



Published in final edited form as:

Cancer Res. 2009 May 15; 69(10): 4407–4414. doi:10.1158/0008-5472.CAN-09-0023.

Persistence of High-Grade Cervical Dysplasia and Cervical Cancer Requires the Continuous Expression of the Human Papillomavirus Type 16 *E7* Oncogene

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Abstract

Several mucosotropic human papillomaviruses (HPV), including HPV type 16 (HPV-16), are etiologic agents of a subset of anogenital cancers and head and neck squamous cell carcinomas. In mice, HPV-16 *E7* is the most potent of the papillomaviral oncogenes in the development of cervical disease. Furthermore, interfering specifically with the expression of *E7* in HPV-positive cell lines derived from human cervical cancers inhibits their ability to proliferate, indicating that the expression of *E7* is important in maintaining the transformed phenotype *in vitro*. To assess the temporal role of *E7* in maintaining HPV-associated tumors and precancerous lesions *in vivo*, we generated *Bi-L E7* transgenic mice that harbor a tetracycline-inducible transgene that expresses both HPV-16 *E7* and firefly luciferase. When we crossed *Bi-L E7* mice to a *K5-tTA* transgene-inducing line of mice, which expresses a tetracycline-responsive transactivator selectively in the stratified squamous epithelia, the resulting *Bi-L E7/K5-tTA* bitransgenic mice expressed *E7* and luciferase in the skin and cervical epithelium, and doxycycline repressed this expression. Bitransgenic mice displayed several overt and acute epithelial phenotypes previously shown to be associated with the expression of *E7*, and these phenotypes were reversed on treatment with doxycycline. Repressing the expression of *E7* caused the regression of high-grade cervical dysplasia and established cervical tumors, indicating that they depend on the continuous expression of *E7* for their persistence. These results suggest that *E7* is a relevant target not only for anticancer therapy but also for the treatment of HPV-positive dysplastic cervical lesions.

Introduction

Human papillomaviruses (HPV) are small, epitheliotropic, dsDNA viruses that cause proliferative lesions, including warts. A subset of mucosotropic HPVs—the “high-risk” HPVs, including HPV type 16 (HPV-16) and HPV-18—is associated etiologically with the vast majority of cervical cancers, other types of anogenital cancers, and approximately a quarter of head and neck squamous cell carcinomas (1). HPV-16, which is a causative agent of over half of cervical cancers (2), encodes three oncogenes: *E5*, *E6*, and *E7* (3–5). *E7* is

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Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

best known for its ability to bind to and degrade the tumor suppressor pRb (6), although its ability to drive carcinogenesis is not due exclusively to this interaction (7).

To characterize the oncogenic properties of E7 *in vivo*, our laboratory previously generated transgenic mice that express E7 under the control of the keratin 14 (K14) promoter (8). These *K14E7* mice display a variety of acute epithelial phenotypes indicative of the ability of E7 to disrupt the cell cycle (9). Furthermore, *K14E7* mice develop high-grade dysplasia and invasive cervical cancer when treated chronically with estrogen for as little as 6 months, whereas nontransgenic mice and *K14E5*³ and *K14E6* (10) transgenic mice do not. These data, coupled with previous demonstrations of the ability of E7 to transform cells in culture (11–13), indicate the importance of E7 in the genesis of cervical cancer.

The observation that cancerous cells depend on the continuous expression of oncogenes for their survival has been described in many murine systems for several oncogenes, including *BCR-ABL* (14), *MYC* (15–17), *H-Ras* (18), and *K-Ras* (19). Whether HPV-associated cancers require the continuous expression of viral oncogenes has been examined in tissue culture by using cell lines derived from HPV-positive human cervical cancers. Repressing the expression of E6 in the HPV-18-positive HeLa cell line by using the papillomaviral E2 transcription factor results in sporadic senescence and widespread apoptosis, whereas silencing the expression of E7 causes senescence only (20). Interfering with the expression of E6 and E7 in HeLa cells using short hairpin RNA (shRNA) induces senescence at low doses of a lentiviral vector containing the shRNA, but at high doses, apoptosis occurs (21). Conflicts in apoptotic versus senescent responses between these and similar studies (22–24) are difficult to reconcile and may reflect differences in the experimental systems being used. It remains unclear whether the requirements for high-risk E6 and E7 observed in such studies are predictive of the *in vivo* dependence of cervical cancers on their presence or specifically reflect their importance in maintaining the growth competence of cells in tissue culture.

