Role of Rb-Dependent and Rb-Independent Functions of Papillomavirus *E7* Oncogene in Head and Neck Cancer

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Abstract

Infection with high-risk human papillomaviruses (HPV) and in particular the expression of the viral proteins E6 and E7 have been associated with the etiology of a subset of head and neck squamous cell cancer (HNSCC). However, the individual consequences of E6 and E7 expression in an *in vivo* model have not been examined in these tissues. We have used transgenes that direct expression of the HPV16 E6 and E7 proteins to the head and neck tissues of mice to dissect the contribution of these proteins to head and neck carcinogenesis. We report here that E7 is the major transforming oncogene in HPV-associated HNSCC, whereas E6 is more likely to play a secondary role in contributing to later stages of carcinogenesis. Furthermore, a conditional deletion of *Rb*, a prominent target for E7, in the same tissues did not recapitulate all E7-mediated phenotypes. Although our results do not preclude an important role for the E7-pRb interaction, they highlight the importance of pRb-independent functions of E7 in head and neck carcinogenesis. [Cancer Res 2007;67(24):11585–93]

Introduction

High-risk human papillomaviruses (HPV), now well accepted to be causative agents for the majority of cervical cancers, more recently have been associated with a subset of head and neck cancers. The oncogenic properties of high-risk human papillomaviruses have been attributed largely to the two early proteins E6 and E7. Expression of E6 and E7 has been detected from both integrated and extrachromosomal genomes in HPV-positive head and neck squamous cell cancer (HNSCC) consistent with the hypothesis that these oncoproteins contribute to these cancers (1–3). E6 and E7 are known to bind and inactivate the tumor suppressors p53 and pRb, respectively (4–9), and these respective activities have been associated with their oncogenic properties (10, 11). Consistent with the contribution of E6 and E7 to HPV-positive HNSCC, their expression correlates with a paucity of mutations in p53 (12), decreased levels of pRb, and increased levels of p16 (13, 14). Conversely, in HPV-negative HNSCC, p53 is often mutated, levels of pRb are normal, and levels of p16 are decreased. The distinct nature of the alterations in the p16/pRb and p53 pathways not only distinguishes the HPV-positive cancers from the HPV-negative ones but also implies roles for both the HPV oncogenes. However, despite the overwhelming evidence from the study of human tumor samples, the individual contributions of these oncogenes has not been studied *in vivo* in the context of head and neck carcinogenesis.

Previous work, with strategies that focused on the use of transgene-directed expression of E6 and E7, has shed light on the *in vivo* roles of these proteins not only in tissues that are considered the natural host for HPV infection, such as the epidermis and the stratified epithelium of the cervix, but also tissues such as the retina, lens, and the central nervous system (15–20). In these mice, the HPV oncogenes are directed in their expression from the keratin 14 (K14) promoter, which targets expression of the viral oncogenes to the same basal compartment of stratified epithelia as observed in natural HPV infections. Furthermore, extensive analyses of these mice have shown that the HPV16 E6 and E7 oncoproteins display the same capacity to inactivate relevant cellular targets, including but not limited to p53 and pRb, respectively, as has been documented to occur in HPV-infected human epithelia (10, 21–25). From our studies, we have learned that these oncogenes not only possess properties that distinguish one from the other, but also that those properties may vary in a tissue-dependent manner. Furthermore, different activities of the oncogenes have been shown to be important for short-term acute phenotypes and/or long-term oncogenic phenotypes (21, 22, 26).

In this study, we have dissected the roles of E6 and E7 in an *in vivo* model for HPV-associated HNSCC. As previously described in a model for cervical cancer (20, 27), E7 was found in this study to be the major transforming oncogene in the head and neck with some evidence for a role of E6 in later stages of carcinogenesis. The HPV E7 oncoprotein is best known for its ability to target the tumor suppressor pRb for degradation. However, we describe here that the conditional deletion of *Rb* in the head and neck epithelia does not fully recapitulate the effects of E7, thus targets of E7 other than the tumor suppressor pRb must be important in its ability to mediate carcinogenesis.

Materials and Methods

Mice. The generation of *K14E6* (originally called *K14E6:E7TTL*) and *K14E7* (originally called *K14E7:E6TTL*) transgenic mice has been previously described (18, 19). Mice used were derived from founder lines, which were extensively characterized and chosen from a larger set of founder lines. These lines were chosen for their physiologic level of expression of the HPV oncogenes (27).[1] All experiments were performed in the hemizygous state to minimize the possibility of effects that might stem from transgene insertion. To obtain bitransgenic *K14E6K14E7* mice, female *K14E6H* mice were crossed with male *K14E7* mice. In all the studies described, heterozygous singly transgenic mice were used. Mice carrying the *K14Cre* transgene were obtained from Dr. Anton Berns at the Netherlands Cancer Institute (Amsterdam, the Netherlands; ref. 28). Mice carrying a conditional *Rb* allele were generated by and

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[1] S.J. Balsitis and P.F. Lambert, unpublished results.

obtained from Drs. Julien Sage (Department of Pediatrics and Genetics, Stanford University, Stanford, CA) and Tyler Jacks (Center for Cancer Research, Massachusetts Institute of Technology, Cambridge, MA; ref. 29). Tail clippings were collected from all the offspring at the time of weaning and DNA was isolated for genotyping by means of PCR.

For studies described in Figs. 1 and 2, all mice were maintained on the *FVB/N* inbred genetic background. For the studies described in Figs. 3 and 4, the studies were performed in a mixed *FVB/N* and *C57BL/6* genetic background. For all the experiments that were performed at a mixed background, the mice were maintained at backcross 6 to *FVB/N* such that all the mice contained a similar degree of genetic heterogeneity. Mice were housed in the Association for Assessment of Laboratory Animal Care–approved McArdle Laboratory Animal Care Unit at the University of Wisconsin Medical School. All protocols for animal work were approved by the University of Wisconsin Medical School Institutional Animal Care and Use Committee.

4-nitroquinoline-*N*-oxide–induced head and neck carcinogenesis study and histologic analysis. The treatment and guidelines for histologic analysis were previously described (30).Briefly, mice were treated with 4-nitroquinoline-*N*-oxide (4-NQO) in their drinking water for 16 weeks and then held off-treatment for 8 weeks. At the end point or when mice became moribund, they were euthanized, tumors in tongue and esophagus were scored, and tissues were collected for pathology.

