Unexpected Roles for pRb in Mouse Skin Carcinogenesis

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Abstract

The mouse skin carcinogenesis represents one of the best models for the understanding of malignant transformation, including the multistage nature of tumor development. The retinoblastoma gene product (pRb) plays a critical role in cell cycle regulation, differentiation, and inhibition of oncogenic transformation. In epidermis, Rb−/− deletion leads to proliferation and differentiation defects. Numerous evidences showed the involvement of the retinoblastoma pathway in this model. However, the actual role of pRb is still unknown. To study the possible involvement of pRb in keratinocyte malignant transformation, we have carried out two-stage chemical skin carcinogenesis on RbF19/F19 (thereafter Rb+/+) and RbF19/F19;K14Cre (thereafter Rb−/−) animals. Unexpectedly, we found that Rb−/− mice developed fewer and smaller papillomas than the Rb+/+ counterparts. Moreover, the small size of the pRb-deficient tumors is associated with an increase in the apoptotic index. Despite this, pRb-deficient tumors display an increased conversion rate to squamous cell carcinomas. Biochemical analyses revealed that these characteristics correlate with the differential expression and activity of different pathways, including E2F/p19arf/p53, PTEN/Akt, c-jun NH2-terminal kinase/p38, and nuclear factor-κB. Collectively, our findings show unexpected and hitherto nondescribed roles of pRb during the process of epidermal carcinogenesis. (Cancer Res 2005; 65(21): 9678-86)

Introduction

pRb is a nuclear phosphoprotein that regulates cell cycle progression mainly through the binding to and inhibition of E2F transcription factors (1). Cell cycle progression requires functional inactivation of pRb by phosphorylation. Serial phosphorylation of pRb by cyclin D/cdk4/6 and cyclin E/cdk2 results in the release of pRb-bound E2F, allowing the progression of cells into the S phase (1). Conversely, cell cycle arrest is mediated through the expression of CIP/KIP and INK4 family members of cyclin-dependent kinase (cdk) inhibitors (2). Inactivation of Rb has been found in several human tumors including hereditary retinoblastoma, osteosarcoma, small cell lung cancer, bladder, and prostate tumors (3). Recent data also suggest an essential tumor suppressor role for the pRb−/−-related proteins p107 and p130 in specific tissues in the absence of pRb (4). Thus far, the vast majority of human tumors display alterations in any of the elements of the so-called Rb pathway. The two-stage mouse skin carcinogenesis is a well-suited model for the understanding of the multistage nature of tumor progression reviewed in ref. 5. In this system, tumor initiation is accomplished through a single topical application of a carcinogen, typically 7,12-dimethylbenz(a)anthracene (DMBA). This produces an irreversible, genetic inheritable change in the H-ras proto-oncogene. Tumor promotion takes place when the initiated cells are expanded due to repeated applications of 12-O-tetradecanoylphorbol-13-acetate (TPA), a hyperproliferative stimulus that promotes the generation of papillomas. Finally, although papilloma regression is a common event, in some cases, conversion occurs and papillomas evolve to squamous cell carcinomas (SCC).

Several lines of evidence suggest that the Rb pathway is involved in the mouse skin carcinogenesis. First, cyclin D1 expression increases at the very early stages of the process and experiments with cyclin D1-null mice showed its requirement during mouse skin carcinogenesis (6). Second, cdk4−/− mice are resistant to skin tumor development (7), whereas transgenic cdk4 expression in epidermis leads to development of papillomas in DMBA-treated skin without promotion (8). Moreover, mice expressing a mutated form of cdk4, which renders this protein insensitive to inhibition by ink4 proteins (R24C mutation), are more susceptible to skin carcinogenesis protocols (9). Third, primary keratinocytes derived from ink4aΔΔ, p16ink4aΔΔ doubly deficient mice are more susceptible to v-rasH1 transformation than their wild-type counterparts (10). Finally, deregulated expression of E2F family members increase the susceptibility to skin tumor development (11–15). Overall, these observations clearly showed that the functional inactivation of pRb is a common event in mouse skin carcinogenesis. Similarly, in human nonmelanoma skin tumors, cyclin D is frequently overexpressed, although the incidence of retinoblastoma gene inactivation is relatively low (16, 17).