To assess the temporal role of E7 in the maintenance of HPV-associated cervical cancers *in vivo*, we generated *Bi-L E7* transgenic mice that harbor a construct containing HPV-16 E7 and firefly luciferase, the expression of which is driven by bidirectional promoters under the control of a tetracycline response element (TRE). When we produced bitransgenic mice by crossing *Bi-L E7* mice to a line of mice that expresses the tetracycline transactivator (tTA) under the control of the keratin 5 promoter (*K5-tTA* mice; ref. 25), they expressed both luciferase and E7 in the skin and in the epithelium of the female reproductive tract. By administering doxycycline, an analogue of tetracycline, to *Bi-L E7/K5-tTA* bitransgenic mice, the expression of E7 and luciferase was eliminated. Bitransgenic mice displayed an array of epithelial phenotypes previously shown to be associated with the expression of E7, and these phenotypes were reversed on treatment with doxycycline. More importantly, we found that repressing the expression of E7 caused the regression of high-grade cervical dysplasia and established cervical tumors, indicating that both depend on the continuous expression of E7 for their persistence. Our findings have important implications for the treatment of HPV-associated cervical disease.

Materials and Methods

For a full description of the Materials and Methods, see Supplementary Information.

³Maufort et al., unpublished data.

Transgenic mice

K14E7 (8) and K5-tTA (25) transgenic mice have been described previously. Bi-L E7 mice were genotyped by PCR using the primers LucF (5'-GAAATGTCCGTTTCGGTTGGCAGAAGC-3') and LucR (5'-CCAAAACCGTGATGGAATGGAACAACA-3').

Treatment with estrogen

To induce persistent estrus and to eliminate cycling through estrus, continuous release pellets delivering 0.05 mg of 17 β -estradiol (estrogen) per 60-d period (Innovative Research of America) were implanted s.c. into the fat pads of the shoulders of adult female virgin mice.

Treatment with doxycycline

Doxycycline-containing chow (2 g/kg; Bio-Serv) was used to repress the expression of the *Bi-L E7* transgene.

Procurement of female reproductive tracts and histologic analysis

One hour before sacrifice, mice were injected with 5-bromo-2'-deoxyuridine (BrdUrd; 12.5 mg/mL in PBS) at 10 μ L/g body weight. Procured female reproductive tracts were fixed overnight at 4°C in 4% paraformaldehyde (w/v), embedded in paraffin, sectioned, and stained with H&E for histologic analysis of neoplastic and dysplastic disease.

Analysis of luciferase

Tissue lysates were analyzed for the activity of luciferase using the Luciferase Assay System (Promega Corp.) according to the manufacturer's instructions.

Analysis of E7

To examine the expression of E7 by Western blot, a mixture of mouse anti-HPV-16 E7 primary antibodies was used (Invitrogen Corp.; Santa Cruz Biotechnology).

Irradiation

Adult mice were mock treated or exposed to 5 Gy of ionizing radiation from a ¹³⁷Cs source and sacrificed 24 h later. One hour before sacrifice, mice were injected with BrdUrd (12.5 mg/mL in PBS) at 10 μ L/g body weight.

Immunohistochemistry

Mouse anti-BrdUrd (Calbiochem Immuno-chemicals) and mouse anti-minichromosome maintenance protein 7 (MCM7; NeoMarkers Corp.) primary antibodies were used for immuno-histochemical analysis.

Quantification of BrdUrd

Nuclei of keratinocytes in 10 (dorsal skin) or 8 (endocervices) visual fields at $\times 40$ magnification were scored as either positive (brown) or negative (blue) for BrdUrd.

Results

The expression of luciferase and E7 in the skin and epithelium of the female reproductive tracts of *Bi-L E7/K5-tTA* bitransgenic mice is controlled by doxycycline

To study the temporal requirement for the expression of E7 in cervical cancers, we generated transgenic mice using a *Bi-L E7* construct. Briefly, this transgene contains a TRE, composed of seven copies of the tetracycline operator (*tetO*) sequence, flanked by two minimal cytomegalovirus promoters in opposite orientations, which drive the expression of firefly luciferase in one direction and HPV-16 E7 in the other (Supplementary Fig. S1A). We initially screened nine lines of *Bi-L E7* mice to determine which lines expressed luciferase under the control of the tTA protein by crossing them to an inducing line of *K5-tTA* mice that expresses tTA specifically in the stratified squamous epithelia (Supplementary Fig. S1B). In eight of the nine lines examined, there was no significant increase in the expression of luciferase in the dorsal skin of singly transgenic *Bi-L E7* mice over background levels observed in nontransgenic mice ($P > 0.05$, two-sided Wilcoxon rank-sum test), indicating that there was no leaky expression of luciferase in the absence of the tTA protein (Supplementary Fig. S2). When we examined lysates from the dorsal skin of *Bi-L E7/K5-tTA* bitransgenic mice, we observed a significant induction of luciferase in seven of the nine lines of *Bi-L E7* mice examined ($P < 0.05$ versus nontransgenic, two-sided Wilcoxon rank-sum test; Supplementary Fig. S2). Based on our results, we chose one line of *Bi-L E7* mice, line 406, which reproducibly expressed luciferase in a manner dependent on the presence of tTA. In all data presented hereafter, we used *Bi-L E7* mice exclusively from line 406.

