The absence of p21^{Cip1/WAF1} alters keratinocyte growth and differentiation and promotes *ras*-tumor progression

Caterina Missero,¹ Ferdinando Di Cunto,¹ Hiroaki Kiyokawa,² Andrew Koff,² and G. Paolo Dotto^{1,3}

¹Cutaneous Biology Research Center, Harvard Medical School and Massachusetts General Hospital, Charlestown, Massachusetts 02129 USA; ²Program in Molecular Biology, Memorial Sloan-Kettering Cancer Center, New York, New York 10021 USA

p21^{Cip1/WAF1} was the first cyclin-dependent kinase (CDK) inhibitor to be identified, as a mediator of p53 in DNA damage-induced growth arrest, cell senescence, and direct CDK regulation. p21 may also play an important role in differentiation-associated growth arrest, as its expression is augmented in many terminally differentiating cells. A general involvement of p21 in growth/differentiation control and tumor suppression has been questioned, as mice lacking p21 undergo a normal development, harbor no gross alterations in any of their organs, and exhibit no increase in spontaneous tumor development. However, a significant imbalance between growth and differentiation could be unmasked under conditions where normal homeostatic mechanisms are impaired. We report here that primary keratinocytes derived from p21 knockout mice, transformed with a *ras* oncogene, and injected subcutaneously into nude mice exhibit a very aggressive tumorigenic behavior, which is not observed with wild-type control keratinocytes nor with keratinocytes with a disruption of the closely related p27 gene. p21 knockout keratinocytes tested under well-defined *in vitro* conditions show a significantly increased proliferative potential, which is also observed but to a lesser extent with p27 knockout cells. More profound differences were found in the differentiation behavior of p21 versus p27 knockout keratinocytes, with p21 (but not p27) deficiency causing a drastic down-modulation of differentiation markers linked with the late stages of the keratinocyte terminal differentiation program. Thus, our results reveal a so far undetected role of p21 in tumor suppression, demonstrate that this function is specific as it cannot be attributed to the closely related p27 molecule, and point to an essential involvement of p21 in terminal differentiation control, which may account for its role in tumor suppression.

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In eukaryotic cells, factors determining whether cells continue to proliferate or cease dividing and differentiate appear to operate mainly during the G₁ phase of the cell cycle (Pardee 1989). Progression through the cell cycle is regulated by the sequential activation and inactivation of cyclin-dependent kinases (CDKs) (Sherr 1994). Inactivation of the cyclin-CDK complex can occur in several different ways: Cyclin levels can be reduced by turning down their transcription or by the degradation of the protein itself, and the catalytic subunit can be inhibited by phosphorylation of the Tyr residue (Tyr-15 in CDK2) or by dephosphorylation of the activating Thr residue (Thr-160 in CDK2) (for review, see Sherr 1994). An additional mechanism of inactivation is provided by the presence of CDK inhibitors (CKIs), proteins that bind to the cyclin-CDK complex or the CDK itself and inhibit the kinase activity (Sherr and Roberts 1995).

On the basis of their structural features and specificity of interactions, the CKIs can be classified into two families. The INK4 family members (p16^{INK4A}, p15^{INK4B}, p18^{INK4C}, p19^{INK4D}) contain tandemly repeated ankyrin motifs and specifically inhibit CDK4 and CDK6 by binding to the catalytic subunit and preventing the formation of the active cyclin-CDK complex (Sherr and Roberts 1995). The Cip/Kip family members (p21^{Cip1/WAF1}, p27^{Kip1}, p57^{Kip2}) share significant sequence homology in their amino-terminal portions and recognize a broad but not identical range of cyclin-CDK targets (Sherr and Roberts 1995). The unique carboxy-terminal domain of p21^{Cip1/WAF1} has been shown to associate with the proliferating nuclear antigen (PCNA), a subunit of DNA polymerase δ , and can inhibit DNA replication directly, without affecting DNA repair (Li et al. 1994; Shivji et al. 1994; Waga et al. 1994). Nothing is known about the unique carboxy-terminal domains of the p27 and p57 proteins.

p21^{Cip1/WAF1} was the first CKI to be identified, as a mediator of p53-induced growth arrest in response to

³Corresponding author.

DNA damage, in cell senescence, and as a direct CDK regulator (El-Deiry et al. 1993; Gu et al. 1993; Harper et al. 1993; Xiong et al. 1993; Noda et al. 1994). The tumor suppressor p53 is a transcription factor required for G₁ arrest and programmed cell death in response to DNA damage (for review, see Hinds and Weinberg 1994; Cox and Lane 1995). Among its transcriptional targets, p21^{Cip1/WAF1} is thought to be a central regulator of the G₁ checkpoint control (El-Deiry et al. 1993). Consistently, embryonic fibroblasts lacking p21 are at least partially deficient in their ability to arrest in G₁ following DNA damage and nucleotide perturbation, whereas apoptosis of p21^{-/-} thymocytes induced by γ -irradiation occurs normally (Brugarolas et al. 1995; Deng et al. 1995). Although p21^{Cip1/WAF1} is involved in certain p53-dependent functions, this CKI is unlikely to be a general mediator of p53 tumor suppression. Mice deficient for p53 develop normally but are susceptible to spontaneous tumors, predominantly lymphomas and soft tissue sarcomas (Donehower et al. 1992; Jacks et al. 1994). In contrast, p21-deficient mice do not develop spontaneous malignancies (Deng et al. 1995).

Independently of p53 function, p21^{Cip1/WAF1} has been implicated in terminal differentiation-associated growth arrest, as its expression is increased during the differentiation of a number of cell types, of hematopoietic, muscular, epithelial, and melanocytic origin (Jiang et al. 1994; Steinman et al. 1994; Halevy et al. 1995; Jiang et al. 1995; Macleod et al. 1995; Missero et al. 1995). During embryogenesis, p21^{Cip1/WAF1} expression pattern correlates with terminal differentiation in several cell lineages, including skeletal muscle, cartilage, skin, and nasal epithelium (Parker et al. 1995). Although compelling, these observations are correlative in nature, and the role played by p21 in differentiation has been questioned by the fact that p21 knockout mice undergo normal development and harbor no gross abnormalities in any of their organs (Deng et al. 1995).

p27^{Kip1} has been implicated in growth arrest triggered by antiproliferative signals such as TGF- β , cAMP, rapamycin, and cell-contact inhibition (Kato et al. 1994; Nourse et al. 1994; Polyak et al. 1994; Reynisdottir et al. 1995). Unlike p21^{Cip1/WAF1}, expression of p27^{Kip1} does not appear to be under p53 control. p27^{Kip1} expression has also been shown to correlate with differentiation in vitro in certain cell types and in vivo in postmitotic neurons during mouse embryogenesis and in luteal cell differentiation (Kranenburg et al. 1995; Kiyokawa et al. 1996; Lee et al. 1996). Like p21 knockouts, mice with a disruption of the p27^{Kip1} gene develop normally, but they display hyperplasia of several organs, owing, at least in part, to increased cell proliferation (Fero et al. 1996; Kiyokawa et al. 1996; Nakayama et al. 1996). Interestingly, p27-deficient mice show spontaneous neoplastic changes limited to the pituitary gland, similar to mice lacking Rb (Williams et al. 1994) and consistent with these two molecules playing a critical role in this particular organ.