Despite these evidences, the actual role of pRb in the mouse skin carcinogenesis has not been established. This is mainly due to the early embryonic lethality displayed by pRb-deficient mice (18–20). To solve this problem, others and we have recently used the Cre/loxP technology to inactivate the Rb gene in epidermis (21, 22). pRb-deficient skin is characterized by increased proliferation and altered differentiation leading to epidermal hyperplasia and hyperkeratosis, a phenotype that is further aggravated by loss of p107 alleles (22) or coexpression of E7 papillomavirus protein (21). These phenotypic alterations are suggestive of a premalignant state. In this work, we studied the possible susceptibility of RbF19/F19;K14Cre (thereafter Rb−/−) mice to the two-stage chemical carcinogenesis. Surprisingly, we found that the absence of pRb leads to reduced tumor development. However, the Rb−/−-deficient tumors displayed an increased rate in the malignant...
conversion to SCC. Moreover, we could correlate these alterations with changes in specific signal transduction pathways, including E2F/p19arf/p53, PTEN/Akt, c-jun NH2-terminal kinase (JNK)/p38, and nuclear factor-κB (NF-κB).

Materials and Methods

Mice and histologic procedures. B6 Tg(TRA) and K14cre animals, in an FVB-enriched background, were genotyped as described (22). Skin and tumor samples were fixed in either 4% buffered formalin or 70% ethanol and embedded in paraffin wax. Sections (5 μm) were stained with H&E or processed for immunohistochemistry. To analyze cell proliferation in vivo, mice were injected i.p. with bromodeoxyuridine (BrdUrd; Roche, Nutley, NJ; 0.1 mg/g weight in 0.9% NaCl), 1 hour before sacrifice (22). Mice were injected i.p. with bromodeoxyuridine (BrdUrd; Roche, Nutley, NJ; 0.1 mg/g weight in 0.9% NaCl), 1 hour before sacrifice (22).

Tumor induction protocols. For the two-stage tumorigenesis, a cohort of 15 Rb+/+ and 15 Rb−/− mice were used. Dorsal skin was shaved in the resting stage of the hair cycle (8-10 weeks of age) and initiated 3 days later with a single dose of DMBA (Sigma, St. Louis, MO; 100 μg/200 μl in acetone). Ten days after initiation, mice were subsequently treated twice a week with TPA (Sigma; 5 μg/200 μl in acetone) for 10 weeks. The number of tumors and their size, monitored with an external caliper, were scored weekly. Tumor samples were harvested 30 weeks after initiation and frozen in liquid nitrogen for protein analysis or processed for histopathologic procedures. Samples with similar stroma/tumor ratio, as determined by histopathology, were used in biochemical analyses. For the one-stage tumorigenesis, dorsal skin of 18 Rb+/+ and 15 Rb−/− mice were treated with a single dose of DMBA (100 μg/200 μl in acetone) and skin analyzed 10 months later.

Immunohistologic procedures. Immunohistochemistry and immunofluorescence were done using standard protocols on deparaffinized sections using antibodies against K5 and K6 (both at 1:500 dilution; Covance, Princeton, NJ), K15 (1:50 dilution; a generous gift from Dr. D. Roop, Baylor College of Medicine, Houston, TX), BrdUrd (1:50 dilution, Roche), E2F1 (1:200 dilution; C-20 polyclonal, Santa Cruz Biotechnology, Santa Cruz, CA), and p53 (1:500 dilution; CM5 polyclonal, Novocastara Laboratory, Newcastle upon Tyne, United Kingdom). Detection of BrdUrd was done as described (22). p53 and E2F1 detection were done after standard unmasking antigens protocols. Biotin and Texas Red–conjugated secondary antibodies were purchased from Jackson Immunoresearch Laboratory (West Grove, PA) and used at 1:4000 and 1:5000 dilutions, respectively. Apoptosis was monitored using In situ Cell Death Detection Kit (Roche) according to manufacturer’s recommendations. Peroxidase was visualized using avidin-biotin complex (Vector, Burlingame, CA). In all cases, control slides were obtained by replacing the primary antibodies with either PBS or preimmune serum (data not shown). To better quantify apoptosis in tumor samples, we did double immunofluorescence using anti K5 (to detect epidermal tumoral cells) and terminal deoxynucleotidyl transferase–mediated nick-end labeling (TUNEL; In situ Cell Death kit, Roche), essentially as described (23). For the quantification of BrdUrd- or apoptotic-positive cells, a total of 10 different papillomas or SCCs derived from Rb+/+ and Rb−/− mice were scored.