Administering chow containing 2 g/kg doxycycline for 1 week to *Bi-L E7/K5-tTA* bitransgenic mice eliminated the expression of luciferase in the skin, indicating that, when the transcriptional activity of the tTA protein was inactivated by doxycycline, the expression of luciferase was abolished (Fig. 1A). We also examined the expression of luciferase in lysates from the lower female reproductive tract, where we observed an induction of luciferase exclusively in bitransgenic mice ($P < 0.05$ versus nontransgenic mice, two-sided Wilcoxon rank-sum test) that was eliminated in mice given as little as 3 days of doxycycline (Fig. 1B). These data indicate that luciferase is induced and can be repressed with doxycycline in the stratified squamous epithelia of *Bi-L E7/K5-tTA* bitransgenic mice.

We next examined the expression of E7 in *Bi-L E7/K5-tTA* bitransgenic mice by performing Western blots on lysates from the dorsal skin and lower female reproductive tract. By using loading controls consisting of known amounts of protein from lysates from *K14E7* mice, a line of transgenic mice that we generated previously to characterize the oncogenic properties of E7 *in vivo* (8), we determined that the level of expression of E7 in the dorsal skin of *Bi-L E7/K5-tTA* mice was slightly higher than that seen in *K14E7* mice (Fig. 1C). In the lower female reproductive tract, the level of expression of E7 in bitransgenic mice was approximately half that observed in *K14E7* mice (Fig. 1D). As expected, we did not detect E7 in lysates from bitransgenic mice given doxycycline for 1 week or in singly transgenic *Bi-L E7* mice.

These data indicate that the expression of luciferase and E7 is induced in the dorsal skin and lower female reproductive tracts of *Bi-L E7/K5-tTA* bitransgenic mice and that this expression is repressed by administering doxycycline.

Bi-L E7/K5-tTA bitransgenic mice display striking overt phenotypes that are reversible on treatment with doxycycline

We noted that *Bi-L E7/K5-tTA* bitransgenic mice presented numerous, fully penetrant overt phenotypes, including alopecia, runting, excessive scratching, and dry and wrinkled

hyperplastic skin (Fig. 2). These phenotypes were apparent by 4 weeks of age and invariably worsened over time. By 6 to 7 months of age, bitransgenic mice often were nearly devoid of hair, and the excessive scratching led to the appearance of flaky, raw, and scabbed skin on and around the shoulders of the animals. To determine whether we could reverse these overt phenotypes by silencing the expression of E7, we administered doxycycline to bitransgenic mice and monitored the amelioration of these phenotypes over the course of 20 days (Fig. 2). After as little as 3 days on doxycycline, the skin of bitransgenic mice was noticeably less dry and hyperplastic when compared with mice that never received doxycycline, and by 6 days on the drug, there was obvious regrowth of hair over the entire body. The overt phenotypes continued to regress as the mice remained on doxycycline, and by 20 days, bitransgenic mice continuously fed doxycycline were nearly indistinguishable from nontransgenic mice. These observations show that the overt phenotypes displayed by *Bi-L E7/K5-tTA* bitransgenic mice are reversed on treatment with doxycycline, regardless of their severity.

