [33] S.C. Helfand, E.B. Dickerson, K.L. Munson, M.L. Padilla, GD3 ganglioside antibody augments tumoricidal capacity of canine blood mononuclear cells by induction of interleukin 12, *Cancer Res.* 59 (1999) 3119–3127.
- [34] N. Barboule, V. Baldin, J.O. S, S. Vidal, A. Valette, Increased level of p21 in human ovarian tumors is associated with increased expression of cdk2, cyclin A and PCNA, *Int. J. Cancer* 76 (1998) 891–896.
- [35] H. Jiang, J. Lin, Z.Z. Su, F.R. Collart, E. Huberman, P.B. Fisher, Induction of differentiation in human promyelocytic HL-60 leukemia cells activates p21, WAF1/CIP1, expression in the absence of p53, *Oncogene* 9 (1994) 3397–3406.
- [36] B. St. Croix, C. Sheehan, J.W. Rak, V.A. Florenes, J.M. Slingerland, R.S. Kerbel, E-Cadherin-dependent growth suppression is mediated by the cyclin-dependent kinase inhibitor p27(Kip1), *J. Cell Biol.* 142 (1998) 557–571.
- [37] A. Kato, H. Takahashi, Y. Takahashi, H. Matsushima, Inactivation of the cyclin D-dependent kinase in the rat fibroblast cell line, 3y1, induced by contact inhibition, *J. Biol. Chem.* 272 (1997) 8065–8070.
- [38] J. Brugarolas, R.T. Bronson, T. Jacks, p21 is a critical CDK2 regulator essential for proliferation control in Rb-deficient cells, *J. Cell Biol.* 141 (1998) 503–514.
- [39] N. Boudreau, Z. Werb, M.J. Bissell, Suppression of apoptosis by basement membrane requires three-dimensional tissue organization and withdrawal from the cell cycle, *Proc. Natl. Acad. Sci. USA* 93 (1996) 3509–3513.