Immunohistochemistry. The protocol for immunohistochemical analysis of tissues has been previously described (22). Briefly, primary incubation with anti-bromodeoxyuridine (BrdUrd) antibody (Calbiochem) was performed at a concentration of 1:50 in blocking solution at 4°C. For other antigens, the primary antibodies were used as follows: Ki67 (Dako, 1:25), Mcm7 (Neomarkers, 1:200), pRb (G3-245, PharMingen, 1:50), and p16 (M156, Santa Cruz Biotechnology, 1:50). Incubation with secondary antibodies was performed in a 1:100 dilution in PBS of biotinylated anti-rat immunoglobulin (PharMingen) for Ki67 or 1:100 Vectastain Universal Elite secondary antibody for all other antigens (Vector) for 30 min at room temperature. The signal was amplified using the ABC reagent from the Vectastain Universal Elite kit according to the manufacturer's instructions. The signal was developed using 3,3′-diaminobenzidine substrate (Vector) for 2 to 9 min.

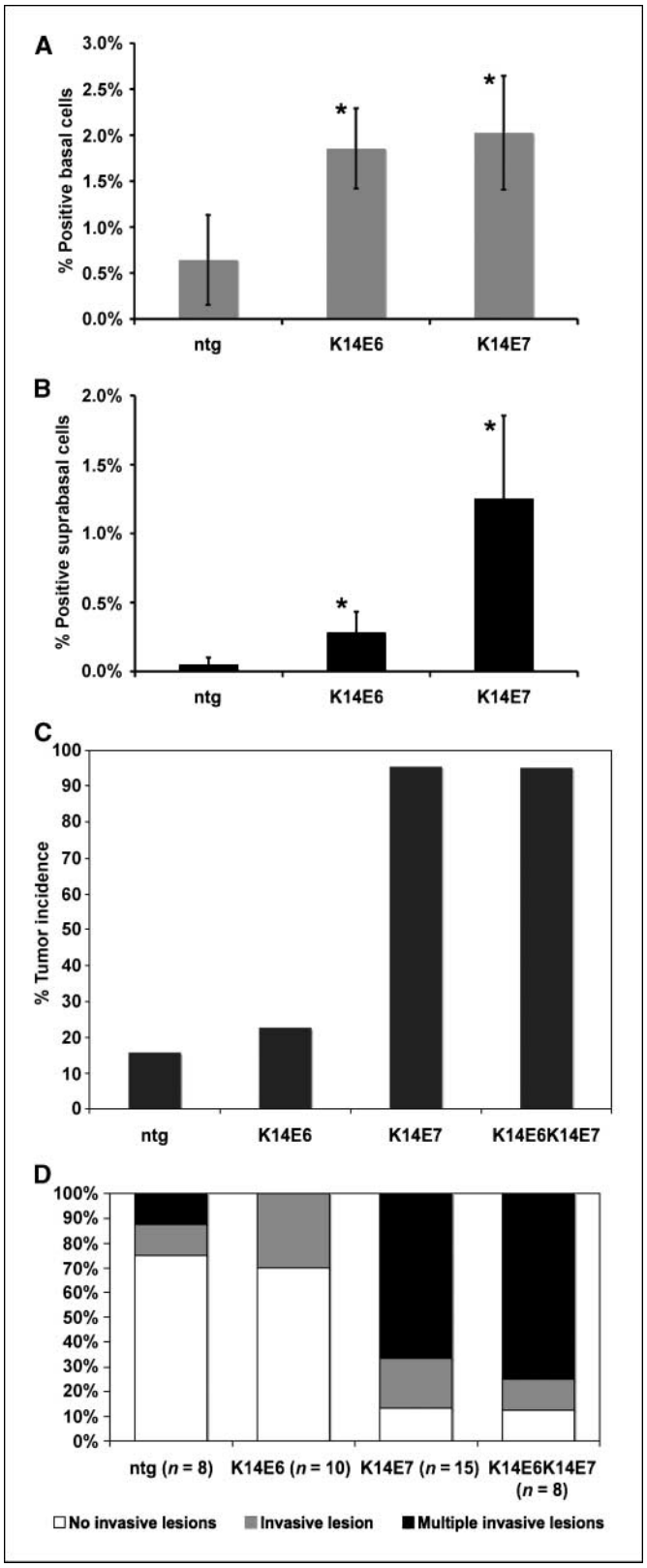

Figure 1. Examination of acute effects of E6 and E7 on DNA synthesis. To determine the DNA synthesis occurring at the basal (*A*) and suprabasal (*B*) compartments, mice were injected with BrdUrd before sacrifice and their tissues were stained for BrdUrd. At least three mice of each genotype, nontransgenic, *K14E6*, and *K14E7h*, were used and ~8 to 10 frames of cells from tongue epithelium were quantified for each mouse. The amount of positive nuclei over the number of total cells was plotted in each case (*columns*); *bars,* SD. *, both in terms of basal and suprabasal DNA synthesis, the increase seen in *K14E6* and *K14E7* animals was increased compared with the amount of DNA synthesis seen in the nontransgenics. The differences were statistically significant according to a two-sided Wilcoxon rank sum test (*K16E6* versus nontransgenic basal $P = 0.01$, suprabasal $P = 0.04$; *K14E7* versus nontransgenic basal $P = 0.009$, suprabasal $P = 0.009$; *K14E6* versus *K14E7* basal $P = 0.6$, suprabasal $P = 0.006$). *ntg,* nontransgenic control animals. *C,* overt tumor incidence in carcinogen-treated mice. Animals represented in this group were treated with 4-NQO for 16 wk and held for a further 8 wk or until they were moribund. At the end point, necropsy was performed and the overt, visible tumors on the mouse tongue and esophagus were counted. The differences in tumor incidence between nontransgenic and *K14E7* groups ($P = 1.4 \times 10^{-7}$), and *K14E6K14E7* groups ($P = 3.0 \times 10^{-7}$), are statistically significant. Also, the differences between *K14E6* and *K14E7* ($P = 1.1 \times 10^{-6}$) and *K14E6K14E7* groups ($P = 1.1 \times 10^{-6}$) are statistically significant. The differences seen between nontransgenic and *K14E6* ($P = 0.7$) and *K14E7* and *K14E6K14E7* ($P = 1$) groups are not statistically significant. All statistical comparisons were performed using a two-sided Fisher's exact test. *D,* multiplicity of cancers. Data from subset of mice randomly chosen for full histopathologic analysis are represented here in terms of cancer multiplicity. Animals with no cancers or benign tumors are assigned to the first group and the other two groups contain animals with one or more than one invasive cancers. The differences between the nontransgenic and *K14E7* ($P = 0.004$) and *K14E6K14E7* ($P = 0.01$) groups are statistically significant. The differences between the *K14E6* and *K14E7* and *K14E6K14E7* groups are also statistically significant. The nontransgenic and *K14E6* ($P = 0.95$) and the *K14E7* and *K14E6K14E7* ($P = 0.72$) comparisons are not statistically significant. All comparisons were performed using the Wilcoxon rank sum test (two-sided). The tumor incidences of the negative (nontransgenic) and positive (*K14E6K14E7*) control groups, which were further characterized in this figure, were previously reported in ref. 30.

