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The Human Papillomavirus E6 Oncogene Dysregulates the Cell Cycle and Contributes to Cervical Carcinogenesis through Two Independent Activities

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Abstract

Cervical cancer is a leading cause of death due to cancer among women worldwide. Using transgenic mice to dissect the contributions of the human papillomavirus (HPV) 16 E6 and E7 oncogenes in cervical cancer, E7 was identified previously to be the dominant oncogene. Specifically, when treated with exogenous estrogen for 6 months, E7 transgenic mice developed cancer throughout the reproductive tract, but E6 transgenic mice did not. E6 contributed to carcinogenesis of the reproductive tract, as E6/E7 double transgenic mice treated for 6 months with estrogen developed larger cancers than E7 transgenic mice. In the current study, we investigated whether the E6 oncogene alone could cooperate with estrogen to induce cervical cancer after an extended estrogen treatment period of 9 months. We found that the E6 oncogene synergizes with estrogen to induce cervical cancer after 9 months, indicating that E6 has a weaker but detectable oncogenic potential in the reproductive tract compared with the E7 oncogene. Using transgenic mice that express mutant forms of HPV16 E6, we determined that the interactions of E6 with cellular α -helix and PDZ partners correlate with its ability to induce cervical carcinogenesis. In analyzing the tumors arising in E6 transgenic mice, we learned that E6 induces expression of the E2F-responsive genes, *Mcm7* and *cyclin E*, in the absence of the E7 oncogene. E6 also prevented the expression of p16 in tumors of the reproductive tract through a mechanism mediated by the interaction of E6 with α -helix partners.

Introduction

Cervical cancer is the second most common type of cancer among women, with high mortality rates worldwide, despite increased screening efforts (1). Human papillomavirus (HPV) infection contributes to nearly all cases of cervical cancer based on the observed presence of HPV DNA within these cancers (2) and more than half of the HPV-associated cervical cancers are attributed to infection with HPV16 (2,3).

Two viral genes, *E6* and *E7*, are commonly expressed in cervical cancer. In tissue culture, *E6* and *E7* display properties of oncogenes, including the ability to immortalize and transform cells (4). To assess the oncogenic properties of these genes *in vivo*, we generated K14E6^{WT} and K14E7^{WT} transgenic mice expressing either the HPV16 E6 or the HPV16 E7 oncogene, respectively. The human keratin-14 promoter was used to direct transgene

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expression to the basal layer of the stratified squamous epithelium lining the skin, oral cavity, and reproductive tract (5–7). K14E6^{WT} and K14E7^{WT} transgenic mice display many of the known activities of each oncogene identified in tissue culture, including the ability of E6 and E7 to inactivate p53 and pRb, respectively. Furthermore, these HPV16 transgenic mice develop tumors in the skin either spontaneously or with increased efficiency when induced chemically with the carcinogens, 7,12-dimethylbenz(a)anthracene and 12-*O*-tetradecanoylphorbol-13-acetate (5,6,8).

Prior studies showed that both the K14E6^{WT}/K14E7^{WT} transgenic mice and the K14E7^{WT} singly transgenic mice developed cervical cancer following estrogen treatment for 6 months. However, similarly treated K14E6^{WT} transgenic mice developed only low-grade dysplasia (7). Estrogen is a cofactor in cervical carcinogenesis in this mouse model, as untreated K14E6^{WT}/K14E7^{WT} or K14E7^{WT} mice did not develop cancer. Subsequent studies indicated that estrogen is required for multiple stages of cervical carcinogenesis (9). Reproductive tumors arising in the K14E6^{WT}/K14E7^{WT} transgenic mice were more aggressive than those arising in the K14E7^{WT} transgenic mice, indicating that the E6 oncogene contributed to the malignant progression.

In the current study, we investigated whether the E6 oncogene could cooperate with estrogen to induce cervical cancer given an extended (9 months) treatment period. To examine the mechanism(s) by which the E6 oncogene contributes to cervical cancer, we monitored cervical carcinogenesis in K14E6^{I128T} and K14E6^{Δ146–151} mice, which express mutant forms of the HPV16 E6 oncogene (10,11). K14E6^{I128T} transgenic mice express a mutant form of E6 greatly reduced in its ability to bind α -helix partners. Specifically, E6^{I128T} protein binds the α -helix partners, E6AP and E6BP, at 1% to 5% the levels of wild-type (WT) E6 protein (12). E6AP or UBE3A belongs to the HECT family of E3 ubiquitin ligases (13) and is normally associated with the human neurologic disorder, Angelman's syndrome (14,15). E6AP is thought to be the primary cellular factor mediating the degradation of p53 by E6 (16) and is thus a potentially important partner in mediating the oncogenic activities of E6. Correspondingly, K14E6^{I128T} transgenic mice are defective for inactivating p53 (17). K14E6^{Δ146–151} transgenic mice encode a mutant E6 protein defective for interacting with PDZ partners (18), such as *DLG* and *Scribble*, two genes known to be tumor suppressors in *Drosophila*. We have previously used K14E6^{I128T} and K14E6^{Δ146–151} transgenic mice to show a role of the α -helix and PDZ domain partners of E6, respectively, in mediating the oncogenic potential of E6 in the skin (10,17,19). These studies provide a framework for our studies of cervical carcinogenesis studies reported herein.

We found that the E6 oncogene synergizes with estrogen to induce cervical cancer after an extended estrogen treatment. Specifically, K14E6^{WT} mice, treated with estrogen for 9 months, developed cervical cancers at an increased frequency compared with nontransgenic mice. Compared with K14E6^{WT} mice, K14E6^{I128T} and K14E6^{Δ146–151} mice in the absence and/or the presence of E7 displayed reduced oncogenic potential in the reproductive tract.

We also evaluated mouse reproductive tracts and their associated tumors for biomarkers commonly used for the diagnosis of human cervical cancers. Biomarkers included the E2F-responsive genes *Mcm7*, involved in DNA replication (20), and *cyclin E*, involved in the G₁-S transition (21,22). We also monitored expression of the cyclin kinase inhibitor, p16. p16 is a biomarker for HPV-associated cervical lesions and cancers (23). Finally, tumors were evaluated for p53 expression. In most cases, biomarker expression in the lesions and tumors from our nontransgenic and transgenic mice mirrored results observed in human cervical samples. Of particular interest, lesions from our K14E6^{WT} and K14E6^{mutant} (refers to both K14E6^{I128T} and K14E6^{Δ146–151} herein) mice showed an up-regulation of the E2F-responsive genes, *Mcm7* and *cyclin E*, even in the absence of E7, but at lower levels than in

tumors arising in E7-expressing mice. In contrast, we observed an inverse relationship between the expression of p16 and pRb in the reproductive tumors from K14E6^{WT} versus K14E7^{WT} mice. Biomarker expression in tumors arising in K14E6^{WT}/K14E7^{WT} mice were similar to tumors arising in K14E7^{WT} mice, showing that E7 is the dominant oncogene in deregulating the p16/pRb pathway during cervical carcinogenesis. In summary, this study shows that two properties of E6 contribute to the development of cervical cancer. These contributions lead to a distinct pattern of dysregulation of cell cycle regulatory genes compared with that seen in E7-expressing tumors.