Although p21^{Cip1/WAF1} and p27^{Kip1} have been implicated in growth/differentiation control in several cellu-

lar systems, their specific roles remain to be established. The limited phenotype of mice lacking either p21^{Cip1/WAF1} or p27^{Kip1} could be attributed to functional compensation between these two CKIs and/or other cell cycle regulatory mechanisms that render their function dispensable. Equally possible, powerful homeostatic mechanisms may override, under normal in vivo conditions, the functional consequences of a p21 or p27 deficiency. Thus, a detailed analysis of different cell types cultured from p21 and p27 knockout mice could be invaluable in detecting general as well as cell type-specific functions of these molecules.

We report here our studies aimed at establishing the specific role played by p21^{Cip1/WAF1} in keratinocyte growth/differentiation control. Primary keratinocytes derived from p21-deficient mice were examined under well-defined culture conditions, as well as in vivo. The most striking alteration of p21 knockout keratinocytes that we have uncovered is their very aggressive tumorigenic behavior after *ras* oncogene transformation, which is not observed with wild-type control keratinocytes nor with keratinocytes with a disruption of the closely related p27 gene. Our findings further suggest that this function of p21 in keratinocyte tumor suppression is likely connected to its specific involvement in terminal differentiation control, rather than simple withdrawal from the cell cycle.

Results

Increased proliferative potential of p21 knockout keratinocytes

Mouse primary keratinocytes provide an excellent system to study growth and differentiation control of normal epithelial cells, both in vitro and in vivo (Missero and Dotto 1996). To establish the role of p21 in this system, we analyzed primary keratinocytes derived from mice with a knockout mutation of the p21 gene (Deng et al. 1995). We first examined the growth properties of these cells under basal proliferating conditions. Primary keratinocytes cultured in medium at low calcium concentrations (0.05 mM, low calcium medium) behave similarly to cells of the basal layer of the epidermis (Henning et al. 1980). Under these conditions, keratinocytes are not susceptible to cell-contact inhibition but proliferate even at confluence, shedding cells into the medium. p21^{-/-} keratinocytes became confluent consistently 1–2 days earlier than genetically matched p21^{+/+} cells (plated at the same numbers: 10⁷ cells/100-mm dish) and reached a two- to threefold higher density. However, like control keratinocytes, p21-deficient cells were unable to grow under sparse conditions (<10⁶/100-mm dish).

To measure the number of actively replicating keratinocytes, asynchronous cultures were pulse-labeled with the thymidine analog bromodeoxyuridine (BrdU) for 3 hr. As shown in Figure 1A, the nuclear labeling index of p21-deficient keratinocytes was twice as high as that of genetically matched wild-type cells (40% vs. 22%). Sim-

Figure 1. Proliferative activity of wild-type vs. p21 or p27 knockout keratinocytes. (A) DNA synthesis was measured by labeling asynchronous primary keratinocyte cultures, obtained from p21 knockout mice and genetically matched wild-type controls with BrdU for 3 hr under proliferating conditions (*left*). The total number of nuclei was evaluated by staining with Hoechst 33258 (*right*). A minimum of 1200 cells in four independent fields were counted in all cases. The percentage of BrdU-positive nuclei is indicated in parentheses. Similar results were obtained in two other independent experiments and were confirmed by [³H]thymidine pulse-labeling experiments. (B) Cell cycle distribution of primary keratinocyte cultures, derived from p21 knockout mice and genetically matched wild-type controls, was determined by FACS analysis as described in Materials and Methods. Cells were analyzed 2 days after plating under growing conditions.

ilar results were obtained under both subconfluent and confluent conditions. Fluorescence-activated cell sorting (FACS) confirmed these findings, showing an approximately twofold higher fraction of p21 knockout keratinocytes in the S phase of the cell cycle than the wild-type controls (Fig. 1B).

Thus, p21-deficient keratinocytes kept under basal growing conditions have an increased propensity to proliferate, even though these cells, like their wild-type counterparts, are unable to grow clonally.

Induction of p21 and p27 expression at very early times of keratinocyte differentiation

Addition of calcium (1.2–2 mM) to primary keratinocyte cultures triggers a well-characterized terminal differentiation program (Hennings et al. 1980), with induction of growth arrest occurring as early as 8–12 hr after calcium addition (Fig. 2A). Increased p21 expression is observed by 4–8 hr after induction of differentiation, in parallel with CDK inhibition and growth arrest (Missero et al. 1995). Decreased p105–Rb phosphorylation occurs in these cells with similar timing (our unpublished observations). We investigated whether modulation of other cell cycle regulatory proteins occurs, which may contribute to the differentiation-associated growth arrest.

Immunoblotting analysis of total keratinocyte cell extracts with antibodies against murine p21 confirmed the very early induction of this CKI after calcium-induced differentiation (Fig. 3). Interestingly, the p21 protein returned to basal levels or even below by 24 hr of calcium treatment, pointing to a possible post-transcriptional regulation, given that p21 mRNA levels remain elevated at this and later times (Missero et al. 1995). Expression of p27 was also found to increase in keratinocyte differentiation, with a similar time course as p21 (Fig. 3). In contrast, CKIs of the unrelated p15 and p16 family did not appear to be induced, and expression of CDK2, CDK4, and CDK6 also remained constant (Fig. 3; data not shown). Analysis of G₁ cyclins indicated that cyclin

D3 is not expressed at detectable levels in mouse primary keratinocytes (data not shown), whereas cyclin D1 and D2 were significantly down-modulated by 24 hr of calcium treatment (Fig. 3). Cyclin A expression remained constant, whereas that of cyclin E decreased only at 48 hrs after induction of differentiation (Fig. 3; data not shown).

Thus, increased p21 and p27 expression are the earliest cell cycle regulatory events that can be detected in differentiating keratinocytes, followed by cyclin D1 and D2 down-modulation. Additional modifications, such as decreased cyclin E expression, occur at later times.

Normal sensitivity of p21 knockout keratinocytes to differentiation- and TGF- β -induced growth arrest

p21 may play a specific function in differentiation that may be only partially compensated by the other cell cycle regulatory proteins that are involved in this process. To test this possibility, we initially compared the sensitivity of wild-type and p21 knockout keratinocytes to differentiation-associated growth arrest. In spite of their enhanced growth capability, asynchronous culture of p21^{-/-} keratinocytes underwent growth arrest upon calcium-induced differentiation with a similar time course as p21^{+/+} cells (Fig. 2A).