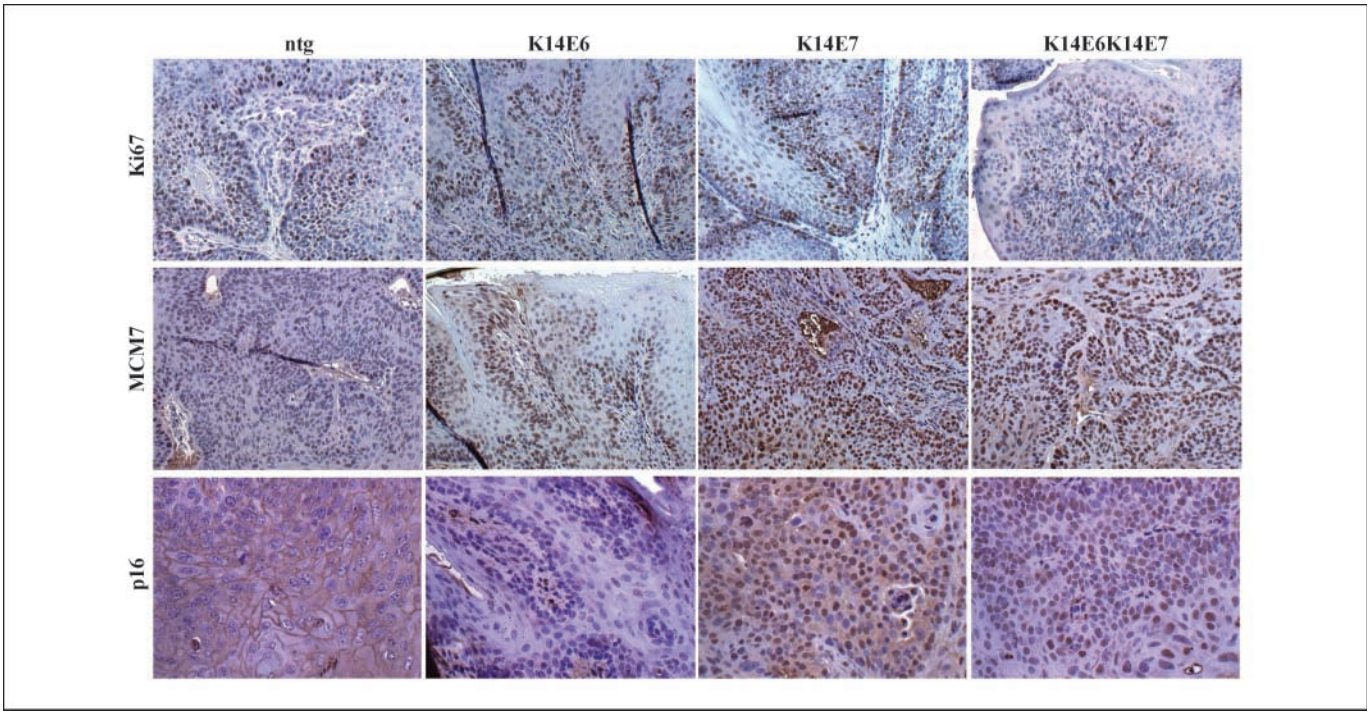

Figure 2. Molecular characterization of cancers arising in treated *K14E6* and *K14E7* mice. To characterize molecular differences between the cancers generated in nontransgenic, *K14E6, K14E7*, and *K14E6K14E7* animals, sets of cancers from these animals were stained for Ki67, MCM7, and p16. Representative images.

Results

Acute phenotypes of E6 or E7 in the head and neck epithelia. In *K14E6/K14E7* bitransgenic mice, we observed an induction of aberrant DNA synthesis in the head and neck epithelia (30). To understand the individual roles of E6 and E7 in contributing to this phenotype, 7-week-old *K14E6* and *K14E7* transgenic mice, expressing either HPV16 E6 or HPV16 E7, respectively, were injected with the nucleotide analogue BrdUrd 1 h before sacrifice. Paraffin-embedded tissues were sectioned and stained with BrdUrd-specific antibodies. Both E6 and E7 led to an increase in the levels of basal DNA synthesis in the head and neck epithelia, suggesting that the expression of each viral oncoprotein in the undifferentiated cells of head and neck epithelia leads to an increase in the number of cells that are in the cell cycle at any given point (Fig. 1*A*). The expression of E6 and E7 each also led to an induction of DNA synthesis in the normally quiescent suprabasal compartment of the stratified epithelia of the head and neck region (Fig. 1*B*).

Contribution of E6 and E7 to tumorigenesis. Short-term phenotypes have in the past served as good indicators for E6 and E7 function in tissues; however, they have not necessarily correlated with the ability of E6 and E7 to contribute to tumorigenesis. We recently established a mouse model for head and neck cancers in which treatment with a low dose of 4-NQO led to the formation of more cancers and cancers of higher grade in bitransgenic *K14E6K14E7* mice compared with nontransgenic mice (30). To evaluate the individual contributions of E6 and E7 to the tumors that formed in the bitransgenic mice, we treated *K14E6* and *K14E7* singly transgenic animals, with 4-NQO as previously described. At the end point or when they became moribund, mice were euthanized, tissues were excised, and the incidence of overt tumors on their tongue and esophagus was scored.

Out of a group of 22 4-NQO–treated *K14E6* mice, only 5 (22%) had overt tumors at the study end point, not significantly higher than the frequency observed in the 4-NQO–treated nontransgenic mice (16%; see Fig. 1*C*). Esophagus and tongues harvested from a random subset of the mice of each genotype were sectioned throughout, and a thorough histopathologic analysis was carried out in which every 20th 5-μm section was stained with H&E and scored under the microscope for the presence of hyperplasia/dysplasia, benign tumors, and grade of carcinoma. For each mouse, the worst phenotype found within the esophagus and tongue tissue was tabulated (Table 1). As seen in the nontransgenic animals, most of the E6 animals had benign disease such as hyperplasia or dysplasia in their tissues. The incidence of carcinoma in these two subgroups was not statistically different. The multiplicity of invasive disease likewise was not different between nontransgenic and *K14E6* mice (Fig. 1*D*). Overall, we observed no significant differences in the nature of the head and neck disease between the nontransgenic and *K14E6* mice based either on scoring the frequency of overt tumors or the histopathologic grade of disease.

