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# Increased E2F1 activity induces skin tumors in mice heterozygous and nullizygous for p53

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**ABSTRACT** **The $p16^{INK4a}$-cyclin D–retinoblastoma tumor suppressor pathway is disrupted in most human cancers, and it has been suggested that the subsequent release of E2F transcription factors from inhibitory complexes may be a key event in tumor development. We described recently the generation of transgenic mice with E2F1 gene expression targeted to squamous epithelial tissues by a keratin 5 (K5) promoter. In the present study, K5 E2F1 transgenic mice were crossed with p53 null mice to examine functional interactions between E2F1 and p53 *in vivo*. We find that E2F1-induced apoptosis of epidermal keratinocytes is reduced in K5 E2F1 transgenic mice lacking p53, whereas E2F1-induced hyperproliferation is unaffected by p53 status. We also find that K5 E2F1 transgenic mice heterozygous or nullizygous for p53 develop spontaneous skin carcinomas, which normally are rare in p53-deficient mice. The timing of tumor development correlates with the level of E2F1 transgene expression and the status of p53. In primary transgenic keratinocytes, the major change in E2F1 DNA-binding activity is the generation of a complex also containing the retinoblastoma tumor suppressor protein. Nevertheless, the expression and associated kinase activity of cyclin E, a known target for E2F transcriptional activity, is elevated significantly in K5 E2F1 transgenic keratinocytes. These findings firmly establish that increased E2F1 expression can contribute to tumor development and suggest that p53 plays an important role in eliminating cells with deregulated E2F1 activity.**

It has been demonstrated that loss of retinoblastoma tumor suppressor (Rb) function can lead to unchecked proliferation and tumorigenesis in a variety of model systems (1–6). In many cases, however, loss of Rb function leads to p53-dependent apoptosis (3–9). In the absence of functional p53, apoptosis as a consequence of Rb inactivation is suppressed. Thus, the status of p53 can determine whether loss of Rb results in apoptosis or deregulated proliferation. This finding likely explains why DNA tumor viruses such as simian virus 40, adenovirus, and human papilloma virus encode proteins that inactivate both Rb and p53. This "guardian of Rb" function for p53 also is consistent with the observation that in many human cancers with Rb mutations, p53 also is inactivated (10).

The Rb protein has been shown to bind and regulate a large number of cellular proteins, including members of the E2F transcription factor family (11). It has been suggested that deregulation of E2F transcriptional activity as a consequence of Rb inactivation contributes to the development of cancer (11–13). This hypothesis is supported by the finding that several members of the E2F gene family can behave as oncogenes in cell culture transformation assays (14–17). However, increased E2F activity has not been shown directly to contribute to tumorigenesis *in vivo*. Deregulation of E2F transcription factors may also play a role in the induction of apoptosis as a result of Rb inactivation. At least one member of the E2F gene family, E2F1, has been shown to induce apoptosis in a p53-dependent manner (18–20).

We described recently the generation of transgenic mice containing the human E2F1 gene under the control of the bovine keratin 5 (K5) promoter (21). Two lines were established (K5 E2F1 lines 1.0 and 1.1), both of which showed E2F1 overexpression in basal cells of the epidermis, hair follicles, and other squamous epithelial tissues. E2F1 overexpression results in epidermal hyperplasia and hyperproliferation but does not appear to block epidermal differentiation. The higher expressing line 1.0 transgenic mice also have a hair growth defect. Examination of the skin by terminal deoxynucleotidyltransferase-mediated dUTP nick end-labeling (TUNEL) assay suggested that the paucity of hair seen in this line is the result of aberrant apoptosis in developing hair follicles. In addition to promoting proliferation and inducing apoptosis, we also found that the K5 E2F1 transgene could cooperate with a v-Ha-*ras* transgene to induce benign skin papillomas.

In this report we describe the phenotype of K5 E2F1 transgenic mice that are also deficient (heterozygous or nullizygous) for p53. We find that aberrant apoptosis in the epidermis of K5 E2F1 line 1.0 mice is reduced in a p53 null background. We also find that K5 E2F1 transgenic mice heterozygous or nullizygous for p53 develop spontaneous skin tumors. The majority of these tumors are squamous cell carcinomas (SCC) or basal cell carcinomas (BCC), which rarely develop in p53-deficient mice. Examination of transgenic keratinocytes suggests that tumor development may require only a modest increase in E2F1 DNA-binding activity and involve up-regulation of the cyclin E gene.

## MATERIALS AND METHODS

**Transgenic Mice.** The generation of K5 E2F1 transgenic mice has been described (21). Briefly, the transgene contains the bovine keratin 5 promoter (22), the rabbit $\beta$-globin intron 2, the human E2F1 cDNA, and the simian virus 40 polyadenylation signal. Transgenic mice were genotyped by PCR amplification of sequences specific for the $\beta$-globin intron and/or the human E2F1 cDNA from tail genomic DNA. The genetic background of the founder mice was C57BL/6 × SJL $F_2$ hybrid. The original p53 heterozygous mice (23, 24) used for breeding were C57BL/6. All mice in this study were at least 75% C57BL/6.