Materials and Methods

Mouse lines and estrogen treatment

The K14E6^{WT} (5), K14E6^{I128T} (17), K14E6^{Δ146–151} (10), and K14E7^{WT} (6) lines were all maintained on a heterozygous FVB background. K14E6^{WT}, K14E6^{I128T}, and K14E6^{Δ146–151} mice were mated to K14E7^{WT} mice to generate double transgenic mice. Female mice were treated with 17-β estradiol as described previously (7) for 9 months. Untreated control mice were held for the same period. All mice were bred and maintained in the American Association for Accreditation of Laboratory Animal Care–approved McArdle Laboratory Animal Care Facility in accordance with an institutionally approved animal protocol.

Quantification of E6 levels

Five-week-old female mice were treated with estrogen, and reproductive tracts were harvested after 6 weeks of treatment to obtain a state of constant estrus in all of the mice. Dorsal skin was also harvested at the time of sacrifice in addition to skin from 9-day-old mice, as transgene expression is highest during this period in the skin. The tissue was placed in cold HNTG lysis buffer [50 mmol/L HEPES (pH 7.5), 150 mmol/L NaCl, 1.1% Triton X-100, 1 mmol/L EGTA, 10% glycerol, 1 mmol/L phenylmethylsulfonyl fluoride, 1× PIC] and homogenized. Protein lysates (100–200 μg) were separated by SDS-PAGE, transferred, and immunoblotted with a monoclonal HPV16 E6 antibody (24) at 5 μg/mL. A mouse IgG secondary conjugated to horseradish peroxidase (Jackson ImmunoResearch, West Grove, PA) was used at 1:10,000. Detection of E6 was achieved by using the Enhanced Chemiluminescence Plus Western Detection kit (Amersham, Piscataway, NJ).

Analysis of reproductive tracts

Reproductive tracts were harvested after 9 months of estrogen treatment and analyzed as described previously (7). The fixed tissue was histologically sectioned and every tenth 5-μm section was stained with H&E and pathologically examined, with the worst lesion scored as the final diagnosis. The tumors were measured with the Zeiss Axiovision (version 3.1) program (Zeiss, Thorwood, NY). Any tumor with an area >2,000 μm² was classified as a large cancer.

Quantification of bromodeoxyuridine

To quantify the amount of basal DNA synthesis, the total number of bromodeoxyuridine (BrdUrd)-positive basal cells was counted and divided by the total number of basal cells and multiplied by 100 to determine the percentage. To quantify the amount of epithelial hyperplasia, the total number of suprabasal BrdUrd-positive cells were counted and divided by the total number of cells and multiplied by 100 to determine the percentage. BrdUrd was counted in eight, ×40 microscopic fields per mouse, with a total of at least three or more mice per genotype group.

Statistical analysis

The Fisher's exact test was used to determine the significance in tumor incidence. The Wilcoxon rank-sum test was used to determine the significance in BrdUrd quantification and in measurements of tumor size and area. Statistical analysis was carried out using the MSTAT program.³

Immunohistochemistry

Sections were prepared for immunohisto-chemical analysis as described previously (7). For BrdUrd, cyclin E, pRb, and p16 analysis, the slides were immersed in 2N HCl for 20 min to unmask further. Primary antibody was applied to the sections at 1:100 for BrdUrd (Calbiochem, Darmstadt, Germany); 1:200 to 1:500 in blocking solution for p53 (CM5; Novocastra, Norwell, PA); and 1:50 for MCM7 (NeoMarkers, Fremont, CA), cyclin E (M-20; Santa Cruz Biotechnology, Santa Cruz, CA), pRb (BD Biosciences, San Jose, CA), and p16 (M156; Santa Cruz Biotechnology) overnight at 4°C. A universal biotinylated secondary antibody was applied and developed.

Results

K14E6^{WT} and K14E6^{mutant} transgenic mice express physiologic levels of E6 protein in the epithelia of the skin and reproductive tract

Prior detection of HPV16 E6 protein was difficult due to an absence of adequately sensitive antibodies. A recently generated HPV16 E6-specific antibody (24) allowed us to detect and compare levels of E6 protein expressed in our transgenic mice with cell lines derived from human precancerous cervical intraepithelial neoplasia (CIN) lesions and cervical cancers (Fig. 1). HPV16 E6 protein was detected in the HPV16-positive SiHa and Caski but not in the HPV18-positive HeLa and HPV-negative C33a cervical cancer cells (Fig. 1A). E6 protein was also detected in W12 clonal cell lines derived from a HPV16-positive CIN1 lesion. Dorsal skin from 9-day-old WT and mutant E6 transgenic mice expressed E6 protein at levels slightly higher than SiHa cells, whereas expression in adult mice were lower (Fig. 1B; data not shown). This result is consistent with our prior observations that K14-directed transgene expression is maximal in young pups and wanes in adults (25). In homozygous K14E6^{WT} transgenic mice, the level of E6 protein was approximately half of the amount of E6 expressed in Caski cells (Fig. 1B). E6 protein was detected in the reproductive tracts of all adult K14E6^{WT} and K14E6^{mutant} mice, at levels lower than in both SiHa and Caski cells (Fig. 1C). Given that <10% of the total protein from the harvested reproductive tract tissues comes from the stratified epithelium, we conclude that our K14E6^{WT} transgenic mice express E6 protein at levels similar to that observed in human cervical cancer. K14E6^{Δ146-151} mice expressed ~1.5 times the amount of E6 protein relative to K14E6^{WT} mice in the reproductive epithelium (Fig. 1C). K14E6^{I128T} transgenic mice (data not shown) expressed mutant E6 protein roughly equal to that in K14E6^{WT} mice.

E6 synergizes with estrogen in the absence of E7 to form reproductive tumors after estrogen treatment for 9 months

Whereas K14E6^{WT} transgenic mice did not develop cancer after 6 months of estrogen treatment, E6 contributed to the severity of tumors arising in K14E6^{WT}/K14E7^{WT} mice when treated with estrogen for either 6 or 9 months (7,9). In the current study, K14E6^{WT} mice were treated with estrogen for an extended 9-month period to investigate whether E6 could induce cervical cancer in the absence of E7. As expected, none of the untreated mice, regardless of genotype, developed cancer (data not shown). After 9 months of estrogen

³<http://mcardle.oncology.wisc.edu/mstat>

treatment, 41% of K14E6^{WT} mice developed tumors in the reproductive tract compared with 6.7% for nontransgenic mice (Table 1). This difference was statistically significant ($P = 0.02$). In contrast, 100% of K14E7^{WT} transgenic mice treated for 9 months developed cancer (9). Thus, E6, in the absence of E7, can induce cervical cancers in cooperation with exogenous estrogen, albeit less robustly than E7.

An E6 mutant reduced in binding α -helix partners has a lower incidence of cancer and develops smaller tumors

E6 binds to numerous cellular proteins. One subset (e.g., the E3 ubiquitin ligase E6AP) binds to E6 via a leucine-rich α -helical (α -helix) motif, whereas another (e.g., Dlg and Scribble) binds through PDZ domains. We used two lines of E6 mutant mice, K14E6^{I128T} and K14E6 ^{Δ 146-151}, defective in binding α -helix and PDZ partners, respectively (10,17), to examine the importance of the interaction of E6 with each subset of partners in mediating the oncogenic properties of E6 in the reproductive tract. After a 9-month treatment period with estrogen, K14E6^{I128T} mice had a marginally significant reduction in tumor incidence relative to K14E6^{WT} mice (19.4% versus 41%; $P = 0.058$; Table 1). In contrast, the K14E6 ^{Δ 146-151} transgenic mice had similar rates of cancer incidence as the K14E6^{WT} mice ($P = 0.25$). Tumors from the reproductive tract of K14E6^{WT} and K14E6^{mutant} mice were variable in size (Fig. 2A). Nonetheless, tumors arising in the K14E6^{I128T} transgenic mice were generally smaller relative to tumors from either K14E6^{WT} or K14E6 ^{Δ 146-151} mice (Table 1). The largest tumor size on average for K14E6^{I128T} mice (1.38 mm² cross-sectional area) was significantly smaller ($P = 0.041$) than that for K14E6^{WT} mice (5.22 mm²). In contrast, there was no significant difference in the largest tumor size for the K14E6 ^{Δ 146-151} mice compared with the K14E6^{WT} mice. Thus, the ability of E6 to interact with α -helix partners contributes to both tumor incidence and tumor size.