In contrast to the *K14E6* mice, the *K14E7* mice, when treated with 4-NQO, had an almost fully penetrant tumorigenic phenotype. Out of a group of 22 animals, 21 had overt tumors at the time of sacrifice (95%). This represents a statistically significant increase over the tumor incidence seen in the control group; however, it is not statistically different from that seen in the *K14E6K14E7* group (95%). The subset of *K14E7* mice that was randomly chosen for histopathologic analysis revealed that the majority of the mice had carcinomas (13 of 15) and, as seen in the *K14E6K14E7* group, a large portion of the carcinomas in those mice were poorly differentiated, grade 3 lesions (7 of 15). The presence of high-grade carcinomas was observed in the *K14E6K14E7* in a higher fraction (6 of 8) of mice analyzed, which suggests a contribution

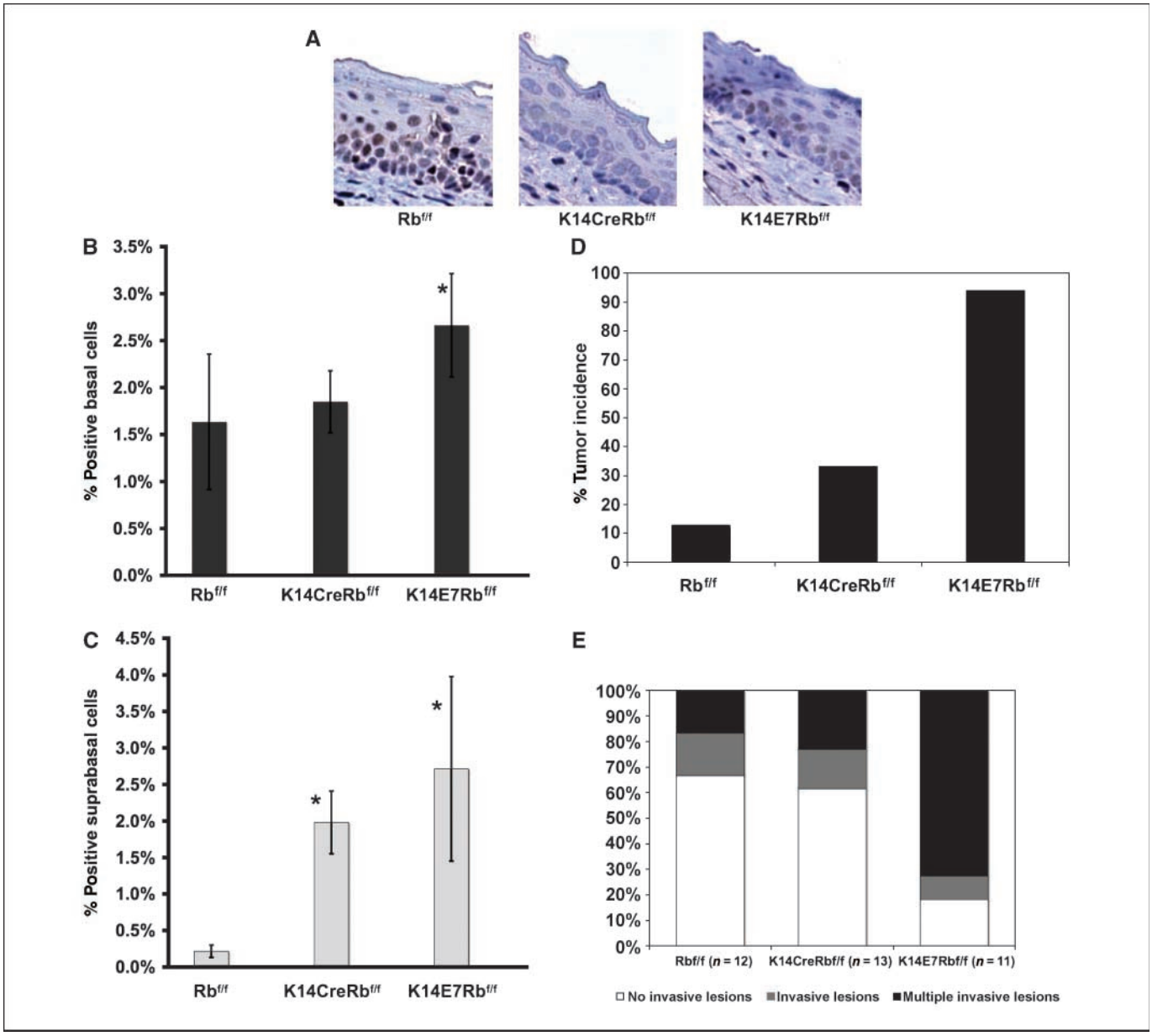

Figure 3. *A,* detection of pRb in control, *Rb*-deleted, and E7-expressing epithelial tongue sections from $Rb^{f/f}$, $K14CreRb^{f/f}$, and $K14E7Rb^{f/f}$ animals stained immunohistochemically for pRb. The results are similar to those seen on sections from esophagus. Although staining is abundantly detectable in $Rb^{f/f}$ controls, it is largely undetectable in $K14CreRb^{f/f}$ mice and greatly diminished in $K14E7Rb^{f/f}$ mice. *B* and *C,* acute effects of *Rb* deletion and E7 expression in epithelia: $Rb^{f/f}$, $K14CreRb^{f/f}$, and $K14E7Rb^{f/f}$animals were injected with BrdUrd before sacrifice and BrdUrd-specific immunohistochemistry was performed on collected tissues. The number of positive basal or suprabasal over the total number of cells is shown in graphs *B* and *C*, respectively. At least three animals per group were quantified and 8 to 10 frames from tongue epithelium per animal were counted. *Bars,* SD. In terms of basal DNA synthesis, the increase was statistically significant only between $K14E7Rb^{f/f}$ and $Rb^{f/f}$ mice ($P = 0.04$, one-sided). In terms of suprabasal DNA synthesis both in $K14CreRb^{f/f}$, and $K14E7Rb^{f/f}$ mice, a statistically significant increase over that seen in the $Rb^{f/f}$ control mice was observed ($P = 0.03$, $P = 0.01$, respectively, Wilcoxon rank sum test, two-sided). *D,* tumor incidence in carcinogen-treated animals: Following treatment with 4-NQO for 16 wk and a hold period for 8 wk, animals of the indicated genotypes were sacrificed and overt tumors in their tongue and esophagus were scored. The increase seen in $K14E7Rb^{f/f}$ compared with $Rb^{f/f}$ animals was statistically significant ($P = 3.1 \times 10^{-9}$), whereas that seen between $K14CreRb^{f/f}$ and $Rb^{f/f}$ animals was also statistically significant ($P = 0.02$). The difference between the $K14E7Rb^{f/f}$ and $K14CreRb^{f/f}$ groups was also statistically significant ($P = 6.7 \times 10^{-5}$). All comparisons were performed using a two-sided Fisher's exact test. *E,* data from subset of mice randomly chosen for full histopathologic analysis are represented here in terms of cancer multiplicity. The differences between the $K14E7Rb^{f/f}$ and the $Rb^{f/f}$ ($P = 0.02$) and $K14CreRb^{f/f}$ ($P = 0.009$) are statistically significant. The difference between the $Rb^{f/f}$ and $K14CreRb^{f/f}$ is not statistically significant ($P = 0.75$). All comparisons were performed using a two-sided Wilcoxon rank sum test.

