



Published in final edited form as:

Cancer Res. 2008 April 15; 68(8): 2622–2631. doi:10.1158/0008-5472.CAN-07-5266.

p53 Loss Synergizes with Estrogen and Papillomaviral Oncogenes to Induce Cervical and Breast Cancers

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Abstract

Whereas the tumor suppressor *p53* gene is frequently mutated in most human cancers, this is not the case in human papillomavirus (HPV)-associated cancers, presumably because the viral E6 oncoprotein inactivates the p53 protein. The ability of E6 to transform cells in tissue culture and induce cancers in mice correlates in part with its ability to inactivate p53. In this study, we compared the expression of the HPV16 E6 oncogene to the conditional genetic disruption of *p53* in the context of a mouse model for cervical cancer in which estrogen is a critical cofactor. Nearly all of the *K14Crep53^{fl/fl}* mice treated with estrogen developed cervical cancer, a stark contrast to its complete absence in like-treated *K14E6^{WT}p53^{fl/fl}* mice, indicating that HPV16 E6 must only partially inactivate p53. p53-independent activities of E6 also contributed to carcinogenesis, but in the female reproductive tract, these activities were manifested only in the presence of the HPV16 E7 oncogene. Interestingly, treatment of *K14Crep53^{fl/fl}* mice with estrogen also resulted in mammary tumors after only a short latency, many of which were positive for estrogen receptor α . The majority of these mammary tumors were of mixed cell types, suggestive of their originating from a multipotent progenitor. Furthermore, a subset of mammary tumors arising in the estrogen-treated, p53-deficient mammary glands exhibited evidence of an epithelial to mesenchymal transition. These data show the importance of the synergy between estrogen and p53 insufficiency in determining basic properties of carcinogenesis in hormone-responsive tissues, such as the breast and the reproductive tract.

Introduction

Infection with “high-risk” mucosal-tropic or human papilloma-viruses (HPV) is etiologically associated with the development of several human cancers, including cervical cancer (1), the second leading cause of cancer-related deaths among women worldwide (2). HPV16, one of the dozen or so high-risk HPVs, is associated with more than half of all cases of HPV-related cervical cancer (3). Two HPV genes, *E6* and *E7*, are consistently up-regulated in human cervical cancers upon integration of the viral genome into the host chromosome (4,5). High-risk HPV *E6* and *E7* are considered potent oncogenes based on their transforming (6) and immortalizing (7) activities in tissue culture systems and their capacities to induce tumors in various animal models (8–11).

High-risk HPV *E6* and *E7* are multifunctional proteins and are best known for their abilities to bind and inactivate p53 (12,13) and pRb (14,15), respectively. We previously investigated the functional contributions of both *E6* and *E7* in cervical cancer in transgenic mice, which

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Note: Supplementary data for this article are available at Cancer Research Online (<http://cancerres.aacrjournals.org/>).

express these genes either individually or in combination. These studies made use of *K14E6^{WT}* and *K14E7^{WT}* transgenic mice in which the HPV16 *E6* and *E7* oncogenes were directed in their expression from the human keratin 14 (K14) promoter, which is specifically active in the basal compartment of stratified epithelia including that of the cervix. Treatment of *K14E6^{WT}* and *K14E6^{WT}E7^{WT}* transgenic mice with exogenous estrogen resulted in cervical cancer after 6 months of treatment with estrogen (16). None of the *K14E6^{WT}* transgenic mice, however, developed high-grade lesions or cancers unless the treatment period was extended to 9 months (17). The ability of E6 to inactivate p53 is thought to be an important contributing factor in cervical carcinogenesis. Consistent with this premise, *K14E6^{I28T}* transgenic mice, which express a mutant form of the E6 protein hindered in degrading p53 (18,19), had fewer and smaller cervical cancers (17). This mutant form of E6, however, is also deficient in other activities, including the ability to bind cellular partners that recognize E6 through leucine-rich motifs, including E6AP and E6BP (19). Because *p53* is an important tumor suppressor and presumably an important target of *E6*, it was of interest to investigate further the specific contribution of *p53* in cervical cancer.

To determine the role of *p53* in cervical carcinogenesis, we treated mice with estrogen for 6 months that had both alleles of *p53* conditionally deleted via the *Cre-lox* system in all stratified epithelia, including the epithelia lining the reproductive tract by using *Cre* driven by the K14 promoter (*K14Cre*). We made use of conditional *p53*-null (*Crep53^{fl/fl}*) mice, as mice homozygous for a germ-line deletion of *p53* succumb to lymphomas and sarcomas early in life (20) and therefore were not predicted to survive the 6 months of treatment with exogenous estrogen, a required cofactor for cervical cancer in HPV transgenic mice (16,21). Moreover, use of *K14Crep53^{fl/fl}* mice permitted us to determine the importance of *p53* inactivation specifically in the relevant epithelial cell type in which HPV infections and associated cancers arise and eliminate any potential influence that inactivation of *p53* in the stroma might confer. Finally, the conditional *p53* mouse model allowed us to investigate the contributions of *p53*-dependent and *p53*-independent activities of HPV E6 in tumorigenesis and whether these activities synergize with those of HPV E7.

Conditional loss of *p53* in stratified epithelia led to the efficient induction of cervical cancer when *K14Crep53^{fl/fl}* mice were treated with estrogen for 6 months. HPV E6 must not completely inactivate *p53* because *K14E6^{WT}* (16) or *K14E6^{WT}p53^{fl/fl}* (this study) mice did not develop cervical cancers when only treated with estrogen for 6 months. *K14Crep53^{fl/fl}* mice developed nonreproductive tumors in the absence of estrogen. These tumors consisted primarily of squamous cell carcinomas and mammary tumors, similar to those found in previous studies that did not include treatment with exogenous estrogen (22–24). Interestingly, mammary tumors developed with a shortened latency in *K14Crep53^{fl/fl}* mice treated with estrogen, were of mixed cell types, and were estrogen receptor α (ER α) positive. These observations suggest that estrogen and the loss of *p53* synergize to result in early-onset tumors that are dependent on estrogen and may arise from multipotent progenitor cells in the mammary gland. Furthermore, a subset of the mammary tumors arising in the *K14Crep53^{fl/fl}* mice treated with estrogen exhibited signs indicative of an epithelial to mesenchymal transition (EMT), including loss of E-cadherin and the expression of vimentin (25,26). Addition of HPV *E6* and/or *E7* onto the *K14Crep53^{fl/fl}* background significantly shortened tumor latency and increased tumor multiplicity in both nonreproductive and reproductive sites, indicating that *p53*-independent effects of E6 act together with or in some tissue contexts independently of HPV E7 to increase its oncogenic potential.

Materials and Methods

Mouse lines

The previously described $p53^{f/f}$ (27), $K14Cre$ (23), $K14E6^{WT}$ (11), $K14E6^{\Delta 146-151}$ ($K14E6^{\Delta}$ herein; ref. 28), and $K14E7^{WT}$ (8) mouse strains were maintained on the inbred FVB background. $K14Cre$ mice were crossed to $p53^{f/f}$ mice to generate $K14Crep53^{f/f}$ mice in which p53 is deleted by Cre-mediated excision in the basal layer of stratified epithelia. $K14E6^{WT}$, $K14E6^{\Delta}$, and $K14E7^{WT}$ transgenic mice were crossed to $p53^{f/f}$ and then intercrossed with $K14Crep53^{f/f}$ mice to generate $K14E6p53^{f/f}$, $K14E6^{\Delta}p53^{f/f}$, $K14E7^{WT}p53^{f/f}$, $K14E6^{WT}E7^{WT}p53^{f/f}$, $K14E6^{WT}Crep53^{f/f}$, $K14E6^{\Delta}Crep53^{f/f}$, $K14E7^{WT}Crep53^{f/f}$, and $K14E6^{WT}E7^{WT}Crep53^{f/f}$ mice. All mice were bred and maintained in the American Association for Accreditation of Laboratory Animal Care–approved McArdle Laboratory Animal Care Facility in accordance with an institutionally approved animal protocol.