**BrdUrd Incorporation.** Mice were injected i.p. with 0.06 mg of BrdUrd per gram of body weight, allowed to incorporate for 30 min, and sacrificed. Skin samples were collected, fixed in formalin, and paraffin-embedded. Tissue sections then were subjected to immunohistochemistry as described below using antibody

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Abbreviations: Rb, retinoblastoma tumor suppressor; K5, keratin 5; SCC, squamous cell carcinoma; BCC, basal cell carcinoma; TUNEL, terminal deoxynucleotidyltransferase-mediated dUTP nick end-labeling.

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(Becton Dickinson) specific for BrdUrd. For determining percentage of incorporation, interfollicular basal cells were examined and the number of unstained and stained cells was determined.

**Immunohistostaining.** Formalin-fixed sections were deparaffinized and fixed in methanol containing 1% hydrogen peroxide for 20 min. Tissue sections were then rinsed with PBS containing 0.1% BSA three times. Slides were boiled for 5–10 min (E2F1 staining) or incubated in HCl (1 M, 40°C) and then 0.05% protease solution (BrdUrd staining) and again rinsed in PBS/BSA. Slides for E2F1 staining were preincubated with normal goat serum and then incubated with primary E2F1 antibody (raised in rabbit, 1:500, Santa Cruz Biotechnology) for 30 min at room temperature. Slides for BrdUrd were incubated with primary BrdUrd antibody (mouse, 1:25, Becton Dickinson) for 1 hr at room temperature. After incubation with primary antibody, slides were rinsed in PBS/BSA, incubated with biotinylated anti-rabbit IgG (E2F1 staining), or biotinylated anti-mouse IgG (BrdUrd staining) for 30 min, and rinsed again. Slides were incubated with streptavidin-horseradish peroxidase conjugate for 30 min, developed with diaminobenzidine tetrahydrochloride solution, rinsed again, and counterstained.

**TUNEL Assay.** Skin sections from transgenic and wild-type sibling controls were fixed in formalin, paraffin embedded, and sectioned. After deparaffinization and hydration, sections were incubated in the TUNEL reaction mixture containing terminal deoxynucleotidyltransferase and fluorescein-dUTP per manufacturer's instructions (Boehringer Mannheim, *in situ* cell death detection kit). Sections were washed and the label incorporated in apoptotic cells was visualized by fluorescence microscopy. Alternatively, the assay was performed by using the ApopTag *in situ* apoptosis detection kit (Oncor) and apoptotic cells were visualized by peroxidase-diaminobenzidine staining.

**Isolation of Protein and RNA from Primary Keratinocytes.** Primary keratinocytes were made from newborn mice by incubating washed skins in 0.25% trypsin/PBS overnight at 4°C. Epidermis then was separated from the dermis, minced in culture media containing 10% fetal bovine serum (FBS) and high calcium (2 mM), and stirred for 20 min. This mixture was filtered through polypropylene mesh and cells were counted and plated at a density of 3 million per 35-mm dish. Media was changed after 2 hr to low-calcium (0.05 mM) medium. Whole-cell protein extract was prepared after 48–72 hr by resuspending washed cells in lysis buffer (50 mM Hepes, pH 7.9/250 mM KCl/0.1 mM EDTA/0.1 mM EGTA/0.1% Nonidet P-40/0.4 mM NaF/0.4 mM $Na_3VO_4$/10% glycerol/1 mM DTT/0.5 mM phenylmethylsulfonyl fluoride/2 $\mu$g/ml aprotinin/2 $\mu$g/ml leupeptin/1 $\mu$g/ml pepstatin), freeze thawing, and collecting supernatant after high-speed centrifugation. Total RNA was isolated by using Tri Reagent (Molecular Research Center, Cincinnati) and the manufacturer's protocol.

**Electrophoretic Mobility-Shift Assays.** Whole-cell extracts were prepared from primary keratinocytes as described above. Eight to ten micrograms of extract was incubated for 20 min at room temperature with an end-labeled DNA fragment containing two E2F sites derived from the adenovirus E2 promoter. Binding reactions also included 10% glycerol/50 ng of sonicated salmon sperm DNA/5 mM EDTA/5 mM EGTA/0.5% Nonidet P-40/100 mM Hepes/200 mM KCl/5 mM $MgCl_2$/5 mM phenylmethylsulfonyl fluoride/5 mM DTT. Ficoll (1.25%) was added to the reactions before running on a nondenaturing 5% polyacrylamide gel. E2F1, p107, and p130 antisera were purchased from Santa Cruz Biotechnology. Rb mAb (14001A) was purchased from PharMingen.