for E6 in late-stage carcinogenesis; however, that difference was just shy of the 95% confidence limit ($P = 0.06$; one-sided Wilcoxon rank sum test). The multiplicity of tumor load in these subgroups of mice histopathologically analyzed was also assessed. In the bitransgenic mice, 75% had multiple invasive lesions, whereas 67% of the *K14E7* mice had multiple lesions (Fig. 1*D*). Again, this difference between the two groups, although it may be indicative of a function for E6 in the context of E7, is not statistically significant.

In this study, analyzing the individual contributions of HPV16 E6 and E7 in head and neck cancers, we found E7 to be the more

potent oncogene. This finding correlates with that seen in our mouse model for cervical cancer, in which E7 also was found to be the dominant oncogene. Although a statistically significant contribution for E6 in the context of head and neck carcinogenesis was not observed under the experimental conditions used in this study, it is possible that under different experimental conditions, a contribution of E6 to carcinogenesis may be evident. A difference in the frequency of high-grade carcinomas between *K14E7* and *K14E6K14E7* mice, although not statistically significant, is consistent with a role for E6 in the later stages of tumor progression as observed in the skin and cervix.

Proliferation and expression of biomarkers in carcinomas arising in *K14E6* and *K14E7* animals. In addition to the differences detected in tumor incidence between animals carrying the E6 versus E7 oncogenes, we wanted to determine whether there were other molecular differences that distinguished the tumors arising in these mice. Up-regulation of p16 has been reported in human cancers containing HPV DNA both in the cervix and the head and neck (13, 14, 31, 32). Our laboratory previously reported that MCM7 is another useful biomarker for carcinogenesis in the cervix of humans and mice (23). We previously described studies that showed that both p16 and MCM7 are induced in head and neck cancers arising in the *K14E6K14E7* bitransgenic mice (30). We therefore carried out immunohistochemical studies on Ki67, p16, and MCM7 in the head and neck–treated tissues (tongue and esophagus) of our 4-NQO–treated bitransgenic, singly transgenic, and nontransgenic mice to determine whether characteristic marker expression was due to one or both oncogenes.

When comparing *K14E6K14E7*-positive cancers to nontransgenic cancers by immunohistochemistry, no marked differences in proliferation were noted using antibodies to Ki67, a marker for cell proliferation (ref. 30; see also Fig. 2). To assess whether tumors singly transgenic for E6 or E7 had any differences in the proliferation index, we stained those cancers with the same

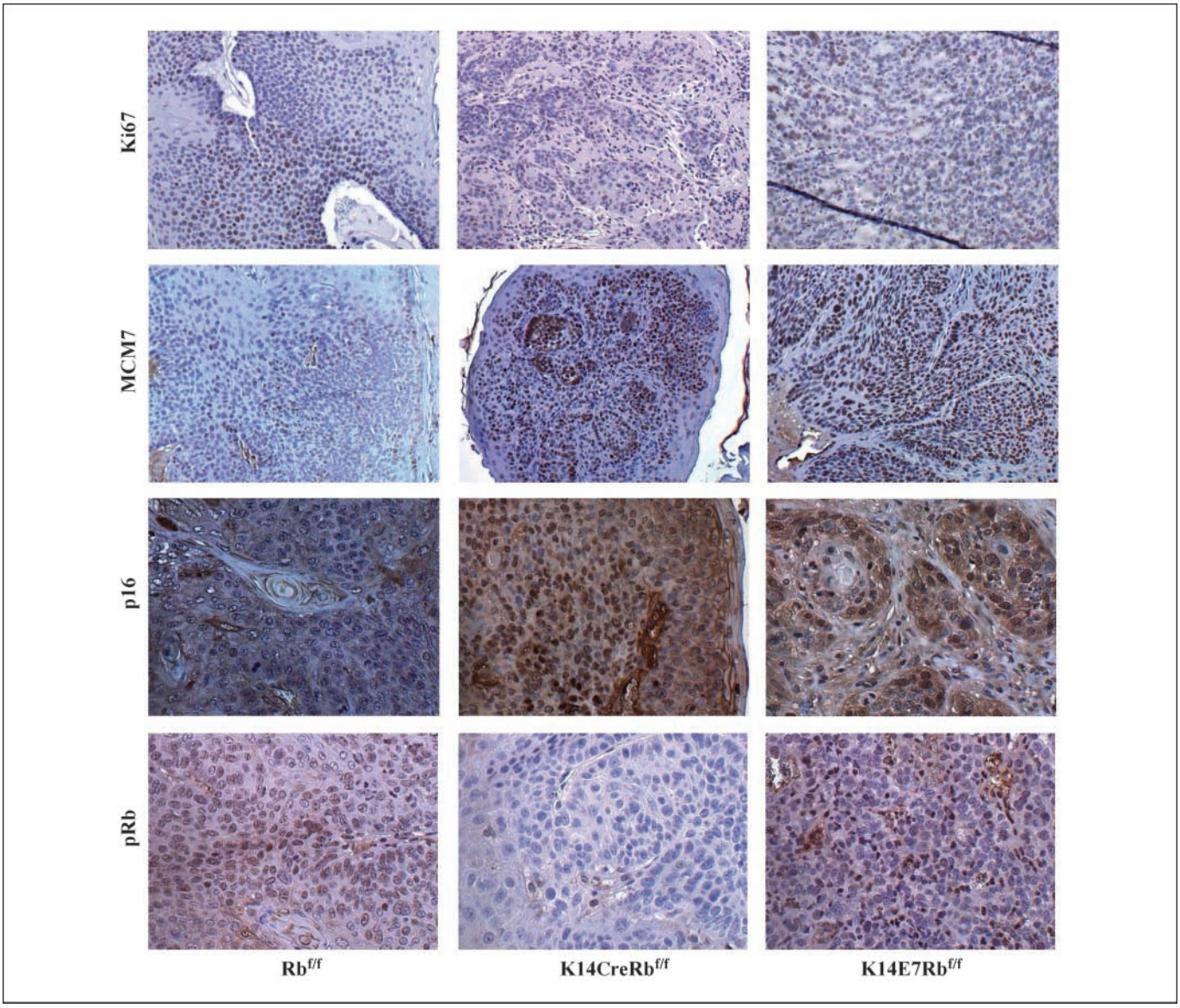

Figure 4. Molecular characterization of cancers arising in *Rb*$^{f/f}$, *K14CreRb*$^{f/f}$, and *K14E7Rb*$^{f/f}$ mice: Cancers from *Rb*$^{f/f}$, *K14CreRb*$^{f/f}$, and *K14E7Rb*$^{f/f}$ animals were immunohistochemically stained and compared for the expression of these markers Ki67, MCM7, p16, and pRb. Representative panels for each genotype and marker.