To test whether the calcium-induced growth arrest occurred at the same point of the cell cycle, primary keratinocytes were synchronized in G₀ by serum and EGF starvation for 36 hr and subsequently restimulated with fully supplemented medium. Calcium was added at various times after release from starvation, and all cells were pulse-labeled with [³H]thymidine for 3 hr at the end of the experiment (Fig. 2B). A parallel experiment with TGF- β was performed, as this agent is a well-established G₁ blocker in keratinocytes (Massague 1990). Time of entry into S phase of both control and p21^{-/-} keratinocytes was determined by measuring their sensitivity to mimosine treatment. Mimosine functions as a specific inhibitor of the initiation but not the elongation phase of cellular DNA synthesis, and resistance to mi-

Figure 2. Differentiation-associated growth arrest in wild-type vs. p21 knockout keratinocytes. (A) Time course of calcium-induced growth arrest of wild-type and p21 knockout keratinocytes. DNA synthesis was measured in asynchronous cultures of p21^{-/-} primary mouse keratinocytes (open bars) or genetically matched p21^{+/+} controls (solid bars) under either growing conditions (0) or at various times (hours) of calcium-induced differentiation, as indicated. Cells in triplicate wells were labeled with [³H]thymidine for the last hour of the calcium treatment. Values are expressed as percentage of untreated controls and the standard error is indicated. Similar results were obtained in three other independent experiments and were confirmed by BrdU nuclear labeling measurements. (B) Sensitivity of wild-type and p21 knockout keratinocytes to calcium- and TGF-β-induced growth arrest as a function of the cell cycle. p21^{+/+} and p21^{-/-} keratinocyte cultures were growth arrested by incubation in serum-free medium for 36 hr. At time 0, cells were released from serum starvation by addition of fully supplemented medium. Cells were either kept in low calcium medium (untreated control) or were exposed to calcium (2 mM; ■), TGF-β (3 ng/ml; △), or mimosine (50 μM; ●) at the indicated times (hours) after release from growth arrest. In all cases, DNA synthesis was measured by a 3-hr [³H]thymidine incorporation assay prior to termination of the experiment (24 hr after serum stimulation). p21^{+/+} (top) and p21^{-/-} keratinocyte cultures (bottom) were similarly analyzed. Values were determined in triplicate wells and were expressed as percentage of the untreated control. The standard error was as indicated.

mimosine treatment provides a reliable indication of the time of entry into S phase (Alexandrow and Moses 1995). In wild-type keratinocytes, differentiation-associated

Figure 3. Modulation of cell cycle regulatory proteins in calcium-induced keratinocyte differentiation. Total cell extracts from primary keratinocyte cultures under growing conditions and at various times after calcium-induced differentiation were analyzed by 12.5% SDS-PAGE and immunoblotting with antibodies specific for the indicated cell cycle regulatory proteins as described in Materials and Methods. The blots probed with antibodies against the most relevant proteins (p21, p27, cyclin D1) were stripped of these antibodies and reprobbed with anti-cdk2 antibodies as an additional control for equal loading conditions. Similar results were obtained in at least two other independent experiments.

growth arrest occurred in G₁ with a time course similar to that induced by TGF-β (Fig. 2B). p21^{-/-} keratinocytes displayed a shortened G₁ phase (as determined by their response to mimosine), in accordance with their ability to grow faster than wild-type cells, but they still arrested in G₁ in the presence of either calcium or TGF-β (Fig. 2B).

Thus, lack of p21 function does not affect cell cycle withdrawal associated with differentiation, neither is it by itself sufficient to interfere with TGF-β-induced growth arrest.

Abnormal differentiation properties of p21 knockout keratinocytes

Even if p21 function is not essential for growth inhibition per se, it may still be involved in other important aspects of the keratinocyte differentiation process. Primary keratinocyte cultures contain a small number of cells that undergo spontaneous differentiation already under basal, low calcium conditions. Differentiation is induced in most if not all cultured cells by calcium addition (Hennings et al. 1980). Keratin 1 (K1) is a marker of the spinous layer of the epidermis, which is expressed at low levels in growing keratinocytes, and is induced upon calcium addition (Dlugosz and Yuspa 1993; Calautti et al. 1995). Remarkably, relative to genetically matched wild-type controls, K1 expression was found to be substantially reduced in p21^{-/-} keratinocytes, both under basal conditions and after calcium treatment (Fig. 4). When keratinocytes derived from heterozygous p21 knockout mice were analyzed in parallel with cells from

Figure 4. Comparative analysis of differentiation marker expression in p21 knockout vs. wild-type keratinocytes. Primary keratinocytes from p21 knockout mice and genetically matched wild-type controls were either kept under growing conditions (0) or induced to differentiate by calcium (2 mM) for the indicated times (hours). Same amounts of total cell extracts were analyzed by 7.5% SDS-PAGE and immunoblotted with antibodies specific for K1, K5, loricrin (Lor), involucrin (Inv), and filaggrin. Filaggrin is synthesized as a high-molecular-weight precursor, profilaggrin, which is subsequently processed. The diffuse bands correspond to the multiple products of this processing. The same blot was sequentially probed, in the order, with antibodies against K1, involucrin, and K5. The same protein extracts were run on a second gel and sequentially blotted with antibodies against loricrin and filaggrin, followed again

with anti-K5 immunoblotting as a control for equal loading conditions. Similar results were observed in at least two other independent experiments.

homozygous littermates, levels of K1 expression in the heterozygous cells were found to be intermediate between those in the homozygous knockouts and the wild-type controls [not shown]. Two other markers of the suprabasal epidermal layers, involucrin and loricrin (Dlugosz and Yuspa 1993), were also expressed at much lower levels in p21^{-/-} keratinocytes than in p21^{+/+} controls (Fig. 4). Like K1, expression of these markers was significantly reduced in keratinocytes already under basal, low calcium conditions, consistent with lack of p21 function affecting the intrinsic ability of keratinocytes to differentiate. In contrast, filaggrin, which is also a marker of the upper epidermal layers and is induced by calcium, exhibited a similar expression pattern in both p21^{-/-} and p21^{+/+} cells (Fig. 4). Normal expression levels were also found with keratin 5 (K5), which is a marker of the basal epidermal layers constitutively expressed in cultured mouse keratinocytes (Caiauti et al. 1995) (Fig. 4).

Thus, lack of p21 results in specific alterations of the keratinocyte differentiation program, with a subset of differentiation markers being significantly reduced in cells already under basal conditions, as well as after calcium exposure.

Aggressive tumorigenic behavior of p21 knockout keratinocytes after ras oncogene transformation

Immunostaining of newborn epidermis revealed a partial reduction of involucrin and K1-positive layers in p21 knockout mice. The histological appearance of the epidermis of newborn animals and of the adults was otherwise normal (data not shown). The absence of additional alterations may be attributable, at least in part, to overriding homeostatic mechanisms that normally operate in the skin. A significant imbalance between keratinocyte growth and differentiation could be unmasked

even in vivo, under conditions where such homeostatic mechanisms are mostly removed. We tested this possibility by evaluating the tumorigenic potential of p21^{-/-} and p21^{+/+} keratinocytes after transformation with a *ras* oncogene and subcutaneous injections into nude mice.

