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Genetic Deletion of p21^{WAF1} Enhances Papilloma Formation but not Malignant Conversion in Experimental Mouse Skin Carcinogenesis

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Abstract

Tumor suppression by p53 is believed to reside in its ability to regulate gene transcription, including up-regulation of p21^{WAF1}. In *p53(−/−)* mice, chemical- or oncogene-induced skin tumors undergo accelerated malignant conversion. To determine the contribution of the *p21*^{WAF1} gene product to epidermal carcinogenesis, animals +/+, +/−, and −/− for a null mutation in the *p21*^{WAF1} gene were treated once with 25 nmol 7,12-dimethylbenz[a]anthracene, followed by 5 μg of TPA two times/week for 20 weeks. Papilloma frequency was higher in the p21^{WAF1}-deficient mice. However, the frequency of malignant conversion was similar among all three genotypes. After TPA treatment, all genotypes developed epidermal hyperplasia, although the labeling index was lower in *p21*^{WAF1} (−/−) epidermis compared with *p21*^{WAF1} (+/+). Furthermore, the expression of differentiation markers was the same across genotypes in untreated or TPA-treated epidermis. Similar frequencies of malignant conversion were also observed in an *in vitro* assay. Thus, p21^{WAF1} suppresses early stages of papilloma formation but not malignant progression in mouse skin carcinogenesis, and decreased levels of p21^{WAF1} do not account for the enhanced malignant conversion of p53 null epidermal tumors.

Introduction

The cyclin-dependent kinase inhibitor p21^{WAF1} (CIP1/CAP20/mda6/sdi1) was one of the first identified targets of p53-mediated gene transcription (1). Up-regulation of p21^{WAF1} after DNA damage participates in cell cycle arrest (2). Up-regulation has also been documented in cells undergoing differentiation, senescence, and apoptosis, all processes that may negatively influence tumor formation or progression (2–5). Both p53-dependent and -independent regulation of this gene product have been described (2, 4, 6, 7). In keratinocytes it has been also suggested that splice variants of p53 show differential regulation of target gene activation, including p21^{WAF1} (8). A role for p21^{WAF1} as a downstream effector of p53-mediated tumor suppression is supported by its ability to block proliferation of p53 deficient tumor cells *in vitro* and *in vivo* (1, 9, 10). Furthermore, both basal and induced levels of p21^{WAF1} are lower in p53-deficient cells (11, 12). However, the function of p21^{WAF1} as a tumor suppressor is unclear. Mutations in the gene are rarely observed in human cancers (2), and although a majority of mice homozygous for a null mutation in the *p53* gene develop spontaneous tumors by 6 months of age (13, 14), p21^{WAF1} null mice do not exhibit any predisposition to spontaneous tumor formation (15). In addition, previous studies from our laboratory have demonstrated that p53-deficient keratinocytes, but not p21^{WAF1}-deficient keratinocytes, display an increased growth rate *in vivo* after transduction with the v-*ras*^{Ha} oncogene and grafting to nude mouse hosts, compared with wild-type keratinocytes (12, 16). Furthermore, in this protocol, keratinocytes derived from all *p21*^{WAF1}genotypes formed papillomas with similar malignant conversion frequencies (16), whereas p53-deficient keratinocytes had a markedly increased conversion frequency over control keratinocytes (12). To test directly whether p21^{WAF1} levels can influence skin tumor formation and progression in an intact animal model, we have examined the incidence and malignant conversion of epidermal papillomas formed in mice expressing a null mutation in the *p21*^{WAF1} gene after a two-stage carcinogenesis protocol. We demonstrate that p21^{WAF1}-deficient mice display an increased predisposition to tumor formation after exposure to chemical carcinogens; however, the benign tumors formed in p21^{WAF1}-deficient mice do not display an increased malignant potential compared with those arising in *p21*^{WAF1} (+/+) mice. These results contrast to earlier studies of chemically induced epidermal carcinogenesis in p53 null mice; thus, the contribution of altered p21^{WAF1} expression to tumorigenesis is distinct from that of disruption of p53-mediated tumor suppression.