Table 1. Tissue pathology of 4-NQO–treated mice

	Hyperplasia/dysplasia (%)	Papilloma (%)	Grade 1 (%)	Grade 2 (%)	Grade 3 (%)
Ntg (n = 8)	50	25	0	25	0
K14E6 (n = 10)	70	0	30	0	0
K14E7 (n = 15)	7	7	27	13	47
K14E6K14E7 (n = 8)	0	13	0	13	75

NOTE: A randomly selected subgroup of mice from each genotype was selected for detailed histopathologic analysis of tongue and esophageal tissue for the phenotypes denoted above. Each mouse was assigned the worst phenotype found in at least two consecutive slides analyzed. Statistically significant differences were found only between the *K14E7* versus nontransgenic groups (P = 0.015), and *K14E6K14E7* and nontransgenic groups (P = 0.002). The other comparisons were not statistically different (nontransgenic versus *K14E6*, P = 0.417, two-sided; *K14E7* versus *K14E6K14E7*, P = 0.062, one-sided, P = 0.125, two-sided). All statistical analyses were made using a Wilcoxon rank sum test.
Abbreviation: Ntg, nontransgenic control mice.

antibody (Fig. 2). Not surprisingly, there were no gross differences seen in the proliferation of the cancers from any of the genotypes; in fact, proliferation correlated with the state of disease rather than the genotype of the mice.

Tissues were also subjected to immunohistochemistry using an antibody specific for MCM7, previously seen to be up-regulated in cancers from *K14E6K14E7* bitransgenic mice (ref. 30; see also Fig. 2). *MCM7* is an E2F-responsive gene and therefore is predicted to be up-regulated owing to the inactivation of pRb by E7. In the *K14E6* animals, MCM7 was slightly increased in cancers as opposed to normal or dysplastic epithelium; however, it was not increased to the same degree observed in bitransgenic cancers (Fig. 2). Not surprisingly, tissues from *K14E7* mice showed a marked up-regulation in MCM7 not only in dysplastic epithelium, papillomas, and carcinomas but also in epithelium that was histopathologically normal (Fig. 2 and data not shown). The difference seen between the *K14E7* animals and the nontransgenic animals was quite striking and analogous to the difference seen between the *K14E6K14E7* animals and the nontransgenic animals (Fig. 2). The high frequency of MCM7-positive cells in the tissues from the *K14E7* animals was greater than the frequency of Ki67-positive cells in the same tissues regardless of the grade of disease (Fig. 2), suggesting that E7 dysregulation of pRb is pervasive and is not restricted to proliferating cells. In contrast, in nontransgenic and *K14E6* animals, MCM7, which is moderately expressed in carcinomas and restricted to the basal and parabasal layers of histopathologically normal epithelium, is more indicative of the proliferation state of the cells as the pattern of expression correlates well with the pattern of Ki67 expression (Fig. 2). We conclude that the ubiquitous expression of MCM7 seen in bitransgenic cancers is largely due to the action of E7 and less so to that of E6. The robust transcriptional activation of the E2F-responsive MCM7 protein is likely a result of the collective action of E7 on all the *Rb* family proteins. Unfortunately, due to the lack of adequate antibodies for immunohistochemistry specific for p107 and p130, we cannot verify that their overall levels are likewise diminished.

HPV-positive human tumors frequently show high levels of staining for p16 (13, 14, 31, 32). We hypothesized that this result is largely due to the action of E7 on pRb, which is downstream of p16 in the same pathway. We therefore performed p16-specific immunohistochemistry on tumors from our mice (Fig. 2). Our results were consistent with this hypothesis as abundant staining for p16 was observed on both *K14E7* and *K14E6K14E7* tumors. Very little p16 was detectable in the nontransgenic or *K14E6* tumors.

Loss of pRb can recapitulate some but not all of the acute effects of E7 in inducing aberrant DNA synthesis in head and neck epithelia. A challenge in evaluating the individual interaction of E7 with pRb is posed by the fact that E7 interacts with all the *RB* family proteins (including p107 and p130) through the same binding pocket, the *LxCxE* binding motif. Thus, the use of a mutant form of E7 that cannot bind pRb is of limited value because this

Table 2. Histopathology of treated tissues

	Hyperplasia/dysplasia (%)	Papilloma (%)	Grade 1 (%)	Grade 2 (%)	Grade 3 (%)
Rbf/f (n = 13)	50	17	33	0	0
K14CreRbf/f (n = 12)	46	15	15	8	15
K14E7Rbf/f (n = 11)	0	18	27	18	36

NOTE: A randomly selected subset of the carcinogen-treated animals was examined in detail throughout the thickness of their tongue and esophageal epithelia for the phenotypes indicated above. Each mouse was assigned the worst phenotype found in two or more consecutive slides. The difference seen between the *K14E7Rb$^{f/f}$* and *Rb$^{f/f}$* groups was statistically significant (P = 0.001) as well as the difference between the *K14E7Rb$^{f/f}$* and *K14CreRb$^{f/f}$* groups (P = 0.01). The difference between the *Rb$^{f/f}$* and *K14CreRb$^{f/f}$* groups was not different (P = 0.56). All comparisons were performed using a two-sided Wilcoxon rank sum test.

mutation will affect E7 binding to not only pRb but also p107 and p130. To determine specifically the importance of pRb as a relevant target in E7-mediated tumorigenesis, we made use of $Rb^{f/f}$ mice homozygous for a conditional null allele of *Rb* (29). In $Rb^{f/f}$ mice, exon 3 of the *Rb* allele is flanked by *loxP* sites. Excision of exon 3 by Cre recombinase leads to a frameshift and the premature termination of translation of pRb. The gene product of this recombined allele was shown to lack any function ascribed to full-length pRb. To direct expression of *Cre* to the same compartment in which we express E7, we obtained *K14Cre* mice in which the expression of the recombinase is driven from the same cytokeratin 14 promoter used in our *K14E7* mice (28).