***Bi-L E7/K5-tTA* bitransgenic mice display several acute, microscopic phenotypes in the stratified squamous epithelia that can be reversed with doxycycline**

We have shown previously that E7 can induce DNA synthesis in the normally quiescent suprabasal compartment of the endocervical epithelium of *K14E7* mice (7). To investigate whether this induction occurs in the endocervical epithelium of *Bi-L E7/K5-tTA* mice, we performed immunohistochemical staining for BrdUrd (Fig. 3A) in sections from mice injected with the nucleotide analogue 1 hour before sacrifice and quantified the results (Fig. 3B). In *Bi-L E7/K5-tTA* bitransgenic mice, we observed a significant induction of DNA synthesis in the suprabasal compartment of the epithelium ($P < 0.005$ versus nontransgenic, two-sided Wilcoxon rank-sum test); this induction was statistically indistinguishable from the level observed in *K14E7* mice ($P = 0.28$, two-sided Wilcoxon rank-sum test) and absent in singly transgenic mice. Administering doxycycline for 1 week to bitransgenic mice eliminated this induction of suprabasal DNA synthesis. We also examined the expression of MCM7, which we previously have shown is up-regulated in the endocervical epithelium of *K14E7* mice and which is a marker in humans for high-grade cervical dysplasia and HPV-associated cancers (26). In nontransgenic mice, cells staining positively for MCM7 were restricted to the basal and parabasal layers of the epithelium; in contrast, we observed the expression of MCM7 throughout the full thickness of the endocervical epithelium of *Bi-L E7/K5-tTA* bitransgenic mice (Fig. 3C). The pattern of expression of MCM7 in singly transgenic *Bi-L E7* mice and in bitransgenic mice given doxycycline for 1 week resembled that seen in nontransgenic mice.

We also investigated whether *Bi-L E7/K5-tTA* bitransgenic mice displayed acute phenotypes in the dorsal skin, where their overt phenotypes had been most obvious. As described fully in Supplementary Information, in the dorsal skin of *Bi-L E7/K5-tTA* mice, we observed an induction of DNA synthesis in the suprabasal compartment of the epidermis (Supplementary Fig. S3A, quantified in Supplementary Fig. S3C), a disruption of epithelial differentiation (Supplementary Fig. S3B), and an abrogation of the response to DNA damage induced by ionizing radiation (Supplementary Fig. S3D), all of which we have observed previously in the epidermis of *K14E7* mice (9, 27, 28). In addition, all of these phenotypes in bitransgenic mice regressed once we administered doxycycline for 1 week (Supplementary Fig. S3).

Taken together, these data indicate that *Bi-L E7/K5-tTA* bitransgenic mice display an array of acute epithelial phenotypes associated with the expression of E7 and that these phenotypes regress on treatment with doxycycline.

***Bi-L E7/K5-tTA* bitransgenic mice develop high-grade cervical dysplasia and cervical cancer when treated chronically with estrogen**

We have shown previously that estrogen is a necessary cofactor for the development (10) and maintenance (29) of cervical cancers in *K14E7* mice. Thus, to induce cervical cancers in female *Bi-L E7/K5-tTA* bitransgenic mice, we treated them for 6 to 7 months with exogenous estrogen. At the end points, we sacrificed the mice, harvested and fixed the reproductive tracts, embedded them in paraffin, sectioned them, and stained the sections with H&E to allow us to score for the presence of dysplastic disease and cancer. To do this, we categorized each mouse based on the most severe state of disease observed. The categories were nondysplastic hyperplasia, low-grade dysplasia [cervical intraepithelial neoplasia 1 (CIN1)], mid-grade dysplasia (CIN2), high-grade dysplasia/carcinoma *in situ* (CIN3/CIS), and cervical cancer. The results are summarized in Table 1. As a positive control, we treated *K14E7* mice with estrogen for 7 months and observed a 40% incidence of cervical cancer. In contrast, mice that did not express E7—singly transgenic *Bi-L E7* mice, *K5-tTA* transgenic mice, and nontransgenic mice—developed nothing worse than sporadic CIN1 lesions. When we examined tracts from *Bi-L E7/K5-tTA* bitransgenic mice, we found that 33% of mice treated for 6 months with estrogen developed cervical cancer, and this proportion increased to 53% after 7 months. Both results were statistically significant compared with nontransgenic mice ($P < 0.05$, two-sided Fisher's exact test). In all bitransgenic mice, regardless of the presence of cancer, mid- to high-grade dysplasia was ubiquitous throughout the female reproductive tract; statistically, this led to an increase in the severity of disease in bitransgenic mice over what was observed in nontransgenic mice ($P < 1 \times 10^{-5}$, two-sided Wilcoxon rank-sum test). Consistent with our prior findings with *K14E7* mice (10,26,29), these results indicate that the expression of E7 in the female reproductive tracts of *Bi-L E7/K5-tTA* bitransgenic mice, in combination with estrogen, leads to the development of high-grade dysplasia and cervical cancer.

