Inactivating All Three Rb Family Pocket Proteins Is Insufficient to Initiate Cervical Cancer

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Abstract

Human papillomavirus-16 (HPV-16) is associated etiologically with many human cervical cancers. It encodes 3 oncogenes, E5, E6, and E7. Of these oncogenes, E7 has been found to be the dominant driver of cervical cancer in mice. More than 100 cellular proteins have been reported to associate with HPV-16 E7, which is thought to dysregulate the cell cycle in part by binding and inducing the degradation of pRb and its related pocket protein family members, p107 and p130. The ability of E7 to inactivate the pRb family correlates with its ability to induce head and neck cancers in mice. We previously showed that the inactivation of pRb is itself not sufficient to recapitulate the oncogenic properties of E7 in cervical carcinogenesis. In this study, we evaluated mice that were deficient in multiple pocket proteins, including mice that lacked pRb, p107, and p130. Strikingly, combined loss of two or all 3 pocket proteins resulted in development of high-grade cervical intraepithelial neoplasia, but not frank cervical carcinoma. These findings strongly argue that the oncogenic properties of HPV-16 E7 in human cervical carcinogenesis may involve disruption of E7 binding proteins beyond simply the pRb family members.

Introduction

Cervical cancer remains a major woman’s health problem worldwide (1, 2). Infections by a subset of human papillomavirus (HPV) are the primary cause of cervical cancer and, among this ‘high-risk’ subset of human mucosal HPVs, HPV type 16 (HPV-16) is responsible for approximately 60% of cervical cancers (3). HPV-16 encodes three major oncogenes: E5, E6, and E7. Of these oncogenes, E7 is commonly found expressed in HPV-associated cervical cancers (6). E7 was previously identified as the predominant oncogene in terms of its ability to induce cervical cancer in transgenic mouse models (7, 8).

In tandem affinity purification/mass spec analyses, HPV-16 E7 protein has been found associated with more than 100 different cellular proteins (9). Through these associations, E7 has been implicated in dysregulating a wide range of cellular processes, including gene transcription, DNA synthesis, protein degradation, epigenetic reprogramming, genomic integrity and cellular metabolism (10). Of these cellular targets of E7, the best known are the tumor suppressor pRb and its related pocket protein family members, p107 and p130 (11). pRb is an important regulator of the cell cycle in the transition point from the G1 phase to the S phase at least in part because it can bind to and inactivate a family of transcription factors called E2Fs. In response to mitogenic stimuli, pRb is posttranslationally modified via phosphorylation by cyclin/cdk complexes, resulting in its release from, and consequent activation of these E2F transcription factors that are key regulators of expression of genes involved in cell cycle (12). E7’s binding to pRb leads to the inactivation of pRb and its degradation through proteosome-dependent degradation (13) resulting in the activation of the E2F transcription factors. Inactivation of pRb by E7 also is thought to contribute to alterations in differentiation, DNA damage responses, centrosome synthesis, and tumorigenesis (14, 15). In various human cancers, genetic or epigenetic inactivation of RB has been reported; these observations and the analysis of genetically engineered mice have identified pRb as a major tumor suppressor (12). These findings support the hypothesis that pRb inactivation by E7 is an important contributor to the oncogenic potential of HPV-16 E7 in cervical carcinogenesis. However, in our prior studies, we discovered that genetic inactivation of pRb is not sufficient to account for E7’s ability to induce cervical cancer in mice (16). This observation led us to the present studies directed at asking what other cellular targets of E7 contribute to cervical carcinogenesis.

Global gene expression analysis of HPV-associated cancers indicated that many of the cell cycle regulatory genes induced in HPV-positive cancers are E2F-responsive genes (17). For this reason we focused our attention on other cellular targets of E7 that are involved in regulating E2Fs, specifically other members of the pocket protein family, p107 and p130 (18). p107 and p130 have similarities with pRb, both in their overall structure, their ability to bind and inactivate E2Fs, as well as sequence...
homology in a large C-terminal domain known to mediate their interaction with viral oncoproteins such as SV-40 LT. Adenovirus E1A, and HPV E7 (19–22). Functional overlap among the pocket proteins has been documented among the pocket protein family members (23). Despite these similarities to pRb, the tumor suppressive activity of p107 and p130 remains largely questioned, as genetic and epigenetic alterations of these genes are not commonly found in human cancers (12, 24). Nevertheless, the concept that p107 and/or p130 function as tumor suppressors in the context of cervical carcinogenesis has remained a popular hypothesis because HPV-16 E7 is able to bind all three pocket proteins. Furthermore, multiple studies in mice have shown that p107 as well as p130 can function as tumor suppressors in different tissue contexts (25–29), including our own studies in the context of head and neck carcinogenesis (30).

In this study, we made use of genetically engineered mice to determine whether the combined loss of function of multiple pocket proteins is sufficient to account for the oncogenic properties of HPV-16 E7 in cervical carcinogenesis. Our results show that the combinatorial inactivation of two or all three pocket proteins is not sufficient to induce cervical cancer but is sufficient to induce high grade cervical intraepithelial neoplasia (CIN), the precursor to cervical cancer. These findings indicate that other cellular target(s) of E7 must contribute to late stages in cervical carcinogenesis.