Transformation of mouse primary keratinocytes with a retrovirally transduced *ras* oncogene is sufficient to induce tumor formation, which can be more or less aggressive depending on number of retrovirus-infected cells, levels of *ras* expression, and conditions of the surrounding cellular environment (Roop et al. 1986; Dotto et al. 1988; Brissette et al. 1992). In particular, infection of keratinocytes with helper-free Harvey Sarcoma virus (HaSV), unlike the helper-associated form, results in lower *ras* expression levels, which in turn correlate with the induction of a much weaker tumorigenic phenotype (Brissette et al. 1992).

p21^{-/-} keratinocytes and genetically matched wild-type controls were infected in vitro with a helper-free Ha-*ras* retrovirus, grown for a week in culture, and injected subcutaneously into nude mice at two different cell concentrations. At 3 weeks after injection, the number and size of tumors formed by *ras*-transformed p21^{-/-} keratinocytes were dramatically different from those formed by similarly transformed control cells (Table 1). p21-deficient keratinocytes consistently produced very large tumors, whereas control keratinocytes gave rise to very small or no lesions. Histologically, control tumors essentially consisted of keratinized cysts surrounded by a limited and well-structured epithelium, with characteristics similar to benign papillomas (Fig. 5A). In contrast, tumors formed by p21^{-/-} cells exhibited the features of aggressive squamous cell carcinomas, with very high cellularity, and areas of squamous cell differentiation next to others of much more pronounced anaplasia (Fig. 5B,C). Threads of undifferentiated kera-

Table 1. Tumorigenic behavior of control vs. p21 and p27 knockout keratinocytes after *ras* oncogene transformation

Cells	Tumorigenicity	Weight	Histology
p21 +/+ (5 × 10 ⁵)	1/10	0.05 g	Benign lesion
p21 -/- (5 × 10 ⁵)	10/10	0.18 g (±0.034)	SCC I-III
p21 +/+ (2 × 10 ⁶)	4/10	0.02 g (±0.005)	Benign lesions
p21 -/- (2 × 10 ⁶)	10/10	0.48 g (±0.11)	SCC I-III
p27 +/+ (5 × 10 ⁵)	0/5		
p27 -/- (5 × 10 ⁵)	0/5		
p27 +/+ (2 × 10 ⁶)	0/4		
p27 -/- (2 × 10 ⁶)	1/9	<0.01 g	NT

Ha-*ras* infected keratinocytes were injected subcutaneously into nude mice. Cells (5 × 10⁵ or 2 × 10⁶) were used for each injection, as indicated. Tumorigenicity is defined as the ratio between the number of tumors obtained and the number of injections. In each experiment the average weight is reported, with the standard error in parentheses. The tumor weight between the groups was significantly different ($P < 0.005$). Histologically, the tumors were very consistent in each category. Benign lesions are defined as very small lesions, which span from benign cysts to highly differentiated papilloma with massive keratin production. Squamous cell carcinoma (SCC) were from well differentiated with keratin production (type I) to highly undifferentiated with very little cellular organization (type III). Given the size reached by p21 knockout tumors, mice were sacrificed at 3 wk after injection. In the experiments with p27 knockout cells, mice were sacrificed at 1 month after injection. Similar results were obtained in two other independent experiments. Very weak tumorigenicity similar to that of genetically matched wild-type keratinocytes was also observed in additional control experiments with Ha-*ras* infected keratinocytes derived from the Sencar strain (a strain selected for "Sensitivity to skin carcinogenesis"). (NT) Not tested.

tin-positive cells invading the surrounding stroma were observed frequently (Fig. 5D).

Thus, loss of p21 function cooperates with *ras* oncogene transformation in the induction of aggressive and relatively undifferentiated tumors in vivo.

No increased tumorigenicity of p27 knockout keratinocytes, with limited differentiation abnormalities

The markedly increased predisposition to tumorigenic conversion of p21 knockout keratinocytes could result from the loss of the cell cycle inhibitory function that p21 normally provides or could be linked to other, more specific functions of p21, not shared with other CKIs. To discriminate between these two possibilities, we tested the tumorigenic potential of primary keratinocytes with a disruption of the closely related p27 gene. As mentioned before, mice with an inactivating mutation of p27

Figure 5. Histological appearance of tumors formed by p21-deficient vs. wild-type keratinocytes after *ras* oncogene transformation. p21 knockout primary keratinocytes and genetically matched wild-type controls were infected with a helper-free Ha-*ras* retrovirus (Ha-*ras*) 1 day after plating and cultivated for 6 days before subcutaneous injection into nude mice (2 × 10⁶ cells/injection). After 3 weeks, mice were sacrificed and tumors were isolated for histological analysis. (A) Papilloma obtained by injection of wild-type keratinocytes infected with Ha-*ras*. Note the well organized structure of this lesion, showing abundant keratin and high level of differentiation. (B) Undifferentiated grade II-III carcinoma obtained by injection of p21 -/- keratinocytes infected with Ha-*ras*. (C) High magnification of a tumor deriving from Ha-*ras*-infected p21 -/- keratinocytes. (D) Immunofluorescence of a similar region of a tumor deriving from Ha-*ras*-infected p21 -/- keratinocytes, with pan-keratin antibodies. The positive staining confirmed the epithelial nature of the highly disorganized bundles of cells. Similar expression of Ha-*ras* in p21 +/+ and p21 -/- keratinocytes was confirmed by immunoblotting. Magnification: (A,B) 100×; (C,D) 400×.

exhibit significant hyperplasia in a number of tissues (Fero et al. 1996; Kiyokawa et al. 1996; Nakayama et al. 1996), even if their epidermis, like that of p21 knockouts, is histologically normal (our unpublished observations).

Primary keratinocytes were derived from the p27 knockouts and genetically matched wild-type controls, infected with the helper-free Ha-*ras* retrovirus 2 days after plating, grown for a week in culture, and injected subcutaneously into nude mice at the same concentrations used for the p21 knockout keratinocytes. In contrast to these cells, which started to form detectable tu-

mors as early as 1–2 weeks after injections, p27 knockout keratinocytes transformed in a similar manner behaved like the controls and formed little or no tumors even at 4 weeks after injection (Table 1).

To gain some insights into the basis for this specificity, we undertook a comparative analysis of the growth/differentiation behavior of p27- versus p21-deficient keratinocytes under well-defined in vitro conditions. Like with p21^{-/-} cells, the BrdU nuclear labeling index of asynchronous cultures of p27 knockout keratinocytes was found to be increased, even if to a lesser extent (32% vs. 25% of genetically matched wild-type controls; similar results were obtained in a second independent experiment). p27-deficient keratinocytes were as sensitive as wild-type or p21 knockout cells to calcium-induced growth arrest (not shown). p21 and p27 knockout keratinocytes were also equally sensitive to TGF- β growth inhibition, at the highest concentrations (Fig. 6). Interestingly, and consistent with previous biochemical studies [Massagué et al. 1994; Polyak et al. 1994; Reynisdottir et al. 1995], partial TGF- β resistance was observed with p27 knockout keratinocytes, in either homo- or heterozygous states, at lower TGF- β concentrations (Fig. 6). p21 knockout keratinocytes also exhibited a partial level of TGF- β resistance but only at the lowest concentration (Fig. 6).

A subset of differentiation markers are significantly reduced in p21-deficient keratinocytes, which are characteristic of cells of the intermediate and upper epidermal layers (Fig. 4). A similar analysis of p27 knockout cells revealed that K1, a marker of the intermediate spinous layer, is down-modulated to a similar extent in these cells as in the p21 knockouts (Fig. 7). In contrast, loricrin and involucrin, markers of the granular and cornified layers that are down-modulated in the p21 knockouts, were found to be expressed at normal or only slightly reduced levels in the p27-deficient cells (Fig. 7). Expression of filaggrin and K5 was also normal in these cells.

