Sensitivity of the Cervical Transformation Zone to Estrogen-induced Squamous Carcinogenesis

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ABSTRACT

Regions where one type of epithelium replaces another (metaplasia) have a predilection for cancer formation. Environmental factors are closely linked to metaplastic carcinogenesis. In particular, cervical cancers associated with human papillomavirus (HPV) infection develop primarily at the transformation zone, a region where metaplastic squamous cells are detected in otherwise columnar epithelial-lined endocervical glands. Previously, we reported estrogen-induced multistage vaginal and cervical carcinogenesis in transgenic mice expressing HPV16 oncogenes in basal squamous epithelial cells. In the present study to investigate the threshold neoplastic response to exogenous estrogen, we treated groups of transgenic mice with lower hormone doses. A 5-fold reduction in estrogen dose induced squamous carcinogenesis solely at the cervical transformation zone compared with other reproductive tract sites. Further study delineated stages of transformation zone carcinogenesis, including formation of hyperplastic lower uterine glands and emergence of multiple foci of squamous metaplasia from individual stem-like glandular reserve cells, followed by neoplastic progression of metaplasia to dysplasia and squamous cancer. We propose that a combination of low-dose estrogen and low-level HPV oncogene expression biases transformation zone glandular reserve cells toward squamous rather than columnar epithelial fate decisions. Synergistic activation of proliferation by viral oncoprotein cell cycle dysregulation and estrogen receptor signaling, together with altered paracrine stromal-epithelial interactions, may conspire to support and promote neoplastic progression and cancer formation.

INTRODUCTION

Epithelial carcinogenesis frequently arises from metaplasia, a process in which a particular cell type, normally resident in a different tissue or organ, is found in another tissue (1). Common features of metaplasia include alterations of stem cell fate decisions and epithelial-stromal tissue remodeling (2). Metaplasia occurs during development and sexual maturation, but its appearance as an adaptive response to noxious environmental stimuli appears to be the first stage of several types of epithelial cancers. Examples of metaplastic epithelial carcinogenesis include (a) columnar cell metaplasia (Barrett's esophagus), chronic gastroesophageal acid reflux, and esophageal adenocarcinoma (3); (b) gastric intestinal metaplasia, Helicobacter pylori infection, and gastric cancer (4, 5); (c) stratified squamous metaplasia, chronic smoking, and lung cancer; and (d) glandular squamous metaplasia, “high-risk” HPV3 infection, and uterine cervical cancer (6, 7). Despite an increasing understanding of the molecular controls of epithelial carcinogenesis in general (8), the precise mechanisms underlying induction of epithelial metaplasia or its predilection for carcinogenesis are obscure.

Cervical carcinogenesis associated with HPV primarily affects metaplastic squamous epithelium in a specific anatomical location, the transformation zone. The topography and natural history of this region is complex and dynamic, affected by age, hormonal status, pregnancy, and parity (1). In adult women, the transformation zone usually is on the vaginal surface of the cervix, an irregular line of demarcation dividing one type of epithelium from another (1). Microscopically, columnar epithelium lines both the endocervical canal and associated endocervical glands, whereas squamous epithelium covers the outer cervix. During cervical squamous metaplasia, foci of squamous cells are detectable among the endocervical glandular columnar epithelium (1). HPV appears to be tropic for the cervical transformation zone during infection and persistent disease. The transformation zone may have a unique estrogen metabolism compared with other reproductive tract epithelial cell types producing hormone metabolites with direct genotoxicity (9).

We investigated mechanisms of multistage HPV-associated squamous carcinogenesis in K14-HPV16 transgenic mice containing the entire HPV16 early region cloned behind the human keratin-14 promoter and expressing viral oncogenes in basal squamous epithelial cells (10–13). Chronic estrogen treatment induced squamous cancers predominantly in the vagina and outer cervix of transgenic mice (14). In the present study, we discovered that a 5-fold reduction in hormone dosage, compared with our original study (14), produced multistage carcinogenesis restricted solely to the cervical transformation zone of transgenic mice. Transformation zone carcinogenesis exhibits a biology distinctive from that of our previous model. Multifocal glandular squamous metaplasia develops in the lower uterus, and metaplastic foci progress to high-grade dysplasia and invasive cancers. Expansion of a basal squamous cell population potentially expressing both estrogen receptor-α and the HPV transgene may also underlie synergism between viral oncoproteins and estrogen.