Materials and Methods

Mice

K14E7 mice have been described previously (8), Rb+/p107−/−, Rb+/p130−/−, and Rb+/p130−/p107−/− mice were previously described (31). KRT14-cre/Esr1 (K14CreErTm) was obtained from Jackson Laboratory. K14CreRbnull mice have been described previously (16). Details on the nature of the genetic crosses made in this study are provided under the Supplemental Data section. For the irradiation studies, mice were exposed to 0 or 12 Gy ionizing radiation from a 137Cs source 24 hours before mice were injected IP with bromodeoxyuridine (BrdUrd; 10 mg/kg) and were managed in accordance with an approved animal protocol.

Inducible Cre-mediated recombination

To generate the Rb-nulligenic state in the cervical stratified epithelia of K14CreErTm Rb+/p107−/− mice, 6-week-old mice were intraperitoneally injected with tamoxifen [4 mg of tamoxifen dissolved in corn oil (Fisher Scientific Inc.) per day] for 5 consecutive days. To generate the Rb-nulligenic and p130-nulligenic states in the cervical stratified epithelia of K14CreErTmRb+/p130−/p107−/− mice, 6-week-old mice 4-hydroxytamoxifen [4-OHT; 0.05 mg of 4-hydroxytamoxifen dissolved in corn oil (Fisher Scientific Inc.) per day] was applied topically to the vaginal cavity for 5 consecutive days. We did not observe Cre activation by estrogen pellet in estrogen-treated K14CreErTmRb+/p107−/− mice (data not shown) consistent with the fact that the Cre-ERtm gene product is selectively responsive to tamoxifen, not estrogen.

Immunohistochemistry

All of immunohistochemistry was conducted as previously described (30, 32). Briefly, the primary antibodies were used in different condition as follows: anti-pRb (1:25, in 5% horse serum, BD Biosciences), anti-p107 (1:125 in 5% nonfat milk/5% horse serum, Santa Cruz Biotechnology), anti-p130 (1:100 in 5% nonfat milk/5% horse serum, BD Biosciences), anti-BrdUrd (1:50 in 5% horse serum, Calbiochem), anti-Mcm7 (1:200 in 5% nonfat milk/5% horse serum, LabVision Neomarkers).

Statistical analysis

A 2-sided Fisher’s exact test was used to determine the significance of differences in the incidence of cervical between each mouse group. To determine the significance of differences in the severity of disease and DNA synthesis level between each mouse group, a 2-sided Wilcoxon rank sum test was conducted.

Results

Conditional inactivation of both Rb and p107 or Rb and p130 in cervical epithelia

To study whether the combined loss of pRb and other pocket protein members, either p107 or p130, contribute to cervical carcinogenesis, we generated mice that were inactivated for both pRb and p130, or pRb and p107 in stratified cervical epithelia. To achieve knockout of pRb and p130, we crossed a conditional knockout allele of Rb with a germline knockout allele for p130. To knock out Rb in the cervical epithelium, we placed the Rb+/p130−/− mice onto the K14Cre transgenic mouse background that expresses Cre recombinase in all stratified squamous epithelia of the adult mouse. The same strategy could not be used to generate mice knocked out both Rb and p107 in the cervix because K14CreRb+/p107−/− mice display high rates of mortality at a very early age, which we and others ascribe to difficulty in the mice drinking water or eating food owing to hyperplasia leading to occlusion of the esophagus (30). Therefore, to generate mice inactivated for both pRb and p107 in cervical epithelium, we crossed Rb+/p107−/− mice to K14CreErTm, a transgenic mouse strain that expresses a tamoxifen-inducible form of Cre in stratified squamous epithelia. We then administered tamoxifen to adult K14CreErTmRb+/p107−/− mice, at 6 weeks of age, well after the age when mortality was observed in the K14CreRb+/p107−/− mice. By this time K14-driven transgenes have become actively expressed in the cervical and vaginal epithelium. Before this age K14-driven transgenes are poorly expressed in the female reproductive tract (data not shown). This strategy eliminated mortality issues presumably because the adult esophagus is sufficiently large that the epithelial hyperplasia resulting from inactivation of both pRb and p107 does not occlude it. To verify the efficiency of Cre or Cre-ERtm-induced disruption of pRb expression in cervical stratified epithelium of the K14CreRb+/p130−/− and K14CreErTmRb+/p107−/− mice, we conducted pRb, as well as p107 and p130 immunohistochemical staining on the female
reproductive tracts of these mice, as well as that of nontransgenic, \( Rbf/p130^{-/-} \), \( Rbf/p107^{-/-} \), \( K14CreRbf^{f/f} \), and \( K14E7 \) transgenic mice (Fig. 1). As expected, pRb staining in cervical epithelium was sharply reduced in the \( K14E7 \), \( K14CreRbf^{f/f} \), \( K14CreRbf^{f/f}p130^{-/-} \), and the tamoxifen-treated \( K14CreER-tmRbf/p107^{-/-} \) mice, whereas still observed in Cre-negative \( Rbf^{f/f} \) mice, such as \( Rbf/p130^{-/-} \), \( Rbf/p107^{-/-} \) mice (Fig. 1, left). These observations indicate that the floxed \( Rb \) allele in cervical stratified epithelium had undergone recombination by the \( K14 \) promoter-driven \( Cre \) or \( CreER \) transgenes. Likewise, we could not detect \( p107 \) in cervical stratified epithelium nulligenic for \( p107 \), such as \( Rbf/p107^{-/-} \) and tamoxifen-treated \( K14CreER-tmRbf^{f/f}p107^{-/-} \) mice, as well as \( K14E7 \) mice, but it was detected in the \( Rbf^{f/f}p130^{-/-} \), \( K14CreRbf^{f/f} \), \( K14CreRbf^{f/f}p130^{-/-} \), and nontransgenic mice (Fig. 1, middle). Levels of \( p107 \) were increased in both \( K14CreRbf^{f/f} \) and \( K14CreRbf^{f/f}p130^{-/-} \) mice over that observed in \( Rb \)-sufficient nontransgenic control mice. Moreover, \( p107 \) positive-stained cells were more uniformly observed in the stratified epithelium of the \( K14CreRbf^{f/f} \) \( p130^{-/-} \) mice compared with that of the \( K14CreRbf^{f/f} \) mice.

