Human Papillomavirus E7 Oncoprotein Overrides the Tumor Suppressor Activity of p21Cip1 in Cervical Carcinogenesis

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Abstract

The E7 oncoprotein of the high-risk human papillomaviruses (HPV) is thought to contribute to cervical carcinogenesis at least in part by abrogating cell cycle regulation. E7 can dysregulate the cell cycle through its interaction with several cellular proteins including the retinoblastoma suppressor protein pRb, as well as the cyclin-dependent kinase inhibitor p21Cip1. Inactivation of pRb in cervical epithelia is not sufficient to explain the ability of E7 to cause cervical cancers in transgenic mice. In the current study, we focused on the role of p21Cip1 in cervical cancer. Cervical disease was significantly increased in p21−/− mice compared with p2γ−/− mice, showing that p21Cip1 can function as a tumor suppressor in this tissue. Importantly, the ability of E7 to induce cervical cancers was not significantly enhanced on the p21-null background, consistent with the hypothesis that the ability of E7 to inhibit p21Cip1 contributes to its carcinogenic properties. Further supportive of this hypothesis, cervical carcinogenesis in mice expressing a mutant form of HPV-16 E7, E7TVQ, which fails to inactivate p21Cip1, was significantly reduced compared with that in K14E7WT mice expressing wild-type HPV-16 E7. However, K14E7TVQ mice still displayed heightened levels of cervical carcinogenesis compared with that in nontransgenic mice, indicating that activities of E7 besides its capacity to inactivate p21Cip1 also contribute to cervical carcinogenesis. Taken together, we conclude that p21Cip1 functions as a tumor suppressor in cervical carcinogenesis and that p21Cip1 inactivation by HPV-16 E7 partially contributes to the contribution of E7 to cervical carcinogenesis. [Cancer Res 2009;69(14):5656–63]

Introduction

Among the mucosotropic human papillomaviruses (HPV), a subset, called the high-risk HPVs, is the major causative factor in cervical cancer, the second most common cancer among women worldwide (1). Of these high-risk HPVs, HPV-16 is the most common, being found in 60% for all cervical cancers (2). High-risk HPVs encode three oncogenes, E5, E6, and E7, which independently and synergistically transform and/or immortalize murine fibroblasts and/or human keratinocytes in tissue culture (3–5) and synergistically transform and/or immortalize murine fibroblasts and/or human keratinocytes in tissue culture (3–5) and induce skin cancers in mice (6–9). Based on studies in mouse models, E7 appears to be the most potent of these three oncogenes in inducing cervical carcinogenesis (10).1 This study focuses on the carcinogenic properties of E7 in the cervix.

HPV-16 E7 is a multifunctional protein with potent transforming and oncogenic properties that is capable of overriding the normal differentiation process and disrupting cell cycle regulation (11). HPV-16 E7 is able to dysregulate the cell cycle by binding to several cellular proteins, including the pocket protein family members, pRb, p107, and p130 and the cyclin-dependent kinase inhibitors p21Cip1 and p27Kip1. Of these, the best-characterized cellular target is retinoblastoma tumor suppressor or pRb (12). pRb, when bound by E7, is dissociated from E2Fs and degraded by the proteasome (3, 13). As a result, the functions of pRb in cell cycle regulation are disrupted by E7 (11, 14). Although these data support the hypothesis that pRb inactivation could account for the ability of E7 to transform/immortlize cells in tissue culture and cause cancer in vivo, we recently learned that pRb inactivation is not sufficient to explain the abilities of E7 to cause cervical dysplasia or invasive cervical cancer, although pRb inactivation by E7 is necessary for the induction of cervical dysplasia (15). Thus, the non-pRb target(s) of E7 is likely important for the contribution of E7 to cervical carcinogenesis.