Estrogen treatment and analysis of reproductive tracts

For acute studies, 5- to 6-wk-old virgin female mice were treated with 17 β -estradiol (0.05 mg, 60-d release pellets) for 6 wk to achieve a state of persistent estrus (29) to eliminate variability in the thickness of the reproductive epithelia. The mice were then either exposed to 12 Gy of ionizing radiation from a ^{137}Cs source or untreated and sacrificed 24 h later. One hour before sacrifice, the nucleotide analogue 5-bromo-2'-deoxyuridine (BrdUrd; 12.5 mg/mL in PBS) was injected i.p. at 10 μ L/g body weight to label any newly synthesized DNA.

For long-term cancer studies, 5-wk-old virgin female mice were either untreated or treated with 17 β -estradiol (0.05 mg, 60-d release pellets) for a period of 6 mo, after which the reproductive tracts were harvested and fixed according to previously described methods (16) and sectioned. Every tenth, 5- μ m section was stained with H&E and evaluated histopathologically, with the worst lesion scored as the final diagnosis.

Quantification of DNA synthesis and DNA damage response

To quantify the levels of DNA synthesis in the epithelium of the cervix, basal and suprabasal BrdUrd-positive cells were counted and divided by the total number of cells and multiplied by 100 to determine the percentage. To quantify the DNA damage response, the total number of BrdUrd-positive cells was counted and divided by the total number of cells in the epidermis and multiplied by 100. BrdUrd was counted in ten 40 \times microscopic fields per mouse, with a total of at least three or more mice per genotype group.

Statistical analysis

The MSTAT software program¹ was used in determining statistical significance. A two-sided Wilcoxon rank sum test was used for determining statistical significance in the quantification of DNA synthesis, tumor multiplicity, and tumor area. A two-sided Fisher's exact test was used in determining significance for cancer incidence, whereas the log-rank test was used for Kaplan-Meier analyses.

Immunohistochemistry and immunofluorescence

Immunohisto-chemistry and immunofluorescence were carried out as previously described (16,17) and described further in Supplementary Methods.

¹<http://mcardle.oncology.wisc.edu/mstat>

Results

p53 is efficiently deleted in the epithelium of the reproductive tract in *K14Cre**p53^{ff}* mice

The primary goal of this study was to determine whether inactivation of p53 in the presence of exogenous estrogen is sufficient for the induction of cervical cancer. Early development of lymphomas and other tumors in germ-line p53-null mice (20) makes it impossible to use these mice in long-term experiments. Thus, we obtained *p53^{ff}* mice that are homozygous for alleles of *p53* in which loxP sites were inserted into introns 1 and 10 (27) and mated to ones that express *Cre* driven by the human K14 promoter (*K14Cre*; ref. 23). *Cre* mediates the excision of sequences flanked by the inverted loxP sites, resulting in a frameshift within the spliced transcript arising from the *p53^{ff}* allele. This frameshift produces a short unstable gene product that is undetectable in all *K14Cre*-expressing tissues (23).

To determine whether p53 was efficiently deleted in the epithelium of the reproductive tract in *K14Cre**p53^{ff}* mice, we tested whether these mice (treated with exogenous estrogen to induce persistent estrus and thereby eliminate variability in proliferative indices due to estrus/diestrus cycling) lacked the ability to mount a DNA damage response, an activity that requires p53 (30). To induce a DNA damage response, mice were either not treated or treated with ionizing radiation and sacrificed 24 h later. One hour before sacrifice, the mice were injected with the nucleotide analogue BrdUrd to label any newly synthesized DNA. The levels of p53 and BrdUrd were assessed by performing immunohistochemistry on reproductive tissue sections. p53 was not detected in the cervical epithelium of *K14Cre**p53^{ff}* mice in contrast to *p53^{ff}* mice on irradiation, indicating that p53 was deleted in these cells (Supplementary Fig. S1A). Furthermore, cervical cancers obtained from *K14Cre**p53^{ff}* mice did not express p53 (data not shown). The number of BrdUrd-positive cells was quantified (Supplementary Fig. S1B; Fig. 1A, left). DNA synthesis was reduced upon DNA damage in *p53^{ff}* mice (1.5% unirradiated versus 0.13% irradiated; $P = 0.02$), whereas in *K14Cre**p53^{ff}* mice the levels of DNA synthesis remained similar to that in unirradiated mice (2.9% unirradiated versus 2.1% irradiated; $P = 0.28$). These data provide functional proof that p53 was efficiently deleted in the cervical epithelia of the *K14Cre**p53^{ff}* mice.

Deletion of p53 in the epithelium of the cervix results in an increase in the levels of DNA synthesis

As evident from the data presented above, the overall frequency of BrdUrd-positive cervical epithelial cells in unirradiated *K14Cre**p53^{ff}* mice (2.9%) compared with unirradiated *p53^{ff}* mice (1.5%) was increased by ~2-fold. This difference was statistically significant ($P = 0.03$). To characterize further the nature of the epithelial hyperplasia in *K14Cre**p53^{ff}* mice, the frequency of BrdUrd-positive cells within the basal versus suprabasal compartments of the cervical epithelium of *K14Cre**p53^{ff}* and *p53^{ff}* mice was compared (Fig. 1A, right). *K14Cre**p53^{ff}* mice treated with estrogen had a marginal increase in the levels of DNA synthesis in the basal layer relative to *p53^{ff}* mice (11.6% versus 5.6%, respectively; $P = 0.07$). The levels of suprabasal DNA synthesis, on the contrary, were significantly increased in *K14Cre**p53^{ff}* relative to *p53^{ff}* mice (0.8% versus 0.3%, respectively; $P = 0.03$). Thus, the epithelial hyperplasia that arose in the cervix of *K14Cre**p53^{ff}* mice is manifested in both compartments. The levels of basal and suprabasal DNA synthesis in the cervix were also quantified in *p53^{WT}* and *p53^{-/-}* mice treated with estrogen and showed similar results as *p53^{ff}* and *K14Cre**p53^{ff}* mice, respectively (data not shown).

Inactivation of p53 in the reproductive epithelium in the presence of exogenous estrogen is sufficient for carcinogenesis

Groups of *p53^{ff}* and *K14Cre**p53^{ff}* mice expressing *E6^{WT}*, *E6^A*, or *E7^{WT}* were either not treated or treated with exogenous estrogen for 6 months to determine whether deletion of p53

contributes to cervical carcinogenesis. Reproductive tracts from these mice were histopathologically analyzed to identify the worst stage of disease present by using a previously established grading system (16). Similar to previous findings, none of the untreated mice irrespective of the genotype developed cervical cancer (data not shown), even in the absence of p53. These results reiterate the observation that estrogen is a necessary cofactor in this mouse model of cervical cancer (21). As expected, none of the p53-sufficient, *p53^{ff}* mice developed high-grade lesions or cancers after 6 months of treatment with estrogen. In contrast, nearly all of the estrogen-treated *K14Crep53^{ff}* mice developed cervical cancer (88%; Fig. 1B; Table 1), which is highly significant compared with estrogen-treated *p53^{ff}* mice ($P < 4 \times 10^{-5}$). Thus, the loss of p53 in the reproductive epithelium in combination with estrogen is sufficient for the induction of cervical cancer. Similar results were observed when we analyzed the lower reproductive tract for the incidence of vaginal intraepithelial neoplasia and cancers. Specifically, *K14Crep53^{ff}* mice had high-grade lesions and cancers (Supplementary Tables S1 and S2) in the lower reproductive tract; however, compared with the cervix, these lesions were much less frequent.