Western blot analysis. Frozen tumors were lysed in 20 mM/L Tris-HCl (pH 7.5), 137 mM/L NaCl, 10% glycerol, 1% Triton X-100, 1 mM/L phenylmethylsulfonyl fluoride, 1 mM/L Na3VO4, 1 μg/ml aprotinin and leupeptin, 1 mM/L/disodium pyrophosphate, and 20 mM/L NaF. Total protein (25 μg) was used for SDS-PAGE, transferred to nitrocellulose (Amersham, Arlington Heights, IL), and probed with antibodies against p53 (1:500; Novocastara Laboratories). Antibodies to Bax, p21, PUMA, 14-3-3σ, E2F1, E2F4, E2F5, p19, CyclD1, CyclE, PTEN, extracellular signal-regulated kinase 1/2 (ERK1/2), phospho-ERK1/2, Akt1/2, p38, phospho-p38, JNK, JNK alpha (IKKα), IKKβ, IkBα, p50, p65, and TRAF2 were purchased from Santa Cruz Biotechnology. Antibodies to phospho-JNK1, phospho-AKT, phospho-p53, and phospho-β-amyloid precursor were purchased from Cell Signaling (Beverly, MA). The monoclonal antibody anti-JNK1 was purchased from PharMingen (San Diego, CA). Horseradish peroxidase–conjugated anti-rabbit, mouse, or goat secondary antibodies were purchased from Jackson Immunoresearch Laboratory and used at 1:5,000, 1:5,000, and 1:10,000 dilution, respective. Actin (1:500 dilution, Santa Cruz Biotechnology) was used for normalization. Detection was done using Western Picosignal Detection kit (Pierce, Rockford, IL).

Band shift analysis. Electrophoretic mobility shift assays (EMSAs) were done by incubating whole cell extracts with labeled oligonucleotide corresponding to E2F, p53, or σB sites essentially as described (24). The sequences of the oligonucleotide-coding strand were p53, 5′-TACAGAAGATTGAATCAGCATGCAGGGG-3′; E2F, 5′-ATTAAATTTCGCGCCTTCTCACATTGG-3′; and σB, 5′-GATGACGGAGTTGGTTTCCCTTCTCATT-3′.

Complexes were separated on either 4% (p53 and E2F) or 5% (σB) native polyacrylamide gels in 0.25× Tris-borate-EDTA buffer, dried, and exposed to Hyperfilm-MP (Amersham) at −70°C. Controls omitting cell extracts and competition with mutated or wild-type oligonucleotides were routinely done.

Results

Decreased tumor formation in pRb-deficient skin. pRb deficiency leads to increased proliferation and aberrant differentiation in epidermis (21, 22). However, no development of spontaneous skin tumors has been observed in a large cohort of Rb−/− mice up to 1.5 years of age when compared with Rb+/+ control littersmates (data not shown). However, the occurrence of an initiation step might be capable of inducing the neoplastic transformation of the hyperproliferative pRb-null keratinocytes. To monitor if these mice were susceptible to tumor formation, we initiated two groups of 18 Rb+/+ and 15 Rb−/− mice with a single dose of DMBA. This treatment induces activating Ras gene mutations. We observed that, 10 months after DMBA application, both groups of animals developed a similar number of papillomas (3 of 18 and 2 of 15, for Rb+/+ and Rb−/−, respectively), suggesting that pRb deficiency can not bypass tumor promotion in mouse skin. Next, we studied tumor susceptibility in Rb+/+ and Rb−/− animals to the two-stage (DMBA/TPA) tumorigenesis protocol. We detected the appearance of tumors 6 weeks after DMBA treatment, with similar latency in both groups (Fig. 1A). However, a percentage of Rb−/− mice remained refractory to tumor development, whereas all the control littersmates developed skin tumors (Fig. 1A). Tumor multiplicity (mean number of tumors per mice) clearly shows a reduced number of tumors in Rb−/− mice compared with their respective control littersmates throughout the experiment (Fig. 1B). In addition, the size of the tumors was significantly reduced in Rb−/− (Fig. 1D) compared with control mice (Fig. 1C). Collectively, these observations show that the susceptibility to skin tumor development is reduced in the absence of pRb.