The cyclin-dependent kinase (CDK) inhibitor p21Cip1 is another cellular target of HPV-16 E7 (16, 17). Induction/overexpression of p21Cip1 results in the inhibition of cyclin E/CDK2 complexes that are involved in the phosphorylation of pocket family proteins (18). This growth-arresting activity of p21Cip1 correlates with its induction during growth arrest by transforming growth factor-β, cell differentiation, and in senescent cells (19, 20). In humans, loss of p21Cip1 expression correlates with breast cancer and oral/esophageal cancers (21–23). In mouse model studies, p21−/− mice have an increased incidence of spontaneous tumors compared with p21-sufficient mice (24) and an increased susceptibility to chemically induced skin carcinogenesis (25, 26). Thus, p21Cip1 is considered to be a tumor suppressor. In addition, p21Cip1 is inhibited by E7 despite that the expression level of p21 is upregulated in E7-expressing cells. HPV-16 E7 can bind to and inactivate the function of p21 to inhibit CDK2 in human keratinocytes (17) and block proliferating cell nuclear antigen-dependent DNA replication in vitro (16). These results implicate p21Cip1 as a relevant target of E7 in its role in HPV-associated carcinogenesis.

In this study, we investigated the role of p21Cip1 in cervical carcinogenesis and examined the importance of p21Cip1 as a target of HPV-16 E7 in cervical cancer using mouse models. Our observations indicate that p21Cip1 has a tumor-suppressive activity in cervical carcinogenesis and that E7 overrides the tumor-suppressive function of p21Cip1; however, the inactivation of p21Cip1 alone is not sufficient to explain fully the oncogenic

potential of HPV-16 E7. These data are consistent with p21\textsuperscript{Cip1} being an important target for E7 in carcinogenesis and for E7 being a multifunctional oncogene.

**Materials and Methods**

**Transgenic mice.** K14E7\textsuperscript{WT} mice (maintained on the inbred FVB/N genetic background), which have been described previously (7), were crossed to p21\textsuperscript{−/−} mice (on a mixed 129/C57 genetic background) and K14E7p21\textsuperscript{−/+} offspring and then crossed to p21\textsuperscript{−/−} mice to generate p21\textsuperscript{+/+}, p21\textsuperscript{−/−}, K14E7p21\textsuperscript{−/+}, K14E7p21\textsuperscript{−/−} mice used in this study. K14E7\textsuperscript{CVQ68-70AAA} (K14E7\textsuperscript{CVQ}) transgenic mice were generated by mutating amino acids 68 to 70. The K14E7\textsuperscript{CVQ} transgene cassette was excised, purified, and microinjected into FVB/N 1-day-old embryos, which were then implanted into pseudo-pregnant female mice. Founder mice and their offspring were screened by PCR and Southern blot to identify lines with single, stable transgene integration sites. K14E7\textsuperscript{CVQ} mice were maintained on the inbred FVB/N genetic background and compared with K14E7\textsuperscript{WT} transgenic mice maintained on the same genetic background. All mice were bred and maintained in the American Association for Accreditation of Laboratory Animal Care-approved McArdle Laboratory Cancer Center Animal Care Facility and were managed in accordance with an approved animal protocol.

**Estrogen treatment and cervical carcinogenesis.** All studies were done with female mice treated with exogenous estrogen pellets (17β-estradiol 0.05 mg, 60-day release pellets) as described previously (10). Treatment was initiated at age 6 weeks. For acute studies, mice were treated for just 6 weeks. For cervical cancer studies, all mice treated with estrogen for 6 months, and mice were sacrificed and the reproductive tracts were harvested. These reproductive tissues were fixed and sectioned as described previously (10). Every tenth 5 μm section was stained with H&E and evaluated histopathologically. For the diagnosis of cervix disease, each mouse was scored blindly for the worst cervical lesion detected in cervix and vagina.

**Statistical analysis.** The MSTAT software program\textsuperscript{2} was used for determining statistical significance. A two-sided Fisher’s exact test was used to determine the significance for cancer incidence between each group. To determine the significance for the tumor multiplicity, tumor size, and disease state, a two-sided Wilcoxon rank-sum test was used.

**E7 Western blot.** Mice were treated with estrogen for 6 weeks as described above. Mice were sacrificed, the lower reproductive tract was purified, and microinjected into FVB/N 1-day-old embryos, which were then implanted into pseudo-pregnant female mice. Founder mice and their offspring were screened by PCR and Southern blot to identify lines with single, stable transgene integration sites. K14E7\textsuperscript{CVQ} mice were maintained on the inbred FVB/N genetic background and compared with K14E7\textsuperscript{WT} transgenic mice maintained on the same genetic background. All mice were bred and maintained in the American Association for Accreditation of Laboratory Animal Care-approved McArdle Laboratory Cancer Center Animal Care Facility and were managed in accordance with an approved animal protocol.