The interactions of E6 with both α -helix and PDZ partners contribute to tumor size and tumor multiplicity in the reproductive tract in the presence of E7

To understand the role of α -helix and PDZ partners of HPV16 E6 in cervical cancer when E6 is expressed together with HPV16 E7, the same K14E6^{mutant} lines were crossed onto the K14E7^{WT} background and treated with estrogen for 9 months. All K14E7^{WT} mice developed cervical cancer in response to estrogen treatment (9). Therefore, it was not surprising that nearly all treated K14E6^{WT}/K14E7^{WT} and K14E6^{mutant}/K14E7^{WT} mice also developed cervical cancer (Table 2). Differences were observed, however, in terms of the size of tumors. Comparing the largest tumor from each mouse, both the K14E6^{I128T}/K14E7^{WT} and the K14E6 ^{Δ 146-151}/K14E7^{WT} transgenic mice developed significantly smaller tumors in contrast to the K14E6^{WT}/K14E7^{WT} mice ($P = 0.005$ and 0.014 , respectively; Table 2). In addition, the total area of tumor invasion of both the K14E6^{I128T}/K14E7^{WT} and the K14E6 ^{Δ 146-151}/K14E7^{WT} transgenic mice were significantly reduced relative to K14E6^{WT}/K14E7^{WT} tumors ($P = 0.003$ and 0.03 , respectively; Table 2). Average tumor size was also reduced in K14E6^{I128T}/K14E7^{WT} mice (data not shown). Differences were also observed in terms of tumor frequency, with both mouse lines of K14E6^{mutant}/K14E7^{WT} having reduced number of tumors compared with K14E6^{WT}/K14E7^{WT} mice (Table 2). This reduction was highly significant ($P = 0.003$) for the K14E6 ^{Δ 146-151}/K14E7^{WT} mice, but less so for K14E6^{I128T}/K14E7^{WT} mice ($P = 0.187$).

E6 tumors develop primarily in the cervix and the cervi-covaginal junction

In prior studies, estrogen-treated K14E7^{WT} transgenic mice efficiently developed tumors in the vagina as well as in the cervix (9). In contrast, nearly all tumors arising in K14E6^{WT} and K14E6^{mutant} lines developed in the cervix or at the junction of the cervix and the vagina. Only 2 of 62 (6%) tumors observed in the three E6 transgenic lines developed in the vagina proper. In the presence of E7, the percentage of tumors arising in the vagina of the

K14E6^{WT}/K14E7^{WT} and K14E6^{mutant}/K14E7^{WT} double transgenic mice increased to 38% to 45%. Thus, E6 predisposes animals primarily to tumors of the cervix. In contrast, E7 alone or in combination with E6 induces tumors in the vagina as well as in the cervix, with the largest tumor area predominantly found in the cervico-vaginal junction (Fig. 2C).

The ability of E6 to interact with PDZ partners contributes to hyperplasia in the cervical epithelium

K14E6^{WT} transgenic mice display epidermal hyperplasia characterized by an induction of DNA synthesis within that suprabasal compartment (5). A similar finding was observed in the cervical epithelium (Fig. 2D), with significant increases in both basal and suprabasal DNA synthesis ($P = 0.02$) in K14E6^{WT} transgenic mice (11.6% and 2.9%, respectively) compared with nontransgenic mice (5.6% and 0.9%, respectively). Suprabasal DNA synthesis in the K14E6^{Δ146–151} transgenic mice was reduced compared with K14E6^{WT} transgenic mice (1.64% versus 2.87%; $P = 0.06$) and not significantly different from that observed in nontransgenic mice ($P = 0.14$). No difference in suprabasal DNA synthesis was observed between K14E6^{I128T} and K14E6^{WT} transgenic mice ($P = 0.26$). Thus, in the cervical epithelium, the E6 oncogene is able to increase DNA synthesis in both basal and suprabasal layers of the cervical epithelium, resulting in hyperplasia. This is comparable with results in the skin, where the ability of E6 to increase suprabasal DNA synthesis was mediated at least partially through interactions with PDZ partners (10).

Mcm7 and cyclin E are up-regulated in E6 epithelia and tumors of the reproductive tract in the absence of E7

Mcm7 is an E2F-responsive gene and robust biomarker expressed in high-grade CINs and cervical cancer in humans as well as in K14E6^{WT}/K14E7^{WT} and K14E7^{WT} mice (26). We evaluated MCM7 expression in the epithelia and in tumors of the reproductive tract arising in nontransgenic, K14E7^{WT} and the three E6 transgenic lines with or without E7 (Fig. 3). Analyses of biomarker expression in both the epithelium and the tumors were limited to immunohistochemistry. Reproductive tract tumors in HPV transgenic mice were often too small and sessile. The tumors tended to grow inwardly into the stroma, thereby prohibiting the ability to dissect tumors for Western analyses. Hyperplastic reproductive epithelium from estrogen-treated nontransgenic mice expressed MCM7 only in the basal layer. In contrast, highly dysplastic reproductive epithelium from K14E6^{WT}/K14E7^{WT} and K14E7^{WT} mice was strongly positive for MCM7 throughout the full thickness of the epithelium, similar to data from our previous studies (26). Unexpectedly, approximately one third to two thirds of the epithelia from all three E6 transgenic lines stained positive for MCM7, beyond the basal layer of staining in the nontransgenic epithelium. Expression of MCM7 in the epithelia of the three E6 transgenic lines was generally uniform. No differences in staining between all doubly transgenic lines were observed, presumably due to the strong induction of MCM7 in the E7-expressing tissues. In agreement with previous studies (26), MCM7 staining patterns predominantly correlated positively with lesion grade. These results indicate that E6 can induce *Mcm7*, an E2F-responsive gene.

In tumors, MCM7 expression had a less consistent pattern compared with the epithelium. Expression of MCM7 in tumors was variable, not correlating with genotype, tumor size, or location. Furthermore, levels varied between tumors arising within the same mouse. The sole tumor arising in the nontransgenic mouse had low MCM7 expression (Fig. 3; Table 3). Nonetheless, all tumors from transgenic mice had some level of MCM7 expression. No tumor was MCM7 negative. Aside from K14E7^{WT} tumors, which were robust for MCM7 staining, all tumors from other genotypes had low to high MCM7 expression.

Cyclin E is another E2F-responsive gene that is also used as a biomarker for dysplastic lesions and cervical cancer (27). We evaluated cyclin E expression using immunohistochemistry in the epithelia and in tumors for all genotypes. In the absence of E7, cyclin E staining was more nuclear and less cytoplasmic. In nontransgenic mice, cyclin E expression was restricted mostly to the basal and parabasal layers of cervical epithelia. In both K14E6^{WT} and K14E6^{mutant} cervical epithelium, cyclin E expression positively correlated with the level of dysplasia (data not shown). Unlike MCM7, cyclin E expression was not uniform, such that not every cell was positive. The sole nontransgenic tumor had low cyclin E expression. Tumors from the singly E6 transgenic lines had variable cyclin E expression similar to that observed for MCM7. Tumors had low to high cyclin E expression with no correlation between staining level, tumor size, or location.