Materials and Methods

Animals. Mice differing in *p21*^{WAF1} gene dose were generated by homologous recombination, as described previously (15). The background strain of the mice used in these studies was 129/SvEv. Breeding pairs of mice differing in *p53* gene dose were obtained from Robert S. Sikorski (National Cancer Institute, Rockville, MD; Ref. 13). DNA was isolated from tail samples by standard phenol-chloroform extraction, and *p53* or *p21*^{WAF1} gene status was determined by PCR using primers described previously (Refs. 12 and 16; Recombinant DNA Laboratory, National Cancer Institute-Frederick Cancer Research and Development Center, Frederick, MD). In experiment 1, a total of 34 *p21*^{WAF1}(+/+), 71 *p21*^{WAF1} (+/−), and 37 *p21*^{WAF1} (−/−) mice, 6–10 weeks of age, were treated following the two-stage carcinogenesis protocol described below. In experiment 2, 15 *p21*^{WAF1} (+/+), 27 *p21*^{WAF1} (+/−), and 17 *p21*^{WAF1} (−/−) mice, 5–7½ months of age, were treated. The first tumors were observed at week 11.

Animal Treatments/Tumor Induction Protocols. For tumor induction, mice were initiated with a single dose of 25 nmol DMBA[2] (6.4 μg; Acros Organics, Somerville, NJ), applied in 200 μl of acetone to the dorsal surface 1 day after shaving. TPA (5 μg in 200 μl of acetone; Alexis Corp., San Diego, CA) was applied twice weekly for 20 weeks, beginning 1 week after initiation. This dose of TPA was determined to sustain continuous hyperplasia in the 129/SvEv strain at 3 weeks of treatments, and the dosage and schedule of treatments were as described by Reiners and Kameshwar (17). In experiment 2, a small number of control mice of each genotype were treated with DMBA or TPA alone. The occurrence of papillomas and carcinomas was monitored by gross appearance and recorded weekly for each mouse. Tumors were measured between 43 and 46 weeks of the study for experiment 1 and between 34 and 37 weeks for experiment 2, and tumor volumes were calculated as

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[2] The abbreviations used are: DMBA, 7,12-dimethylbenz[a]anthracene; BrdUrd; 5-bromo-2′-deoxyuridine; TPA, 12-O-tetradecanoylphorbol-13-acetate; MNNG, *N*-methyl-*N*′-nitro-*N*-nitrosoguanidine.

length × width × height of each individual tumor, as measured with calipers. At 52 weeks, or if the animal became moribund, mice were euthanized and tumor samples were collected in 70% ethanol or buffered formalin solution. Tumors with a gross carcinoma phenotype and a sampling of papillomas were confirmed by histological analysis of H&E-stained sections. In some experiments mice were treated either once with TPA (5 μg) and euthanized 48 h later, or treated three times at 3–4-day intervals and euthanized 3 days after the final treatment. Additional mice were pretreated with 50 μg of dexamethasone 5 min before TPA treatment. Control animals were treated in parallel with acetone alone. For determination of *in vivo* labeling index, mice received i.p. injections of 200–250 mg/kg BrdUrd (Sigma Chemical Co., St. Louis, MO) in saline 1 h before being euthanized.

Immunostaining. BrdUrd incorporation was detected in ethanol-fixed sections by sequential incubation with mouse antibody to BrdUrd (Becton Dickinson, San Jose, CA) and biotinylated goat antimouse IgG (Kirkegaard and Perry Laboratories, Gaithersburg, MD). Labeled nuclei were visualized with AEC chromogen (BioGenex, San Ramon, CA) and counterstained with Meyer's Hematoxylin (Sigma Chemical Co.). Differentiation-specific proteins keratin 1 and 10, loricrin, and filaggrin were recognized by incubation with monospecific rabbit polyclonal antibodies (BabCO, Richmond, CA) and biotinylated goat antirabbit IgG (Kirkegaard and Perry Laboratories), using AEC (BioGenex) or 3,3′-diaminobenzidine (Sigma Chemical Co.) as chromogen.