As evaluated by pRb-specific immunohistochemistry (Fig. 3*A*), pRb is expressed abundantly throughout the thickness of the head and neck epithelia of adult $Rb^{f/f}$ mice that are homozygous for conditional-null *Rb* allele but express no Cre recombinase. In these same epithelia in age-matched $K14CreRb^{f/f}$ animals, pRb was undetectable, validating that Cre was efficient at targeting recombination of the floxed allele of *Rb* in these tissues. When E7 is expressed in these same tissues in age-matched $K14E7Rb^{f/f}$ mice, pRb protein was diminished in its abundance but still detectable, consistent with E7 being able only partially to degrade pRb.

We have previously shown that the expression of E7 in head and neck epithelia can lead to an increase in DNA synthesis in the normally proliferating basal compartment of the epithelium, and induce unscheduled DNA synthesis in the normally quiescent, suprabasal compartment (Fig. 1). These same phenotypes were observed in same tissues of adult $K14E7Rb^{f/f}$ mice (Fig. 3*B* and *C*). In $K14CreRb^{f/f}$ mice, we observed a similar, statistically significant increase in DNA synthesis in the suprabasal (Fig. 3*C*) compartment of the head and neck tissues, but, interestingly, no significant increase in the basal compartment (Fig. 3*B*). It is unclear why the loss of pRb positively induces DNA synthesis in the suprabasal compartment of the epithelium yet leaves it unaffected in the basal compartment of the same tissue. A possible explanation may involve the amounts of the other pocket proteins that are expressed in the two compartments. Whereas in the head and neck epithelia, loss of *Rb* recapitulated some but not all E7-mediated effects on DNA synthesis, loss of *Rb* fully recapitulated the effects of E7 on DNA synthesis in the epidermis (22). Interestingly, loss of *Rb* recapitulated none of the E7-mediated phenotypes in the epithelium of the female reproductive tract (26). These observations highlight the significance of a particular tissue in studying the role of pRb in proliferation and differentiation. The differences observed between the tissues may offer clues to the biology of these different tissues, and, perhaps, specifically, to the pool of available p107 and/or p130 in each tissue.

The loss of pRb does not fully recapitulate the oncogenic phenotypes of E7. To compare the consequences of loss of *Rb* versus expression of E7 on the susceptibility of mice to HNSCC, we treated groups of $Rb^{f/f}$, $K14CreRb^{f/f}$, and $K14E7Rb^{f/f}$ animals using the same 4-NQO carcinogen treatment protocol. At the study end point, the animals from each group were evaluated for overt tumors and tissues from random subsets of mice were subjected to detailed histopathologic analyses (Fig. 3*D* and *E*; Table 2). Not surprisingly, the 4-NQO–treated $K14E7Rb^{f/f}$ animals had a high incidence of overt tumors (16 of 17 animals or 94%; Fig. 3*D* and *E*) similar to that seen in the *K14E7* animals on the $Rb^{wt/wt}$ background (Fig. 1*C*). In the subset of $K14E7Rb^{f/f}$ mice for which we performed detailed histopathology, 9 of the 11 mice had invasive carcinomas, and the other two had noninvasive papillomas (Table 2). Furthermore, 8 of these 11 mice had multiple invasive lesions, and four of those had at least one high-grade carcinoma (grade 3; Table 2). The 4-NQO–treated $Rb^{f/f}$ animals had a low incidence (6 of 46 mice or 13%) of overt tumors (Table 2), similar to that observed in $Rb^{wt/wt}$ *FVB/N* animals. Of the 4-NQO–treated $Rb^{f/f}$ mice subjected to detailed histopathology, 8 of 12 (67%) of the animals had no invasive lesions (75% of *FVB/N* mice were free of invasive tumors and the difference between the two groups is not statistically significant). Furthermore, of the $Rb^{f/f}$ mice, none had high-grade lesions and only 2 of 12 had more than one invasive tumor. The 4-NQO–treated $K14CreRb^{f/f}$ animals had a statistically significant increase in the incidence of overt tumors (12 of 36 or 33%) over that observed in the in the 4-NQO–treated $Rb^{f/f}$ mice (Fig. 3*D*). However, this increase in tumor incidence seen in the $K14CreRb^{f/f}$ animals was significantly less than the incidence of tumors observed in the $K14E7Rb^{f/f}$ animals. Histopathologic analysis indicated that the tumor multiplicity (Fig. 3*E*) and grade of carcinoma (Table 2) was not statistically different to that observed in the 4-NQO–treated $Rb^{f/f}$ mice. The absence of a statistically significant difference in this analysis between the $K14CreRb^{f/f}$ mice and the $Rb^{f/f}$ group could be attributed to the small sample size of mice undergoing full histopathology. However, it is important to note that though the loss of *Rb* leads to a significant increase in tumor susceptibility, it is not nearly as dramatic as the susceptibility seen in the same tissues upon expression of E7, and that, interestingly, is despite the fact that E7 does not abolish detectable pRb as the conditional deletion does.

Expression of molecular markers in cancers. We stained treated epithelia and cancer tissue from $Rb^{f/f}$, $K14E7Rb^{f/f}$, and $K14CreRb^{f/f}$ animals with an antibody specific to pRb (Fig. 4). In the control $Rb^{f/f}$ mice, pRb was detectable although the nuclear signal is quite modest. Positive staining for pRb in the $Rb^{f/f}$ mouse tissue can best be appreciated by comparing the signal with the complete absence of signal in the $K14CreRb^{f/f}$ mouse sample (Fig. 4). In $K14E7Rb^{f/f}$ tissues, detection of pRb either was diminished compared with $Rb^{f/f}$ mouse tissue (example shown in Fig. 4) or in some cases abolished (example not shown). Indeed, most cancers from $Rb^{f/f}$ mice and about half of the $K14E7Rb^{f/f}$ showed detectable pRb, although, in the case of the E7+ cancers, pRb still seemed diminished compared with that in the nontransgenic cancers. These findings are all consistent with prior findings in the mouse skin (22), mouse cervix,[1] and in human keratinocytes (33) that all have shown that E7 only incompletely degrades pRb and further suggest that the complete loss of pRb is not necessary for the formation of cancers induced by E7. No $K14CreRb^{f/f}$ cancers had any detectable pRb as expected.