**Immunohistochemistry.** Quantification of DNA synthesis and immunohistochemistry were done as described previously (10, 15, 27). Briefly, anti-p21 (1:25 in 5% nonfat milk/5% horse serum; BD Pharmingen), anti-pRb (1:50 BD in 5% horse serum; BD Pharmingen), anti-Mcm7 (1:200 in 5% horse serum; LabVision Neomarkers), anti-phospho-CDK2 (1:20 in 4% horse serum; Cell Signaling Technology), anti-p16 (1:50 in 5% nonfat milk/5% horse serum; Santa Cruz Biotechnology), anti-bromodeoxyuridine (BrdUrd; 1:50 in 5% horse serum; Calbiochem), anti-CDK2 (1:20 in 4% horse serum), anti-p21 (1:500 in 1% PBS-Tween 20 with 0.1% bovine serum albumin; Santa Cruz Biotechnology) were used for immunohistochemistry in these studies [for the latter case, mice were injected intraperitoneally with BrdUrd (10 μL/g body weight of 12.5 mg/mL solution) 1 h before sacrifice].

**Results**

**p21 expression level is induced by HPV-16 E7 in murine cervix.** The levels of expression of p21 are known to be up-regulated in HPV-16 E7-expressing human keratinocytes (16, 17) as well as in high-grade cervical intraepithelial neoplasia (CIN) squamous epithelium of human (28), and this appears to be mediated by the inactivation of pRb by E7 (29). To examine whether p21 expression is increased by HPV-16 E7 in the murine cervix, we performed p21 immunohistochemical staining on the cervical epithelium from nontransgenic and K14E7 transgenic mice (Fig. 1). The level of expression of p21 was significantly increased in the stratified epithelium of the K14E7 mice compared with that of the nontransgenic mice. This induction was most evident in the suprabasal compartment of the stratified epithelium of the cervix, much like what was observed in the skin of these mice (29), and in the raft cultures of E7-positive human keratinocytes (16, 30). In addition, we confirmed (data not shown) that p21 was induced in HPV-positive human cervix tissues both in CIN lesions and in squamous cervical carcinoma (28, 31). These data show that HPV-16 E7 induces p21 expression in the cervix of transgenic mice as is apparent in the cervix of HPV-positive lesions in women.

**p21 functions as a tumor suppressor in the mouse cervix, but its tumor-suppressive activity is suppressed by HPV-16 E7.** It previously has been shown that p21 functions as a tumor suppressor in skin carcinogenesis (25, 26). We wanted to determine whether p21 has a tumor-suppressive effect on cervical

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\textsuperscript{2}http://www.mcardle.wisc.edu/mstat
carcinogenesis, especially in light of the fact that E7 is known to inactivate p21 function. To address this question, we evaluated the incidence of cervical cancer and precancerous cervical lesions in p21−/−, p21+/−, K14E7p21−/−, and K14E7p21+/− mice treated with exogenous estrogen for 6 months. Estrogen has been shown to be a critical cofactor in HPV-associated cervical carcinogenesis in mouse models (10, 32).

As predicted based on prior studies (10), p21+/− mice did not develop high-grade cervical dysplasia or cervical cancer (Table 1). In contrast, a subset of p21−/− mice developed CIN3, carcinoma in situ (CIS), or cervical cancer (Table 1). This increase in cervical disease in the p21−/− mice compared with p21+/− mice was significant (P = 0.05), consistent with p21 functioning as a tumor suppressor in this tissue. Given this finding, it was notable that the incidence of cervical disease in the K14E7p21−/− was not significantly higher than that observed in the K14E7p21+/− mice (P = 0.3). These data are consistent with the hypothesis that E7 overcomes the tumour-suppressive effect of p21. Also of note, the p21−/− mice had a reduced incidence of cervical disease compared with that observed in the K14E7p21−/− mice (P = 0.06), consistent with the hypothesis that E7 possesses oncogenic activities in addition to its ability to suppress the tumour-suppressive activities of p21.