In the presence of E7, cyclin E diffusely stained both nuclei and cytoplasm (Fig. 3; Table 3). K14E7^{WT} reproductive epithelium had at least 50% of cells staining positive for cyclin E. In general, E7-positive tumors had medium to high cyclin E expression. Cyclin E expression in epithelia and tumors of doubly transgenic mice was generally high, with at least 60% of cells staining positive.

Reproductive tract tumors are more likely to express p53 and in greater intensities in the presence of E7

p53 is generally undetectable in normal tissue unless induced in response to DNA-damaging agents. The reproductive tract is a p53-responsive tissue, in which a DNA damage response can be mounted in response to ionizing radiation if WT p53 is intact. Dominant-acting, missense mutations in p53, causally associated with tumorigenesis, often lead to the stabilization and accumulation of p53, providing a useful indicator of p53 status and the disease state in most tumor types. In HPV-associated cancers, p53 is thought to be inactivated through E6-induced, ubiquitin-mediated protein degradation. However, p53 mutations have been detected in both premalignant lesions and human cervical tumors at low frequencies. We did p53 immunohistochemistry to monitor levels of p53 protein in the reproductive tumors of our various HPV transgenic mice (Fig. 4; Table 3). The sole nontransgenic tumor was p53 negative. Tumors from K14E7^{WT} transgenic mice were p53 positive and had low to medium expression, whereas tumors from K14E6^{WT} transgenic mice were almost completely p53 negative. These observations were consistent with prior studies showing the destabilization of p53 by E6- and E7-induced accumulation of p53 (28–30). K14E6^{I128T}-expressing epithelium had elevated p53 expression compared with K14E6^{WT} mice, consistent with the reduced ability of K14E6^{I128T} to degrade p53. Tumors from K14E6^{I128T} mice, however, had clearly less intense levels of p53 expression than tumors from K14E7^{WT} mice. In agreement with our predictions, the K14E6^{Δ146–151} transgenic tumors were p53 negative. Expression of p53 in the tumors arising in K14E6^{WT}/K14E7^{WT} transgenic mice was variable, with a range from nil to sporadic highly positive (Fig. 4). Tumors from K14E6^{mutant}/K14E7^{WT} transgenic mice (data not shown) had similar levels of p53 expression relative to K14E6^{WT}/K14E7^{WT} tumors but were generally less intense. We also noted that vaginal tumors had reduced expression of p53 relative to tumors from the cervix or the cervicovaginal junction.

p16 expression is inversely correlated with retinoblastoma expression in reproductive tumors from HPV transgenic mice

p16 is up-regulated in several cervical cancer cell lines as well as in human cervical samples (23). This cyclin kinase inhibitor is a marker for high-risk HPV infection in human dysplastic lesions and cancers of the reproductive tract as well as cancers of the head and neck (31,32). We evaluated p16 status in the tumors from our estrogen-treated mice. Expression of p16 was uniformly diffuse with both nuclear and cytoplasmic expression in all

mice. There was a general cytoplasmic expression pattern, with nuclear accumulation localized predominantly to the bottom one third to one half of the epithelium (data not shown). The sole nontransgenic tumor had low levels of p16 expression. Tumors from both K14E6^{WT}/K14E7^{WT} and K14E7^{WT} mice displayed high levels of p16 (Fig. 4; Table 3), similar to the patterns observed in human cervical samples. Presumably, due to the ability of E7 to induce p16 strongly (33), differences in expression between K14E6^{mutant}/K14E7^{WT} tumors were not observed (data not shown). Conversely, expression of p16 in tumors from the K14E6^{WT} and K14E6^{Δ146-151} transgenic mice was either low or negative. This decrease in p16 expression was clearly more pronounced in the tumors compared with the neighboring epithelium, which was variable (data not shown). In contrast, tumors from K14E6^{I128T} transgenic mice displayed a strong increase in the expression of p16 relative to K14E6^{WT} and K14E6^{Δ146-151} tumors. The intensity of p16 expression in K14E6^{I128T} tumors was generally less robust relative to tumors containing the E7 oncogene.

Because expression of p16 was reduced in K14E6 reproductive tumors and given that pRb contributes to the regulation of p16, we measured the expression levels of pRb via immunohistochemistry (Fig. 4; Table 3). The sole nontransgenic tumor had low expression levels of pRb. In agreement with the known ability of E7 to induce the degradation of pRb, E7-expressing tumors had little to no detectable pRb regardless of E6 mutational status (Fig. 4; Table 3; data not shown). In contrast, tumors from K14E6^{WT} and K14E6^{Δ146-151} transgenic mice expressed high levels of pRb. K14E6^{I128T} tumors, however, displayed low levels of pRb relative to K14E6^{WT} and K14E6^{Δ146-151} tumors. Hence, HPV E6 is able to alter pRb and p16 levels in tumors in a manner distinguishable from HPV E7 and is dependent on its interaction with α -helix partners, as the expression pattern of p16 and pRb no longer resembles WT E6, but more like E7-expressing tumors.

Discussion

In this study, we dissected the contributions of HPV E6 in both the presence and the absence of HPV E7 in cervical carcinogenesis by focusing on specific properties of E6 and extending the estrogen treatment period. E6, in the absence of E7, induces primarily cervical tumors in the reproductive tract. The abilities of E6 to interact with both α -helix and PDZ partners contribute to this role in cervical carcinogenesis. Furthermore, E6 induces a pattern of cellular gene expression that is overlapping yet distinct from that induced by E7. Specifically, E6 leads to a dysregulation of the p16/pRb pathway in a manner different from that of E7 yet led to a similar though less robust induction of E2F-responsive genes.

HPV16 E6 has a weaker oncogenic potential than HPV16 E7 in the reproductive tract

In our prior studies, K14E6^{WT} transgenic mice did not develop cervical cancer or even high-grade dysplasias after 6 months of estrogen treatment. K14E7^{WT} transgenic mice on the hand developed multiple high-grade dysplastic lesions and tumors throughout the entire reproductive tract. In this study, we extended the estrogen treatment period to 9 months. A large fraction (41%) of K14E6^{WT} transgenic mice developed cancer. The majority of the remaining K14E6 mice developed at least one high-grade dysplastic lesion (Table 4). This represented a significant increase in tumorigenesis compared with nontransgenic mice (6.7%) yet significantly less than that observed in K14E7^{WT} mice (100% tumor incidence). Likewise, tumor multiplicity was significantly reduced in K14E6^{WT} mice compared with the K14E7^{WT} mice (1.67 versus 6.67). Furthermore, the K14E6^{WT} transgenic mice did not develop the extensive dysplastic pathology that occurred throughout the entire reproductive epithelial lining of K14E7^{WT} mice. Thus, HPV16 E6 has a demonstrable yet clearly weaker oncogenic activity than HPV16 E7 in the reproductive tract. In contrast, E6 is the more potent oncogene in the skin, contributing to both the promotion and the progression stages of

skin carcinogenesis and induces primarily malignant tumors (8). Therefore, the relative potency of the HPV16 E6 and E7 oncogenes differs depending on the tissue evaluated.