These results indicate that compensatory increases in expression of \( p107 \) can arise in cervical epithelium when the tissue is deficient in the expression of \( pRb \) alone, and more so when it is deficient in expression of both \( pRb \) and \( p130 \). A similar trend was observed for \( p130 \). It was not detectable in cervical epithelium from both \( Rbf^{f/f}p130^{-/-} \) and \( K14CreRbf^{f/f}p130^{-/-} \) mice, which are harboring the \( p130 \) null allele (Fig. 1, right), but was increased in \( pRb \)-deficient tissue, although not nearly as strongly as was seen for \( p107 \). Taken together, these data indicate that the \( K14 \)-driven \( Cre \) and \( CreERtm \) transgenes were effective at inducing recombination of the floxed \( Rb \) allele in the cervical epithelia within the female reproductive tract, and show that compensatory increases in expression of functional pocket proteins can arise in cervical stratified epithelium.

**Suprabasal DNA synthesis in cervical stratified squamous epithelium deficient in pocket proteins**

HPV-16 E7 confers acute changes to the cervical epithelium including the reprogramming suprabasal cells to support DNA synthesis, and disrupting DNA damage-induced cell-cycle
arrest, activities that may contribute to the accumulation of mutations and carcinogenesis (10). As previously described (16), pRb inactivation alone was not sufficient to cause these same phenotypes in cervical epithelium. We asked if the combinatorial inactivation of pRb and p130 or pRb and p107 could cause induction of DNA synthesis in suprabasal cells. To address this, we analyzed by immunohistochemistry (16, 32) the frequency of BrdUrd-positive suprabasal cells in sections of tissue from mice injected with this nucleoside analog 1 hour before sacrifice. In Rb(+/−)p130−/−, tamoxifen-treated Rb(+/−)p107−/−, and K14CreRb(+/−) mice, there was no significant induction of suprabasal DNA synthesis in cervical stratified epithelia compared with nontransgenic mice (Fig. 2A and B), indicating that inactivation of any one pocket protein is not able to induce a suprabasal DNA synthesis in cervical stratified epithelia. The data obtained with the K14CreRb(+/−) mice was consistent with our prior study (16). In contrast, in K14CreRb(+/−)p130−/− mice, we observed a significant increase in suprabasal DNA synthesis compared with either Rb(+/−)p130−/− or K14CreRb(+/−) mice although this induction of suprabasal DNA synthesis was significantly lower than that observed in K14E7 mice (Fig. 2A). Similarly, we observed an increase of suprabasal DNA synthesis in tamoxifen-treated K14CreERTmRb(+/−)p107−/− mice compared with either nontransgenic or tamoxifen-treated Rb(+/−)p107−/− mice, but the frequency of BrdUrd-positive cells was less than that observed in K14E7 mice (Fig. 2B). These observations show that the combined loss of pRb and either p107 or p130 partially accounts for the DNA synthesis in suprabasal compartment induced by E7.

**Abrogation of DNA damage response in irradiated cervical epithelia for pRb and p130 or pRb and p107**

One of the hallmarks of E7 is its ability to abrogate DNA damage response in epithelia including the cervical epithelium (16). To determine if the combinatorial inactivation of pRb and either p107 or p130 is sufficient to account for HPV-16 E7’s ability to inhibit DNA damage response, mice were exposed to ionizing radiation.