pRb deficiency increases the tumor malignant conversion during the two-stage carcinogenesis. In the mouse skin carcinogenesis system, papilloma regression is a common event, and in some cases, conversion occurs and papillomas evolve to SCCs. This is clearly observed in Rb−/−, as the mean tumor number peaks and then decreases (Fig. 1B). However, this is less evident in Rb-null mice (Fig. 1B). This would mean that papillomas do not regress in the absence of Rb thus suggesting increased conversion rates. To determine the possible consequences of pRb loss in tumor progression, we studied the histopathology of the tumors at the end of the protocol. We considered the dysplastic and anaplastic changes, as well as the infiltrative behavior, to classify the tumors in papillomas, premalignant papillomas, and SCC. Strikingly, a significant percentage (23%) of pRb-deficient papillomas were classified as premalignant papillomas (Fig. 2A and B). In contrast, most of papillomas observed in control animals were regularly or moderately dysplastic (Fig. 2B). The percentage of malignant

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expression of K13, a marker of premalignant conversion (25) was positive for TUNEL staining in pRb-deficient SCCs (Fig. 3D, D).

A model for skin carcinogenesis in Rb+/+ and Rb−/− mutant mice. Incidence (A) and multiplicity (B) of tumors in Rb+/+ and Rb−/− mice during a two-stage carcinogenesis. Data are expressed as percentage of mice that developed tumors (A, incidence; P ≤ 0.05) and average number of tumors per mouse (B, multiplicity; P ≤ 0.01) as a function of weeks after DMBA initiation. C–D, differences in tumor growth. All the tumors obtained in Rb+/+ (C) and Rb−/− (D) were classified by their size and plotted as total number of the tumors versus weeks after initiation (P ≤ 0.01 for tumors larger than 0.9 mm and between 0.6 and 0.9 mm; P ≤ 0.05 for tumors smaller than 0.3 mm and between 0.3 and 0.6 mm).

The connection between the retinoblastoma and the p53 pathways has been well shown. In particular, E2F1 activation promotes a very mild increase in CycD1 expression. However, the levels of cyclin E did not show significant changes in tumors (Fig. 4A), although it is up-regulated in p5b-deficient keratinocytes (data not shown). Finally, we also analyzed the expression of several E2F-responsive genes. On one hand, the levels of the apoptotic-related p19ARF and Apaf1 proteins display a clear increase in apoptotic-related p19ARF and Apaf1 proteins display a clear increase in apoptosis rather than altered proliferation.

E2F and p53 pathways in Rb−/− mouse skin tumors. To understand the molecular mechanisms responsible for the altered skin tumor susceptibility and progression observed in pRb-deficient mice, we carried out a comprehensive analysis of the different pathways potentially involved in mouse skin carcinogenesis.

Many of the functional activities of pRb lie on its ability to interact with E2F family of transcription factors. Indeed, pRb-deficient keratinocytes showed elevated levels of E2F activity (22). In agreement, we found increased E2F activity in pRb-deficient tumors (Fig. 4A). In parallel, we observed increased E2F1 and E2F4 protein expression (Fig. 4A), whereas E2F5 is only moderately increased in pRb-deficient papillomas (Fig. 4A). The increased E2F1 expression in the absence of pRb is also detected by immunostaining (Fig. 4B and B′). To determine the role of the E2F increased activity in the phenotype of pRb-deficient tumors, we analyzed the expression of several E2F-responsive genes. On one hand, the levels of the apoptotic-related p19ARF and Apaf1 proteins display a clear parallelism with E2F1, being increased in pRb-deficient tumors. On the other hand, the levels of cyclin E did not show significant changes in tumors (Fig. 4A), although it is up-regulated in Rb−/− keratinocytes (data not shown). Finally, we also analyzed the expression of cyclin D1, because this protein is involved and necessary for skin tumor development (6). In papillomas, pRb loss promotes a very mild increase in CycD1 expression. However, the overall levels of this protein seem similar in SCCs irrespective of the genotype (Fig. 4A).