The ability of E6 to bind to α -helix partners contributes to cervical carcinogenesis

K14E6^{I128T} transgenic mice had a reduction in the incidence and size of reproductive tract tumors compared with K14E6^{WT} transgenic mice. Given the reduced tumorigenic phenotype of the K14E6^{I128T} transgenic mice, we hypothesize that the inactivation of p53 by E6 contributes to cervical carcinogenesis. Consistent with this hypothesis, slightly elevated levels of p53 protein were seen in tumors arising in K14E6^{I128T} mice compared with K14E6^{WT} mice.

Whereas reduced in their incidence of tumors compared with K14E6^{WT} mice, K14E6^{I128T} transgenic mice retained a significant increase in their tumorigenic phenotype compared with nontransgenic mice (Table 1). This increase was evident in tumor multiplicity (1.14 versus 0.07) and average tumor size (1.39 mm² versus 0.029 mm²). Such residual oncogenic activity in K14E6^{I128T} transgenic mice could reflect the ability of the I128T mutant protein to bind α -helix partners, albeit at 1% to 5% the level of WT E6 protein, or it may reflect an activity of E6 distinct from its ability to bind α -helix partners. Tumors arising in K14E6^{I128T} mice also displayed a distinct pattern of expression of p16 and pRb relative to tumors of K14E6^{WT} mice. Whether this alternative dysregulation of the p16/pRb pathway contributes to the tumorigenesis observed in the K14E6^{I128T} transgenic mice is unclear. Regardless, this finding supports the hypothesis that residual oncogenic activity in the K14E6^{I128T} mice reflects a distinct activity of E6 and not a partial retention in the binding capacity to α -helix partners. The absence of a reduction in the tumorigenic phenotype of K14E6 ^{Δ 146–151} compared with K14E6^{WT} mice indicates that PDZ domain partners are not relevant in the context of these experiments or that their contribution is modest.

The contribution of E6 to cervical carcinogenesis in the presence of E7 is dependent on interactions with both α -helix and PDZ partners

The above experiments were all carried out in the absence of E7. Similar studies in the presence of E7 (Table 2) revealed that the interaction of E6 with α -helix partners is important for cervical carcinogenesis. The most obvious difference in the tumorigenic phenotypes between K14E6^{I128T}/K14E7^{WT} and K14E6^{WT}/K14E7^{WT} mice was tumor size. In contrast to observations in the absence of E7, the interactions of E6 with PDZ partners also contributed to cervical carcinogenesis in the presence of E7. Specifically, we observed a reduction in tumor size in the K14E6 ^{Δ 146–151}/K14E7^{WT} mice compared with K14E6^{WT}/K14E7^{WT} mice. Tumors arising in the K14E6^{mutant}/K14E7^{WT} mice were not significantly different in size from those arising in the K14E7^{WT} singly transgenic mice. Tumor multiplicity also was reduced in both K14E6^{mutant}/K14E7^{WT} lines relative to K14E6^{WT}/K14E7^{WT} mice. This reduction was only statistically significant for the K14E6 ^{Δ 146–151}/K14E7^{WT} transgenic mice. Thus, in the presence of E7, E6 contributes to cervical carcinogenesis through at least two distinct mechanisms. This finding is consistent with what we have observed previously in the skin, where the ability of E6 to bind both α -helix and PDZ domain partners contributed to carcinogenesis (10,17,19).

It is unclear which PDZ partner(s) of E6 contributes to both tumor size and tumor multiplicity. The E6 oncogene interacts with numerous cellular partners, which contain PDZ motifs, such as Dlg, Scribble, and Magis (34–37). DLG and/or *Scribble* are attractive candidates given that both are tumor suppressors in *Drosophila*. Mutations in either of these genes in *Drosophila* result in the development of epithelial hyperplasia, loss of cell-cell contacts (38,39), and tumorigenesis of the imaginal discs and brain lobes (40). In the human cervix, both hDlg and hScrib are gradually altered in cellular localization and expression is

lost as low-grade lesions progress to invasive cervical carcinomas (41–43). Reductions in hDlg and hScrib are also seen in human cervical cancer cell lines (19). Because Dlg and Scribble are both expressed in the septate junction (44) and seem to have similar functions, both genes may contribute to the oncogenic potential of E6 in the reproductive tract. Until analysis of targeted individual PDZ deletion mutants can be done, the exact E6-PDZ interaction(s) responsible for the oncogenic potential of E6 remains unclear.

The E6 oncogene induces the E2F-responsive genes, *MCM7* and *cyclin E*, in reproductive epithelia and tumors in the absence of E7

All E6 transgenic lines expressed the E2F-responsive genes, *MCM7* and *cyclin E*, in both the epithelium and the tumors of the reproductive tract. Expression of these genes was above the levels seen in nontransgenic mice. These results in the E6 mice were somewhat unexpected given that the E7 oncogene was absent in these mice and therefore not available to induce the expression of E2F-responsive genes through pRb inactivation. Hence, the E6 oncogene must be activating the transcription of these E2F-responsive genes by a mechanism different than E7. In the epithelium of the K14E6^{WT} mice, this up-regulation of E2F-responsive genes correlated with high levels of p16 and low levels of pRb, as seen in the epithelium and tumors in the K14E7^{WT} mice. However, there is an inverted pattern of expression of p16 and pRb in the K14E6^{WT} tumors (Fig. 4; Table 3). Specifically, levels of p16 were low and levels of pRb were high in the reproductive tumors of K14E6^{WT} mice. This result indicates that the alteration of the cell cycle during progression to malignancy in K14E6^{WT} mice differs from that observed in K14E7^{WT} mice. Interestingly, the pattern seen in the tumors of K14E6^{WT} mice is consistent with the low expression levels of p16 and high levels of hyperphosphorylated pRb observed in fibroblast and epithelial cell lines immortalized with the HPV E6 oncogene (33,45–47). Thus, E6-dependent immortalization *in vitro* and E6-dependent tumorigenesis *in vivo* arise through means that lead to a similar dysregulation of the p16/pRb pathway opposite of that observed in E7-dependent tumorigenesis (this study) or in human cervical cancers (48). The inactivation of p53 by E6 and consequent inhibition of p53-induced expression of the cyclin-dependent kinase (CDK) inhibitor p21 might lead to higher CDK activity and thereby increased hyperphosphorylated pRb. Alternatively, E6 could induce phosphorylation of pRb by up-regulating the levels of CDK4/6 (49). How E6 induces levels of CDK4/6 is unknown. Thus, it remains unclear whether these two hypotheses reflect the same or distinct mechanisms. Regardless, a role of p53 inactivation in mediating the dysregulation of p16/pRb pathway by E6 is supported by the reversed pattern of p16 expression in K14E6^{I28T} tumors, which encode a mutant E6 protein defective for inactivating p53 compared with K14E6^{WT} tumors (Fig. 4; Table 3).