![Graph](image_url)

**Figure 2.** Acute phenotypes in cervical epithelium conferred by deficiency in pocket proteins. A and B, quantification of percentage of suprabasal cells supporting DNA synthesis in cervical epithelium of 12-week-old mice of each genotype (n = 3) that were injected BrdUrd 1 hour before sacrifice, and paraffin-embedded sections from these mice were stained with anti-BrdUrd. A, analysis of mice deficient for p130 alone, pRb alone, or both p130 and pRb in comparison to nontransgenic and E7 transgenic mice. *, DNA synthesis level in suprabasal compartment is significantly different compared with either K14E7 mice or nontransgenic (NTG) mice (P = 0.0495, 2-sided Wilcoxon rank sum test). B, analysis of mice deficient for p107 alone or both p107 and pRb in comparison to nontransgenic and E7 transgenic mice. **, DNA synthesis level in suprabasal compartment is significantly different compared with either in K14E7 mice or NTG mice (P = 0.0495, 2-sided Wilcoxon rank sum test). C and D, quantification of epithelial cells supporting DNA synthesis in cervical epithelium of 12-week-old mice of each genotype (n = 3 for each genotype) that were exposed to 0 Gy versus 12 Gy ionizing radiation 24 hours before BrdUrd administration. The ratio of BrdUrd-positive staining in basal cells is graphed. Stars indicate that basal DNA synthesis in 12 Gy is significantly lower than that counted in 0 Gy. C, analysis of mice deficient for p130 alone, pRb alone, or both p130 and pRb in comparison to nontransgenic and E7 transgenic mice. D, analysis of mice deficient for p107 alone or both p107 and pRb in comparison to nontransgenic and E7 transgenic mice. See Supplementary Fig. S1 for representative images of BrdUrd-specific immunohistochemistry for each of the genotypes p mice ±/− irradiation.
ionizing radiation from a $^{137}$Cs source, injected with BrdUrd 24 hours later, and sacrificed 1 hour after injection. The prevailing effect of ionizing radiation on cervical epithelia is an arrest in DNA synthesis within the normally proliferating basal layer of the cervical epithelium, which is maximally observed at 24 hours postirradiation. We scored the frequency of BrdUrd-positive basal cells at this time point in the cervical epithelia of the different mouse strains. There was a significant radiation-induced arrest in DNA synthesis after irradiation in nontransgenic mice, but this effect of radiation was absent in the K14E7 mice (Fig. 2C and D), consistent with prior studies showing that E7 abrogates this DNA damage response (16). In mice singly deficient for 1 pocket protein, we also observed a significant decrease in DNA synthesis after irradiation, indicating that inactivation of individual pocket proteins does not inhibit DNA damage responses. For the K14CreRbf/f mice, this result was consistent with our prior study (16). However, in both K14CreRbf/f $p130^{-/-}$ and tamoxifen-treated K14CreERtmRbfp107$^{-/-}$ mice, we saw an abrogation of the DNA damage response much like that seen in K14E7 mice. Thus, combined loss of pRb and either p107 or p130 is sufficient to recapitulate E7's ability to inhibit DNA damage response in cervix, supporting the premise that there is some degree of functional overlap among pocket proteins in the context of radiation-induced cessation in cellular DNA synthesis.

**Contribution of combinatorial inactivation for either pRB and p107 or pRB and p130 on cervical carcinogenesis**

To determine if combined loss of pRb and either p107 or p130 is also able to recapitulate the oncogenic potential of E7 in cervical carcinogenesis, we exposed mice of the different genotypes described above to exogenous estrogen for 6 months. Exogenous estrogen sufficient to induce continuous estrus acts as a cocarcinogen in the mouse cervix, leading to high incidence of progressive neoplastic disease culminating in cervical cancer in HPV-16 transgenic mice. E7 transgenic mice are particularly sensitive to estrogen induced cervical cancers (8). After treatment with estrogen for 6 months, we evaluated the incidence of cervical cancer and precancerous cervical lesions in cohorts of mice of the following genotypes: nontransgenic, Rb$^{+/+}$/p130$^{-/-}$, tamoxifen-treated Rb$^{+/+}$/p107$^{-/-}$, K14CreRbf/f, K14CreRbf/p130$^{-/-}$, tamoxifen-treated K14CreERtmRbfp107$^{-/-}$, and K14E7 mice. Because mice deficient for pRb and p130 were generated on one mixed genetic background, and mice deficient for pRb and p107 were generated on a slightly different genetic background (see Supplementary data), separate sets of positive and negative control groups (e.g., nontransgenic, E7 transgenic, and mice disrupted for individual pocket proteins) were generated on each of these 2 genetic backgrounds, and the histopathology results for each genetic background are reported in separate tables (Tables 1 and 2). Frequencies of cervical cancer and overall severity of disease between the negative controls (i.e., comparing nontransgenics in Table 1 to nontransgenics in Table 2 $P = 1.1$) and between the positive controls (i.e., comparing K14E7 in Table 1 to K14E7 in Table 2, $P = 0.7722, 0.9921$) on the 2 genetic backgrounds were not significantly different (see Tables 1 and 2), indicating that these 2 genetic backgrounds had little effect on susceptibility to cervical carcinogenesis. Consistent with our prior studies (8, 16), nearly all K14E7 mice developed severe cervical dysplasia and/or invasive cervical cancers, whereas most nontransgenic mice failed to develop high-grade cervical dysplasia or cancer (Tables 1 and 2). Neither high-grade dysplasia nor cervical cancer was observed in estrogen treated Rb$^{+/+}$/p130$^{-/-}$, K14CreRbf/f (Table 1), and tamoxifen-treated Rb$^{+/+}$/p107$^{-/-}$ mice (Table 2), indicating that inactivation of single pocket proteins is not sufficient to induce either severe CIN or cancer. Interestingly, in the K14CreRbf/p130$^{-/-}$ and tamoxifen-treated K14CreERtmRbfp107$^{-/-}$ mice, we also failed to see a significant increase in frequencies of cancer compared with that observed in the single inactivation of pocket proteins (Tables 1 and 2), despite the fact that pRb was absent in virtually all cells in these tissues (Fig. 1), indicative of efficient Cre recombination.