Generation of a transgenic mouse expressing a form of HPV-16 E7 unable to inactivate p21. The above-described data are consistent with HPV-16 E7 directly suppressing the tumour-suppressive activity of p21; however, it is also possible that HPV-16 E7 is overriding the function of p21 via the ability of E7 to inactivate pRB and the other pocket proteins, the phosphorylation of which is normally regulated by p21. To distinguish between these two hypotheses, we generated transgenic mice expressing the HPV-16 E7CVQ68-70AAA mutant (hereafter called E7CVQ, consistent with prior literature), which in tissue culture is able to bind and target pRB for degradation in our transgenic mice, we performed pRB immunohistochemistry. Both wild-type E7 and E7CVQ mutant proteins were able to cause decreased steady-state levels of pRB (Fig. 3A) as well as the other two pocket proteins p107 (Supplementary Fig. S1A) and p130 (Supplementary Fig. S1B). Not surprisingly, we also could not detect phospho-pRB (S807/S811) in the cervical epithelium of either K14E7WT or K14E7CVQ mice (data not shown). These data are consistent with tissue culture-based studies, indicating that this mutant retains the ability of wild-type E7 to bind and degrade the human pocket proteins (33, 34). The ability of E7 to induce the expression of the E2F-responsive gene MCM7 is largely the consequence of its inactivation of pRB (15). Therefore, the induction of MCM7 can be used as a marker to assess the ability of E7 to inactivate pRB. Consistent with the ability

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**Table 1. Incidence of cervical disease in p21-deficient or p21-sufficient mice treated 6 months with estrogen**

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<th>Genotype</th>
<th>Grade of cervical disease (no. mice)</th>
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<td>p21+/− (n = 8)</td>
<td>7</td>
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<tr>
<td>p21−/− (n = 10)*</td>
<td>5</td>
</tr>
<tr>
<td>K14E7p21−/− (n = 9)</td>
<td>2</td>
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<tr>
<td>K14E7p21+/− (n = 12) †</td>
<td>8</td>
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Abbreviations: MIC, microinvasive cancer; LIC, large invasive cancer.

*P = 0.05, comparing incidence of cervical disease in p21−/− versus p21+/− mice.

†P = 0.06, comparing incidence of cervical disease in K14E7p21−/− versus p21+/− mice.

‡P = 0.3, comparing incidence of cervical disease in K14E7p21+/− versus K14E7p21−/− mice.
Figure 2. E7 expression in K14E7WT and K14E7CVQ mice. Mouse lower reproductive tract protein lysates were made as described in Materials and Methods and analyzed by E7-specific Western blot analyses. 

A, representative experiment. Top, E7 Western blot; bottom, K14 blot to verify equal loading. Equivalent amounts of protein from two K14E7WT and two K14E7CVQ mice were analyzed in this Western blot. 

B, quantification of multiple E7 Western blots (n = 3 for each genotype). Intensities of E7-specific bands were quantified and normalized to K14 expression.

C and D, quantification for DNA synthesis in stratified cervical epithelium of mice treated with estrogen for 6 weeks or 6 months. Estrogen-treated mice of each genotype (n = 3 for each genotype) were injected with BrdUrd 1 h before sacrifice, and paraffin-embedded sections from these mice were stained with anti-BrdUrd. Percentage BrdUrd incorporation in mice treated with estrogen for 6 weeks (C) and 6 months (D). Asterisks, Wilcoxon rank-sum test.

We next examined whether K14E7CVQ mice have a deficiency in their ability to inactivate p21 in stratified epithelia of female reproductive tract. In previous studies, it has been shown that p21 activity is associated with dephosphorylation of CDK2 (36) and also reported that p21 blocks the phosphorylation of CDK2 by CDK-activating kinase (36, 37). Thus, we looked at phospho-CDK2 expression in the epithelia of reproductive tracts in p21−/− and K14E7CVQ mice to confirm whether phospho-CDK2 expression is regulated by p21 function in vivo. As expected, phospho-CDK2 expression is up-regulated in p21−/− mice compared with wild-type mice (Supplementary Fig. S2). Next, we evaluated both total CDK2 and phospho-CDK2 expression in the epithelia of reproductive tracts in nontransgenic mice, K14E7CVQ and K14E7WT mice. Whereas total CDK2 was present in all three genotypes (Fig. 3C), phospho-CDK2 was only evident in the cervical epithelium of K14E7WT mice, not that of either nontransgenic or K14E7CVQ mice (Fig. 3D). This is consistent with the previous findings by Helt and Galloway that the HPV-16 E7CVQ mutant is deficient in inactivating p21 (33, 34).