Increased apoptosis in pRb-deficient papillomas. To investigate the biological determinants that are responsible for the reduced number and size observed in pRb-deficient tumors, we monitored possible alterations in the processes of proliferation and apoptosis. We determined the number of cells in the S phase by BrdUrd incorporation analysis. These experiments showed that the BrdUrd-labeling index was similar between the two groups of papillomas (Fig. 3A, A′, and E) and SCCs (Fig. 3B, B′, and E; P ≤ 0.5, Student’s t test analysis). The percentage of apoptotic cells in papillomas and SCCs was monitored by TUNEL labeling in parallel with K5 expression. The percentage of apoptotic cells in wild-type papillomas was 8 ± 2%, whereas pRb-deficient papillomas showed a marked increase in this percentage (16.5 ± 0.5%; Fig. 3C, C′, and F; P ≤ 0.001). The opposite situation was observed in the case of SCCs. Rb−/−-derived tumors showed an average of 18 ± 5% of apoptotic cells, whereas only 5 ± 0.2% of K5-positive cells were also positive for TUNEL staining in pRb-deficient SCCs (Fig. 3D, D′, and F; P ≤ 0.005). These results indicate that the observed decrease in number and growth of papillomas in Rb mice might be due to the increase in apoptosis rather than altered proliferation.

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induces the expression of p19ARF leading to p53 stabilization and p53-dependent apoptosis (27). In fact, the reduced tumor development with increased malignant behavior observed in pRb-deficient papillomas are similar to the results obtained upon two-stage carcinogenesis in p53-deficient mice (28). We thus studied the expression and activity of p53 and the p53-dependent signaling in the tumors. p53 activity, measured by EMSA, was increased in pRb-deficient papillomas compared with their tumor controls (Fig. 5A). Conversely, Rb-null SCCs showed reduced p53 activity compared with control SCCs (Fig. 5A). The analysis of p53 protein levels showed a general mild increase in pRb-deficient papillomas. However, in the case of Rb-null SCCs, we detected reduced protein levels, or even absence of p53 (Fig. 5A). To further substantiate these observations, and discriminate the p53 expression in tumoral and stromal cells, we analyzed p53 expression and localization by immunohistochemistry in 15 papillomas and eight SCCs from control mice and 12 papillomas and 10 SCCs from Rb−/− mice (representative results are shown in Fig. 5B, B′, C, and C′). We found that in the vast majority of control papillomas, p53 staining was absent or restricted to few epidermal cells (13 of 15 samples, Fig. 5B). On the contrary, only few pRb−/−-deficient papillomas displayed absence of p53 expression in the tumoral cells (3 of 12 samples, Fig. 5B′). However, a similar percentage of SCCs showed no p53 staining regardless their Rb+/+ or Rb−/− origin (6 of 8 and 8 of 10 samples, respectively, Fig. 5C and C′). These data suggest that tumor induction in the absence of pRb leads to increased p53 levels and activity in papillomas, probably through E2F-dependent
expression of p19^{ARF}. In addition, E2F1 can activate p53 by promoting the phosphorylation of Ser^{20}, in a p19^{ARF}-independent mechanism (29). The analysis of p53Ser^{20}-P showed that p53 is only phosphorylated in Rb^{+/+} papillomas (Fig. 5A). We also studied the expression of several downstream elements of the p53-dependent pathway. We observed that p21^{waf} and 14-3-3, two proteins involved in p53-dependent cell cycle arrest, displayed similar levels irrespective of the type of the tumor or genotype analyzed (Fig. 5A). In contrast, the levels of PUMA and Bax, which are involved in p53-dependent apoptosis, were increased in parallel with the levels and activity of p53 (Fig. 5A). These observations might explain the changes in apoptosis and the absence of proliferative alterations observed in pRb-deficient tumors compared with their respective Rb^{+/+}-derived tumors. Moreover, these results indicate that induction of p53 in Rb^{−/−} papillomas promote a p53-dependent apoptosis.