MATERIALS AND METHODS

Mouse Breeding, Care, and Hormone Treatment. Subcutaneous continuous release pellets that deliver 17β-estradiol at doses of 0.25, 0.10, 0.05, and 0.01 mg over 60 days (Innovative Research of America, Sarasota, FL) were implanted in the dorsal back skin of heterozygous 1-month-old K14-1203#1 transgenic mice (15). Groups of mice were treated with hormone for 1, 3, 4, 5, and 6 months. Those groups treated for longer than 2 months had two or three separate pellet insertions. Mice were housed in a pathogen-free barrier facility, and all procedures were approved by the University of California, San Francisco, Committee on Animal Research.

Animal Sacrifice, Perfusion, and Dissection. As described previously (13, 15), mice received i.p. injections of 100 mg/kg BrdUrd, and under Avertin anesthesia, were sacrificed 2 h later by perfusion of the ascending aorta with 3.75% paraformaldehyde. Reproductive tracts and surrounding soft tissue, including any lymph nodes, were dissected and postfixed overnight at 4°C. The posterior wall of the vagina was removed, and the tissues were rinsed in PBS, dehydrated through graded alcohols and xylene, oriented with the cut vaginal
surface facing downward, embedded in paraffin, and 5-μm sections were stained with H&E.

Keratin Immunohistochemistry. Immunoperoxidase staining for keratin-14 expression was performed as described previously (11, 15). Sections were incubated with a rabbit antimouse K14 antibody (BAbCO), 1:10,000 in 3% BSA, overnight at 4°C. Immunoperoxidase staining was performed using 3,3′-diaminobenzidine (cat. no. D5637; Sigma), and the sections were counterstained with hematoxylin.

Tissue DNA Synthesis. Immunohistochemistry to detect BrdUrd incorporation was performed as described previously (13, 15). The number of diamino-

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**Table 1**  
Total number of study mice and subset of mice treated with 17β-estradiol for 6 months

<table>
<thead>
<tr>
<th>Dose 17β-estradiol (mg/60-day release)</th>
<th>Entire study</th>
<th>6-month treatment</th>
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<tr>
<td></td>
<td>TG(^a)</td>
<td>NTG</td>
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<tr>
<td>0.25 mg/60-day 17β-estradiol</td>
<td>25</td>
<td>13</td>
</tr>
<tr>
<td>0.10 mg/60-day 17β-estradiol</td>
<td>38</td>
<td>33</td>
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<tr>
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<td>45</td>
<td>41</td>
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<td>21</td>
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<tr>
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<td>12</td>
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<td>0.10 mg/60-day placebo</td>
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<td>0.01 mg/60-day placebo</td>
<td>8</td>
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\(^a\) TG, transgenic; NTG, nontransgenic.  
\(^b\) Percentage of TG and NTG groups.  
\(^c\) Percentage of TG and NTG groups treated with 17β-estradiol for 6 months.

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Fig. 1. Different doses of estrogen induce cancers distributed throughout the reproductive tract. *a*, schematic of the murine female reproductive tract, indicating regions analyzed. *b*, reproductive tract histopathology after 6 months of treatment with different doses of 17β-estradiol. *Panels 1 and 2*, vulvar and vaginal cancers in transgenic mice treated with 0.25 mg/60-day 17β-estradiol (arrowheads delineate the extent of cancer). *Panel 3*, an invasive cancer replacing the entire outer cervix in a transgenic mouse treated with 0.10 mg/60-day 17β-estradiol. *Panel 4*, multifocal transformation zone cancer (arrowheads) and extensive glandular hyperplasia and multifocal squamous metaplasia in a transgenic mouse treated with 0.05 mg/60-day 17β-estradiol (panel 5) or placebo (mouse in estrus; panel 6) demonstrate mild squamous epithelial thickening but no glandular hyperplasia or invasion of underlying stroma by branching lower uterine glands as in panel 4. Bars in all panels = 200 μm; bars in insets in panels 5 and 6 = 20 μm. *c*, frequency distribution of cancers induced by different continuous doses of 17β-estradiol at each anatomical site in the reproductive tract. The total number of mice with cancer in each dosage group is indicated in the key at the top left of the panel. Neither cancer nor dysplasia developed in any mouse treated with 0.01 mg/60-day 17β-estradiol, or in any nontransgenic mouse.

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Estrogen Receptor Expression. Paraffin sections were melted at 55°C for 20 min, immediately deparaffinized, rehydrated, and microwave-boiled for 10 min in 10 mM citrate buffer (pH 6.0). After cooling to room temperature, sections were blocked in 10% BSA, incubated overnight at 4°C with ER-21 antibody (1:15,000 in 3% BSA; Geoffrey Greene, University of Chicago, Chicago, IL), biotinylated goat antirabbit serum (1:200 in BSA) for 1 h, followed by ABC-alkaline phosphatase reagent for 1 h (Vector Labs, Burlingame, CA). Slides were washed in 0.1 M Tris (pH 9.5) and incubated in the dark for 1.5 h in 5-bromo-4-chloro-3-indolyl phosphate/nitroblue tetrazolium substrate reagent (SK-5400; Vector) with levamisole.