HPV-16 E7CVQ mutant that is deficient in its ability to inactivate p21 has a lower incidence of cancer in murine cervix. To determine if p21 inactivation by HPV-16 E7 contributes to cervical carcinogenesis, we evaluated the incidence of cervical cancer in K14E7CVQ and K14E7WT mice when treated with exogenous estrogen pellet for 6 months. Consistent with our previous study (10), all K14E7WT mice developed high-grade cervical dysplasia and/or invasive cervical cancer (Table 2). In contrast, K14E7CVQ mice displayed a significantly reduced incidence of frank cancer compared with K14E7WT mice (P = 0.008; Table 2). Tumor multiplicity in K14E7CVQ mice was also reduced (P = 0.001) compared with that in K14E7WT mice (Table 2). The mean size of the cancers (mean cross-sectional area) was significantly smaller (P = 0.0012) in the K14E7CVQ mice (0.39 mm2) compared with the K14E7WT mice (2.55 mm2); correspondingly, K14E7CVQ mice also had a significantly lower incidence of large invasive cancers when compared with K14E7WT mice (P = 0.004; Table 2). In addition, we observed less severe overall disease in K14E7CVQ mice compared with that in K14E7WT mice (P = 0.001; here, we compared the worst grade of disease among each cohort of mice). All of these results together indicate that the ability of E7 to inactivate p21 correlates with its oncogenic properties.

Our data also support the conclusion that the inactivation of p21 alone is insufficient to fully account for the oncogenic potential of E7, as K14E7CVQ mice displayed a significant increase in overall cervical disease (P = 0.000001) and a marginally significant increase in cancer incidence (P = 0.07) compared with nontransgenic mice. We conclude that the inactivation of p21 by E7 contributes to, but alone is insufficient to account fully for, the role of E7 in cervical carcinogenesis.

HPV-16 E7CVQ mutant induces expression level of the E2F-responsive gene Mm7 and CDK inhibitor p16 in cervical cancer similar to that observed in HPV-16 E7WT–induced cervical cancers. Both MCM7 and p16 have been used as robust biomarkers for HPV-associated cervical cancer in humans and

mice (27, 38), and their induction correlates strongly with the expression of E7 (27). We therefore compared the expression patterns of these same two biomarkers in the cancers arising in the K14E7CVQ and K14E7WT mice. Similar to the prior analysis of cervical epithelia (Fig. 3), MCM7 expression was induced in the tumors arising in K14E7CVQ mice much like that in tumors from K14E7WT mice (Fig. 4A). Likewise, we saw heightened levels of p16 in tumors from mutant E7 transgenic mice (Fig. 4B), although it was slightly reduced compared with tumors in wild-type E7 transgenic mice.

Discussion

p21 is a tumor suppressor in cervical cancer. In mice treated with estrogen for 6 months, we observed that some of p21+/− mice developed cervical cancer in contrast to none of p21+/+ mice (Table 1). Furthermore, disease progression in the cervix was significantly increased in p21−/− mice (Table 1). These results are consistent with studies on chemically induced skin carcinogenesis in which p21 was found to function as a tumor suppressor in the epidermis (25, 26). However, we also found that the disruption of the tumor-suppressive activity of p21 alone is not sufficient to account fully for cervical carcinogenesis induced by HPV-16 E7, because K14E7p21−/− mice had a significantly higher incidence of cervical disease compared with p21−/− mice (Table 1). That we observed that the tumor-suppressive activity of p21 was not effective in the context of E7-expressing mice (K14E7p21+/+ versus K14E7p21−/− mice; Table 1) is consistent with the hypothesis that E7 inactivates the tumor-suppressive activity of p21 (16, 17).

In a prior study, pRb inactivation together with exogenous estrogen treatment was found to be insufficient to cause the development of high-grade cervical dysplasia or cervical cancer.
(15). One possible explanation for this finding is that inactivation of multiple pocket proteins (e.g., pRb, p107, and p130), all of which are normally targeted by E7, are necessary for tumorigenesis in the cervix, and studies are under way to test this possibility. In contrast, p21 inactivation together with exogenous estrogen treatment was sufficient to cause the development of high-grade cervical dysplasia or cervical cancer, albeit at lower penetrance compared with that in E7 transgenic mice. This observation and the fact that E7 could override the tumor-suppressive activity of p21 indicate that p21 is a critical but certainly not the sole target for E7.