**Table 1.** Histopathology summary in cervix for the both pRb and p130 conditionally deficient mice treated with estrogen for 6 months

<table>
<thead>
<tr>
<th>Genotype$^a$</th>
<th>H</th>
<th>CIN1</th>
<th>CIN2</th>
<th>CIN3</th>
<th>CC</th>
<th>Cancer incidence, %</th>
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<tbody>
<tr>
<td>NTG ($n = 22$)</td>
<td>19</td>
<td>2</td>
<td>1</td>
<td></td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>K14E7$^{WT}$ ($n = 42$)</td>
<td>33</td>
<td>9</td>
<td></td>
<td>23</td>
<td>9</td>
<td>28.1</td>
</tr>
<tr>
<td>K14CreRbf/f ($n = 42$)</td>
<td>26</td>
<td>7</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Rb$^{+/+}$/p130$^{-/-}$ ($n = 33$)</td>
<td>6</td>
<td>11</td>
<td>10</td>
<td></td>
<td>1</td>
<td>3.4</td>
</tr>
</tbody>
</table>

$^a$All mice were on the same FVB/129/C57 mixed genetic background (see Materials and Method for details on breeding scheme).
$^bP = 0.014$ comparing the incidence of cervical cancer in K14CreRbf/p130$^{-/-}$ versus K14E7.
$^cP = 1.7 	imes 10^{-10}$ comparing the severity of cervical disease in K14CreRbf/p130$^{-/-}$ versus K14E7.
$^dP = 8.2 	imes 10^{-6}, 7.7 	imes 10^{-7}, 1.3 	imes 10^{-5}$ comparing the severity of cervical disease in K14CreRbf/p130$^{-/-}$ versus NTG, Rb$^{+/+}$/p130$^{-/-}$, K14CreRbf/f, respectively.
In K14CreRb\(^{f/f}\)p130\(^{-/-}\) mice, only one out of 29 mice developed invasive cervical cancer (Table 1); this incidence of cervical cancer was not significantly different compared with that in either Rb\(^{f/f}\)p130\(^{-/-}\) (P = 0.4677), K14CreRb\(^{f/f}\) (P = 0.4085), or the nontransgenic (P = 1) mice, whereas it was significantly lower than that seen in the K14E7 mice (P = 0.014). The severity of cervical disease, which takes into account the frequencies of not only cancer but also precancerous lesions, was significantly worse in K14CreRb\(^{f/f}\)p130\(^{-/-}\) mice compared with that in Rb\(^{f/f}\)p130\(^{-/-}\) (P = 7.7 x 10\(^{-3}\)), K14CreRb\(^{f/f}\) (P = 1.3 x 10\(^{-2}\)), and nontransgenic (P = 8.2 x 10\(^{-5}\)) mice, but still not as severe as that observed in K14E7 mice (P = 1.7 x 10\(^{-10}\)). These results indicate that the combined loss of pRb and p130 contributes to development of precancerous lesions in cervix, but is not sufficient to cause cancer to develop.