**Histone H1 Kinase Activity.** Primary keratinocytes were scraped into ice-cold Nonidet P-40 buffer (50 mM Tris/0.5% Nonidet P-40/150 mM NaCl/5 mM EDTA) containing protease inhibitors phenylmethylsulfonyl fluoride (1 mM), DTT (1 mM), and aprotinin (20 $\mu$g/ml), and subjected to one round of freeze–thaw lysis, and insoluble material was pelleted by centrifugation at 12,000 $\times$ *g*. Equivalent amounts of protein then were reacted with cyclin E antibody (Santa Cruz Biotechnology, M-20) for 1 hr. Protein G agarose (GIBCO) then was added to the antibody lysate for 30 min. The antibody bead complexes were washed five times in Nonidet P-40 lysis buffer. For kinase reactions, immunocomplexes were resuspended in 30 $\mu$l of lysis buffer containing 2 $\mu$g of histone H1 (Sigma), 10 $\mu$Ci (1 $\mu$Ci = 37 kBq) of [$\gamma$-$^{32}$P]ATP, and 20 $\mu$M unlabeled ATP. Kinase reactions were incubated at 30°C for 30 min, after which 2$\times$ loading buffer was added and the samples were boiled. Samples were separated by electrophoresis on an SDS/12% polyacrylamide gel, and the gel was dried and exposed to film.

## RESULTS

**Phenotype of K5 E2F1 Mice Lacking p53.** A transgenic line (line 1.0) expressing high levels of E2F1 under the control of the bovine K5 promoter was found previously to have a sparse hair phenotype (21). Examination of skin from 6-day-old line 1.0 mice suggested that the hair growth defect may be the result of aberrant apoptosis occurring in the hair follicles. Aberrant apoptosis also can be detected in the interfollicular epidermis of adult line 1.0 mice (Fig. 1*A*). In some cell culture systems it has been shown that E2F1-induced apoptosis largely depends on wild-type p53 activity (18, 19). To determine whether aberrant apoptosis in K5 E2F1 transgenic mice depended on wild-type p53, we introduced the K5 E2F1 transgene into a p53 null background by crossing K5 E2F1 transgenic males with females heterozygous for p53. K5 E2F1 males heterozygous for p53 obtained from this cross then were crossed again to p53 heterozygous females to obtain K5 E2F1 transgenic mice null for p53.

The absence of wild-type p53 reduced the level of epidermal apoptosis in line 1.0 transgenic mice by 3-fold (Figs. 1*B* and Fig. 2*A*). Line 1.0 transgenic mice with wild-type p53 have an average

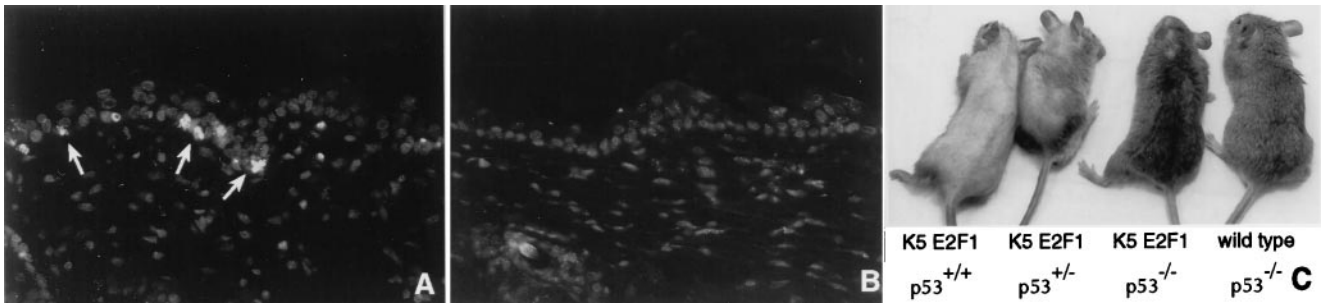


FIG. 1. Reduced apoptosis and rescued hair growth defect in K5 E2F1 transgenic mice lacking p53. (*A*) Skin section from a line 1.0 transgenic mouse with wild-type p53 was subjected to a TUNEL assay by using fluorescein-dUTP. Several brightly stained nuclei can be seen in the epidermis. (*B*) Skin section from a line 1.0 transgenic mouse null for p53 was subjected to a TUNEL assay. (*C*) A line 1.0 male mouse heterozygous for p53 was mated with a p53 heterozygous female. The genotypes of resultant siblings pictured are, from left to right: K5 E2F1 positive, $p53^{+/+}$; K5 E2F1 positive, $p53^{+/-}$; K5 E2F1 positive, $p53^{-/-}$; K5 E2F1 negative, $p53^{-/-}$.

of seven apoptotic cells per 10 mm of skin epithelium whereas transgenic mice null for p53 have an average of 2.3 apoptotic cells per 10 mm of skin epithelium. In addition, the hair growth defect in line 1.0 mice was at least partially rescued by the absence of p53 function (Fig. 1*C*). Immunohistochemistry of dorsal epidermis was done to confirm expression of the E2F1 transgene in mice with a rescued phenotype (data not shown).