**Characterization of the phenotype of HPV-16 E7\(^{CVQ}\) mutant in the cervical stratified epithelium of transgenic mice.** In previous studies in human foreskin keratinocytes, HPV-16 E7\(^{CVQ}\) mutant was shown to destabilize pRb and its related pocket proteins as efficiently as wild-type E7 while being defective for its ability to inactivate p21 (33, 34). A recent study has shown that the cullin-2 ubiquitin-ligase complex contributes to HPV-16 E7-mediated pRb destabilization and that the E7\(^{CVQ}\) mutant was reduced in its association with the cullin-2 ubiquitin-ligase complex (39). In our study, the E7\(^{CVQ}\) mutant retained the ability to cause decreased steady-state levels of pRb (Fig. 3A). Furthermore, the heightened level of MCM7 expression, which is known to correlate with pRb inactivation, was not greatly different between wild-type E7 and E7\(^{CVQ}\) mutant-expressing mouse tissues (Fig. 3B). These results are consistent with the hypothesis that E7\(^{CVQ}\) mutant is able to efficiently inactivate pRb and supports the hypothesis that an additional E7-associated ubiquitin ligase(s) contributes to HPV-16 E7-mediated pRb degradation or that the E7\(^{CVQ}\) protein retains sufficient ability to bind cullin-2 to allow it to efficiently degrade pRb.

The relationship between E7 and p21 is paradoxical. On one hand, E7 causes an increased level of p21 in human and mouse cells/tissues (16, 29, 30). On the other hand, E7 can inactivate p21 (16, 17). In our study, we observed that the ability of E7\(^{CVQ}\) to inactivate p21 was compromised based on the fact that the activated form of CDK2, phospho-CDK2, was not induced in K14E7\(^{CVQ}\) mouse tissues, in contrast to what we observed in K14E7\(^{WT}\) tissues. This observation in mouse tissues is consistent with the prior observation that E7\(^{CVQ}\) is defective for its ability to inactivate p21 in human cells (33). However, we still noted an increase in the steady-state levels of p21 in K14E7\(^{CVQ}\) mouse tissues. This indicates that the induction of p21 by E7 is separable from the ability of E7 to inactivate p21. This finding is consistent with the hypothesis (29) that the induction of p21 by E7 is a consequence of the ability of E7 to inactivate pRb, an activity that is retained by the E7\(^{CVQ}\) mutant (Fig. 1).

**Oncogenic potential of HPV-16 E7\(^{CVQ}\) mutant in cervical carcinogenesis.** By using p21-null mice, we observed a tumor-suppressive activity of p21 in cervical carcinogenesis. Thus, we investigated the importance of the inactivation of p21 by E7 in cervical carcinogenesis by performing a phenotypic comparison between K14E7\(^{WT}\) and K14E7\(^{CVQ}\) mice. In K14E7\(^{WT}\) mice treated with estrogen for 6 months, all of K14E7\(^{WT}\) mice developed high-grade squamous intraepithelial lesions as described previously (10). K14E7\(^{CVQ}\) mice showed a significant reduction in the incidence of both cervical cancer and disease progression compared with that in K14E7\(^{WT}\) mice (Table 2). Furthermore, tumor multiplicity in K14E7\(^{CVQ}\) mice was also reduced compared with that in K14E7\(^{WT}\) mice (Table 2) and the mean size of the cancers was significantly smaller in the K14E7\(^{CVQ}\) mice compared with the K14E7\(^{WT}\) mice. These observations are consistent with the hypothesis that the

<table>
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<th>Genotype</th>
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<th>LIC incidence (%)</th>
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<td>CIN3/CIS</td>
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<td>23</td>
<td>11</td>
<td>5</td>
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\(^{*}\)P = 0.008, comparing incidence of cervical cancer in K14E7\(^{CVQ}\) versus K14E7\(^{WT}\) mice.

\(^{1}\)P = 0.004, comparing incidence of large invasive cancers (LIC) in K14E7\(^{CVQ}\) versus K14E7\(^{WT}\) mice.

\(^{2}\)P = 0.001, comparing tumor multiplicity in K14E7\(^{CVQ}\) versus K14E7\(^{WT}\) mice.

\(^{3}\)Data from prior study (32).