Thus, the striking tumorigenic behavior of p21-deficient keratinocytes is specifically linked to loss of this

CKI, as it is not observed with keratinocytes with a disruption of the closely related p27 gene. This specific susceptibility to tumorigenic conversion correlates with a block of late keratinocyte marker expression in the p21 but not p27 knockout cells.

Discussion

The most striking alteration of p21 knockout keratinocytes that we have uncovered is their very aggressive tumorigenic behavior after *ras* oncogene transformation. Although p21 is thought to be one of the primary mediators of p53-induced growth inhibition [El-Deiry et al. 1993], its role in p53 tumor suppression has been questioned, as p21 knockout mice do not exhibit an increased rate of spontaneous tumor formation [Deng et al. 1995], as it is instead observed with mice lacking p53 [Donehower et al. 1992; Jacks et al. 1994]. However, the spontaneous tumors formed in the p53 knockouts do not involve the epidermis, suggesting that p53 may exert different tumor suppressing functions in this tissue. In fact, mutation and/or loss of the p53 gene has been shown to play an important role in chemically induced mouse skin carcinogenesis, but only in the late stages of benign to malignant tumor conversion [Kemp et al. 1993]. The possibility that p53 and p21 genes have at least partially overlapping tumor suppressing functions in keratinocytes is indicated by the highly aggressive tumorigenic behavior of *ras*-transformed p21 knockout keratinocytes, which is similar to that previously reported for *ras*-transformed keratinocytes with a p53 deletion [Weinberg et al. 1994].

Given their central role in cell cycle control, the various CDK inhibitors would be expected to fulfill a rather general function in tumor suppression. Mutations of CKIs of the p15 and p16 family are common in a large number of malignancies of various origin [Sherr and Roberts 1995], and spontaneous development of various tumors has been reported in mice with a disruption of the INK4a locus [Serrano et al. 1996]. In contrast, CKIs of the p21/p27 family appear to have a much more restricted

Figure 6. Differential sensitivity to TGF- β growth inhibition of p27 vs. p21 knockout keratinocytes and wild-type cells. Primary keratinocytes derived from mice with a homozygous ($-/-$) or heterozygous ($+/-$) disruption of the p27 (dark shaded bars) or p21 (light shaded bars) genes and wild-type (wt) controls (open bars) were tested for their sensitivity to the growth inhibitory effects of increasing TGF- β concentrations (0.1, 0.5, 2.5 ng/ml). DNA synthesis was measured at 24 hr after TGF- β exposure in triplicate wells, by a 1-hr [3 H]thymidine incorporation assay. Values are expressed as percentage of untreated controls and the standard error is indicated. Similar results were obtained in two other independent experiments.

Figure 7. Comparative analysis of differentiation marker expression in p27 knockout vs. wild-type keratinocytes. Primary keratinocytes from p27 knockout mice and genetically matched wild-type controls were either kept under growing conditions (0) or induced to differentiate by calcium (2 mM) for the indicated times (hours). Same amounts of total cell extracts were analyzed by 7.5% SDS-PAGE and sequentially blotted with antibodies against K1, involucrin (Inv), loricrin (Lor), filaggrin, and K5 as described in Fig. 4.

role as tumor suppressors. Mutations of either gene have very rarely if ever been detected in any types of tumors, and spontaneous neoplastic development is observed in p27 knockout mice at only one site, the intermediate lobe of the pituitary gland (Fero et al. 1996; Kiyokawa et al. 1996; Nakayama et al. 1996). The absence of spontaneous tumor development in p21 knockout mice (Deng et al. 1995), coupled with the strikingly increased tumorigenicity of keratinocytes with a knockout mutation of the p21 but not p27 gene, points to a similarly restricted and specific role of p21 in tumor suppression. Our findings further suggest that this function of p21 in keratinocyte tumor suppression is probably linked to its specific involvement in terminal differentiation control rather than simple withdrawal from the cell cycle.

Primary keratinocytes derived from p21 knockout mice and cultured for no more than 3–4 days were found to have a significant increase in S-phase fraction relative to the wild-type controls. Thus, at least in this particular epithelial cell type, p21 plays a significant inhibitory function already under basal cycling conditions and in the absence of additional stimuli known to enhance its expression, such as prolonged cultivation and senescence (Noda et al. 1994; Deng et al. 1995). However, this effect does not appear to be specific for lack of p21, as a p27 deficiency resulted in a similar, although lesser, increase in S-phase fraction. Both p21 and p27 knockout keratinocytes still undergo a G₁ block after calcium-induced differentiation similar to that of control cells. Thus, function of these individual CKIs is not essential for the differentiation-associated growth arrest, as lack of one may be compensated by the other, and/or other changes in cell cycle regulatory proteins that we have found during this process (such as cyclin D1 and D2 down-modulation). p21 knockout keratinocytes showed a partial resistance to TGF- β growth inhibition, but only at very low concentrations. p27-deficient keratinocytes exhibited also a partial TGF- β resistant phenotype, which was evident even at higher concentrations. Trans-

formation with the *ras* oncogene increased the resistance of p21 knockout keratinocytes to TGF- β growth inhibition, but to levels that were similar to those of p27 knockout cells (data not shown). Thus, the striking tumorigenicity of p21 but not p27 knockout keratinocytes after *ras* oncogene transformation cannot be accounted for by a differential sensitivity to TGF- β -mediated tumor suppression (Missero et al. 1991).

In contrast to its inability to specifically interfere with differentiation- or TGF- β -induced growth arrest, the loss of p21 function was found to drastically affect expression of a specific subset of keratinocyte differentiation markers, in a manner that does not result from a p27 deficiency. Expression of these markers is reduced already in cells under basal conditions, pointing to an intrinsic impairment of their ability to differentiate, not only in response to calcium. Comparative analysis of p21 and p27 knockout keratinocytes raised the very interesting possibility that two distinct differentiation/cell commitment pathways exist: one for K1-expressing cells, for which both p21 and p27 functions are required, and the other for loricrin and involucrin expressing cells, in which only p21 function is specifically involved (Fig. 8A). It is noteworthy that control of K1 and involucrin/loricrin expression can be pharmacologically dissociated, as all of these markers are induced by calcium, although only involucrin and loricrin are induced by phorbol esters, whereas K1 is suppressed (Dlugosz and Yuspa 1993). K1 is expressed in cells of the intermediate epidermal layers (stratum spinosum), whereas loricrin and involucrin are expressed by the more committed cell populations of the upper layers (stratum granulosum and stratum corneum). Thus, whereas expression of a transforming *ras* oncogene may promote increased keratinocyte growth and tumorigenesis, p21 function may fulfill a counteracting and opposite role, by maintaining or shifting the balance toward more committed terminally differentiated populations (Fig. 8B). Loss of this critical CKI or its decreased expression as a consequence of p53

Figure 8. Specific function of p21 in keratinocyte differentiation control and tumor suppression. (A) Diagram of the keratinocyte differentiation pathway and how it is differently influenced by p21 and p27 function. (B) Counteracting effects of *ras* transformation and p21 function in determining keratinocyte tumor development.

mutation would alter such a balance and be an essential step in keratinocyte tumor development. This regulatory loop may be operative in other cellular systems as well, for instance, melanoma cells, where overexpression of the p21 protein induces not only growth arrest but also some aspects of differentiation as well as tumor suppression (Yang et al. 1995).