***In Vitro* Conversion Assay.** Keratinocytes from *p53* null or *p21*^{WAF1} null mice and isogenic controls were isolated from epidermis of newborn mice of each genotype as described previously (12, 16), plated on matrix-coated dishes (18), and cultured in medium containing 0.05 mM Ca^{2+}, 8% fetal bovine serum (Gemini Bioproducts, Inc., Calabasas, CA), and 1% penicillin-streptomycin (Life Technologies, Inc., Grand Island, NY). A previously described assay for malignant conversion potential was followed (19). Each group consisted of at least 5 and up to 14 dishes, and the experiment was repeated twice with essentially the same results.

Results

Mice 6–10 weeks of age, differing in *p21*^{WAF1} gene dose, were treated once with DMBA as initiating agent and promoted twice weekly with 5 μg of TPA. Animals were scored each week for papilloma and carcinoma formation. As shown in Fig. 1, in experiment 1 when young mice were used, the average papilloma yield was ~3-fold higher in mice homozygous for a null mutation in the *p21*^{WAF1} gene compared with wild-type control mice, although heterozygotes demonstrated an intermediate response. The latency period for tumor eruption was the same in all groups. Survival was similar across genotypes up to 40 weeks of treatment (Fig. 1, *bottom*). The decline in the number of surviving *p21*^{WAF1} (+/−) and (−/−) mice past 40 weeks presumably reflects the increased tumor burden on these animals, resulting in increased mortality. Because age can modulate tumor development, the study was also performed in mice 5–7½ months of age, as shown in experiment 2. The increased papilloma formation in p21^{WAF1} null mice was observed in these mice as well, although the differences were smaller, and the heterozygotes responded more similarly to the wild-type controls. The difference in results between experiments 1 and 2 seems to reflect an increase in tumor formation in older wild-type animals, rather than a decrease in tumor incidence in null or heterozygous mice. No tumors were observed in older mice of any genotype treated with a single dose of DMBA or 20 weeks of TPA promotion alone. There seemed to be a trend for increased mean volume of tumors with decreasing *p21*^{WAF1} gene dose (data not shown); however, due to large-size variability among tumors, the median tumor size was not statistically significantly different across genotypes (Kruskal-Wallis test).

To determine whether p21^{WAF1} deficiency causes an imbalance in cell proliferation *in vivo* that might contribute to the increased papilloma yield, mice received injections of BrdUrd 1 h before being euthanized, and BrdUrd-labeled nuclei were counted. The *in vivo* labeling index of untreated adult skin was similar among the three genotypes (Table 1), with slight variations depending on the hair cycle. Mice of all genotypes developed hyperplasia after a single treatment with the tumor promoter TPA (Fig. 2), and this was sustained after three treatments. The basal cell labeling index was variable within and between animals of each genotype after TPA treatment, presumably reflecting differences in the uniformity of treatment. However, p21^{WAF1}-deficient epidermis displayed a moderately, but significantly, lower labeling index compared with *p21*^{WAF1}(+/+), in contrast to expectations if the absence of p21^{WAF1} led to increased responsiveness to TPA (Table 1). Thus, an increased