E2F1-induced hyperplasia and hyperproliferation also were examined in K5 E2F1 transgenic mice with and without functional p53 (Fig. 2). Proliferation of interfollicular basal keratinocytes was measured by BrdUrd incorporation assays. In nontransgenic mice, approximately 3% of interfollicular basal keratinocytes were in S phase regardless of the status of p53 (Fig. 2*B*). As has been shown (21), line 1.0 transgenic mice have a 5-fold increase in the percent of basal keratinocytes in S phase. Lack of p53 did not alter significantly the level of E2F1-induced hyperproliferation observed in the epidermis (Fig. 2*B*). In contrast, p53 status did have an effect on E2F1-induced hyperplasia as measured by epidermal thickness and increased cellularity (Fig. 3). In the K5 E2F1 transgenic population, p53 null mice exhibited a 22% increase in epidermal thickness and a 23% increase in epidermal cellularity over that of K5 E2F1 mice with wild-type p53 (Fig. 2 *C* and *D*). These findings suggests that p53 does not affect the number of cells in the proliferative compartment but does affect the survival of K5 E2F1 transgenic keratinocytes.

**Skin Carcinomas in K5 E2F1 Mice Deficient in p53.** K5 E2F1 transgenic mice from both lines (1.0 and 1.1) that were also heterozygous and nullizygous for p53 were maintained to determine whether overexpression of E2F1 could cooperate with loss of p53 function in tumorigenesis. Transgenic mice heterozygous or nullizygous for p53 indeed did develop spontaneous skin carcinomas often with more than one independent tumor on a single animal (Table 1). Spontaneous skin carcinomas are rare (1–3% of tumors) in p53 heterozygous or knockout mice (2, 23, 24). Instead, p53-deficient mice commonly develop lymphomas or sarcomas. Immunohistochemistry revealed overexpression of E2F1 in all tumors listed in Table 1 supporting the involvement of the K5 E2F1 transgene in tumor development (Fig. 4).

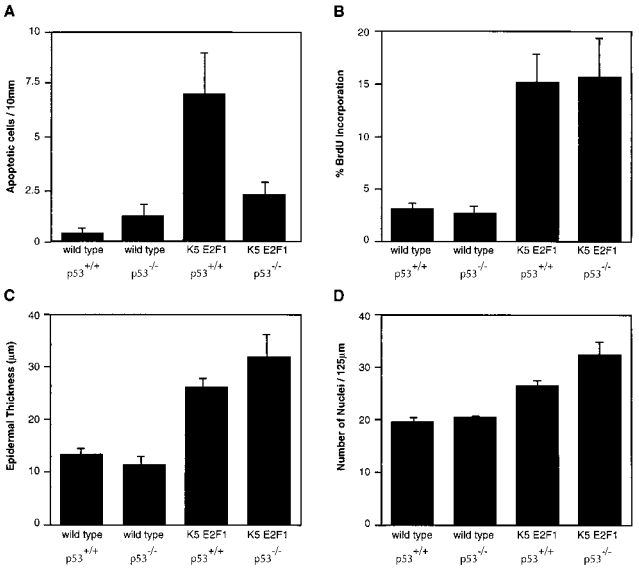


FIG. 2. Effect of p53 on apoptosis, proliferation, and hyperplasia in epidermis of K5 E2F1 transgenic mice. (*A*) Skin sections from line 1.0 transgenic and nontransgenic mice with and without wild-type p53 were subjected to TUNEL assays. Sections from four (nontransgenic) or five (transgenic) mice in each group were used to calculate the average number of apoptotic cells per 10 mm of interfollicular epidermis. (*B*) Line 1.0 transgenic and nontransgenic mice with and without p53 were injected with BrdUrd 30 min before sacrifice. At least 200 cells were counted to calculate the labeling index of interfollicular epidermis from two to four mice (2–13 weeks of age) in each group. (*C*) Skin samples were taken from line 1.0 transgenic and nontransgenic mice either with or without p53, ages 2–20 weeks. At least 10 measurements were taken from three to five different mice in each group to calculate the average thickness of the epidermis. (*D*) The same skin samples used in *C* were used to determine the average number of nuclei per 125 $\mu$m of epidermis.

The timing of tumor development correlated with the level of E2F1 transgene expression and the degree of p53 deficiency. Tumor development in the six line 1.1 (lower expressing) mice heterozygous for p53 occurred between 35 and 46 weeks of age (an average of 39.8 weeks). The 12 line 1.0 (higher expressing) mice heterozygous for p53 developed tumors between 21 and 39 weeks of age for an average of 27.2 weeks. Except for a few line 1.0 mice that had to be sacrificed because of an undefined wasting syndrome, all K5 E2F1 adult mice heterozygous for p53 ultimately developed skin tumors. Three line 1.0 mice nullizygous for p53 also developed skin tumors between 9 and 15 weeks of age (Table 1). Four other K5 E2F1 transgenic mice nullizygous for p53 developed lymphomas or sarcomas apparently unrelated to the transgene between 8 and 18 weeks of age as did p53 knockouts lacking the K5 E2F1 transgene. This timing of lymphoma or sarcoma development in p53 knockout mice is consistent with previous reports (2, 23, 24). At present, no spontaneous skin carcinomas have been found in K5 E2F1 transgenic siblings with normal p53 gene status.