It is still possible that loss of p21 function favors the development of a third mutation that in turn cooperates with *ras* in keratinocyte tumor development. However, we consider this as an unlikely possibility, as primary keratinocytes were infected on mass with a high titer *ras*-transducing retrovirus 2 days after plating and were maintained in culture without splitting for only 6 days prior to injection into mice. There was no external selection applied. The likelihood of rare third-site mutants having grown during this period into large enough populations to generate tumors is low, given that tumor formation was apparent already by 7–10 days after injection and that relatively low numbers of cells were injected.

p21 is composed of two functional domains, one involved in cyclin/CDK association and the other in direct DNA polymerase inhibition (Chen et al. 1995; Luo et al. 1995). Very recently, the p21 protein has been found to exert an additional biochemical function as a noncompetitive inhibitor of stress-activated kinases (SAPKs), which control a number of specific transcription factors with a key role in the control of cell growth and differentiation (Shim et al. 1996). The amino-terminal domains of p21 and p27 share an unexpected structural and functional homology with the cyclin/CDK-binding domain of the Rb-related p107 protein (Zhu et al. 1995). As it has been proposed on the basis of this homology, it is possible that p21^{Cip1/WAF1} function in keratinocyte differentiation and tumor suppression involves the establishment of a specific bridge between signaling com-

plexes such as cyclin/CDKs or SAPKs and other multiprotein apparatuses, such as the transcription and/or DNA synthesis machinery.

Materials and methods

Cells

In each case, primary keratinocyte preparations were derived from pools of 6–10 newborn mice with a homozygous p21 or p27 knockout mutation and corresponding genetically matched wild-type controls (Deng et al. 1995; Kiyokawa et al. 1996). Cells were cultivated in minimal essential medium with 4% Chelex-treated fetal calf serum (Hyclone), epidermal growth factor (EGF; 10 ng/ml; Collaborative Research, Inc., Cambridge, MA), and 0.05 mM CaCl₂ (low calcium medium), as described (Hennings et al. 1980; Missero et al. 1995). For all experiments, cells were used 1 week after plating. Culture medium was changed for the last time 24 hr before the experiment. Keratinocyte differentiation was induced by addition of CaCl₂ (to 2 mM). TGF-β1 was purchased from R&D, and mimosine from Sigma.

DNA synthesis assays

BrdU-labeling experiments Primary keratinocytes were labeled with BrdU (1:500 dilution; cell proliferation reagent; Amersham) for 3 hr and subsequently fixed in 2% paraformaldehyde in phosphate-buffered saline (PBS). After fixation, cells were permeabilized with NP-40 0.1% and the DNA was denatured with 50 mM NaOH. BrdU was detected with mouse monoclonal antibodies (1:4; Becton-Dickinson) and anti-mouse IgG antibodies conjugated to fluorescein isothiocyanate (FITC; Capell). DNA staining was obtained with 1 μg/ml solution of Hoechst dye 33258.

[³H]Thymidine incorporation assays Primary keratinocytes were plated onto 24-well trays and tested 5 days later. DNA synthesis was measured by [Methyl-³H]thymidine (3.3 μCi/ml, 40–60 Ci/mmol; ICN) incorporation as described previously (Missero et al. 1991).

To monitor cell cycle progression into S phase, cells grown in 24-well racks were synchronized by serum and EGF starvation for 36 hr. Calcium (2 mM), TGF-β (3 ng/ml), and mimosine (50 μM) were added to the cultures at indicated times after serum and EGF stimulation. Cells in triplicate wells were labeled at 21 hr after stimulation with [³H]thymidine for 3 hr.

Cell cycle analysis

Mouse primary keratinocytes were plated at high density (4 × 10⁷/100-mm dish), cultivated for 2 days in low calcium medium, collected by trypsinization, washed in PBS, and resuspended (2 × 10⁶/ml) in the staining solution [0.1% sodium citrate (pH 7.8), 3% (wt/vol) PEG 6000, 0.1% (vol/vol) Triton X, 50 μg/ml of propidium iodide, 1 mg/ml of RNase], and incubated at 37°C for 20 min. Equal volume of the salt solution [0.376 M NaCl, 3% (wt/vol) PEG 6000, 0.1% (vol/vol) Triton X, 50 μg/ml of propidium iodide, at final pH 7.2] was added, and cells were stored in the dark at 4°C for 12–18 hr. DNA fluorescence was measured with a FACScan flow cytometer (Becton-Dickinson, San Jose, CA). Cell debris was gated out, and fractions of cells in G₁, S, and G₂/M were quantitated using ModFit LT software.

Immunoblotting and antibodies

Total cell extracts were normalized for protein concentration (Bradford assay; Bio-Rad Laboratories), and same amounts of

proteins (40 μ g) were separated by SDS-PAGE and transferred to Immobilon-P membranes (Millipore). Equal loading conditions were verified by Coomassie blue and Ponceau staining of gels and blots, respectively. Immunoblots were probed with the following antibodies: Rabbit antisera against cdk2, cdk4, and cyclin D2 and mouse monoclonals against cyclin D1 and D3 were purchased from Santa Cruz; mouse monoclonals against cyclin A and p27 were purchased from UBI and Transduction Laboratories, respectively; rabbit antisera against murine p21 and cyclin E were generously provided by Drs. C. Schneider (Laboratorio Nazionale Consorzio Interuniversitario Biotechnologie, Trieste, Italy) and R.A. Weinberg (Whitehead Institute, Cambridge, MA), respectively. Affinity-purified rabbit antisera against K1, K5, loricrin, and filaggrin were generous gifts of Dr. S.H. Yuspa (National Cancer Institute, Bethesda, MD). Anti-involucrin antisera was purchased from Berkeley Antibodies Company. Blots were developed using horseradish peroxidase-conjugated secondary antibodies and the ECL detection system (Amersham).

Tumorigenicity assays

Knockout and wild-type control keratinocytes were infected 2 days after plating with a high titer helper-free recombinant retrovirus based on the LXCN vector (Palmer et al. 1987) and transducing the v-Ha-ras oncogene (Dotto et al. 1985). At 6 days after infection, cells were collected by trypsinization, counted, and injected subcutaneously into nude mice at the indicated concentrations. To minimize individual animal variations, ras-transformed knockout and wild-type keratinocytes were injected in parallel in the same animals, on the right and left sides, respectively. Each tumor was separated from surrounding normal tissues, weighed, and formalin fixed for histological analysis. Sections of frozen tumors were used for immunohistochemical analysis with rabbit anti-keratin wide spectrum antibodies (1:1000; Axell) and subsequently stained with an avidin-biotin immunofluorescence technique, as described previously (Calautti et al. 1995).