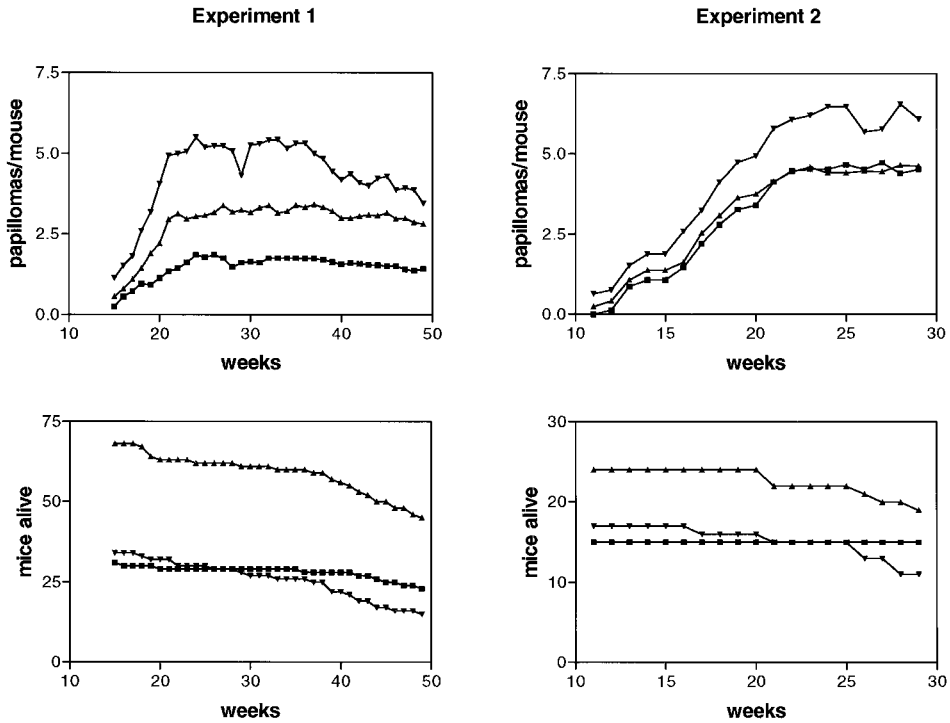

Fig. 1. p21^{WAF1}-deficient mice display increased papilloma yield after chemical carcinogenesis. Mice 6–10 weeks of age (*Experiment 1*) and 5–7½ months of age (*Experiment 2*) were initiated with DMBA (25 nmol) and promoted twice weekly for 20 weeks with 5 μg of TPA, by topical application to dorsal skin. Animals were examined weekly for tumor formation (*top*), and the number of animals alive in each group was documented (*bottom*). ■, *p21*^{WAF1}(+/+); ▲, *p21*^{WAF1}(+/−); ▼, *p21*^{WAF1}(−/−).

Table 1 *p21^{WAF1} deficiency does not increase labeling index of untreated or TPA-treated adult epidermis*

	Labeling index (number of BrdUrd positive cells/100 basal cells)		
	p21^{WAF1} genotype		
	+/+	+/−	−/−
Age of mice[a]			
10–12 weeks	3.7 (2)[c]	2.3 (2)	2.7 (3)
19–22 weeks	3.0 (1)	5.3 (1)	4.5 (1)
25–29 weeks	2.9 (2)	2.0 (2)	3.0 (2)
Treatment[b]			
TPA once	22.2 (2)	13.1[d] (2)	17.7[d] (2)
TPA 3 times	26.9 (2)	25.7 (3)	18.8[d] (3)

[a] Control mice were analyzed at each of three ages, and 750–2300 nuclei were counted for each group.
[b] Mice were treated with TPA, as noted, and 750–3400 nuclei were counted for each genotype and treatment.
[c] Number in parentheses indicates number mice/genotype analyzed.
[d] Significantly different from *p21^{WAF1}* (+/+) (two-tailed $P < 0.0001$ by χ^2 test). There was no statistically significant difference in labeling index of untreated skins of p21^{WAF1}-deficient mice compared with *p21^{WAF1}* (+/+), summed across all age groups, by Mantel-Haenszel extension of χ^2 test.

sensitivity to growth-promoting effects of TPA in epidermis of the deficient mice does not explain the higher papilloma incidence of this genotype. Moreover, topical exposure to 100 μg of DMBA did not stimulate or inhibit DNA synthesis in any genotype when BrdUrd incorporation was examined at 8 h after application (data not shown). The expression of differentiation-specific markers in untreated epidermis of all three genotypes was identical, and strong suprabasal expression of keratins 1 and 10, filaggrin, and loricrin was also consistent across genotypes after one or three exposures of TPA (Fig. 2).