Histological analysis of these skin lesions found them to be a heterologous group of tumors (Table 1 and Fig. 4). All appear to be derived from the epidermis or hair follicles. Tumor morphologies ranged from well differentiated SCC to poorly differentiated, anaplastic SCC (Fig. 4 *A* and *B*). Several tumors resembled human BCC or other hair follicle-derived neoplasias (Fig. 4 *C* and *D*). Dysplastic hair follicles also were observed in these mice (Fig. 4 *E* and *F*), further suggesting a follicular origin for some of these tumors. One additional tumor that arose in the lower jaw was found to be an odontogenic carcinoma resembling an ameloblastoma. The K5 E2F1 transgene likely played a role in the development of this tumor as well because this tumor type has not been reported in p53-deficient mice and the K5 promoter has been shown to be expressed in the odontogenic epithelia (22).

**E2F1 DNA-Binding Activity in Primary Transgenic Keratinocytes.** Previously we demonstrated that primary keratinocytes isolated from K5 E2F1 newborn mice expressed 47- to 80-fold more E2F1 protein than nontransgenic keratinocytes by Western blot analysis (21). However, because E2F1 requires heterodimerization with a DP protein for high-affinity DNA binding and because phosphorylation can inactivate E2F factors, it was unclear how much of this exogenous E2F1 protein was functional. In addition, it was not known whether the exogenous E2F1 was complexed with Rb protein. Association of Rb with E2F1 inhibits E2F1's transcriptional activation capacity and in some cases may convert E2F1 from an activator to a repressor of transcription (25–28).

To examine the effect of transgene expression on E2F DNA-binding activity, primary keratinocytes were isolated from newborn line 1.1 transgenic mice and wild-type siblings. Extracts from these asynchronously growing cells were then used in electrophoretic mobility shift assays. In nontransgenic primary keratinocytes, two E2F-site-specific DNA-binding complexes were observed (Fig. 5). These correspond to a faster migrating, "free" E2F complex and a slower migrating complex containing the Rb-related p107 protein as demonstrated by antibody supershift experiments. In K5 E2F1 transgenic keratinocytes, a modest increase in E2F DNA-binding activity is observed, and this is primarily the result of a new complex that migrates between the free E2F and the p107-containing complex. Addition of antisera specific for either E2F1 or Rb resulted in a supershift of this new complex, demonstrating that both E2F1 and Rb are present in this complex. Further-

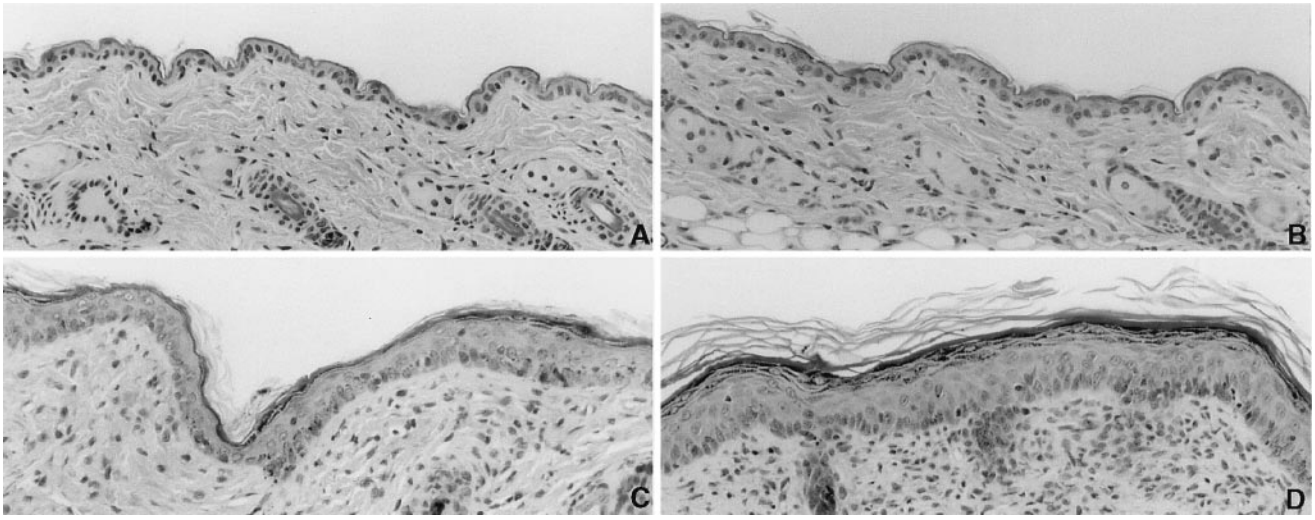


FIG. 3. Histological appearance of skin from K5 E2F1 transgenic mice with or without p53. Hematoxylin/eosin stain of skin sections from nontransgenic sibling with wild-type p53 (*A*), nontransgenic sibling null for p53 (*B*), line 1.0 transgenic with wild-type p53 (*C*), and line 1.0 transgenic null for p53 (*D*).

more, only in transgenic keratinocytes did addition of the anti-E2F1 antisera appear to diminish significantly the free E2F band. The level and pattern of E2F DNA-binding were similar in primary keratinocyte extracts from line 1.0 mice (data not shown).