Continuous expression of E7 is required for the maintenance of high-grade dysplasia and cervical cancer in *Bi-L E7/K5-tTA* bitransgenic mice

To evaluate the dependence of cervical cancers on the continuous presence of E7, we treated a subset of bitransgenic mice with doxycycline for the final month of the 7-month treatment with estrogen. By comparing the incidence of cancers in this group with the two groups of bitransgenic mice that did not receive doxycycline, we were able to determine the effect on cervical cancers of turning off the expression of E7. As shown in Table 1 and Fig. 4A, bitransgenic mice receiving 1 month of treatment with doxycycline had no cervical cancers, indicating that cervical cancers in these mice regressed after we administered doxycycline. Furthermore, we observed a complete regression of high-grade dysplasia in bitransgenic mice given doxycycline for 1 month (Fig. 4B), although in a subset of these mice we still observed isolated CIN1 lesions. This was in stark contrast to what we observed in bitransgenic mice not given doxycycline, where we saw dysplasia throughout the female reproductive tract. To ensure that these results were not due to an effect of the doxycycline other than the silencing of the *Bi-L E7* transgene, we treated a subset of *K14E7* mice with doxycycline for the final month of a 7-month treatment with estrogen and observed no difference in the severity of dysplastic disease or the incidence of cervical cancer between this group and a group never given doxycycline (Table 1).

To determine how quickly the dysplasia in bitransgenic mice regressed once we administered doxycycline, we treated mice given 6 months of estrogen with doxycycline for 3 days. Even after this brief treatment with doxycycline, we observed a complete regression of all high-grade dysplasia in the reproductive tracts of female *Bi-L E7/K5-tTA* mice (Fig. 4B), although cancers still remained (Fig. 4A). We also performed immunohistochemical staining for BrdUrd (Fig. 4C) and MCM7 (Fig. 4D) to verify that the expression of the *Bi-L*

E7 transgene was silenced within 3 days of administering doxycycline, and these results, coupled with the data from luciferase assays (Fig. 1B), indicated that this was the case. In summary, these findings show that both high-grade cervical dysplasia and cervical cancer depend on the continued presence of *E7* for their maintenance.

Mechanism of regression

Studies in tissue culture have argued that suppressing the transcription of HPV oncogenes in cell lines derived from human cervical cancers leads to the rapid induction of senescence or apoptosis and halts cellular growth (20–24). In addition, apoptosis and senescence have been observed within 1 to 8 days of inducing the regression of cancers in many other animal models in which the expression of oncogenes or tumor suppressors can be regulated temporally (14,15,18,19,30). In our model, however, we were unable to detect an increase in apoptosis (Supplementary Fig. S4; data not shown) or an induction of senescence-associated β -galactosidase (SA- β -gal) activity (Supplementary Fig. S5) in either cervical cancers or the endocervical epithelium after administering doxycycline to *Bi-L E7/K5-tTA* bitransgenic mice for 1, 3, or 6 days following prolonged treatment with estrogen. The explanation for the observed regression of cervical disease simply may be that silencing *E7* leads to the restoration of the normal cellular program for proliferation and differentiation (Fig. 4B–D), resulting in the elimination of high-grade cervical dysplasia and cervical cancers; however, it remains possible that senescence is occurring in our samples but is not manifesting as an induction of SA- β -gal in this tissue.⁴

Discussion

The hypothesis that interfering with the expression of HPV oncogenes might trigger the regression of HPV-associated tumors has been bolstered by several lines of evidence obtained from studying HPV-positive cell lines derived from human cervical cancers (20–24). Although it has been shown *in vivo* that an array of cancers requires the continuous expression of their respective oncogenes for their maintenance (14–19), our results (Table 1; Fig. 4A and B) are the first demonstration in the context of an animal model that the expression of HPV *E7* is required for the maintenance of high-grade cervical dysplasia and cervical cancer.

Oncogenic addiction, oncogenic amnesia, and HPV-associated cervical cancer

The hypothesis of oncogenic addiction (31) asserts that the intracellular circuitry in cancerous cells is altered significantly when compared with that of normal cells. In the context of this altered circuitry, an oncogene may fulfill a different and more important role than it does in normal cells; therefore, cancerous cells become “addicted” to the presence of this oncogene for their survival. Recently, the hypothesis of oncogenic amnesia (32) was proposed to explain differently the reason that tumors depend on the continuous presence of an oncogene. It states that no single oncogenic lesion can inactivate all of the checkpoints that regulate cellular proliferation and genomic integrity; instead, a given oncogene will inactivate only some of these checkpoints. Further genomic changes will continue to inactivate additional pathways and eventually will lead to carcinogenesis. As long as oncogenes are expressed, a cell remains “ignorant” of the further accumulation of genomic damage. Once an oncogene is silenced, however, the pathways with which it interfered become activated, recognize the genomic damage that has occurred, and initiate a response—senescence, apoptosis, or differentiation, depending on the reactivated pathways—to deal with the accumulated genomic insults. Unless the pathways with which the oncogene interfered become redundantly inactivated genetically or epigenetically while the oncogene

⁴J. Ewald, personal communication.

is expressed, they will be reactivated on silencing the oncogene and will drive the regression of tumors.