Tumor promotion can be blocked by topical treatment with dexamethasone (20), and glucocorticoids have been shown to up-regulate p21^{WAF1} (21). To determine whether this sensitivity to steroids could influence promotion in different *p21^{WAF1}* genotypes, 50 μg of dexamethasone was applied to dorsal skin of *p21^{WAF1}* (+/+), (+/−), and (−/−) mice before TPA treatment. All genotypes responded equally to dexamethasone inhibition of TPA-induced hyperplasia (data not shown).

Skin tumor induction in *p53* (−/−) mice is characterized by rapid appearance of carcinomas and a high frequency (43%) of malignant conversion (22). To assess if this characteristic is common to *p21^{WAF1}*-deficient mice, tumors arising in each genotype were observed over 52 weeks for conversion to a malignant phenotype. Like p53 (−/−) mice, an increased percentage of p21^{WAF1} null mice developed carcinomas compared with wild-type control mice (Table 2). However, because the papilloma yield was substantially increased in the p21^{WAF1}-deficient mice, the percentage of papillomas converting to carcinomas was low and not significantly different across genotypes (Table 2). The conversion frequency in these mice was similar to that seen in multiple strains of conventional mice, including 129/SvEv (17). In no instance was a mouse observed to develop more than one carcinoma, despite the increased papilloma burden of p21^{WAF1}-deficient mice.

The ability of initiated p21^{WAF1}-deficient cells to convert to malignancy was also evaluated in an *in vitro* model in which keratinocytes were transduced with a retrovirus encoding an activated *ras* oncogene and exposed to 3 μg/ml MNNG for 1 h as a converting agent, followed by selection in medium containing 0.5 mM Ca^{2+}. The elevated [Ca^{2+}] will inhibit growth of benign but not malignant keratinocytes (19). No colonies formed in keratinocytes from mice differing in *p21^{WAF1}* gene dose in the absence of MNNG. After exposure to MNNG, an average of <1 focus developed/dish [0.8, 0.3, and 0.1 in *p21^{WAF1}* (+/+), (+/−), and (−/−) cultures, respectively]. Thus, consistent with the *in vivo* results, there was no increased tendency toward focus formation in p21^{WAF1}-deficient cultures.

Discussion

Previous studies from our laboratory have demonstrated that p21^{WAF1} deficiency does not recapitulate the malignant phenotype associated with p53 loss of function in keratinocytes transduced with the v-*ras*^{Ha} oncogene and grafted onto nude mouse hosts (16). In contrast, Missero *et al.* (23) have reported an enhanced growth potential and malignant phenotype of p21^{WAF1} null keratinocytes reminiscent of those observed in our previous study of p53 null mice (12). The xenograft approach used in these studies does not allow the discrimination of tumor multiplicity, and the outcome of such experiments could be influenced by a number of technical factors, such as the level of expression of exogenous *ras* oncogene, and stromal influences. Therefore, in the present study, we have used an intact animal model to evaluate the contribution of *p21^{WAF1}* gene dose to both tumor development and progression. The results presented here demonstrate that p21^{WAF1} loss enhances the formation of benign epidermal papillomas after exposure to chemical carcinogens, suggesting that p21^{WAF1} normally acts to suppress tumor formation. Tumors did not arise in any experimental genotype treated with DMBA or TPA only, suggesting that p21^{WAF1} deficiency will not substitute for initiation or promotion. Despite an increase in benign tumor formation, we found no increased frequency of malignant conversion in these tumors, consistent with our earlier grafting studies (16).

The increased incidence of benign tumors after carcinogen exposure observed in this study is similar to that observed in transgenic mice expressing a gain of function p53 mutant targeted to the epidermis, where p21^{WAF1} expression was also decreased in the resulting papillomas (24). However, chemical induction of skin tumors on p53 null mice resulted in a low papilloma yield relative to wild-type controls (22). In addition, benign skin tumor formation is inhibited when p53 null mice are bred with mice carrying an activated v-*ras* transgene targeted to the epidermis (25). Together, these results suggest that some p53 function may be required for papilloma formation, whereas other functions, such as p21^{WAF1} induction, may inhibit papilloma formation. In contrast, epidermal tumors induced by both chemicals and UVB arising in a p53 null background demonstrate a high malignant potential (22, 26), suggesting that alternate p53-mediated actions regulate initiation, premalignant progression, and malignant conversion.