In tamoxifen-treated K14CreERtmRb\(^{f/f}\)p107\(^{-/-}\) mice, 2 of 23 mice developed cervical cancer (Table 2); this incidence of cervical cancer was not significantly different compared with that in tamoxifen-treated nontransgenic (P = 0.49) or tamoxifen-treated Rb\(^{f/f}\)p107\(^{-/-}\) (P = 0.49) mice. However, the severity of cervical disease in tamoxifen-treated K14CreERtmRb\(^{f/f}\)p107\(^{-/-}\) mice was significantly worse than that observed in either tamoxifen-treated nontransgenic (P = 1.2 x 10\(^{-5}\)) or tamoxifen-treated Rb\(^{f/f}\)p107\(^{-/-}\) (P = 5.0 x 10\(^{-5}\)) mice. All of tamoxifen-treated K14CreERtmRb\(^{f/f}\)p107\(^{-/-}\) mice developed moderate and/or severe dysplasia (Table 2), whereas most of K14CreRb\(^{f/f}\)p130\(^{-/-}\) mice developed only low to moderate-grade dysplasia (Table 1). This difference was significant (P = 3.49 x 10\(^{-7}\)), suggesting that inactivation of pRb and p107 leads to a greater propensity for neoplastic progression than does inactivation of pRb and p130. Nevertheless, the severity of disease in tamoxifen-treated K14CreERtmRb\(^{f/f}\)p107\(^{-/-}\) mice was not nearly as severe as that in tamoxifen-treated K14E7 mice (P = 5.0 x 10\(^{-5}\)). To verify that the short-term (consecutive 5 days) treatment with tamoxifen used to induce CreERtm activity before initiating treatment with estrogen does not, in of itself, have an effect on cervical carcinogenesis, we also examined both the incidence of cervical cancer and severity of cervical disease from each of non tamoxifen-treated mouse of following genotypes: nontransgenic, Rb\(^{f/f}\)p107\(^{-/-}\), K14CreERtmRb\(^{f/f}\)p107\(^{-/-}\), and K14E7 mice. No effect of short-term treatment with tamoxifen was observed in terms of either severity of disease or frequency of cancers (Supplementary Table S1). Together, these data show that combined loss of pRb and either p107 or p130 is not able to recapitulate the oncogenic potential of E7 in cervical carcinogenesis, but it partially accounts for the potential of HPV-16 E7 to develop moderate and/or severe grade of cervical intraepithelial neoplasia.

Treatment of K14E7 mice with estrogen induces not only cervical cancers but also vaginal cancers consistent with the role of high risk HPV's in vaginal cancer in women. We therefore evaluated the susceptibility of the above-described cohorts of mice inactivated for 1 or 2 pocket proteins to vaginal neoplastic disease. A similar result was observed as seen for the cervix; inactivation of two pocket proteins was not sufficient to induce vaginal neoplasia efficiently as seen in K14E7 transgenic mice (Supplementary Tables 2 and 3).

### Table 2. Histopathology summary in cervix for the pRb/p107, pRb/p107/p130 conditionally deficient mice treated with estrogen for 6 months

<table>
<thead>
<tr>
<th>Genotype(^{a,b,c})</th>
<th>Grade of cervical disease (# of mice)</th>
<th>Cancer incidence, %</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>H</td>
<td>CINI</td>
</tr>
<tr>
<td>NTG (n = 22)</td>
<td>19</td>
<td>3</td>
</tr>
<tr>
<td>K14E7(^{f/f}) (n = 24)</td>
<td>15</td>
<td>5</td>
</tr>
<tr>
<td>Rb(^{f/f})p107(^{-/-}) (n = 20)</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>K14CreERtmRbf/fp107(^{-/-}) (n = 23(^{d,e}))</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>K14CreERtmRbf/fp130(^{-/-}) (n = 15)</td>
<td>1</td>
<td>1</td>
</tr>
</tbody>
</table>

\(^{a}\)All mice were on the same CD1/129/C57 mixed genetic background (see Materials and Method for details on breeding scheme).

\(^{b}\)All of NTG, K14E7, Rb\(^{f/f}\)p107\(^{-/-}\), and K14CreERtmRb\(^{f/f}\)p107\(^{-/-}\) mice were treated with tamoxifen at starting point of this study.

\(^{c}\)All of K14CreERtmRbf/fp130\(^{-/-}\) mice were treated with 4-hydroxy tamoxifen at starting point of this study.

\(^{d}\)P = 0.072 comparing the incidence of cervical cancer in K14CreERtmRb\(^{f/f}\)p107\(^{-/-}\) versus K14E7.

\(^{e}\)P = 5.3 x 10\(^{-3}\) comparing the severity of cervical disease in K14CreERtmRb\(^{f/f}\)p107\(^{-/-}\) versus K14E7.

\(^{f}\)P = 1.2 x 10\(^{-9}\), 5.0 x 10\(^{-9}\) comparing the severity of cervical disease in K14CreERtmRb\(^{f/f}\)p107\(^{-/-}\) versus NTG, Rb\(^{f/f}\)p107\(^{-/-}\), respectively.

\(^{g}\)P = 0.022 comparing the severity of cervical disease in K14CreERtmRb\(^{f/f}\)p130\(^{-/-}\)p107\(^{-/-}\) versus K14E7.