**Signal transduction pathways in Rb^{−/−} mouse skin tumors.** Besides the E2F- and p53-dependent pathways, the development and progression of epidermal tumors is affected by the expression and activity of different signal transduction pathways. Those include the different families of the mitogen-activated protein kinases (MAPK: ERK, JNK, and p38; ref. 30) and the PTEN/Akt pathway (23, 31). As shown in Fig. 6A, we did not find any significant changes in the expression of ERK1/2, JNK1, and p38 or ERK1/2 activity (Fig. 6A). However, we found that JNK1 and p38 were predominantly phosphorylated in Rb^{−/−} papillomas (Fig. 6A), suggesting increased activity. Such p38 phosphorylation was reduced or absent in pRb-deficient SCCs compared with control SCCs (Fig. 6A). Besides the possible function in proliferation, MAPK signaling in mouse skin carcinogenesis can induce survival or promote apoptosis. It has been suggested that ERK activity promotes tumor cell survival, whereas JNK1 and p38 are apoptotic.

**Figure 4.** E2F pathway in pRb-deficient tumors. A, protein extracts obtained from different tumor types of Rb^{+/+} and Rb^{−/−} mice were used in band shift and Western blot analysis to study the E2F activity and the expression of the quoted proteins (see Materials and Methods). Representative E2F1 staining in papillomas derived from Rb^{+/+} (B) and Rb^{−/−} (B′) tumors. Note the increased E2F1 staining in Rb^{−/−}-deficient tumors. Dashed lines, tumor-stroma(s) boundaries. Bar, 100 μm.
prone (32, 33). Therefore, we can assume that the increased JNK1 and p38 activity observed in Rb-deficient papillomas also contributed to the increased apoptosis and thus to the reduced tumor development observed.

Our previous data have shown that Akt-mediated signaling is an essential mediator increasing proliferation and decreasing apoptosis in mouse skin carcinogenesis (23). Moreover, the reported functional link of Akt/PTEN pathway with pRb (34, 35) and p53 (36) suggests its involvement in the apoptotic induction observed in Rb-deficient tumors. We found that the levels of expression of Akt and its activity, analyzed by Ser473 phosphorylation, were increased in Rb-deficient papillomas compared with controls (Fig. 6A). We also observed increased Akt phosphorylation in pRb-deficient SCCs. In agreement with this observation, PTEN expression was increased in Rb−/− papillomas and in control SCCs (Fig. 6A). These data might indicate that the antiapoptotic function of Akt is not sufficient to overcome the proapoptotic signals in pRb-deficient papillomas but clearly can contribute to the reduced apoptotic rate in SCC.

Finally, NF-κB signaling has been implicated in p53- (37) and in E2F-1-induced apoptosis (38). Moreover, the importance of NF-κB signaling in the epidermis has been highlighted in recent years (39). However, the possible role of this signaling in mouse skin tumorigenesis is still a matter of debate. In this regard, under specific settings, increased NF-κB activity leads to epidermal hypoplasia and growth inhibition (40) and the repression of NF-κB activity produces epidermal hyperplasia and spontaneous SCCs (41). Nevertheless, increased endogenous NF-κB activity during chemically induced mouse skin tumorigenesis has been reported (42). We thus studied the expression and activity of this pathway. Using EMSA analysis, we detected decreased NF-κB activity in Rb−/− papillomas (Fig. 6B). The canonical activation of NF-κB requires the phosphorylation of the inhibitory IκB protein by a high molecular weight complex that includes IKKα, IKKβ, and IKKγ proteins. This leads to IκB degradation and allows the NF-κB to move to the nucleus and activate target genes (43). In agreement, we also observed increased of the inhibitory protein IκBα expression in these tumors (Fig. 6B). We also found decreased p50 and IKKα expression, whereas no significant changes in p65 and IKKβ levels were observed. Moreover, a partial increase in IKKγ expression was displayed in the Rb−/− papillomas (Fig. 6B).