Although basal p21^{WAF1} levels are decreased in p53 null keratinocytes (12), p21^{WAF1} levels can be regulated in epidermal keratinocytes independently of p53, after induction of terminal differentiation (4, 6) and during TPA promotion (25). Because cell cycle arrest is linked to the onset of the differentiated phenotype, a loss of this potential growth regulatory control might be expected to affect the commitment of cells to terminal differentiation and, thus, influence the neoplastic phenotype. It has been reported previously that p21^{WAF1} null keratinocytes display an increased proliferation potential *in vitro* and that expression of differentiation-specific markers is blocked in the absence of or by overexpression of p21^{WAF1} (23, 27). However, other investigators have reported no change in differentiation-specific gene expression in normal human keratinocytes overexpressing p21^{WAF} (28). In this study, we found no evidence *in vivo* of an increase in cell proliferation in p21^{WAF1}-deficient epidermis, as determined by BrdUrd incorporation of untreated and TPA-treated skin. Furthermore, untreated and TPA-treated mice of each genotype showed similar expression patterns of differentiation markers *in vivo*.

Because p21^{WAF1} deficiency does not seem to increase the *in vivo*

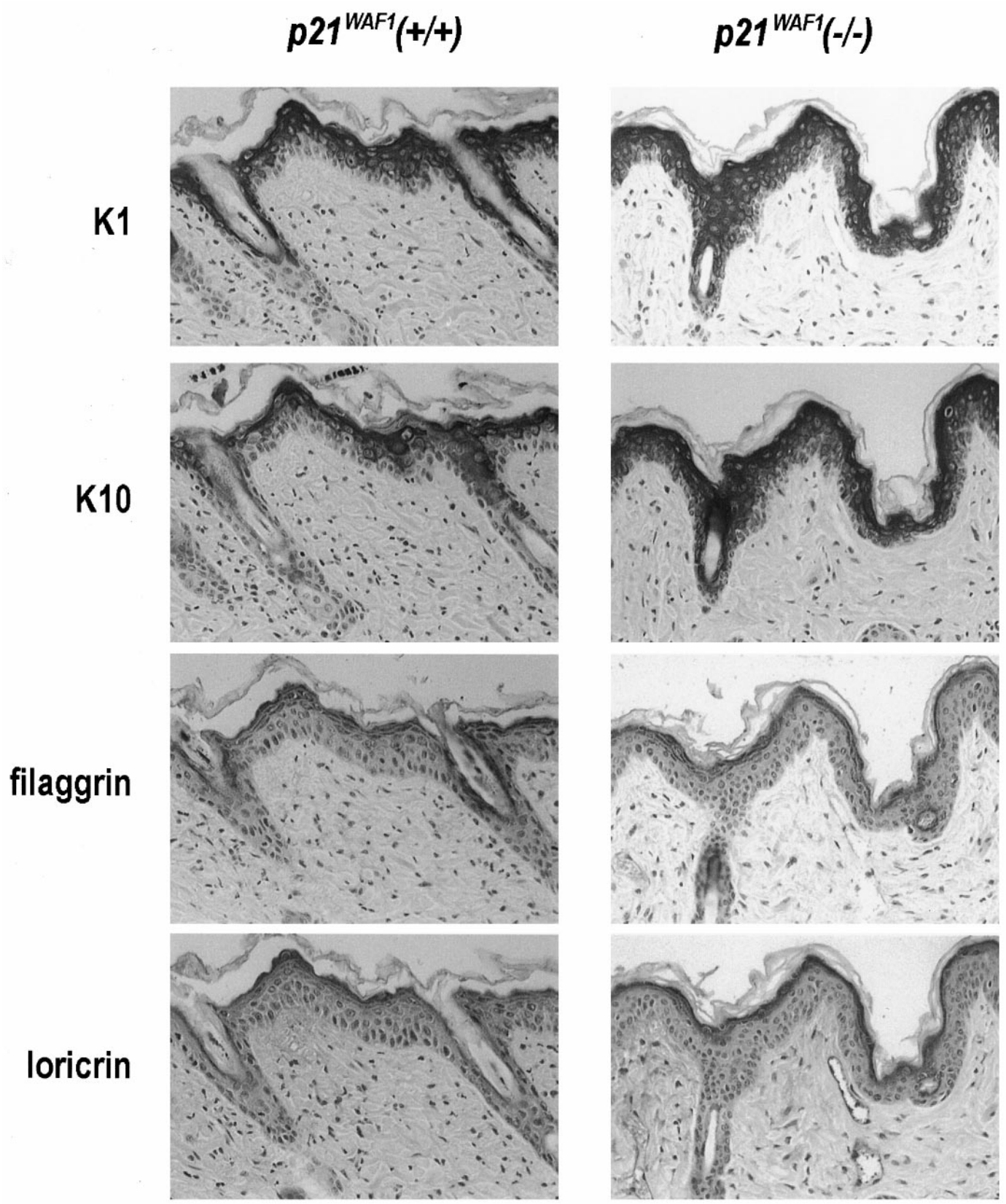

Fig. 2. *In vivo* expression of differentiation markers is independent of *p21*^{WAF1} genotype. Skins from adult mice sacrificed 48 h after treatment with 5 μg of TPA were fixed in 70% ethanol and processed for immunohistochemical staining of keratin 1 (*K1*), keratin 10 (*K10*), filaggrin, and loricrin (×200).

Table 2 *Papillomas arising in p21^{WAF1}-deficient mice do not demonstrate increased frequency of malignant conversion*

p21^{WAF1} genotype	% mice with papillomas	Maximum no. of papillomas observed	% mice with carcinomas[a]	Conversion frequency (no. of carcinomas/ maximum no. of papillomas × 100)