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References

Alexandrow, M.G. and H.L. Moses. 1995. Transforming growth factor β 1 inhibits mouse keratinocytes late in G1 independent of effects on gene transcription. *Cancer Res.* **55**: 3928–3932.

Brissette, J., C. Missero, S.H. Yuspa, and G.P. Dotto. 1992. Different levels of v-Ha-Ras-p21 expression in primary keratinocytes transformed with Harvey sarcoma virus correlate with benign versus malignant behavior. *Mol. Carcinogenesis* **7**: 21–25.

Brugarolas, J., C. Chandrasekaran, J. Gordon, D. Beach, T. Jacks, and G. Hannon. 1995. Radiation-induced cell cycle arrest

compromised by p21 deficiency. *Nature* **377**: 552–557.

Calautti, E., C. Missero, P.L. Stein, R.M. Ezzell, and G.P. Dotto. 1995. Fyn tyrosine kinase is involved in keratinocyte differentiation control. *Genes & Dev.* **9**: 2279–2291.

Chen, J., P. Jackson, M. Kirschner, and A. Dutta. 1995. Separate domains of p21 involved in the inhibition of Cdk kinase and PCNA. *Nature* **374**: 386–388.

Cox, L.S. and D.P. Lane. 1995. Tumor suppressors, kinases and clamps: How p53 regulates the cell cycle in response to DNA damage. *BioEssays* **17**: 501–508.

Deng, C., P. Zhang, J. Harper, S. Elledge, and P. Leder. 1995. Mice lacking p21^{CIP1/WAF1} undergo normal development, but are defective in G1 checkpoint control. *Cell* **82**: 675–684.

Dlgosz, A.A. and S.H. Yuspa. 1993. Coordinate changes in gene expression which mark the spinous to granular cell transition in epidermis are regulated by protein kinase C. *J. Cell Biol.* **120**: 217–225.

Donehower, L.A., M. Harvey, B.L. Slagle, M.J. McArthur, C.A. Montgomery Jr., J.S. Butel, and A. Bradley. 1992. Mice deficient for p53 are developmentally normal but susceptible to spontaneous tumors. *Nature* **356**: 215–221.

Dotto, G.P., L.F. Parada, and R.A. Weinberg. 1985. Specific growth response of ras-transformed embryo fibroblasts to tumor promoters. *Nature* **318**: 472–475.

Dotto, G.P., R.A. Weinberg, and A. Ariza. 1988. Malignant transformation of mouse primary keratinocytes by HaSV and its modulation by surrounding normal cells. *Proc. Natl. Acad. Sci.* **85**: 6389–6393.

Dulic, V., E. Lees, and S.I. Reed. 1992. Association of human cyclin E with a periodic G1-S phase protein kinase. *Science* **257**: 1958–1961.

El-Deiry, W.S., T. Takashi, V.E. Velculescu, D.B. Levy, R. Parsons, J.M. Trent, D. Lin, W.E. Mercer, K.W. Kinzler, and B. Vogelstein. 1993. WAF1, a potential mediator of p53 tumor suppression. *Cell* **75**: 817–825.

Fero, M.L., M. Rivkin, M. Tasch, P. Porter, C.E. Carow, E. Firpo, K. Polyak, L. Tsai, V. Broudy, R.M. Perlmutter, K. Kaushansky, and J.M. Roberts. 1996. A syndrome of multiorgan hyperplasia with features of gigantism, tumorigenesis, and female sterility in p27^{Kip1} deficient mice. *Cell* **85**: 733–744.

Gu, Y., C.W. Turek, and D.O. Morgan. 1993. Inhibition of CDK2 activity in vivo by an associated 20KD regulatory subunit. *Nature* **366**: 707–710.

Halevy, O., B. Novitch, D. Spicer, S. Skapek, J. Rhee, G. Hannon, D. Beach, and A. Lassar. 1995. Correlation of terminal cell cycle arrest of skeletal muscle with induction of p21 by MyoD. *Science* **267**: 1018–1021.

Harper, J.W., G.R. Adami, N. Wei, K. Keyomarsi, and S.J. Elledge. 1993. The p21 cdk-interacting protein Cip1 is a potent inhibitor of G1 cyclin-dependent kinases. *Cell* **75**: 805–816.

Hennings, H., D. Michael, C. Cheng, P. Steinert, K. Holbrook, and S.H. Yuspa. 1980. Calcium regulation of growth and differentiation of mouse epidermal cells in culture. *Cell* **19**: 245–254.

Hinds, P.W. and R.A. Weinberg. 1994. Tumor suppressor genes. *Curr. Opin. Genet. Dev.* **4**: 135–141.

Jacks, T., L. Remington, B.O. Williams, E.M. Schmitt, S. Halachmi, R.T. Bronson, and R.A. Weinberg. 1994. Tumor spectrum analysis in p53-mutant mice. *Curr. Biol.* **4**: 1–7.

Jiang, H., J. Lin, S. Zao-zhong, F. Collart, E. Huberman, and P. Fisher. 1994. Induction of differentiation in human promyelocytic HL-60 leukemia cells activates p21, WAF1/CIP1, expression in the absence of p53. *Oncogene* **9**: 3397–3406.