Although the hypotheses of oncogenic addiction and oncogenic amnesia are not mutually exclusive and both have their caveats (31,32), the assertions of oncogenic amnesia seem especially relevant in the context of HPV-associated cervical carcinogenesis. There is an average latency of 15 years between infection with HPV and the appearance of cervical carcinoma (33), suggesting that cervical carcinogenesis results from the cooperation of HPV oncoproteins with other genetic and epigenetic alterations. Whereas most human tumors contain mutations in the p53 (34) or pRb (35) pathways, these networks are unaltered in HPV-positive cell lines (36); presumably, mutations in the p53 or pRb pathway do not confer any advantage in growth to a cell in which these pathways already are dysregulated by E6 and E7 and do not enhance tumorigenesis. In fact, despite their aneuploidy (37), HeLa cells retain functional p53 and pRb pathways, and on silencing *E6* and *E7*, both pathways are induced and can trigger an arrest in cellular growth (38). Our immunohistochemical data show that the expression of MCM7, an E2F-responsive gene negatively regulated by pRb (39), is lost in suprabasal endocervical cells and in tumors once *E7* is silenced (Fig. 4D), suggesting that the pRb pathway remains functional in the cancers and high-grade dysplasia arising in *Bi-L E7/K5-tTA* bitransgenic mice. Thus, oncogenic mutations that cooperate with HPV to drive tumorigenesis spare the cellular pRb and p53 pathways, and the reactivation of these pathways likely plays an important role in mediating the regression of cancers on the silencing of HPV oncogenes.

Regression of high-grade dysplasia on silencing *E7*

The results presented here are the first to show in any context that high-grade cervical dysplasia depends on the presence of *E7* for its persistence. The elimination of high-grade dysplasia was complete after just 3 days of treatment with doxycycline (Fig. 4B), although cancers still remained (Fig. 4A); thus, even brief interference with the expression of *E7* can cause the rapid regression of high-grade cervical dysplasia, the development of which generally requires several years (33). However, even after the treatment of *Bi-L E7/K5-tTA* bitransgenic mice with doxycycline for 1 month, sporadic CIN1 lesions remained in about half of the mice (Table 1). Currently, it is unclear whether these low-grade lesions represent remnants of high-grade lesions that still are in the process of regressing, lesions whose regression has stalled, or emerging *E7*-independent lesions that potentially could develop into high-grade lesions and even cancer. Determining the nature of these residual CIN1 lesions will have important therapeutic implications.

Implications for the treatment of HPV-positive cervical disease

Results from our (Table 1; Fig. 4A) and previous (20–24) studies reinforce the notion that anti-HPV therapy that targets the expression or function of *E7* holds great promise for the treatment of invasive cervical disease. Currently, the treatment of cervical cancer often involves radiotherapy, chemotherapy, or a partial or total hysterectomy, which can be especially devastating for women of childbearing age. The benefits of potential antiviral therapies over these current methods of treatment, provided these therapies have minimal side effects, are obvious.

Currently, the most common treatment for mid- to high-grade cervical dysplasia—CIN2, CIN3, and CIS—is loop electrosurgical excision procedure (LEEP; reviewed in ref. 40). Although LEEP does not have deleterious effects on fertility, it does carry an overall rate of complication of 9.7%. Although LEEP is considered very effective for the treatment of CIN, the effectiveness is reduced for patients who test positively for HPV DNA at follow-up and varies inversely with the severity of dysplasia at the time of treatment. Our results suggest

that the development of antiviral therapy targeting the expression or function of E7 may be a rapid and effective treatment for HPV-associated high-grade cervical dysplasia (Table 1; Fig. 4B). The benefits over LEEP of targeting HPV E7 potentially include eliminating the need for surgery and its associated complications to remove CIN lesions, increasing the effectiveness of treating the highest-grade dysplastic lesions, and reducing the recurrence of lesions if HPV DNA persists.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

Grant support: National Cancer Institute grants CA022443 and CA098428.