**Up-Regulation of Cyclin E in K5 E2F1 Keratinocytes.** It has been demonstrated that the cyclin E gene is deregulated in $Rb^{-/-}$ cells (6, 29, 30). Moreover, the cyclin E promoter has been shown to contain several E2F DNA-binding sites and to respond to overexpressed E2F transcription factors (31–33). To determine the effect of K5 E2F1 transgene expression on the regulation of the endogenous cyclin E gene, RNA and protein were isolated from primary keratinocytes made from both transgenic lines and from nontransgenic siblings. The cyclin E gene was overexpressed 8-fold in primary keratinocytes from line 1.0 mice and 6-fold in line 1.1 keratinocytes

Table 1. Spontaneous skin tumors in K5 E2F1/p53-deficient mice

| Mouse | Line | p53 status | Age, wk | Site | Histological appearance |
|---|---|---|---|---|---|
| 1 | 1.1 | $p53^{+/-}$ | 35 | Neck | w.d. SCC |
| | | | 42 | Leg | Keratoacanthoma |
| | | | 42 | Face | BCC |
| 2 | 1.1 | $p53^{+/-}$ | 46 | Face | w.d. SCC |
| 3 | 1.1 | $p53^{+/-}$ | 39 | Back | p.d. SCC |
| | | | 39 | Back | BCC |
| 4 | 1.1 | $p53^{+/-}$ | 41 | Flank | BCC |
| | | | 41 | Abdomen | p.d. SCC |
| 5 | 1.1 | $p53^{+/-}$ | 41 | Back | w.d. SCC |
| 6 | 1.1 | $p53^{+/-}$ | 36 | Side | p.d. SCC |
| | | | 36 | Chest | p.d. SCC |
| 7 | 1.0 | $p53^{+/-}$ | 21 | Back | m.d. SCC |
| 8 | 1.0 | $p53^{+/-}$ | 20 | Head | p.d. to m.d. SCC |
| | | | 20 | Mouth | Ameloblastoma |
| 9 | 1.0 | $p53^{+/-}$ | 39 | Head | m.d. SCC |
| 10 | 1.0 | $p53^{+/-}$ | 24 | Back | m.d. SCC |
| | | | 26 | Leg | p.d. SCC |
| 11 | 1.0 | $p53^{+/-}$ | 20 | Back | m.d. SCC |
| 12 | 1.0 | $p53^{+/-}$ | 24 | Back | w.d. SCC |
| | | | 34 | Leg | BCC |
| | | | 36 | Flank | p.d. SCC |
| 13 | 1.0 | $p53^{+/-}$ | 29 | Back | m.d. to p.d. SCC |
| 14 | 1.0 | $p53^{+/-}$ | 32 | Flank | m.d. to p.d. SCC |
| | | | 33 | Back | p.d. SCC |
| 15 | 1.0 | $p53^{+/-}$ | 30 | Flank | p.d. SCC |
| | | | 30 | Head | Spindle cell carcinoma |
| 16 | 1.0 | $p53^{+/-}$ | 27 | Back | BCC |
| 17 | 1.0 | $p53^{+/-}$ | 18 | Back | Tricoepithelioma |
| 18 | 1.0 | $p53^{+/-}$ | 26 | Back | p.d. SCC |
| 19 | 1.0 | $p53^{-/-}$ | 15 | Mouth | p.d. SCC |
| 20 | 1.0 | $p53^{-/-}$ | 9 | Groin | p.d. SCC |
| 21 | 1.0 | $p53^{-/-}$ | 14 | Back | p.d. SCC |

Age is in weeks at time of tumor detection. Site is where on the skin the tumor was detected. w.d., well differentiated; m.d., moderately differentiated; p.d., poorly differentiated.