The high penetrance of cervical cancer in *K14Crep53^{ff}* mice is in sharp contrast to *K14E6^{WT}p53^{ff}* mice, which did not develop any cancers in the reproductive tract after treatment with estrogen for 6 months (Table 1; Supplementary Table S1). The latter result is consistent with prior studies in which *K14E6^{WT}* mice on *p53^{WT/WT}* background also failed to develop cervical cancers after treatment with estrogen for 6 months (16). Furthermore, the incidence of cervical cancer in *K14E6^{WT}* mice was only partially penetrant (41%) when treatment with estrogen was extended to 9 months (17). The weaker oncogenic potential observed in *K14E6^{WT}* mice compared with *K14Crep53^{ff}* mice suggests that HPV16 E6 is only partially inactivating p53.

HPV E6 and E7 oncogenes synergize with the deletion of p53 to shorten tumor latency

Although conditional deletion of p53 in stratified epithelia extended the life span of these mice compared with that of germ-line p53-null mice, the absence of this important tumor suppressor in other epithelial tissues resulted in the development of nonreproductive tumors that contributed to early-onset morbidity/mortality (see below and Discussion). In particular, *K14E7^{WT}* and *K14E6^{WT}E7^{WT}* transgenic mice on the *K14Crep53^{ff}* background were highly susceptible to early-onset morbidity/mortality aside from expected problems of bladder distension (a phenotype induced by exogenous estrogen; refs. 9,29) and thymic hyperplasia due to the expression of E7 (8). A subset of the *K14E7^{WT}Crep53^{ff}* and *K14E6^{WT}E7^{WT}Crep53^{ff}* mice consequently died or had to be sacrificed prematurely after only 3.5 to 4 months of treatment with estrogen due to the added tumor burden in addition to the aforementioned problems. Reproductive tracts were harvested at the time of sacrifice and histologic examination revealed that 75% of *K14E7^{WT}Crep53^{ff}* and 100% of *K14E6^{WT}E7^{WT}Crep53^{ff}* mice had already developed cervical cancers that were quite aggressive as evidenced by the large areas of invasion. This aggressive cancer phenotype worsened with increasing duration of treatment with estrogen (Fig. 1C and D). This led us to investigate whether cancer was also developing in *K14Crep53^{ff}* and *K14E6^{WT}Crep53^{ff}* mice during this time frame. Therefore, mice from these two genotypes were specifically sacrificed at 4 months and histopathologically examined (Fig. 1C). None of the *K14Crep53^{ff}* mice sacrificed at 4 months had developed cancer, which is significantly different than *K14E7^{WT}Crep53^{ff}* mice ($P = 0.007$). One *K14E6^{WT}Crep53^{ff}* mouse did develop a small microinvasive cancer (MIC) in the lower reproductive tract. These results suggest that the HPV oncogene E7, and to a lesser extent E6, can shorten tumor latency in the cervix in the absence of p53.

Loss of *p53* results in the development of nonreproductive tumors in the skin and mammary glands, the latter with phenotypes of EMT

A previous study showed that conditional deletion of *p53* in the basal layer of the epithelium was sufficient for the development of spontaneous tumors, such as fibrosarcomas and squamous cell carcinomas, in the skin (23). We monitored various mice over a 15-month period for the development of spontaneous tumors and observed a similar spectrum of nonreproductive tumors in our study (Fig. 2A). In addition, we observed the development of two possible cases of “collision” tumors, which are rare occurrences when two or more histologically distinct neoplasms develop adjacent to one another. To look for potential gene dosage effects on tumorigenesis and latency, we monitored mice containing one or both copies of the *p53* gene disrupted. As expected, we observed an overall increase in cancer incidence (Fig. 2A) and shorter tumor latency ($P=10^{-6}$; Fig. 2B, *i*) when both alleles of *p53* were deleted. There was a highly significant reduction in median tumor latency in *K14Crep53^{ff}* mice relative to *p53^{ff}* mice (Fig. 2B, *i*), where the latter did not develop any tumors by the end point (median tumor latency, $T_{50} = 10.5$ versus >15 months; $P < 10^{-24}$).

Other studies have shown that spontaneous mammary carcinomas develop when *BRCA2* (23) or *BRCA1* (24) is conditionally deleted in combination with *p53*, with a median latency of 10 and 7.5 months, respectively. We also observed the development of mammary tumors in females on the *K14Crep53^{ff/+}* and *K14Crep53^{ff/ff}* background, without concomitant deletion of *BRCA*. Interestingly, mammary tumors developed more quickly in mice treated with exogenous estrogen, appearing as early as 3 to 4 months. These tumors were large and aggressive as surgical removal of the tumor(s) provided a minor reprieve of ~2 weeks before a quick resurgence of growth. Furthermore, treatment with estrogen resulted in tumors that were often ER α positive (Fig. 3B), in contrast to tumors obtained in the absence of exogenous estrogen (24). In the absence of exogenous estrogen, the breeding or virgin females displayed a longer tumor latency (data not shown). Various histologic types of mammary tumors (adenocarcinomas, myoepithelial carcinomas, and spindle cell carcinomas; data not shown) were observed in estrogen-treated mice on the *K14Crep53^{ff/ff}* background, many of which differentially expressed markers of both myoepithelial (K14) and luminal (keratin 8) cell types (Fig. 3A).

In addition to a shortened latency, a subset of mammary tumors arising in the estrogen-treated mice displayed spindle cell type morphology or contained mixtures of both epithelial and mesenchymal cell types. We were therefore curious to determine whether there was an occurrence of EMT in these tumors. In the course of EMT, expression of E-cadherin is often reduced, and N-cadherin increased (25). Thus, the expression of both N-cadherin and E-cadherin was evaluated by immunofluorescence. The majority of these tumors expressed both N-cadherin and E-cadherin; however, a subset of these tumors irrespective of the presence of either HPV E6 or E7 displayed a loss of E-cadherin. These tumors also expressed vimentin, an intermediate filament marker up-regulated in EMT (Fig. 3C and D; ref. 26).

Cervical cancers arising in *p53* conditionally deficient mice display alterations in the p16/pRb pathway

The cyclin kinase inhibitor *p16* has been established as a marker for high-risk HPV-positive infection in dysplastic lesions and cancers in the reproductive tract in both humans (31) and mice (17). Moreover, HPV E7 accounts for most of the induction of *p16*, as tumors from *K14E6^{WT}* transgenic mice express reduced levels of *p16* and exhibit an inverse staining pattern compared with tumors arising in *K14E7^{WT}* mice (17). The “E7” staining pattern, however, was regained when the ability of E6 to degrade *p53* was hindered in *K14E6^{I128T}* mice. Therefore, we hypothesized that cervical cancers arising in *K14Crep53^{ff}* mice would express low levels of *p16*. Immunohistochemistry for *p16* was performed on tumors from mice on the

K14Crep53^{fl/fl} background in the presence or absence of E6 and E7. Unexpectedly, tumors from *K14Crep53^{fl/fl}* mice predominantly overexpressed p16 (Fig. 4; Supplementary Table S3), a result opposite to that observed in tumors from *K14E6^{WT}* mice (17). A small subset of *K14Crep53^{fl/fl}* tumors, however, expressed lower amounts of p16, resembling more the pattern observed in *K14E6^{WT}* mice (data not shown). Tumors from *K14E6^{WT}Crep53^{fl/fl}* and *K14Crep53^{fl/fl}* mice had similar levels of p16, suggesting that deletion of p53 is dominant with regard to the induction of p16. As expected, tumors from *K14E7^{WT}Crep53^{fl/fl}* and *K14E6^{WT}E7^{WT}Crep53^{fl/fl}* mice expressed robust levels of p16 presumably due to the presence of HPV E7.