We thank Amy Liem for technical assistance, Dr. Henry Pitot for consultations about pathology, Drs. Bill Sugden and Shannon Kenney for critical reading of this manuscript, Drs. Jonathan Ewald and David Jarrard for positive control samples for our analysis of senescence, and Dr. Ewald for helpful discussions about this analysis.

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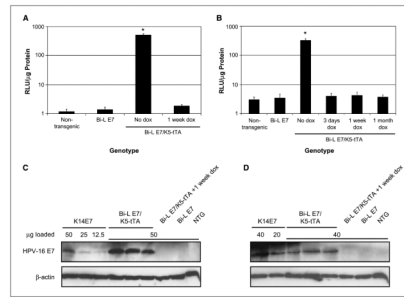


Figure 1.

The expression of luciferase and E7 in the dorsal skin and female reproductive tracts of *Bi-L E7/K5-tTA* bitransgenic mice is eliminated on treatment with doxycycline (*dox*). *A* and *B*, luciferase assays done on lysates from dorsal skin (*A*) or female reproductive tracts (*B*) from mice of the indicated genotypes. *Columns*, mean; *bars*, SD. *, $P < 0.05$ versus nontransgenic mice by a two-sided Wilcoxon rank-sum test, and $n \geq 3$ for all groups of mice. *C* and *D*, Western blots to examine the expression of E7 in lysates from dorsal skin (*C*) or female reproductive tracts (*D*). To compare the level of expression of E7 in line 406 *Bi-L E7/K5-tTA* bitransgenic mice to that seen in *K14E7* mice, equivalent amounts of lysates from three *K14E7* mice were pooled, and the indicated amounts of this pool were analyzed in parallel with lysates from mice of the indicated genotypes. Detection of β -actin was used as a loading control.

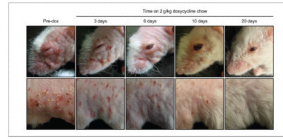


Figure 2. Bi-L E7/K5-tTA bitransgenic mice display striking overt phenotypes that are reversible on treatment with doxycycline. Shown are photographs of the head (top) and flank (bottom) of the same mouse, either before treatment with doxycycline (Pre-dox) or after the indicated times on doxycycline. Although images from only one mouse are shown, similar results were obtained with all 10 mice treated and followed for 20 d.

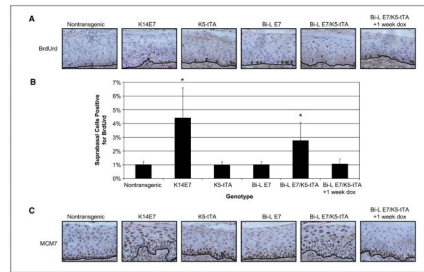


Figure 3.

The endocervical epithelium of *Bi-L E7/K5-tTA* bitransgenic mice displays aberrant proliferation. *A*, immunohistochemical analysis of the incorporation of BrdUrd in sections of the endocervical epithelium from mice of the indicated genotypes. Brown nuclei represent cells positive for BrdUrd, and the black line delineates the basement membrane. *B*, quantification of nuclei positive for BrdUrd in sections shown in *A*. Eight visual fields at $\times 40$ magnification were scored for nuclei positive (*brown*) or negative (*blue*) for BrdUrd. Data are presented as the mean \pm SD. *, $P < 0.005$ versus nontransgenic mice by a two-sided Wilcoxon rank-sum test, and $n \geq 3$ for all groups of mice. *C*, immunohistochemical analysis of the expression of MCM7 in sections of the endocervical epithelium from mice of the indicated genotypes. Brown nuclei represent cells positive for MCM7, and the black line delineates the basement membrane.