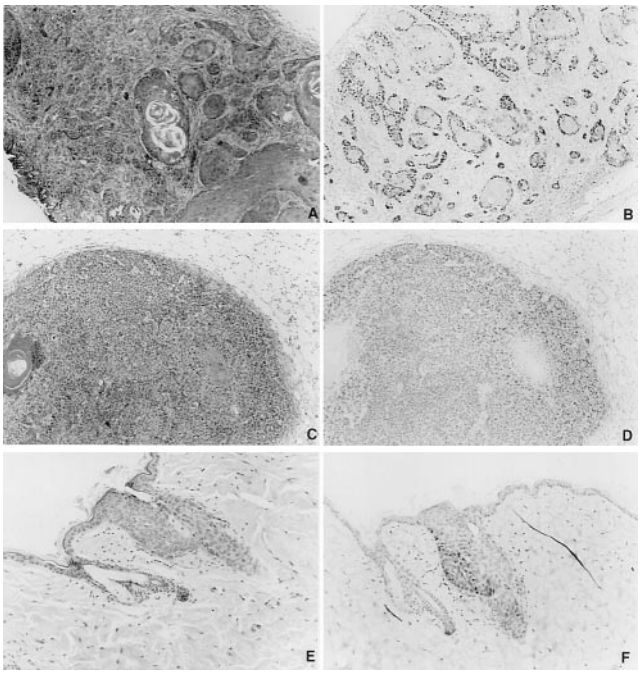


FIG. 4. Histological appearance of skin lesions from K5 E2F1 transgenic mice deficient for p53. (*A*) SCC from a line 1.0 K5 E2F1, $p53^{+/-}$ mouse stained with hematoxylin/eosin. (*B*) SCC from a line 1.0 K5 E2F1, $p53^{+/-}$ mouse immunostained with E2F1 antisera. (*C*) BCC from a line 1.0 K5 E2F1, $p53^{+/-}$ mouse stained with hematoxylin/eosin. (*D*) The same BCC immunostained with E2F1 antisera. (*E*) Dysplastic hair follicle from a line 1.1 K5 E2F1, $p53^{+/-}$ mouse stained with hematoxylin/eosin. (*F*) The same dysplastic hair follicle immunostained with E2F1 antisera. (All micrographs are at ×20 magnification.)

compared with the level seen in nontransgenic keratinocytes (Fig. 6*A*). These increases in cyclin E message correlate with similar increases in cyclin E protein levels (Fig. 6*B*). Moreover, primary keratinocytes from K5 E2F1 transgenic mice had elevated cyclin E-associated kinase activity compared with wild-type keratinocytes (Fig. 4*C*). The steady-state expression levels of several other E2F target genes, including B-*myb* and c-*myc*, showed little or no change (data not shown). These findings suggest that up-regulation of cyclin E may be involved in E2F1-induced proliferation and tumorigenesis.

## DISCUSSION

Since the cloning of the Rb gene considerable research has focused on understanding the molecular mechanism by which loss of Rb function contributes to tumorigenesis. Much of this work has been directed at the identification of cellular proteins that interact with the Rb protein. Many different cellular proteins have been demonstrated to bind Rb *in vivo*. To our knowledge, however, only three of these—cyclin D1, MDM2, and now E2F1—have been shown to contribute to tumorigenesis in their wild-type form (34–37). It is believed that cyclin D1 is an important upstream regulator of Rb protein function through its ability to activate cdk4 and cdk6 and promote Rb phosphorylation (11, 38). MDM2 also appears to be a regulator of Rb function as well as playing a critical role in regulating the activity of p53 (39, 40). On the other hand, it is clear that E2F1 is a downstream target for Rb's activity as a growth suppressor (14, 41). Thus, E2F1 is the first downstream target of Rb shown to directly contribute to tumorigenesis when expressed in a deregulated manner.

Interestingly, mice lacking E2F1 also are prone to tumor development (42). E2F1 therefore is unique in that it can function as both an oncogene and a tumor suppressor gene. It has been suggested that the ability of E2F transcription factors both to activate and repress transcription may explain how

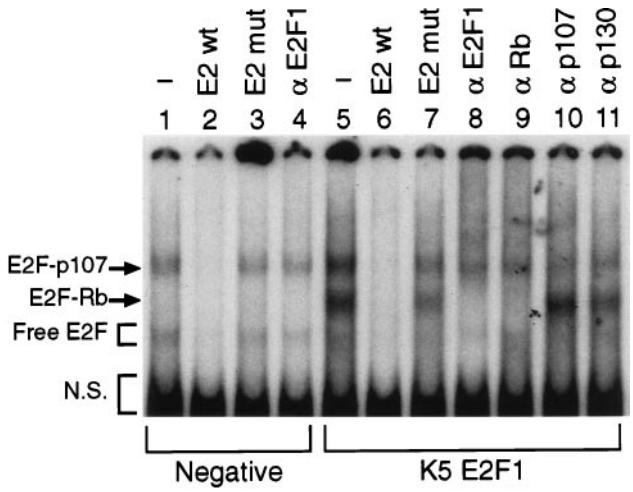


FIG. 5. E2F DNA-binding activity in primary keratinocytes. Whole-cell extracts were prepared from primary keratinocytes derived from line 1.1 transgenic and nontransgenic newborn mice. E2F electrophoretic mobility-shift assay reactions contained 10 μg of either nontransgenic (Negative, lanes 1–4) or transgenic (K5 E2F1, lanes 5–11) extract and an end-labeled DNA fragment derived from the adenovirus E2 promoter. Excess (20 ng) double-stranded oligonucleotide containing either wild-type E2F sites (E2 wt, lanes 2 and 6) or mutated E2F sites (E2 mut, lanes 3 and 7) were added to the binding reaction to distinguish specific complexes from nonspecific (N.S.) complexes. Antibody specific for either E2F1 (lanes 4 and 8), Rb (lane 9), p107 (lane 10), or p130 (lane 11) was added to the binding reactions where indicated to identify proteins in specific complexes.