We and others have shown that the levels of p16 and pRb are inversely correlated in both tumors and tissue culture (32,33). Thus, immunohistochemistry for pRb was also performed to determine if the inverse correlation with p16 is maintained in tumors without p53 (Fig. 4). pRb was low but detectable in tumors from *K14Crep53^{fl/fl}* and *K14E6^{WT}Crep53^{fl/fl}* mice. Consistent with the ability of E7 to inactivate and degrade pRb, the levels of pRb were decreased further or undetectable in tumors from *K14E7^{WT}Crep53^{fl/fl}* and *K14E6^{WT}E7^{WT}Crep53^{fl/fl}* mice. These results suggest that the inverse relationship between p16 and pRb is maintained in tumors without p53.

Bmi-1 has been reported to regulate *p16* through targeting of the *INK4a* locus (34). *Bmi-1* is overexpressed in many different cancers (35,36) and can immortalize certain epithelial cells when expressed exogenously (37). We performed immunohistochemistry to assess the status of *Bmi-1* in cervical tumors (Fig. 4). Tumors from *K14Crep53^{fl/fl}*, *K14E6^{WT}Crep53^{fl/fl}*, *K14E7^{WT}Crep53^{fl/fl}*, and *K14E6^{WT}E7^{WT}Crep53^{fl/fl}* mice all expressed *Bmi-1*. *Bmi-1* was inversely correlated with the expression of p16 in *K14Crep53^{fl/fl}* and *K14E6^{WT}Crep53^{fl/fl}* tumors. In the presence of E7 (*K14E7^{WT}Crep53^{fl/fl}* and *K14E6^{WT}E7^{WT}Crep53^{fl/fl}*), the levels of *Bmi-1* were increased further but primarily retained an inverse relationship with p16.

The E2F-responsive gene *MCM7* has also been established as a biomarker for dysplastic lesions and cancers positive for high-risk HPV infections (38). Induction of *MCM7* likely occurs in part through the ability of E7 to target pRb for degradation, resulting in an up-regulation of E2F target genes. HPV E6 can also dysregulate the pRb pathway (39), in a manner distinct from HPV E7, leading to the up-regulation of expression of E2F target genes, such as *MCM7* (17). We assessed the status of *MCM7* by immunohistochemistry on tumors from this study (Fig. 4; Supplementary Table S3). The majority of tumors from *K14Crep53^{fl/fl}* mice expressed intermediate to high levels of *MCM7*, consistent with the pattern observed in cervical tumors from *K14E6^{WT}* mice (17). We hypothesized that the ability of E6 to hyperphosphorylate pRb (39) results in the up-regulation of the E2F-responsive gene *MCM7*. To determine whether E6 can hyperphosphorylate pRb in the tumors that arise in our mice, immunohistochemistry was performed with an antibody recognizing phosphorylated pRb specifically at Ser⁸⁰⁷ and Ser⁸¹¹, two sites that when phosphorylated can disrupt the binding of E2F to pRb (40). Tumors from both *K14Crep53^{fl/fl}* and *K14E6^{WT}Crep53^{fl/fl}* expressed phosphorylated pRb, suggesting that the inactivation of pRb by phosphorylation drives the up-regulation of E2F target genes, such as *MCM7*, in these tumors in the absence of E7. *K14E6^{WT}Crep53^{fl/fl}* tumors expressed a modest qualitative increase in the levels of phosphorylated pRb relative to *K14Crep53^{fl/fl}* tumors. This increase suggests that the ability of E6 to hyperphosphorylate pRb is in part independent of its ability to inactivate p53, consistent with previous findings (39). In the presence of E7, almost all of the pRb is degraded and only occasional pRb and phosphorylated pRb-positive cells were detected (Fig. 4).

Discussion

In this study, we compared the oncogenic potential of the HPV16 *E6* oncogene to the genetic disruption of the tumor suppressor *p53* in the context of a mouse model for cervical carcinogenesis that relies on treatment with exogenous estrogen. Conditional deletion of *p53* was sufficient for the induction of reproductive (cervical and vaginal) cancers only in the presence of estrogen and the addition of HPV E6 and E7 shortened tumor latency and produced more aggressive tumors. In the course of this study, we also discovered that estrogen and the loss of *p53* synergized to induce early onset of mammary tumors. These mammary tumors are ER α positive, composed of mixed cell types, thus suggestive of a multipotent progenitor and displayed evidence of EMT. Below, we discuss the implications of our studies in the context of understanding cervical carcinogenesis associated with HPVs as well as mammary tumorigenesis.

HPV E6 does not completely inactivate p53

We have previously shown that *K14E6^{WT}* transgenic mice treated with exogenous estrogen do not succumb to the development of high-grade lesions or cancers unless the estrogen treatment period was extended from 6 to 9 months (17). In contrast, *K14E7^{WT}* transgenic mice efficiently developed cancer after only 6 months of treatment with estrogen, suggesting that HPV E6 has a weaker oncogenic potential in the reproductive tract (16). These results are in stark contrast to previous observations in the epidermis of the skin, where E6 was more potent relative to E7 (10). Similar findings were observed in this study. None of the *K14E6^{WT}p53^{ff}* mice developed high-grade lesions or cancers after 6 months of treatment with estrogen. Treatment of *K14Crep53^{ff}* mice with estrogen, however, resulted in a wide spectrum of high-grade lesions, including cancers in both the cervix (Table 1) and the lower reproductive tract (Supplementary Tables S1 and S2). Furthermore, the incidence of large invasive cancer (LIC) in the cervix of *K14E7^{WT}Crep53^{ff}* mice (57.1%) was higher than in *K14E6^{WT}E7^{WT}p53^{ff}* mice (33%). Whereas this increase was not highly significant ($P = 0.40$), the increase in LIC in the lower reproductive tract of *K14E7^{WT}Crep53^{ff}* mice (57.1%) compared with *K14E6^{WT}E7^{WT}p53^{ff}* mice (11%) was statistically significant ($P = 0.04$). These results not only suggest that the conditional inactivation of *p53* in the reproductive epithelium is sufficient to give rise to cancer in concert with exogenous estrogen but also support the hypothesis that HPV E6 is not completely inactivating *p53* in the reproductive tract.

Similar observations were drawn from monitoring the development of nonreproductive tumors. *K14E6^{WT}p53^{ff}* mice were significantly less tumor prone as evidenced by a reduced hazard ratio (0.17), cancer incidence (25.6%), and tumor multiplicity (0.3) compared with that observed in *K14Crep53^{ff}* mice (all $P < 0.0001$; Table 2). Thus, HPV E6 is incompletely inactivating *p53* at all evaluated tissue sites. The analysis of human cervical cancers has led to the same conclusion in that some human cervical cancers still express detectable *p53* protein, indicative of E6 not completely degrading it (41,42). Thus, our mouse model for cervical cancer recapitulates a feature of human cervical cancer in that *p53* is not completely inactivated by E6.