**Contribution of the inactivation for 3 pocket proteins, pRb, p107, and p130 on cervical carcinogenesis**

To learn if inactivation for all 3 pocket proteins is able to recapitulate the oncogenic properties of E7, we treated K14CreERtmRb\(^{f/f}\)p130\(^{-/-}\)p107\(^{-/-}\) mice topically with low doses of 4-OHT to recombine the floxed alleles of pRb and p130 selectively in the lower female reproductive tract. This topical treatment was necessary because systemic delivery of even low doses of tamoxifen to K14CreERtmRb\(^{f/f}\)p130\(^{-/-}\)p107\(^{-/-}\) mice led to high morbidity/mortality (data not shown). Topical treatment with 4-OHT led to efficient disruption of expression of pRb and p130 throughout the lower reproductive tract including the vagina and the majority of the cervix without causing morbidity/mortality. In the cervical septum, which is higher up...
in the female reproductive tract and consequently more difficult to expose to 4-OHT, the pattern of loss of pRb and p130 expression was mosaic (approximately 50% of cells losing expression, compared with 100% in all other areas of the cervix). Surprisingly, when these mice were treated for 6 months with estrogen, none of 4-OHT–treated K14CreERtmRbf/ffp130f/ffp107f/C0 mice developed cervical cancer, although a high percentage of developed high-grade dysplasia (CIN3; Table 2). A similar result was observed in the vagina (Supplementary Table S3). Thus, inactivation of all 3 pocket proteins is not sufficient to account for HPV-16 E7’s potent ability to induce cancers in the lower female reproductive tract pointing to the likely importance of other cellular targets of E7 in mediating oncogenic potential. That we observed a high incidence of CIN3 and VIN3 shows that disruption of all 3 pocket proteins drives precancerous neoplastic development in the lower female reproductive tract (Table 2, Supplementary Table S3).

Expression of MCM7 in cervical stratified epithelium from the mice deficient for pRb and p107, pRb and p130, and all 3 pocket proteins

MCM7 is a potent biomarker for the detection and diagnosis of progressive cervical disease in HPV-16 transgenic mice and cervical cancer in women (33). MCM7, a component of a cellular DNA helicase (34), is upregulated by E2F-transcription factors (35) that are negatively regulated by pocket proteins (36). MCM7 induction by E7 correlates at least in part with pRb inactivation (16). We conducted MCM7 immunohistochemical staining on the cervical epithelium from mice deficient for multiple pocket proteins (Fig. 3). Consistent with our previous observations, the expression of MCM7 was robustly upregulated throughout the cervical stratified epithelium in K14E7 mice, whereas expression of MCM7 in nontransgenic mice was only detected in basal cells. MCM7 staining in cervical epithelium of both Rbf/fp107f/C0 and Rbf/fp130f/C0 mice also was mainly restricted to basal cells with some of positive staining in parabasal cells. MCM7 staining was more strongly upregulated in cervical epithelium of K14CreRbf/f/C0 mice compared with that detected in either Rbf/fp107f/C0 or Rbf/fp130f/C0 mice, but not as strong and even throughout the whole epithelium compared with that seen in K14E7 mice. MCM7 staining in the K14CreERtmRbf/fp107f/C0 and K14CreRbf/fp130f/C0 mice was more abundant than seen in K14CreRbf/f/C0 mice, particularly for the K14CreERtmRbf/fp107f/C0 mice. MCM7 staining in the K14CreERtmRbf/fp130f/C0 mice was most comparable to that seen in K14E7 mice. These observations indicate that each pocket protein can partially regulate the expression of MCM7, but inactivation of single or combinations of 2 pocket proteins is not sufficient to recapitulate fully the induction of MCM7 expression seen with HPV-16 E7.

Correlation of combinational inactivation of pocket proteins, arising from leaky CreERtm activity, with cervical dysplasia

Low level Cre activity was previously documented in the absence of tamoxifen induction in the skin of K14creERtm mice (37). Consistent with this observation, we observed a mosaic pattern of the expression of pRb (data not shown) and MCM7 (Fig. 4A) expression in cervical epithelium from 6-month estrogen-treated K14CreERtmRbf/fp107f/C0 mice that were not treated with tamoxifen. The areas with high MCM7-positive cells displayed moderate- to high-grade cervical dysplasia (Fig. 4C and 4F), whereas areas largely negative for MCM7 staining did not show this grade dysplasia. No cancers arose in these mice. The frequency of the dysplasia in the
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**K14CreERtmRbf/fp107−/−** mice that were not treated with tamoxifen (Supplementary Table S1) was not as high as in the same genotype treated with tamoxifen (Table 2). These findings provide further evidence for the leakiness of the K14CreERtm allele, and further show that inactivation of multiple pocket proteins is sufficient to drive efficient development of dysplasia, but not cancer.

**Discussion**

**The consequence of inactivating all pocket proteins in cervical carcinogenesis**

In this study, we observed that the inactivation of 2 or even all 3 pocket proteins failed to result in the efficient development of invasive cervical cancer in estrogen-treated mice. However, inactivation of pRb and p107, or all 3 pocket proteins was sufficient to drive development of high-grade dysplasia (Tables 1 and 2). These findings provide evidence that p107, and perhaps p130 to a lesser degree, act as tumor suppressors in the mouse cervix in concert with pRb. Furthermore, the combined loss of 2 pocket proteins, either pRb/p107 or pRb/p130, recapitulated at least in part the acute phenotypes induced by E7 in cervical stratified epithelium (Fig. 2). We interpret these results to indicate that the acute phenotypes of E7 are driven by inactivation of pRb and at least 1 other pocket protein, and correlate with the development of precancerous lesions, but not cancer itself. These observations support a model in which inactivation of pocket proteins drive development of benign lesions, but other activities of E7 are necessary for malignant progression. Consistent with this hypothesis, HPV-16 E7 was observed to cause cervical cancer in mice expressing a mutant form of pRb that E7 could not bind (16). Cancers did not form in these same Rb mutant mice without the expression of E7, arguing that an Rb-independent activity(s) of E7 contributes to cervical carcinogenesis. We conclude that pocket proteins-independent activities of E7 contribute to cervical carcinogenesis.