E2F1 can both contribute to and inhibit oncogenic transformation (42). In this model, complexes containing E2F1 and Rb function to transcriptionally repress important genes involved in proliferation. In E2F1 null cells, Rb, which has been shown to contain a potent transcriptional repression domain (25–28), cannot be tethered to promoters through association with E2F1. The lack of E2F1-Rb complexes might lead to derepression of these growth-control genes and deregulated cell proliferation in E2F1 knockout mice.

In K5 E2F1 transgenic keratinocytes we observe the generation of an E2F1-Rb complex as the major change in E2F DNA-binding activity. However, we find no evidence to sup-

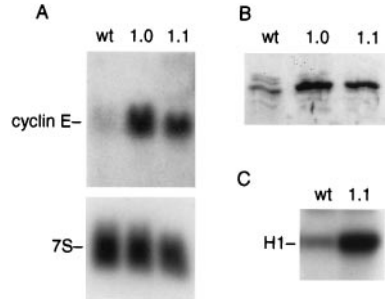


FIG. 6. Cyclin E is up-regulated in K5 E2F1 transgenic keratinocytes. Primary keratinocytes were isolated and cultured from the epidermis of nontransgenic (wt) and K5 E2F1 transgenic (line 1.0 and line 1.1) newborn mice. Total RNA and protein was extracted after 48 to 72 hr. (*A*) Twenty micrograms of total RNA was subjected to Northern analysis by using a random primed-labeled fragment of the mouse cyclin E cDNA as probe. The blot then was striped and reprobed for 7S RNA. (*B*) Fifteen micrograms of protein from nontransgenic (wt) and transgenic (line 1.0 and 1.1) keratinocytes was subjected to Western analysis by using polyclonal antisera specific for cyclin E. (*C*) One hundred and forty micrograms of whole-cell extract from K5 E2F1 transgenic keratinocytes (1.1) or keratinocytes derived from nontransgenic littermates (wt) were immunoprecipitated with 0.5 μg of antibody specific for cyclin E. A kinase assay then was performed with histone H1 as the substrate.

port the hypothesis that this complex functions to repress transcription. Instead, we find that cyclin E is up-regulated whereas the expression of several other E2F-regulated genes is unchanged. It is possible that the target genes for repression by this complex have not yet been examined or that this repressing complex requires an additional component not present in primary keratinocytes.

Another model to explain the dual activity of E2F1 in tumorigenesis that is not necessarily mutually exclusive of the "activation versus repression" model involves the ability of E2F1 to induce both proliferation and apoptosis. A role for endogenous E2F1 in an apoptotic pathway is suggested by the finding that T cells from E2F1 knockout mice are more resistant to apoptosis *in vitro* (43). E2F1 also may play a protective role in eliminating cells that have undergone cell cycle alterations, such as loss of Rb, through the induction of apoptosis. The absence of this protective pathway may predispose E2F1 knockout mice to cancer. Tumor development in K5 E2F1 transgenic mice would require a block in this apoptotic signal to allow continued proliferation as a result of deregulated E2F1 expression. Our studies suggest that a block in E2F1-mediated apoptosis to allow tumorigenesis can occur through at least two mechanisms: loss of p53 function or expression of an activated H-*ras* gene (21). This is consistent with *in vitro* studies demonstrating that apoptosis induced by the E1A oncoprotein, an activator of E2F, can be inhibited by either p53 inactivation or activated H-*ras* expression (8, 9, 44).

In addition to impairing a protective apoptotic pathway, loss of other p53 functions may also cooperate with E2F1 in tumorigenesis. Genomic instability as a result of p53 loss could combine with forced proliferation by E2F1 to drive the tumorigenic process. In addition, p53 may participate in negatively regulating E2F1 activity by stimulating the expression of the p21 cyclin-dependent kinase inhibitor. p21 can maintain Rb in an active state for E2F1 binding through the inhibition of $G_1$ cyclin kinases (11, 13). In p53 null cells, p21 induction is impaired, and this could result in a further increase in E2F1-dependent transcription and deregulated proliferation in K5 E2F1 transgenic cells. It has also been shown that p53 can directly bind E2F1, as well as DP1, and that coexpression of p53 can inhibit transcriptional activation by E2F1 (45, 46). However, p53 does not appear to play a major role in directly inhibiting E2F1 activity, at least not under unstressed conditions, because the level of epidermal hyperproliferation in K5 E2F1 transgenic mice is similar in the presence or absence of p53. The mechanism by which p53 deficiency cooperates with increased E2F1 activity in carcinogenesis should become more clear as other cooperating factors are identified using K5 E2F1 mice. This transgenic model may also be useful in the future development of cancer therapies targeting E2F.

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