Differences in the oncogenic potential of E6 among epithelial tissues

Whereas E6 (*K14E6^{WT}p53^{ff}*) did induce nonreproductive tumors (Table 2), it failed to induce any reproductive tumors (Table 1; Supplementary Table S1). This indicates that E6 has stronger oncogenic potential in nonreproductive sites. Tumor development in nonreproductive sites, in part, may require a lower threshold of inactive *p53*. Alternatively, *p53*-independent activities of E6 may be more influential for tumor development in nonreproductive sites compared with that in the reproductive tract. The latter possibility is supported by the earlier onset of tumors

in nonreproductive sites in *K14E6^{WT}Crep53^{ff}* compared with that in *K14Crep53^{ff}* mice (Fig. 2B, ii).

The E7 oncogene synergizes with the loss of p53 to induce cancer

p53 is regarded as the “guardian of the genome” (43) because it mediates the response of DNA damage and other stress response pathways. Our studies herein show that the deletion of *p53* is sufficient to cause the induction of LICs of the reproductive tract in the presence of exogenous estrogen. Prior studies of HPV transgenic mice, however, determined that induction of LIC in the reproductive tract was highest when both HPV E6 and E7 were expressed (16). Thus, we investigated whether placement of HPV E7 onto the *K14Crep53^{ff}* background led to a further increase in LIC. Indeed, the incidence of LIC in the cervix (Table 1) was significantly higher in *K14E7^{WT}Crep53^{ff}* relative to *K14E7^{WT}p53^{ff}* (57.1% versus 12.5%, respectively; $P = 0.02$). The difference in the incidence of LIC in the lower reproductive tract (Supplementary Table S2) was even greater (57.1% versus 0%; $P = 0.002$). In addition, HPV E7 shortened tumor latency and fostered more aggressive tumor progression when placed on the *p53*-deficient genetic background (Fig. 1C and D). Thus, HPV E7 cooperates with the loss of *p53* to increase cancer incidence, shorten tumor latency, and promote extensive tumor invasion.

p53-independent activities of HPV E6 contribute to the development of nonreproductive tumors independent of HPV E7 but only contribute to cervical carcinogenesis in the presence of HPV E7

The spectrum of histopathology observed in the *K14E6^{WT}Crep53^{ff}* and *K14Crep53^{ff}* mice was quite similar for the cervix, indicating that E6 is not contributing any *p53*-independent effects. The *p53*-independent activities of E6, however, became apparent in the presence of E7. Whereas only 57% of the *K14E7^{WT}Crep53^{ff}* mice developed LIC, 100% of the *K14E6^{WT}E7^{WT}Crep53^{ff}* mice developed LIC (Table 1). These results indicate that the *p53*-independent activities of E6 contribute to cervical carcinogenesis but only do so in synergy with some function(s) of HPV E7. This synergy was observed previously with mice on the *p53*-sufficient background (17). Interestingly, in the lower reproductive tract, the *p53*-independent activities of E6 by itself were weakly evident, as evidenced by the development of LIC in *K14E6^{WT}Crep53^{ff}*, which was not observed in the *K14Crep53^{ff}* mice, and a significantly higher tumor multiplicity ($P = 0.05$) in *K14E6^{WT}Crep53^{ff}* mice (Supplementary Table S2). In the presence of E7, the cooperative effects of the *p53*-independent activities of E6 were again manifested in the lower reproductive tract, as evidenced by the higher incidence of LIC in *K14E6^{WT}E7^{WT}Crep53^{ff}* mice (Supplementary Table S2). These observations reiterate the synergy between the *p53*-independent activities of E6 and some function(s) of E7.

Although *p53*-independent effects of E6 were only weakly evident in the lower reproductive tract and completely absent in the cervix, we clearly observed these effects, independent of E7, in nonreproductive sites. Because deletion of *p53* resulted in a high incidence of cancers at other sites (Fig. 2A), we did not observe dramatic increases in incidence in the presence of E6 and E7. However, we did note *p53*-independent effects of E6 when we compared median tumor latency ($T_{50} = 8$ versus 10.5 months; $P < 0.0001$; Table 2; Fig. 2B, ii) and tumor multiplicity in *K14E6^{WT}Crep53^{ff}* mice relative to that in *K14Crep53^{ff}* mice (3.9 versus 2.6, respectively; $P = 0.04$). Similar to the cervix, these *p53*-independent effects of E6 are likely to reflect interactions with PDZ partners given that *K14E6^ΔCrep53^{ff}* mice do not display the same increase in tumor multiplicity or shortened tumor latency. Tumor multiplicity in these mice was 2.6 and median tumor latency was 11 months (Fig. 2B, iii), which were not significantly different from that observed in the *K14Crep53^{ff}* mice ($P > 0.05$ for both comparisons). These results suggest that in nonreproductive sites *p53*-independent effects of HPV E6 enhance the oncogenic potential of E6 even in the absence of E7, whereas in the

reproductive tract p53-independent effects of E6 largely (in the lower reproductive tract) or entirely (in the cervix) require the presence of HPV E7.

As indicated above, these p53-independent activities of E6 likely include the interactions of E6 with PDZ partners. Specifically, we previously found that *K14E6^A* mice, which express a form of E6 unable to bind PDZ domain partners, have similar incidences of cancer and tumor sizes as *K14E6^{WT}* mice when treated with estrogen for 9 months. *K14E6^AE7^{WT}* mice, however, were reduced in both tumor multiplicity and tumor size when compared with *K14E6^{WT}E7^{WT}* mice (17). Thus, the interactions of E6 with PDZ partners synergize with some activity of HPV E7 in inducing cervical cancer. The specific activities of E7 necessary for this synergy with the p53-independent activities of E6 have yet to be determined. Human cervical cancers have altered localization and expression of Dlg and Scribble (44,45), two PDZ partners of E6. Based on the aforementioned findings, the roles of *Dlg* and *Scribble* warrant further investigation to determine whether they are indeed modulated by E6 as part of its p53-independent effects that synergize with E7 in cervical carcinogenesis.

Modulation of the p16/pRb pathway

Analysis of various biomarkers surprisingly revealed that cervical cancers from *K14Crep53^{ff}* mice have similar expression patterns as tumors expressing HPV E7. From previous results, we expected an expression pattern similar to *K14E6^{WT}* tumors, where levels of p16 were low and levels of pRb were high. Instead, we observed predominantly elevated levels of p16 and low levels of pRb. Observations from this study show that HPV E6 does not completely inactivate p53. We conclude that the complete inactivation of p53 in *K14Crep53^{ff}* mice alters the mechanism of regulating the p16/pRb pathway differently from that caused by HPV E6, which only partially inactivates p53. Tumors in the *K14Crep53^{ff}* mice also express low levels of Bmi-1 and pRb, indicating that there is a lack of cell cycle checkpoints and regulation of p16 activity, at least by Bmi-1. An inverse relationship between Bmi-1 and p16 has been observed in some (35,36) but not all (46) human cancers. In our studies of cervical tumors on a p53-deficient background, we also saw an inverse relationship but with converse results (i.e., we observed heightened levels of p16 and modest levels of Bmi-1). Furthermore, pRb that was observed in the p53-deficient tumors included forms that were phosphorylated and correlated with heightened levels of the E2F-responsive gene *MCM7*. Thus, in tumors arising in p53-deficient epithelial tissues of mice treated with estrogen, the p16/pRb pathway is grossly dysregulated.