Specific to the cancers was the ubiquitous expression of p16 (Fig. 4) only in $K14E7Rb^{f/f}$ and $K14CreRb^{f/f}$ cancers. P16 silencing in human HPV-negative HNSCC is a frequent occurrence; other models using 4-NQO as an oral carcinogen have reported the inactivation of p16 in *Rb* wild-type animals (34). However, HPV-positive cancers are usually noted for their increased expression of p16 (13, 14). Given this information, we were not surprised to see the selective detection of p16 in E7-expressing cancers. The detection of p16 in *Rb*-deleted cancers was expected given the known role of pRb in the methylation of the *INK4a* locus (35). Thus, it makes sense that in both $K14E7Rb^{f/f}$ and $K14CreRb^{f/f}$ tissues in which pRb levels are at least diminished, methylation and silencing of the p16 locus does not occur. An interesting observation is that the loss of *Rb* in head and neck epithelia led to the expansion in the

expression of MCM7 in the suprabasal compartment as was also observed in *K14E7Rb*$^{f/f}$ mice. Furthermore, MCM7 was abundantly expressed in tumors from *K14CreRb*$^{f/f}$ animals and not *Rb*$^{f/f}$ animals (Fig. 4). Although there was a correlation in the activation of this E2F-responsive gene between tissues expressing E7 and lacking pRb, and both types of tissues had increased tumor incidence over that seen in the *Rb*$^{f/f}$ animals, the severity of the tumor phenotypes was not similar between the two types of tissues; it was much higher in E7-expressing tissue. This finding would suggest that E7 does not lead to tumorigenesis merely via the activation of E2F targets, or at least not only through those targets controlled primarily by pRb.

Discussion

It is interesting to note the parallels and differences in terms of oncogene function in the various stratified epithelial tissues. In multistage carcinogenesis studies performed in the skin of E6 and E7 transgenic mice, E6 was shown to contribute weakly to the promotion stage to benign disease, whereas its primary role was in the progression to malignant lesions. In contrast, E7 played a major role in promotion, an early step in tumorigenesis (36). In the cervix of mice, E6 in addition to E7 led to an increase in the formation of large and more invasive cancers (20). In the studies described here, the combination of E6 and E7 seems to lead to a higher grade of disease and slightly increased multiplicity if tumors. The emerging pattern from studies in other tissues implicates E6 most prominently in the late stages of carcinogenesis, although the evidence is still weak for the head and neck model. The p53 tumor suppressor, an important target for E6, was also shown to be important at the late stages of carcinogenesis. When p53$^{+/-}$ mice were treated using multistage skin carcinogenesis protocols, the loss of p53 was shown to be important in the progression to more malignant disease (37). In addition, we have recently shown that inactivation of p53 in the cervical epithelia predisposes mice to cervical cancer.[2] It is also informative that in human HPV-positive HNSCC, p53 mutations are notably absent, which supports an essential role for E6 in these cancers (12). Therefore, we predict that E6, at least through its inactivation of p53 if not its other properties, contributes to HNSCC. Supporting this view, our preliminary data indicate that by reducing the time of treatment with the cocarcinogen, 4NQO, a significant role of E6 in HPV-associated HNSCC becomes apparent.[3]

The E7 oncogene is more potent in inducing malignancies in both the head and neck and the cervix than the E6 oncogene in contrast to the skin. These results probably reflect not only the distinct roles of E6 and E7 at different stages of carcinogenesis but also the different behaviors of E6 and E7 depending on the tissue context. It should be noted that the different behaviors of the oncogenes cannot be merely attributed to differing levels of expression of the oncogenes. Both E6 and E7 expression in various epithelial tissues of these transgenic mice have been shown to be comparable with or lower than the levels expressed in HPV-positive cancer-derived cell lines (27).[1]

The results observed from the deletion of *Rb* in the head and neck epithelia lead to several conclusions about the action of E7 as an oncogene in these tissues. Because the induction of DNA synthesis in postmitotic cells seen in *Rb*-deleted head and neck epithelia does not correlate fully with oncogenic phenotypes, we conclude that the E7 oncoprotein cannot induce tumorigenesis purely through its ability to cause aberrant DNA synthesis in the differentiated compartment of these epithelia, although this is an activity that is thought to be key to the role of E7 in the viral life cycle (33). Furthermore, the activation of E2F-responsive genes that is seen in both pRb-deleted and E7-expressing tissue does not correlate quantitatively with oncogenesis in these tissues, indicating, at the very least, that activating E2F transcription is not sufficient to account for the role of E7 in carcinogenesis. The deletion of pRb leads to an activation of MCM7 as effectively as the expression of E7; however, it elevates the risk for cancer to a lesser degree than does expression of E7. It is still reasonable to posit that the dysregulation of at least some E2F-responsive genes are necessary for each outcome (aberrant DNA synthesis, tumorigenesis).

Evaluating the targets of the E7 oncoprotein that mediate its biological properties, pRb remains an important target because the loss of pRb contributes to ectopic proliferation as previously observed in stratified epithelia of the skin (Fig. 3), and in part also contributes to carcinogenesis (Fig. 3; Table 2). However, these studies show that E7 does not induce cancers merely by targeting pRb for degradation. This observation does not preclude the possibility that pRb is still an important target but clearly shows that its inactivation is not sufficient to recapitulate E7 activities. E7 can in fact interact with all of the pocket proteins, and it could be the combined effects of E7 on two or all of the pocket proteins that lead to its oncogenic effects. This would be in line with previously mentioned studies that implicate other pocket proteins with the combined loss of *p107* and *Rb*, or *p130* and *Rb*, resulting in tumorigenesis (29, 38, 39). However, the facts that there are still some acute phenotypes in the head and neck and increased tumor incidence upon the loss of *Rb* indicate that if there is indeed some functional redundancy and/or compensation, it cannot be complete; otherwise, no phenotypes would be observed.

Another explanation for the inability of loss of pRb to recapitulate the effects of E7 could involve a nonpocket protein target of E7 such as p21 or p27, either alone or in combination with other targets including pRb. The ability of E7 to bind p21 has been shown to be crucial for its *in vitro* transforming abilities (40).

In summary, we have performed studies to examine the individual contribution of the HPV16 oncogenes E6 and E7 in head and neck carcinogenesis. We have determined that E7 is the dominant oncogene in this model with the tumor incidence and biomarker expression seen in the bitransgenic *K14E6K14E7* mice largely reflected in *K14E7* mice. The effects of E7 in these tissues are not likely to be the singular outcome of the targeted degradation of the pRb tumor suppressor. Although the loss of pRb does contribute to HNSCC, it is likely that E7 mediates its cumulative effects through its action on one or more additional targets.

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[2] A. Shai, H.C. Pitot, and P.F. Lambert. The role of p53 in HPV-associated cancers, submitted for publication.

[3] K. Strati and P.F. Lambert, unpublished studies.

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