Jiang, H., J. Lin, S. Zao-zhong, M. Herlyn, R. Kerbel, B. Weiss-

- man, D. Welch, and P. Fisher. 1995. 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* **11**: 1855–1864.
- Kato, J., M. Matsuo, K. Polyak, J. Massagué, and C.J. Sherr. 1994. Cyclic AMP-induced G1 phase arrest mediated by an inhibitor (p27^{Kip1}) of cyclin-dependent kinase-4 activation. *Cell* **79**: 487–496.
- Kemp, C.J., L.A. Donehower, A. Bradley, and A. Balmain. 1993. Reduction of p53 gene dosage does not increase initiation or promotion but enhances malignant progression of chemically induced skin tumors. *Cell* **74**: 813–822.
- Kiyokawa, H., R.D. Kineman, K.O. Manova-Todorova, V.C. Soares, E.S. Hoffman, M. Ono, D. Khanam, A.C. Hayday, L.A. Frohman, and A. Koff. 1996. Enhanced growth of mice lacking the cyclin-dependent kinase inhibitor function of p27^{Kip1}. *Cell* **85**: 721–732.
- Kranenburg, O., V. Scharnhorst, A. Van der Eb, and A. Zantema. 1995. Inhibition of cyclin-dependent kinase activity triggers neuronal differentiation of mouse neuroblastoma cells. *J. Cell Biol.* **131**: 227–234.
- Lee, M.H., M. Nikolic, C.A. Baptista, E. Lai, L.H. Tsai and J. Massagué. 1996. The brain-specific activator p35 allows Cdk5 to escape inhibition by p27^{Kip1} in neurons. *Proc. Natl. Acad. Sci.* **93**: 3259–3263.
- Li, R., S. Waga, G.J. Hannon, D. Beach, and B. Stillman. 1994. Differential effects by the p21 CDK inhibitor on PCNA dependent DNA replication and repair. *Nature* **371**: 534–537.
- Luo, Y., J. Hurwitz, and J. Massagué. 1995. Cell-cycle inhibition by independent CDK and PCNA binding domains in p21^{Cip1}. *Nature* **375**: 159–161.
- MacLeod, K.F., N. Sherry, G. Hannon, G. Beach, T. Tokino, K.W. Kinzler, B. Vogelstein, and T. Jacks. 1995. p53-dependent and independent expression of p21 during cell growth, differentiation, and DNA damage. *Genes & Dev.* **9**: 935–944.
- Massagué, J. 1990. The transforming growth factor- β family. *Annu. Rev. Cell Biol.* **6**: 597–641.
- Massagué, J., K. Polyak, M.H. Lee, H. Erdjument-Bromage, A. Koff, J.M. Roberts, and P. Tempst. 1994. Cloning of p27^{Kip1}, a cyclin-dependent kinase inhibitor and a potential mediator of extracellular antimitogenic signals. *Cell* **78**: 59–66.
- Missero, C. and G.P. Dotto. 1996. p21^{WAF1/Cip1} and terminal differentiation control of normal epithelia. *Mol. Cell. Diff.* **4**: 1–16.
- Missero, C., S. Ramon y Cajal, and G.P. Dotto. 1991. Escape from TGF- β control and oncogene cooperation in skin tumor development. *Proc. Natl. Acad. Sci.* **88**: 9613–9617.
- Missero, C., E. Calautti, R. Eckner, J. Chin, L.-H. Tsai, D.M. Livingston, and G.P. Dotto. 1995. Involvement of the cell cycle inhibitor Cip1/WAF1 and the *Ela*-associated p300 protein in terminal differentiation. *Proc. Natl. Acad. Sci.* **92**: 5451–5455.
- Nakayama, K., N. Ishida, M. Shirane, A. Inomata, T. Inoue, N. Shishido, I. Horii, D.Y. Loh, and K. Nakayama. 1996. Mice lacking p27^{Kip1} display increased body size, multiple organ hyperplasia, retinal dysplasia, and pituitary tumors. *Cell* **85**: 707–720.
- Noda, A., Y. Ning, S.F. Venable, O.M. Pereira-Smith, and J.R. Smith. 1994. Cloning of senescent cell-derived inhibitors of DNA synthesis using an expression screen. *Exp. Cell Res.* **211**: 90–98.
- Nourse, J., E. Firpo, W.M. Flanagan, S. Coats, K. Polyak, M.H. Lee, J. Massagué, G. Crabtree, and J. Roberts. 1994. Interleukin 2-mediated elimination of p27^{Kip1} cyclin-dependent kinase inhibitor prevented by rapamycin. *Nature* **372**: 570–573.
- Palmer, T.D., R.A. Hock, R.A. Osborne, and A.D. Miller. 1987. Efficient retrovirus-mediated transfer and expression of a human adenosine deaminase gene in diploid skin fibroblasts from an adenosine deaminase-deficient human. *Proc. Natl. Acad. Sci.* **84**: 1055–1059.
- Pardee, A.B. 1989. G1 events and regulation of cell proliferation. *Science* **246**: 603–608.
- Parker, S., G. Eichele, P. Zhang, A. Rawls, A. Sands, A. Bradley, E. Olson, J. Harper, and S. Elledge. 1995. p53-independent expression of p21^{Cip1} in muscle and other terminally differentiating cells. *Science* **267**: 1024–1027.
- Polyak, K., M. Lee, H. Erdjument-Bromage, A. Koff, J.M. Roberts, P. Tempst, and J. Massagué. 1994. Cloning of p27^{Kip1}, a cyclin-dependent kinase inhibitor and a potential mediator of extracellular antimitogenic signals. *Cell* **78**: 59–66.
- Reynisdottir, R., K. Polyak, A. Lavarone, and J. Massagué. 1995. Kip/Cip and INK4 CDK inhibitors cooperate to induce cell cycle arrest in response to TGF- β . *Genes & Dev.* **9**: 1149–1163.
- Roop, D.R., D.R. Lowy, P. Tambourin, J. Strickland, J.R. Harper, Balaschak, E. Spangler, and S. Yuspa. 1986. An activated Harvey *ras* oncogene produces benign tumours on mouse epidermal tissue. *Nature* **323**: 822–824.
- Serrano, M., H.-W. Lee, L. Chin, C. Cordon-Cardo, D. Beach, and R.A. Depinho. 1996. Role of the INK4a locus in tumor suppression and cell mortality. *Cell* **85**: 27–37.
- Sherr, C.J. 1994. G1 phase progression: Cycling on Cue. *Cell* **79**: 551–555.
- Sherr, C.J. and J.M. Roberts. 1995. Inhibitors of mammalian G₁ cyclin-dependent kinases. *Genes & Dev.* **9**: 1149–1163.
- Shim, J., H. Lee, J. Park, H. Kim, and E.-J. Choi. 1996. A non-enzymatic p21 protein inhibitor of stress-activated protein kinases. *Nature* **381**: 804–807.
- Shivji, M.K.K., S.J. Grey, U.P. Strausfeld, R.D. Wood, and J.J. Blow. 1994. Cip1 inhibits DNA replication but not PCNA-dependent nucleotide excision-repair. *Curr. Biol.* **4**: 1062–1068.
- Steinman, R., B. Hoffman, A. Iro, C. Guillouf, D. Liebermann, and M. El-Houseini. 1994. Induction of p21 (WAF-1/CIP1) during differentiation. *Oncogene* **9**: 3389–3396.
- Waga, S., G.J. Hannon, D. Beach, and B. Stillman. 1994. The p21 inhibitor of cyclin-dependent kinases controls DNA replication by interaction with PCNA. *Nature* **369**: 574–578.
- Weinberg, R.A. and T. Jacks. 1994. Extensive contribution of Rb-deficient cells to adult chimeric mice with limited histopathological consequences. *EMBO J.* **13**: 4251–4259.
- Weinberg, W.C., C.G. Azzoli, N. Kadiwar, and S.H. Yuspa. 1994. p53 gene dosage modifies growth and malignant progression of keratinocytes expressing the v-ras^{Hs} oncogene. *Cancer Res.* **54**: 5584–5592.
- Williams, B.O., E.M. Schmitt, L. Remington, R.T. Bronson, D.M. Albert, R.A. Weinberg, and T. Jacks. 1994. Extensive contribution of Rb-deficient cells to adult chimeric mice with limited histopathological consequences. *EMBO J.* **13**: 4251–4259.
- Xiong, Y., G.J. Hannon, H. Zhang, D. Casso, R. Kobayashi, and D. Beach. 1993. p21 is a universal inhibitor of cyclin kinases. *Nature* **366**: 701–704.
- Yang, Z.Y., N.D. Perkins, T. Ohno, E.G. Nabel, and G.J. Nabel. 1995. The p21 cyclin-dependent kinase inhibitor suppresses tumorigenicity in vivo. *Nature Med.* **1**: 1052–1056.
- Zhu, L., E. Harlow, and B.D. Dynlacht. 1995. p107 uses a p21^{CIP1}-related domain to bind cyclin/cdk2 and regulate interactions with E2F. *Genes & Dev.* **9**: 1740–1752.)

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C Missero, F Di Cunto, H Kiyokawa, et al.

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