The degree of inactivation for Rb family proteins may be subtly different between two groups: floxed Rb allele/germline Rb-null allele mice versus E7 transgenic mice. Previously, it has been shown that E7 cannot completely inactivate Rb family proteins; in contrast, Cre expression in Rb-floxed allele mice results in more than 90% loss of detectable pRb in cervical epithelium (16). We suspect that this difference does not significantly affect our results for 2 reasons: (i) all of Rb family proteins were scarcely detected in Rb-knockout allele mice as well as K14E7 mice (Figs. 1 and 2) acute phenotypes and biomarker studies indicate that the effect of inactivation for Rb-knockout allele equates to that observed in K14E7 mice (Figs. 2 and 3).

**Redundancy of pocket protein family members in cervix**

Studies have reported functional overlap between pocket protein family members in the context of development and cell-cycle regulation. This functional overlap may explain both the genetic redundancy and the functional compensation among pocket protein family members (23). We observed that the expressions of p107 and to a lesser degree p130 are increased in cervical epithelium deficient for pRb (Fig. 1). A compensatory induction of p107 in response to the inactivation of pRb has been reported in several other mouse tissues (28, 38, 39). It remains unclear whether compensatory induction in expression of pocket proteins in cervical epithelium necessarily informs us on functional compensation.
Role of other cellular target(s) of E7 in cervical carcinogenesis

Using tandem affinity purification/mass spec analyses, more than 100 different cellular factors have been found associated with E7 protein (9). Which of these contribute to E7-mediated cervical carcinogenesis remains largely unclear. We have previously shown a role of inactivation of p21, one such target of E7 and a known cellular tumor suppressor, in cervical carcinogenesis (32). Thus, there is at least 1 nonpocket protein target of E7 that contributes to E7's oncogenic potential in this tissue. But, there are likely more. E7 can associate with Mi2β and histone deacetylase (40), and these interactions have led to the prediction that E7 can broadly dysregulate transcription of cellular genes through chromatin remodeling. More recently, HPV-16 E7 was discovered to associate with E2F6-containing polycomb transcriptional repressor complexes (41) that control the expression of a variety of genes through histone modification (42), and to induce the expression of histone demethylase (43). Aberrant DNA methylation patterns are known to be frequent events in cancers. DNA methylation is thought to be important in many processes including DNA repair, genome stability, and chromatin structure (44, 45). Recently, it was reported that E7 directly associates with and alters the activity of Dmnt1, a DNA methyltransferase (46). Dmnt1 is highly overexpressed in HPV-associated cancers compared with that normal tissue (17). We propose that E7's modulation of the epigenome contributes to its role in cervical cancer in a manner that synergizes with its other capabilities of dysregulating the cell cycle. Epigenetic reprogramming is thought to be a relatively late step in neoplastic progression (10, 47). Consistent with this observation, the number of genes dysregulated in the progressive disease leading to cervical cancer most dramatically increases in the transition between CIN3 and cervical cancer (Ahlquist et al., in preparation).

Differences in the importance of pocket proteins in E7-driven carcinogenesis in different tissues

The findings of this study differ greatly from our prior results in the context of head and neck cancer, another cancer type caused by high risk HPV's in which E7 is again the primary driver (48). Inactivation of pRb and p107 together were found to induce susceptibility to head and neck cancers as efficiently as did E7 (30). A less dramatic synergy was seen with the inactivation of both pRb and p130. This is in striking contrast to findings in the current study investigating cervical carcinogenesis, in which inactivation of all three, let alone 2 pocket proteins was unable to efficiently induce cervical cancer. What can explain this difference? One possibility is the manner in which cancer is induced in these different tissues. For cervical cancer, we use estrogen that likely acts primarily as a reversible promoter (49) through its activation of its receptor ERα (50). In contrast, in the head and neck mouse model we use a mutagen, 4-NQO, that likely is driving the accumulation of genetic changes in cells. Another possibility is that this difference in susceptibility reflects differences in the tissue type, not the carcinogen. For instance, p107 is able to act as a tumor suppressor in the context of both retinoblastoma and nonsmall cell lung cancer in an Rb-deficient background (25, 27). Whereas, p130 can act as a tumor suppressor in the context of small cell lung carcinoma as well as non–small cell lung carcinomas in an Rb/p53-deficient background (26, 28). These findings support our hypothesis that tumor susceptibility may differ depending on the tissue context.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Authors’ Contributions

Conception and design: M.-K. Shin, P.F. Lambert
Development of methodology: M.-K. Shin, J. Sage
 Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): P.F. Lambert
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): M.-K. Shin, P.F. Lambert
 Writing, review, and/or revision of the manuscript: M.-K. Shin, J. Sage, P.F. Lambert
 Study supervision: P.F. Lambert

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