In summary, we report the first *in vivo* study dissecting the mechanism of E6 action in cervical carcinogenesis. The E6 interactions with two groups of cellular partners contributed to cervical carcinogenesis. Additionally, our study revealed that the ability of E6 to induce E2F-responsive genes is likely through the dysregulation of the p16/pRb pathway by mechanisms distinct from that of E7.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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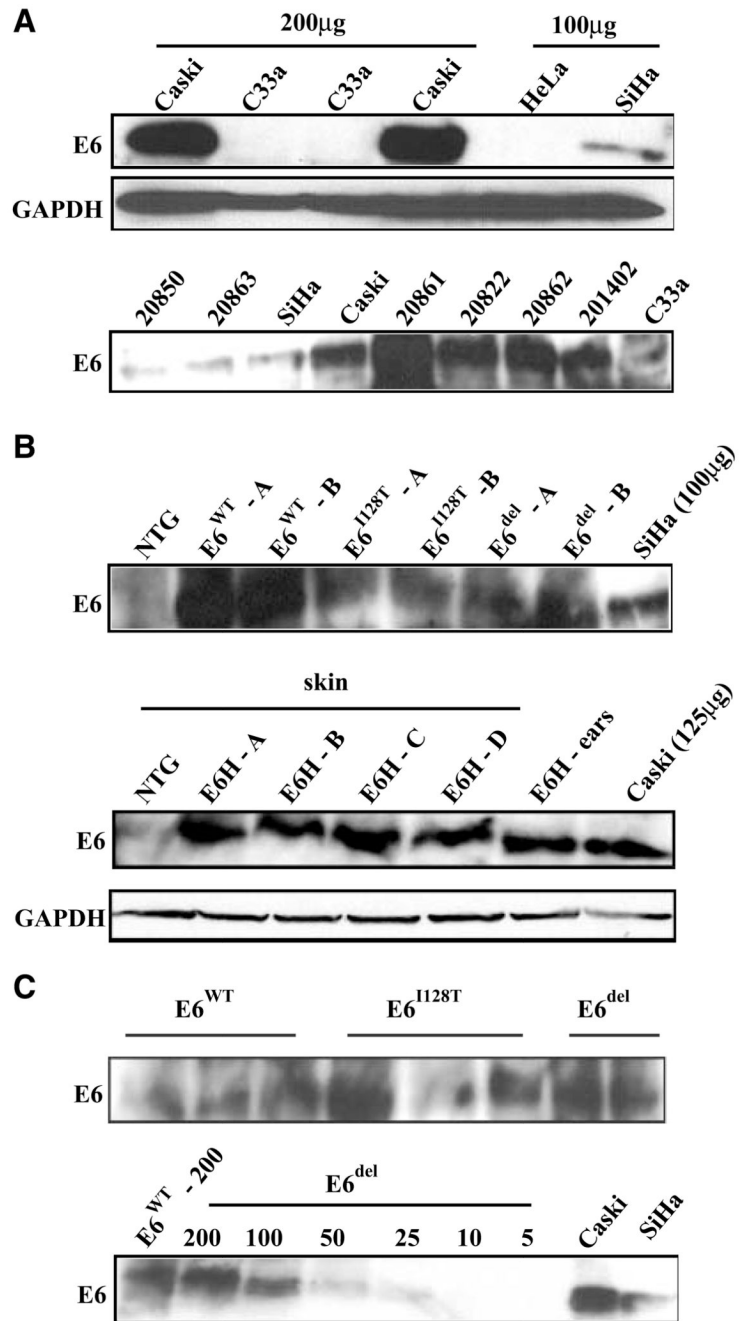
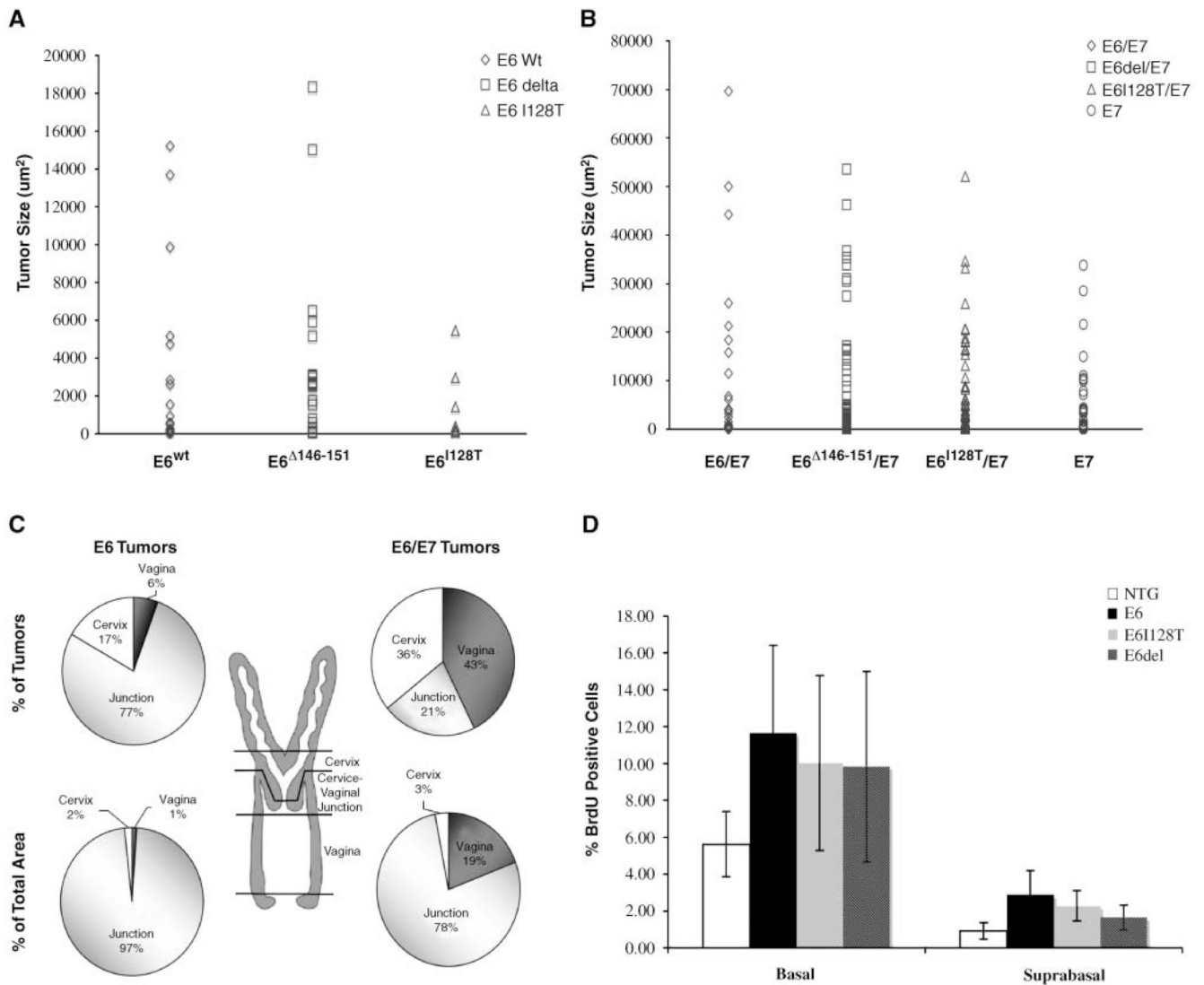


Figure 1.