Finally, increased E2F activity may mediate reduced NF-κB activity

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through the down-regulation of TRAF2 protein levels (38). In agreement, TRAF2 expression was diminished in Rb+/−/− papillomas (Fig. 6B). Of note, in SCCs, we observed increased NF-κB activity and no major alterations among the different elements of the pathway analyzed regardless the genotype. These observations indicate that the inhibition of NF-κB signaling might account for the increase in apoptosis observed in pRb-deficient papillomas.

Collectively, these data show that in the absence of pRb, several signal transduction pathways, besides those dependent on p53 and E2F, are altered and converge to promote apoptotic induction at early stages of epidermal oncogenic progression thus rendering reduced tumor susceptibility. Such apoptotic induction is overcome during the malignant conversion.

Discussion

Role of pRb in epidermal tumorigenesis. There is compelling evidence that disabling the so-called Rb pathway is essential for the formation of cancer cells in human and mouse models, including the two-stage mouse skin carcinogenesis (see Introduction). This is contrast with the reported low frequency of mutations and deletions of Rb gene in human nonmelanoma skin tumors (16, 17).

The precise roles of pRb in mouse epidermal carcinogenesis have not been yet analyzed. Here we used mice bearing epidermal-specific pRb inactivation to study the possible involvement of this protein in tumor development in vivo. Contrary to other tissues, like pituitary (20), the absence of pRb does not induce spontaneous formation of tumors in epidermis. Although there is compelling evidence that the H-ras gene acts upstream of pRb (44), we did not detect cooperation between Ha-ras activation and pRb loss in spontaneous tumor development after a single oncogenic insult with DMBA. This is in contrast with data obtained in K5-cdk4 transgenic mice, which developed skin papillomas upon DMBA initiation without promotion (8). This suggests that cdk4 could act through different targets and could be related to the specific up-regulation of p107 observed upon pRb deletion (22). Our data also seemed in disagreement with the reported cooperation between v-rasH1 and E2F1 to induce skin tumors in mice (14). In this regard, it is important to remark that E2F1 overexpression differs from the possible alleviation of E2F transcriptional activities due to pRb depletion. Moreover, increased E2F1 activity can either promote or suppress skin tumorigenesis depending on the experimental conditions. In two-stage carcinogenesis experiments done with K5-E2F1 transgenic mice, a near-complete resistance to tumor development has been reported in association with apoptosis induction mediated by the TPA treatment (13). In contrast, the expression of E2F4 (12) or DP1 (11) leads to increased production of epidermal tumors in similar experiments, suggesting that different members of the E2F family can also have different functions in the context of mouse skin carcinogenesis (45).

The reduced papilloma formation and the reduced size of these tumors are clearly due to the altered apoptosis induction rather to altered proliferation (Fig. 3). These disturbances are in contrast with the observed increased proliferation in epidermis in absence of apoptosis in intact skin of these mice (22). The induction of apoptosis upon loss of pRb or all pRb family proteins has been well established in several tissues such embryonic central nervous system (46) and embryonic lens (47). Interestingly, similarly to pRb-deficient papillomas, brain tumors obtained by the inactivation of the pRb proteins induced by the expression of an NH2-terminal form of SV40T antigen showed growth retardation due to extensive p53-dependent apoptosis (48).