Loss of p53 synergizes with estrogen to induce a shortened latency for the development of mammary tumors, some with properties of EMT

Originally, mammary tumors were not detected in *K14Crep53^{ff}* mice without conditional deletion of *BRCA2* (23). However, recent follow-up studies determined that spontaneous breast cancers do develop in *K14Crep53^{ff}* mice, with a median latency of 11 months (22), or when either a MMTV- or WAP-directed Cre was used to disrupt the floxed p53 allele (47). In our studies, we also observed mammary tumors in female *K14Crep53^{ff}* in the absence of any deletion of *BRCA2* or *BRCA1*. Importantly, we also observed that treatment of *K14Crep53^{ff}* mice with exogenous estrogen greatly shortened the time of onset of mammary tumors. Similar findings for p53 and breast cancer have been observed in transplantation studies of p53-null mammary glands into p53 wild-type BALB/c mice where exogenous hormones, such as estrogen, significantly shortened tumor latency relative to untreated mice (48). These observations suggest that exogenous estrogen is driving proliferation and enhancing tumorigenicity in the mammary gland deleted for p53.

Approximately 70% of all human breast cancers are ER α positive (49) and many of these tumors are estrogen dependent. Estrogen dependency is not frequently observed in mouse

models for human breast cancer. In our study, exogenous estrogen not only shortened tumor latency but also resulted in a shift in the nuclear hormone receptor ER α status from completely negative (23) to predominantly positive (this study; Fig. 3B). These results lead us to predict that our mouse model system produces estrogen-dependent mammary tumors. The mammary tumors displayed characteristics of both myoepithelial and luminal cell types consistent with the tumors arising from a multipotent progenitor cell. Furthermore, the loss of p53 and exogenous estrogen resulted in phenotypes suggestive of an EMT occurrence in a subset of the mammary tumors. EMT is generally associated with a more aggressive disease, as cancer cells can migrate and metastasize to other sites to establish secondary tumors. These results suggest that estrogen not only increased tumor incidence and shortened tumor latency but also altered the tumor phenotype to one more representative of human breast cancer.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

Grant support: CA098428, CA022443, and CA009135.

We thank Drs. Caroline Alexander, Norman Drinkwater, and Bill Sugden for critical reading of this manuscript; members of the Lambert laboratory for insightful discussions; Dr. Caroline Alexander for assistance in the analysis of mammary tumors; Dr. Chris Bradfield for access to equipment for tumor measurements; and Dr. Anton Berns for sharing the *K14Cre* and *p53^{fl/fl}* mouse strains.

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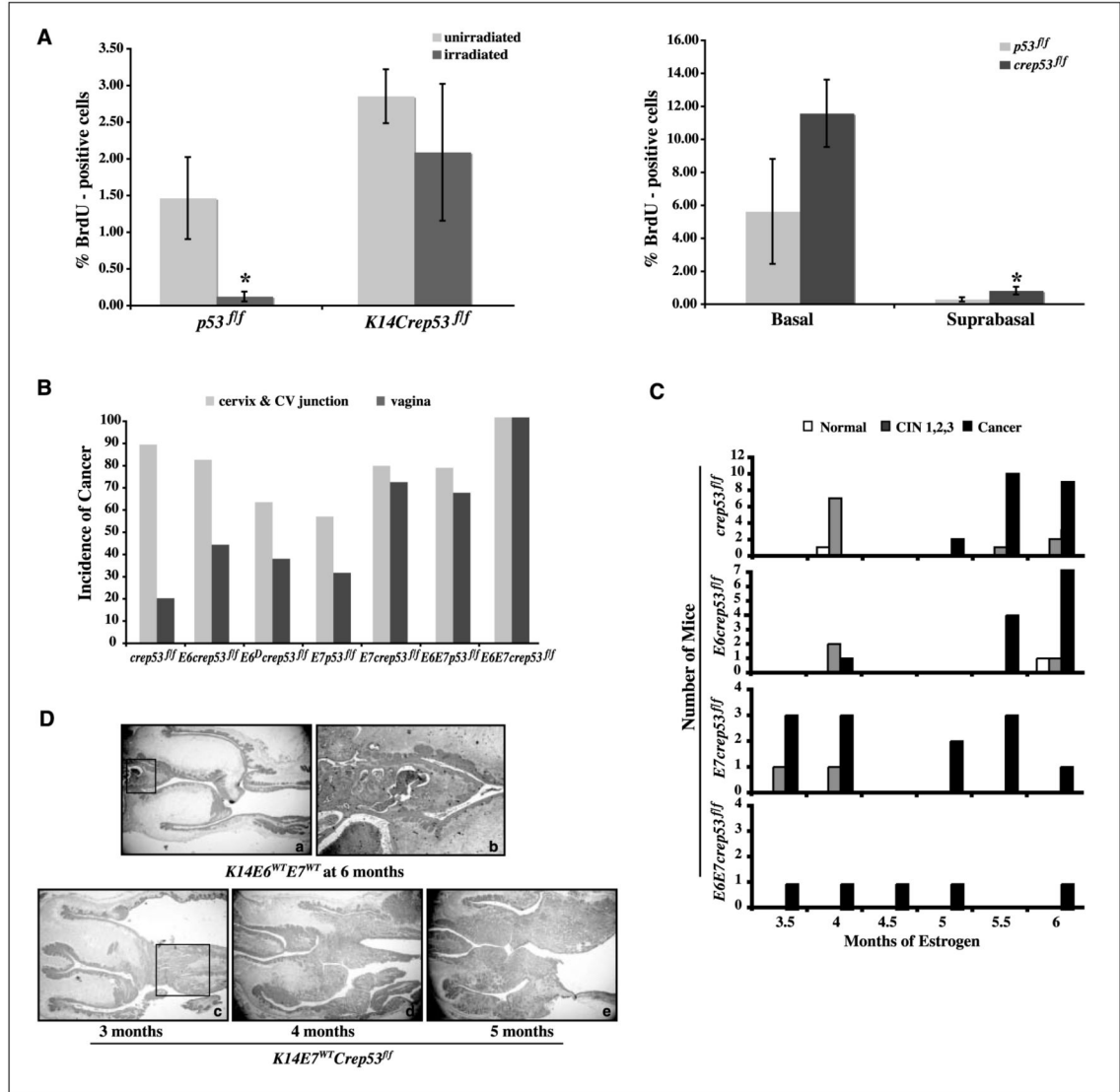


Figure 1. Acute and long-term carcinogenic properties of p53 insufficiency in the mouse cervix. *A*, conditional deletion of p53 results in loss of activity and the induction of epithelial hyperplasia in the cervix. *Left*, quantification of BrdUrd-positive cells in the cervical epithelium in response to radiation. *p53^{fl/fl}* mice show a significant reduction in the amount of BrdUrd-positive cells. In contrast, *K14Cre p53^{fl/fl}* mice maintained similar levels of DNA synthesis on irradiation. The decrease in DNA synthesis on radiation in *p53^{fl/fl}* is significant ($P = 0.02$), whereas no significant change is observed in *K14Cre p53^{fl/fl}* mice ($P = 0.28$). *Right*, quantification of BrdUrd-positive cells in both the basal and suprabasal layers of the cervix. There is a significant increase in the levels of DNA synthesis in the suprabasal layers of *K14Cre p53^{fl/fl}* ($P = 0.03$), whereas the increase in the basal layers is marginal compared with *p53^{fl/fl}* mice ($P = 0.07$). All acute mice were treated with estrogen for 6 wk. For pictures of immunohistochemistry, see Supplementary Fig. S1. *B*, comparison of cancer incidence in both the cervix and lower reproductive tract in various genotypes of mice treated with exogenous estrogen for 6 mo. *C*, comparison of tumor latency in various genotypes treated with estrogen and sacrificed at the indicated time points. Large aggressive cervical cancer arises after 3.5 mo of estrogen in both *K14E7Cre p53^{fl/fl}* and