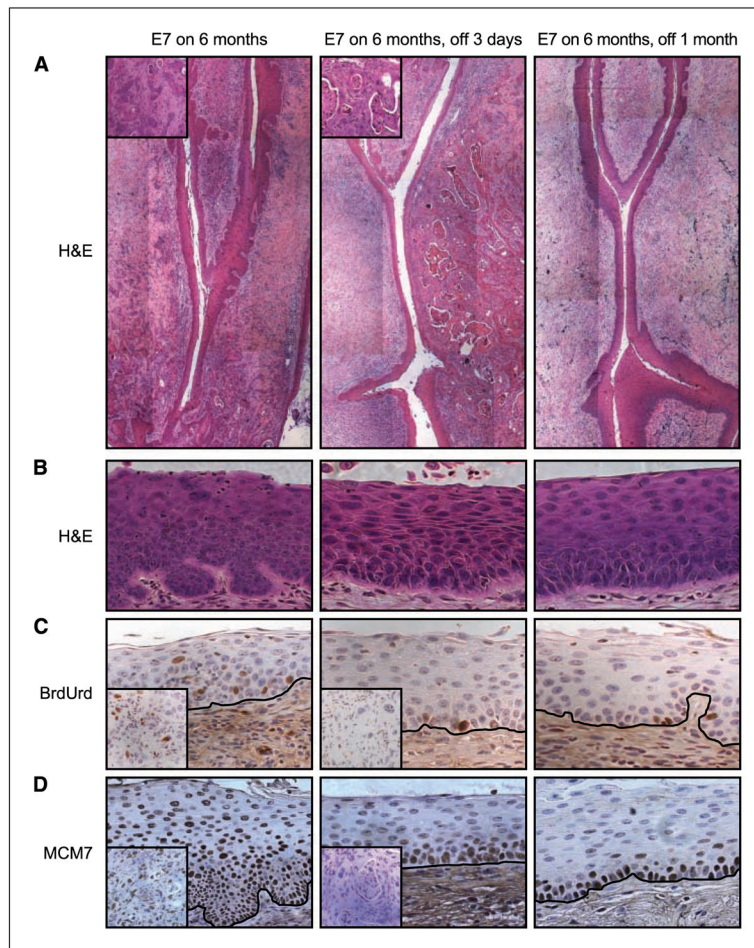


Figure 4. The expression of E7 is required for the maintenance of high-grade cervical dysplasia and cervical cancer in *Bi-L E7/K5-tTA* bitransgenic mice. Shown in all panels are images of sections of the endocervix from bitransgenic mice expressing E7 for the indicated duration. **A**, composite images at $\times 10$ magnification of sections of the endocervix stained with H&E. *Insets*, $\times 40$ magnification of cancers in mice receiving no doxycycline (*left*) or 3 d of doxycycline (*middle*). **B**, images at $\times 40$ magnification of sections from the endocervical epithelium stained with H&E, illustrating a region of high-grade dysplasia observed in a bitransgenic mouse receiving no doxycycline (*left*) and the regression of high-grade dysplasia on treatment of bitransgenic mice with doxycycline for 3 d (*middle*) or 1 mo (*right*). **C** and **D**, immunohistochemical analysis of BrdUrd (**C**) and MCM7 (**D**) in the endocervical epithelium of mice receiving no doxycycline (*left*), 3 d of doxycycline (*middle*), or 1 mo of doxycycline (*right*). The black line delineates the basement membrane. *Insets*, results from tumors from the same animals.

Table 1

Incidence of cervical disease in mice treated chronically with estrogen

| Genotype | Estrogen (mo) | Doxycycline (mo) | Grade of cervical disease | | | | | |
|-----------------------|---------------|------------------|---------------------------|------|------|----------|-----------------------|--|
| | | | Hyperplasia | CIN1 | CIN2 | CIN3/CIS | Cervical cancer (%) | |
| Nontransgenic | 6 | — | 5 | 8 | | | | |
| <i>K14E7</i> | 7 | — | | | 6 | | 4 (40) ^{*†} | |
| <i>K14E7</i> | 7 | 1 | | | 4 | | 6 (60) ^{*†} | |
| <i>K5-tTA</i> | 6 | — | 7 | 8 | | | | |
| <i>Bi-L E7</i> | 6 | — | 8 | 8 | | | | |
| <i>Bi-L E7/K5-tTA</i> | 6 | — | | 5 | 5 | | 5 (33) ^{*†} | |
| <i>Bi-L E7/K5-tTA</i> | 7 | — | | 3 | 6 | | 10 (53) ^{*†} | |
| <i>Bi-L E7/K5-tTA</i> | 7 | 1 | 8 | 9 | | | | |

NOTE: Mice of the indicated genotypes were treated with estrogen for the duration listed and either treated or not treated (—) with doxycycline for 1 mo. Based on the most severe state of disease observed in each animal, mice were placed into the following categories: nondysplastic hyperplasia, CIN1, CIN2, CIN3/CIS, and cervical cancer. The percentage of animals with cervical cancer is listed in parentheses.

* $P < 1 \times 10^{-5}$, when comparing the average severity of disease with that of nontransgenic mice by a two-sided Wilcoxon rank-sum test.

† $P < 0.05$, when comparing the incidence of cancers with that of nontransgenic mice by a two-sided Fisher's exact test.