Comparison of E6 expression in K14E6 transgenic mice and human cervical cancer cell lines. *A*, levels of E6 in HPV-positive and HPV-negative cervical cell lines. Immunoblots probed with antibodies specific for either E6 or glyceraldehyde-3-phosphate dehydrogenase (GAPDH). The GAPDH-specific immunoblot was done to confirm equivalence in loading and was done for all experiments displayed in this figure, although shown only for the top-most one. For each sample, 200 μ g of total soluble protein were analyzed. The top blot was loaded with samples from HPV-negative C33A, HPV18-positive HeLa, and HPV16-positive SiHa and Caski human cervical cancer-derived cell lines. The second blot was loaded with samples from various clonal populations of W12E (20850 and 20963) and W12I (20861,

20822, 20862, and 201402) cell lines derived from a HPV16-positive CINI lesion. W12E clones harbor the viral genome in the extrachromosomal state. W12I clones harbor the genome in a chromosomally integrated state. *B*, levels of E6 in the dorsal skin of K14E6^{WT} and K14E6^{mutant} transgenic mice at age postnatal day 9 or 10. In this immunoblot, 200 µg (*top*) or 250 µg (*bottom*) of total cellular protein from each mouse tissue sample and 100 or 125 µg of SiHa and Caski extracts were analyzed. Extracts from different animals (*A* and *B*) of the same genotype were loaded to assess reproducibility of findings. *Bottom*, levels of E6 protein in E6 homozygous transgenic mice in both skin and ear. *C*, levels of E6 in the reproductive tract of K14E6^{WT} and K14E6^{mutant} transgenic mice. *Top*, 200 µg of total cellular protein from each mouse tissue sample were analyzed. As above, extracts from different animals (*A*, *B*, and *C*) of the same genotype were loaded to assess reproducibility of findings. In the bottom blot assessing relative amounts of E6 protein in K14E6^{WT} and K14E6^{Δ146-151} mice, the amount of protein loaded is indicated in each lane. Mice were treated with estrogen to synchronize them in estrus, thereby eliminating variability in cervical epithelial thickness.

**Figure 2.**

Characterization of reproductive tumors and the proliferative index of the cervix. *A*, comparison of tumor sizes between K14E6^{WT}, K14E6^{I128T}, and K14E6^{Δ146-151} transgenic mice. *B*, comparison of tumor sizes between K14E7^{WT}, K14E6^{WT}/K14E7^{WT}, and K14E6^{mutant}/K14E7^{WT} transgenic mice. *C*, classification of reproductive tumors by location. *Middle*, a cartoon representation of the murine reproductive tract and identifies the approximate borders for determining tumor location used in histopathologic diagnosis. *Left* (for K14E6^{WT}) and *right* (for K14E6^{WT}/K14E7^{WT}), the breakdown of total percentage of tumor development by location (top) and the percentage of total area these tumors encompassed (bottom). In K14E6^{I128T} and K14E6^{Δ146-151}, the percentage of tumors arising in the vagina was 0% and 4%, respectively (data not shown). In K14E6^{I128T}/K14E7^{WT} and K14E6^{Δ146-151}/K14E7^{WT} transgenic mice, the tumors that developed in the vagina were 45% and 38%, respectively (data not shown). *D*, quantification of epithelial hyperplasia in the cervix. The average percentage of basal and suprabasal BrdUrd-positive cells was obtained from eight ($\times 40$) microscopic fields per mouse. An average of at least three mice per genotype were used to calculate the percentage.

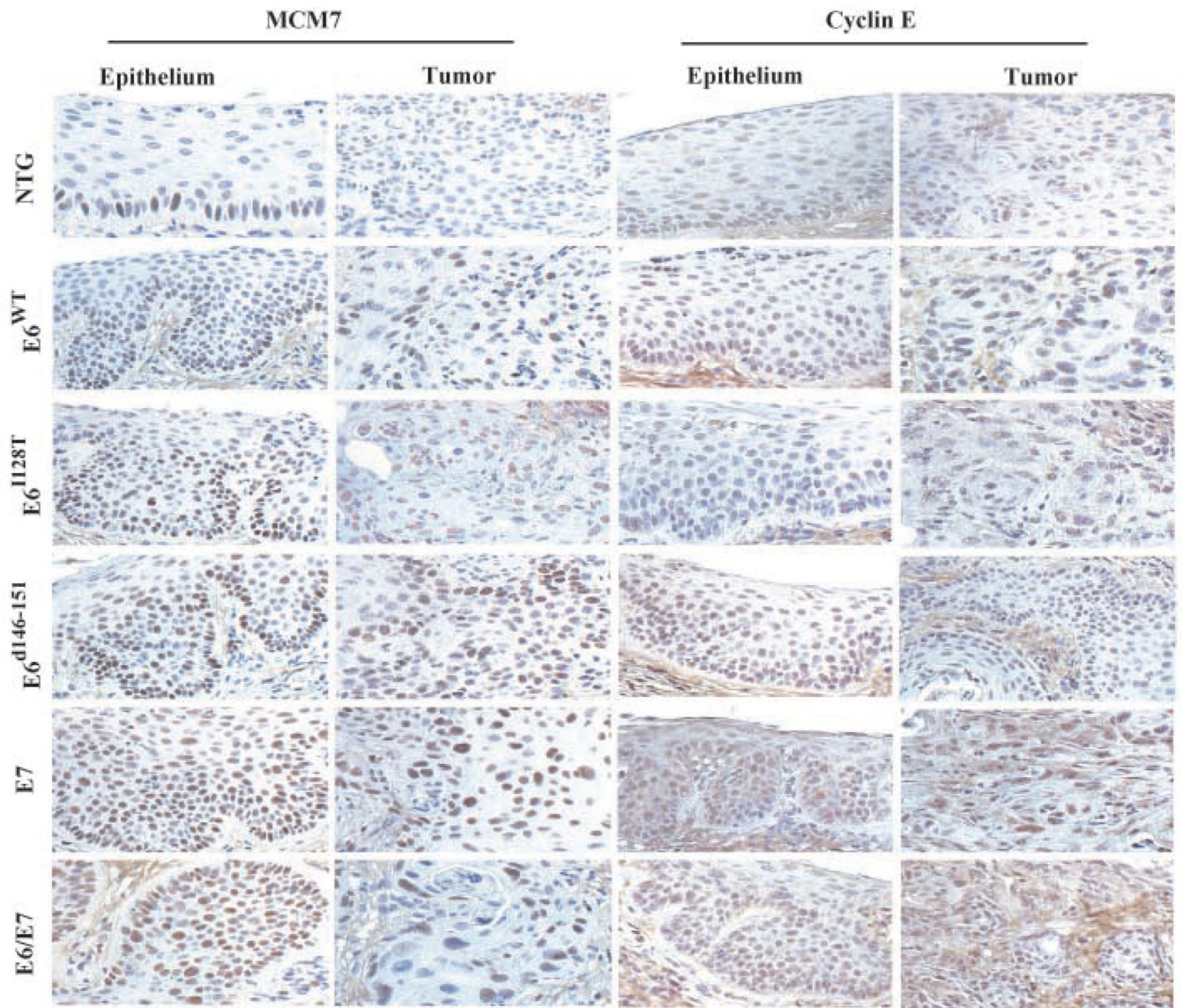


Figure 3. Evaluation of E2F-responsive gene expression in the estrogen-treated epithelium and tumors from the reproductive tract. *Columns 1 and 2*, MCM7 staining (brown staining nuclei), which is up-regulated in singly or double transgenic reproductive epithelia and tumors, whereas MCM7 expression is restricted to the basal and parabasal layers of nontransgenic epithelium. *Columns 3 and 4*, cyclin E staining (brown nuclei). Cyclin E expression, similar to MCM7 expression, is also up-regulated in both single and double transgenic mice. Magnification, $\times 40$.