K14E6^{WT}E7^{WT}Crep53^{ff} mice. *K14Crep53^{ff}* mice do not develop any cancer until after 5 mo of estrogen. There is one small MIC in *K14E6Crep53^{ff}* mice at 4 mo. *D*, comparison of the aggressive nature of cancers in *K14E7Crep53^{ff}* mice relative to *K14E6^{WT}E7^{WT}*. *Top row*, large cancer from a *K14E6^{WT}E7^{WT}* mouse filling the cervical septum after 6 mo of estrogen. The tumor in *a* is demarcated by the black square and is magnified in *b* ($\times 40$). *Bottom row*, cancers from *K14E7Crep53^{ff}* mice taken after 3 (*c*), 4 (*d*), or 5 (*e*) mo of estrogen. Note the large areas of tumor invasion by 4 mo (*d*) and almost the entire area in the reproductive tract is covered with tumors by 5 mo (*e*). All images, except *b*, are taken with a 1.25 \times objective.

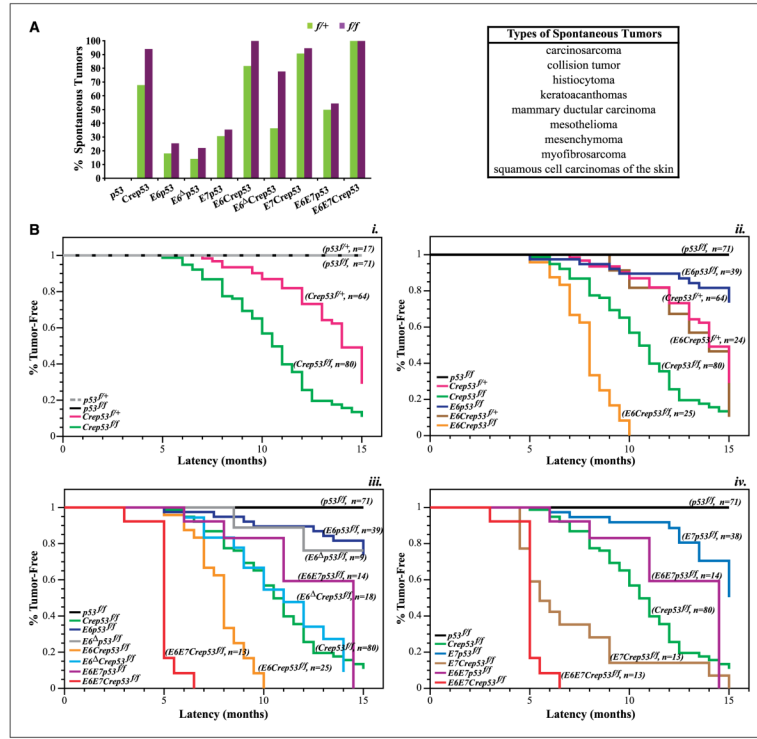


Figure 2. Loss of p53 results in the development of spontaneous nonreproductive tumors. *A*, comparison of spontaneous tumor formation in mice of various genotypes containing either one or two floxed p53 alleles in the presence or absence of K14Cre. Cancer incidence increases when both alleles of p53 are excised. *Right*, list of the various types of tumors that develop in these mice. *B*, Kaplan-Meier curves of tumor-free survival in mice of various genotypes. *i*, comparison of tumor-free survival in p53^{fl/+} (gray dashed line), p53^{fl/fl} (black), K14Crep53^{fl/+} (magenta; T₅₀ = 14 mo), and K14Crep53^{fl/fl} (green; T₅₀ = 10.5 mo). Deletion of both alleles of p53 (K14Crep53^{fl/fl}) decreases the median tumor-free survival from 14 to 10.5 mo ($P = 10^{-6}$). *ii*, comparison of genotypes from the previous graph plus K14E6^{WT}p53^{fl/fl} (blue), K14E6^{WT}Crep53^{fl/+} (brown; T₅₀ = 14 mo), and K14E6^{WT}Crep53^{fl/fl} (orange; T₅₀ = 8 mo). Again, deletion of both alleles of p53 significantly shortens tumor latency in the presence of HPV E6 (K14E6^{WT}Crep53^{fl/fl} versus K14E6^{WT}Crep53^{fl/+}; $P = 1.24 \times 10^{-8}$). *iii*, additional comparison of K14E6^Ap53^{fl/fl} (gray), K14E6^ACrep53^{fl/fl} (turquoise), K14E6^{WT}E7^{WT}p53^{fl/fl} (fuchsia), and K14E6^{WT}E7^{WT}Crep53^{fl/fl} (red). HPV E6 synergizes with loss of p53 to shorten tumor latency in K14E6^{WT}Crep53^{fl/fl} (orange; T₅₀ = 8 mo) relative to K14Crep53^{fl/fl} (green; T₅₀ = 10.5 mo; $P = 4 \times 10^{-8}$). Loss of PDZ interactions in K14E6^ACrep53^{fl/fl} does not result in a shortened latency (turquoise; T₅₀ = 11 mo) as in K14E6^{WT}Crep53^{fl/fl} (orange; T₅₀ = 8 mo; $P = 0.0003$) and is similar to K14Crep53^{fl/fl} mice ($P = 0.82$). *iv*, comparison of tumor-free survival in K14E7^{WT}p53^{fl/fl} (blue), K14E7^{WT}Crep53^{fl/fl} (brown), K14E6^{WT}E7^{WT}p53^{fl/fl} (fuchsia), and K14E6^{WT}E7^{WT}Crep53^{fl/fl} (red) mice. HPV E7 synergizes with loss of p53 to shorten tumor latency in K14E7^{WT}Crep53^{fl/fl} compared with K14E7^{WT}p53^{fl/fl} (T₅₀ = 5.5 mo; $P = 5 \times 10^{-9}$).

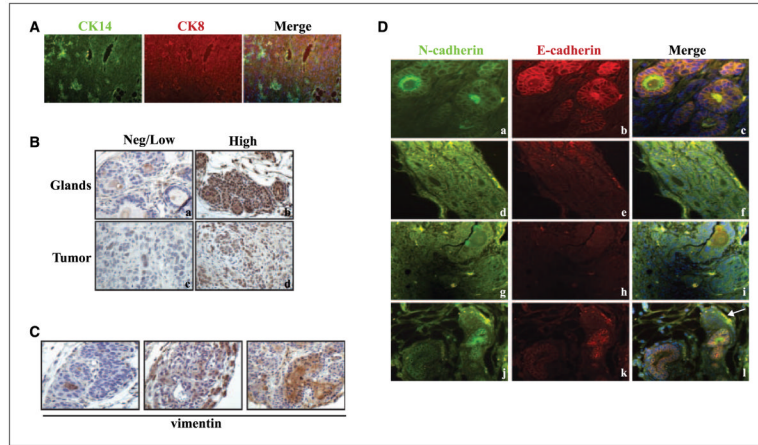


Figure 3.