Possible cooperative functions of pRb with p53 in skin tumors. In mouse skin carcinogenesis, the conversion from papilloma to SCC has been associated with loss or mutations in the Tp53 gene (49). Consequently, p53-null animals showed a reduced yield of papillomas, but these underwent a much more rapid malignant conversion upon two-stage carcinogenesis experiments (28). Similarly, pRb-deficient papillomas also suffer a higher rate of conversion to SCCs suggesting a functional link between pRb and p53 in the process of mouse skin tumorigenesis. Such connection has been reported in mouse models of medulloblastoma (50), ovarian carcinoma (51), and small cell lung carcinoma (52) and is mediated, at least in part, by the E2F1-dependent induction of p19ARF. We also observed increased expression E2F1 and p19ARF.
and increased p53 activity leading to the expression of proapoptotic effectors (Figs. 4 and 5). These data can explain the increase in the apoptosis observed in pRb-deficient papillomas and associated with the tumor growth retardation (Fig. 3). In contrast, the level of apoptosis in pRb-deficient SCCs is lower when compared with their control SCCs (Fig. 3). Strikingly, we were not able to detect p53 expression in some of the pRb-deficient SCCs (Fig. 5). These results, together with the fact that pRb-deficient tumors have an increased malignant progression, suggest that p53-dependent apoptosis induced by the loss of pRb might promote a selective pressure in these tumors leading to premature p53 inactivation. One might speculate that, in agreement with the data obtained in transgenic mice expressing E2F-1 (15), the simultaneous epithelial-specific inactivation of both Rb1 and Tp53 would lead to spontaneous skin tumor development.

**Alterations in signal transduction pathways in pRb<sup>−/−</sup> deficient skin tumors.** The DMBA/TPA mouse skin tumorigenesis protocol predominantly generates mutations in the H-ras gene. This activates several signal transduction pathways including the Raf/MAPK and the phosphatidylinositol 3-kinase/Pten/Akt pathways. These two pathways are highly relevant in the process of tumor promotion and progression in mouse skin carcinogenesis affecting not only proliferation but also apoptosis of the tumor cells (23, 30, 31). We have also analyzed these pathways to find possible explanations for the altered apoptosis observed in Rb<sup>−/−</sup>-deficient tumors. We did not detect significant changes in ERK activation (Fig. 6). However, we observed increased activation of Jnk1 and p38 in pRb-deficient papillomas (Fig. 6). The activation of Jnk1 might be highly relevant as the results obtained in Jnk1 knockout mice upon two-stage mouse skin showed that Jnk1 can suppress skin tumor development (53). The possible mechanism of such increased activation in absence of pRb might be related to the reported ability of pRb to physically interact with Jnk/stress-activated protein kinase (SAPK), inhibiting intracellular signals and also abrogating the apoptotic cell death mediated by Jnk/SAPK (54). We also observed increased PTEN expression in pRb-deficient papillomas. This might also be related to PTEN increased expression in pRb-deficient papillomas, which is probably due to p53 induction (36). The absence of PTEN expression in pRb-deficient SCCs also reinforces the above commented hypothesis suggesting the premature loss of p53 in pRb-deficient tumors (Fig. 6). On the other hand, a moderate increase in Akt expression and activity was observed in pRb-deficient papillomas (Fig. 6). This is clearly insufficient to overcome apoptosis. The activation of Akt, in agreement with the reduced PTEN expression, is much higher in pRb-deficient SCCs and would help to reduce the apoptotic index in these tumors. The mechanism of increased activation of Akt is still unknown but could be related to the recently reported ability of E2F to promote Akt activation (35).

A major player controlling apoptosis relays in the NF-κB transcription factor signaling activity. Interestingly, NF-κB activation is a putative target of different transduction pathways such as MAPK and Akt and has been implicated in p53- (37) and E2F-1-induced apoptosis (38). We detected decreased NF-κB activity in pRb-deficient papillomas probably through the reduced expression of IKK subunits and TRAF2 protein levels. These data are in agreement with the reduced NF-κB activity due to E2F activation (38), which also accounts for the down-regulation of TRAF2 (38). This may contribute, along with the other pathways commented above, to the observed increased apoptosis in these tumors.

Collectively, the results presented here indicate that the absence of retinoblastoma tumor suppressor gene leads to decreased papilloma formation in mouse skin due to increased apoptosis, showing that regulation of cell survival is a crucial mechanism for crippling cellular defense against epidermal neoplasia. Such increased apoptosis is due to the activation of E2F, which leads to induction of p53 activity. In this scheme, the activation or repression of specific signaling pathways, such as Jnk, p38, Akt/PTEN, and NF-κB, mediated either by E2F and/or p53 activation, contribute to this increased apoptotic rate. As an overall consequence, this apoptosis induction generates a selective pressure in papillomas leading to increased rate of malignant conversion probably mediated by the p53 inactivation.

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