Experiment 1[b]				
+/+	56	61	11 (19)	3.3
+/−	76	217	20 (54)	5.1 [p = 0.74][c]
−/−	81	174	27 (30)	4.6 [p = 1.00]
Experiment 2[b]				
+/+	100	75	7 (15)	1.3
+/−	82	97	27 (22)	6.2 [p = 0.14]
−/−	100	97	18 (17)	3.1 [p = 0.63]

[a] Number of mice with carcinomas/tumor-bearing mice × 100; number in parentheses indicates maximum number tumor bearing mice in each genotype (mice at risk).

[b] Mice in experiments 1 and 2 were 6–10 weeks and 5–7½ months of age, respectively, at time of initial treatment.

[c] Comparison of conversion frequency in *p21*^{WAF1} (+/+) *versus* genotype shown (two-tailed *P* from Fisher's exact test).

proliferation rate or alter the expression of differentiation markers in untreated or TPA-treated epidermis, the increased benign tumor incidence observed in p21^{WAF1}-deficient mice may reflect a specific effect of p21^{WAF} deficiency on initiated cells. In addition to its participation in cell cycle arrest mediated by inhibition of cyclin-dependent kinase activity, the p21^{WAF1} protein interacts with proliferating cell nuclear antigen and, through this interaction, can influence both replicative DNA synthesis and nucleotide excision repair (reviewed in Ref. 2). p21^{WAF1}-deficient tumor cell lines have been reported to display DNA repair defects (2). If such a defect is present in normal keratinocytes, the increased incidence of papillomas in p21^{WAF1}-deficient mice may reflect decreased repair of carcinogen-induced DNA damage, resulting in a growth advantage to a higher proportion of initiated cells. Additional experiments are required to clarify whether p21^{WAF1} influences *in vivo* tumor formation directly via its ability to inhibit cyclin-dependent kinase activity, its interaction with proliferating cell nuclear antigen affecting DNA replication or repair processes, or some other mechanism.

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