Loss of p53 in the presence of estrogen results in spontaneous ER α -positive and ER α -negative mammary tumors. *A*, cross-sections of mammary tumors. Sections were stained by immunofluorescence for cytokeratin 8 (CK8; red) and cytokeratin 14 (CK14; green). Nuclei are shown by 4',6-diamidino-2-phenylindole (DAPI) stain. *B*, cross-sections of mammary glands (*a* and *b*) attached to solid tumors (*c* and *d*) taken from mice on the *K14Cre*p53^{fl/fl} background and stained for ER α (1:100; brown nuclei). Both low and high levels of ER α were observed. *C*, cross-sections of mammary glands from tumors that had reduced levels of E-cadherin and evaluated for the expression of vimentin by immunohistochemistry (brown). *D*, cross-sections of mammary glands attached to solid tumors taken from *K14Cre*p53^{fl/fl} mice. Sections were stained by immunofluorescence for N-cadherin (green; *a*, *d*, *g*, and *j*) and E-cadherin (red; *b*, *e*, *h*, and *k*). Nuclei are shown in DAPI. Both N-cadherin and E-cadherin were observed in the majority of glands as shown in *c*. In a subset of tumors, expression of E-cadherin was decreased or lost (*e*, *h*, and *k*). The arrow in *l* points to a section of the gland that has lost expression of E-cadherin, whereas the connected neighboring tissue retains expression of both N-cadherin and E-cadherin.

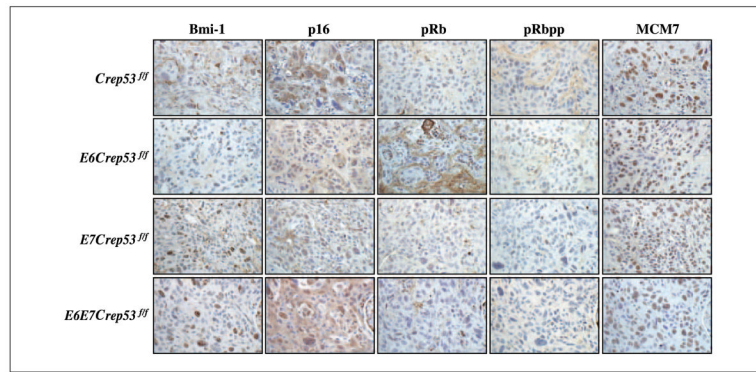


Figure 4.

Evaluation of the p16/pRb pathway in tumors without p53. Images are cross-sections of cervical tumors taken from mice of the indicated genotypes and evaluated for levels of Bmi-1, p16, pRb, phospho-Rb (*pRbpp*), and MCM7 (*brown nuclei*) expression. Both tumors from *K14Crep53^{fl/fl}* and *K14E6^{WT}Crep53^{fl/fl}* mice have low expression of Bmi-1, pRb, and phospho-Rb, whereas expression of p16 and MCM7 was higher. Both tumors from *K14E7^{WT}Crep53^{fl/fl}* and *K14E6^{WT}E7^{WT}Crep53^{fl/fl}* mice have similar expression patterns, such that Bmi-1, p16, and MCM7 were high and expression of pRb and phospho-Rb was low (more detailed description in Supplementary Table S3).

Table 1

Cervical histopathology from mice treated with exogenous estrogen

Genotype	NH	CIN1	CIN2	CIN3	MIC	LIC	Cancer incidence (%)	LIC incidence (%)	Tumor multiplicity
<i>p53^{fl/fl}</i> (n = 7)	5	1	1				0	0	0.00
<i>K14Cre;p53^{fl/fl}</i> (n = 25)	1			2	13	9	88	36	2.32
<i>K14E6^{WT}p53^{fl/fl}</i> (n = 22)	10	9	3				0	0	0.00
<i>K14E6^Δp53^{fl/fl}</i> (n = 10)	3	5	1	1			0	0	0.00
<i>K14E6^{WT}Cre;p53^{fl/fl}</i> (n = 16)	2			1	8	5	81.3	31.3	2.00
<i>K14E6^ΔCre;p53^{fl/fl}</i> (n = 16)	2		1	3	8	2	62.5	12.5	2.25*
<i>K14E7^{WT}p53^{fl/fl}</i> (n = 16)	1	1	1	4	7	2	56.3	12.5	1.50
<i>K14E7^{WT}Cre;p53^{fl/fl}</i> (n = 14)	1	1	1	1	3	8	78.6	57.1 [†]	4.18 [‡]
<i>K14E6^{WT}E7^{WT}p53^{fl/fl}</i> (n = 9)				2	4	3	77.7	33	3.00
<i>K14E6^{WT}E7^{WT}Cre;p53^{fl/fl}</i> (n = 5)					5	5	100	100	ND

NOTE: All mice were treated with estrogen. None of the *K14E6^{WT}p53^{fl/fl}* mice developed any high-grade lesions or cancers, which is significantly different than *K14Cre;p53^{fl/fl}* mice ($P < 10^{-9}$, two-sided Fisher's exact test). Tumor multiplicity was not determined in *K14E6^{WT}E7^{WT}Cre;p53^{fl/fl}* mice due to difficulty in delineating individual tumors.

Abbreviation: ND, not determined.

* Tumor multiplicity in *K14E6^ΔCre;p53^{fl/fl}* mice is not significant relative to either *K14Cre;p53^{fl/fl}* or *K14E6^{WT}Cre;p53^{fl/fl}* ($P > 0.05$, two-sided Wilcoxon rank sum test).

[†] Incidence of LIC in *K14E7Cre;p53^{fl/fl}* is significant relative to *K14E7p53^{fl/fl}* ($P = 0.02$, two-sided Fisher's exact test).

[‡] Tumor multiplicity in *K14E7Cre;p53^{fl/fl}* mice is marginally significant relative to *K14E7p53^{fl/fl}* ($P = 0.06$, two-sided Wilcoxon rank sum test).

Table 2

Comparison of spontaneous nonreproductive tumor development

Genotype	Cancer incidence (%)	Tumor multiplicity	Median tumor latency (mo)
<i>p53^{ff}</i> (n = 71)	0	0	0
<i>K14Crep53^{ff}</i> (n = 80)	94.1	2.41	10.5
<i>K14E6^{WT}p53^{ff}</i> (n = 39)	25.6*	0.30*	-*
<i>K14E6^Ap53^{ff}</i> (n = 9)	22.2*	0.25*	-
<i>K14E6^{WT}Crep53^{ff}</i> (n = 25)	100	3.88 [†]	8 [†]
<i>K14E6^ACrep53^{ff}</i> (n = 18)	77.8 [‡]	2.57	11 [‡]
<i>K14E7^{WT}p53^{ff}</i> (n = 38)	35.5 [§]	1.27 [§]	-. [§]
<i>K14E7^{WT}Crep53^{ff}</i> (n = 24)	94.7	2.33	5.5*
<i>K14E6^{WT}E7^{WT}p53^{ff}</i> (n = 14)	54.5	1.40	-
<i>K14E6^{WT}E7^{WT}Crep53^{ff}</i> (n = 13)	100	4.17	5

NOTE: All statistics were two sided (further descriptions in Materials and Methods). These mice were not treated with estrogen.

* Significant relative to *K14Crep53^{ff}* ($P < 0.0001$).[†] Significant relative to *K14Crep53^{ff}* ($P = 0.04$).[‡] Significant relative to *K14E6^{WT}Crep53^{ff}* ($P = 0.02$).[§] Significant relative to *K14E7^{WT}Crep53^{ff}* ($P < 0.001$).