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Table 2. Incidence of cervical disease in K14E7\(^{WT}\) versus K14E7\(^{CVQ}\) mice treated 6 months with estrogen

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**Figure 4.** Evaluation of MCM7 and p16 expression in tumors from the reproductive tract of female mice treated with estrogen for 6 months. Representative images from sections stained with anti-MCM7 (A) or anti-p16 (B) antibody (brown) and counterstained with hematoxylin (blue).
ability of E7 to inactivate p21 contributes to its oncogenic potential. Although our data with the K14E7CVQ mouse tissues are consistent with a role of the inactivation of E7 of p21 contributing to cervical carcinogenesis, other interpretations cannot be discounted. For instance, wild-type E7 is known also to inhibit another CDK inhibitor that inhibits CDK2 activity, p27. It is not known whether the CVQ mutant retains the ability to inhibit p27. Also, E7 can directly interact with CDK2 and this could lead to the modulation by E7 of the activity of this kinase. It is not known whether the CVQ mutation alters the direct interaction of E7 with CDK2. Regardless, our results indicate that an alteration in the activity of CDK2 by E7, whether by its inhibition of p21 and p27 and/or its direct modulation of CDK2, correlates with its role in cervical carcinogenesis.

Another interesting finding is that cervical disease and incidence of cancer in K14E7CVQ mice was significantly higher than in nontransgenic mice. These data support the hypothesis that other activities of E7 also contribute to cervical carcinogenesis. Current studies are attempting to determine if these other activities include the abilities of E7 to inactivate multiple pocket proteins.

**Molecular function of p21 on cervical carcinogenesis.** Previous studies have shown that p21 both inhibits CDKs (18) and regulates proliferating cell nuclear antigen-dependent DNA replication (42, 43). The CDK2-inhibitory activity of p21 correlates with Rb-dependent E2F transcription regulation (44). In the current study, we observed that expression of Mcm7, an E2F-responsive gene, was induced in cervical epithelium of p21KO mice, consistent with the hypothesis that p21 indirectly regulates E2F activity, but this induction was less than that seen in K14E7WT mice. Given that E7 not only inactivates p21 but also the pocket proteins and thereby modulates E2F activity by multiple means, this result suggests that pRb remains partially active in p21 nulligenic cells. We also observed that MCM7, while induced in the K14E7CVQ mouse tissues, was not induced in the most terminally differentiated cells (Fig. 3B). This observation is consistent with the hypothesis that the inactivation of p21 by E7 contributes partially to the activation of E2Fs by E7. Loss of pRb protein alone is not sufficient to completely account for the ability of E7 to up-regulate E2F-dependent transcription, particularly in the suprabasal compartment because MCM7 expression was less frequently observed in the suprabasal cells of the cervix of K14E7WT mice compared with that in K14E7 mice (15). These data raise the possibility that the inactivation of p21 by E7 may play a more important role in increasing the ability of E7 to suppress the E2F inhibitory function of p107 and/or p130. Alternatively, the influence of the inactivation of p21 by E7 may be independent of a disruption of pocket protein function. p21 can also inhibit proliferating cell nuclear antigen-dependent DNA synthesis and E7 can override this inhibition (16). Levels of proliferating cell nuclear antigen expression were higher in E7-expressing keratinocytes in tissue culture (17) and also in human patient samples (45). It is also possible that the inhibition of the CDK-inhibitory activity of p21 alters the phosphorylation status on proteins in addition to the pocket proteins. Of potential relevance in this context is the observation that p16 induction is attenuated in tumors arising in K14E7CVQ mice. Recently, it has been described that E7 associates with polycomb group proteins including E2F6 and Bmi1, the latter a transcriptional repressor of p16, and E7’s interaction with E2F6 was unaffected by the CVQ68-70AAA mutation (46). This raises the interesting possibility that p21 inactivation by E7 contributes to the modulation of epigenetic regulation of cellular genes by E7. Therefore, it is likely that the inhibition of p21 by E7 has multiple effects on the cell cycle that are not restricted to the modulation of pocket protein function.

In summary, our data show that that p21 functions as a tumor suppressor in cervical carcinogenesis, that the inactivation of p21 by E7 correlates with the induction of carcinogenesis by E7, but that the inactivation of p21 cannot account fully for the oncogenic potential of E7.

**Disclosure of Potential Conflicts of Interest**

No potential conflicts of interest were disclosed.

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**References**


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Human Papillomavirus E7 Oncoprotein Overrides the Tumor Suppressor Activity of p21<sup>Cip1</sup> in Cervical Carcinogenesis

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