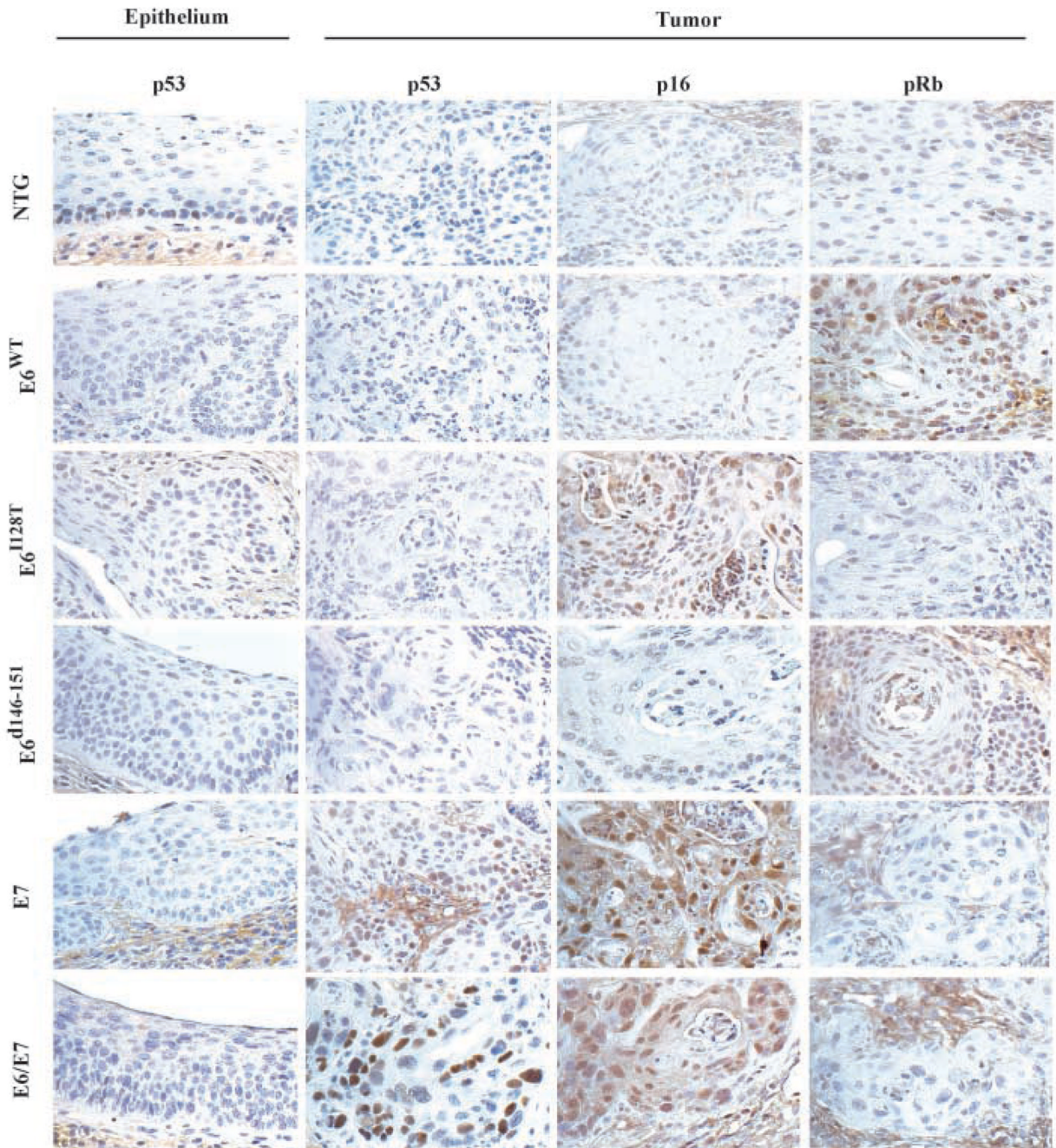


Figure 4.

p53, p16, and pRb status in the reproductive tract. Images from sections stained with antibodies to p53, p16, or pRb (brown) and counterstained with hematoxylin (blue). *Column 1*, p53 expression in the cervical epithelium from various genotypes. The nontransgenic (NTG; top) sample is from a mouse exposed to 5 Gy of ionizing radiation and is used as a positive control to show p53-positive staining, which is primarily observed in the basal and parabasal strata. The cervical epithelium from unirradiated nontransgenic mice (data not shown) is p53 negative. All other panels are from unirradiated mice. *Columns 2 to 4*, the status of p53, p16, and retinoblastoma (*pRB*) expression in reproductive tumors from various genotypes. Tumors expressing the E7 oncogene generally displayed variable positivity for

p53. Shown in the panel from the E6/E7 tumor is an area of high sporadic p53 positivity. p16 was up-regulated in tumors expressing E7 or K14E6^{I128T}. Retinoblastoma expression was inversely correlated to p16 in tumors expressing either HPV E6 or E7. Magnification, ×40.

Table 1

Comparison of tumors from the reproductive tract of nontransgenic, K14E6^{WT} and K14E6^{mutant} transgenic mice

Genotype	Cancer incidence (%)	Tumor multiplicity	Largest tumor (mm ²)
NTG (<i>n</i> = 15)	6.7	0.07	0.029
K14E6 ^{WT} (<i>n</i> = 27)	41	1.64	5.22
K14E6 ^{I128T} (<i>n</i> = 36)	19.4 [*]	1.14	1.38 [†]
K14E6 ^{Δ146-151} (<i>n</i> = 28)	53.6	1.60	4.79

Abbreviation: NTG, nontransgenic.

* Cancer incidence in K14E6^{I128T} was marginally significant compared with K14E6^{WT}, *P* = 0.058, Fisher's exact test.

[†] K14E6^{I128T} tumors were significantly different than K14E6^{WT} tumors, *P* = 0.04, Wilcoxon rank-sum test.

Table 2

Comparison of tumors from the reproductive tract of nontransgenic, K14E7^{WT}, K14E6^{WT}/K14E7^{WT}, and K14E6^{mutant}/K14E7^{WT} transgenic mice

Genotype	Cancer incidence (%)	Tumor multiplicity	Total area of tumor invasion (mm ²)	Largest tumor (mm ²)
NTG (n = 15)	6.7	0.07	0.029	0.029
K14E7 (n = 11)	100	6.64	18*	12.6*
K14E6 ^{WT} /K14E7 ^{WT} (n = 6)	100	7.00	49	37.36
K14E6 ^{I128T} /K14E7 ^{WT} (n = 24)	100	5.79	19*	13.72*
K14E6 ^{Δ146-151} /K14E7 ^{WT} (n = 21)	90.5	3.85*	24*	16.81*

* Compared with K14E6^{WT}/K14E7, $P < 0.05$, Wilcoxon rank-sum test.

Table 3

Summary of biomarker expression in tumors from the reproductive tract of estrogen-treated mice

Genotype	p53	MCM7	cyclin E	p16	pRb
NTG	-	±	±	±	±
E6 ^{WT}	-	+/++	±/+++	-/+	±/++
K14E6 ^{U28T}	-±	+/++	±/++	+/+++	-±
K14E6 ^{Δ146-151}	-	+/++	±/++	-/+	±/++
K14E7 ^{WT}	-/+	+++	+++	+++	-
K14E6 ^{WT} /E7 ^{WT}	-/+	+++	+++	+++	-
K14E6 ^{U28T} /E7 ^{WT}	-/+	+/+++	+++	+++	-
K14E6 ^{Δ146-151} /E7 ^{WT}	-/+	+/+++	+++	+++	-

NOTE: -, negative; ±, <5%; +, 5% to 20%; ++, 20% to 50%; +++, >50%.

Table 4

Summary of histopathologic diagnosis in K14E6^{WT} and K14E6^{mutant} transgenic mice

Genotype	NH	CIN I	CIN II	CIN III/CIS	Cancer
K14E6 ^{WT} (n =27)	0	3	6	7	11
K14E6 ^{L28T} (n =36)	0	0	10	19	7
K14E6 ^{Δ146-151} (n = 28)	0	3	0	10	15

Abbreviations: CIS, carcinoma *in situ*; NH, normal hyperplasia.