Serum Estrogen Levels. Blood was collected by peribortal puncture into heparinized tubes and centrifuged; serum estrogen was assayed using a RIA (Diagnostic Products, Los Angeles, CA), modified for mouse serum (16). In situ Hybridization. In situ hybridization for expression of HPV16 E6/E7 and control mRNAs was performed as described previously (14, 17). Sections were exposed for 1 month, developed, and counterstained with H&E.

Microscopic Imaging and Statistical Analysis. Microscopic images were captured using a Leica DMRXA microscope equipped with a Spot digital camera (Diagnostic Instruments, Inc.). Images were composed using Adobe Photoshop 5.5 (San Jose, CA). Numerical data are presented as the mean ± SD, and were analyzed statistically by two-way ANOVA and χ² analysis, using Graph Pad 2 software (San Diego, CA).

RESULTS

Location of Squamous Carcinogenesis Varies According to Estrogen Dose. To investigate the threshold of estrogen-induced reproductive tract squamous carcinogenesis, different groups of K14-HPV16 transgenic and nontransgenic mice were treated for intervals up to 6 months with the same hormone doses used in previous experiments (14) and lower hormone doses (Table 1 and Fig. 1). Table 1 delineates the total number of transgenic and nontransgenic mice starting treatment in each group and the subset of transgenic and nontransgenic mice treated with each dose of 17β-estradiol for 6 months. This part of the study was designed to delineate the location of reproductive tract cancers; thus, derivation of cancer incidence versus dose was not available. The frequency distribution of the numbers of cancers occurring at different levels of the reproductive tract was significantly different for mice treated with either 0.25 or 0.10 mg/60-day 17β-estradiol compared with transgenic mice treated with 0.05 mg/60-day 17β-estradiol (Fig. 1). Transgenic mice treated with either 0.25 or 0.10 mg/60-day 17β-estradiol predominantly developed squamous cancers of the vulva, vagina, and lower/outer cervix (Fig. 1, a and c, and Fig. 1b, panels 1–3, respectively). In contrast, 0.05 mg/60-day 17β-estradiol induced squamous cancers that were almost exclusively localized to the transformation zone situated between the upper cervix and the lower uterus (Fig. 1b, panel 4, and Fig. 1c). Despite extensive invasion into underlying stromal tissues, none of the transgenic mice treated with any dose of 17β-estradiol developed lymph node metastasis. The 0.01 mg/60-day 17β-estradiol dose did not affect the reproductive tract squamous epithelium of either transgenic or nontransgenic mice when compared with placebo-treated controls matched for stages of estrus (Fig. 1b, panels 5 and 6, and insets therein). Nontransgenic mice treated with each 17β-estradiol dose developed squamous epithelial thickening consistent with benign hyperplasia (Fig. 1). None of the nontransgenic mice treated with any estrogen dose developed neoplasia or malignancy (data not shown and Fig. 1). Spontaneous reproductive tract pathology did not develop in placebo-treated transgenic mice.

Similar to our previous study (14), estrogen treatment caused mortality in both transgenic and nontransgenic mice (Table 1). Mortality during estrogen treatment was due to urinary bladder dilation in both transgenic and nontransgenic mice. Mice severely affected by bladder dysfunction were sacrificed immediately for reproductive tract histopathological analysis and were designated as a “mortality.” Deaths in the 0.25 mg/60-day 17β-estradiol groups occurred as early as after 2.5 months of treatment, whereas all unscheduled deaths in the 0.1 and 0.05 mg/60-day 17β-estradiol groups occurred after 5–6 months of treatment. As such, calculation of mortality for the entire group of mice encompassing the entire study diluted mortality estimates for the lower estrogen doses because of inclusion of early time points (Table 1). Therefore, we analyzed a small subset of transgenic and nontransgenic mice treated with estrogen for 6 months (Table 1). Mortality in the 0.25 and 0.10 mg/60-day 17β-estradiol groups was greater than that reported previously for this model (14) and may have been due to modifier effects of the FVN/n in-bred strain (see “Discussion”). Although there was no statistically significant difference in mortality between groups, there was a clear trend toward reduction in mortality in mice treated with 0.05 mg/60-day 17β-estradiol (see “Discussion”). Despite step-sectioning of the bladder and the urethra, our analysis did not reveal a discrete point of bladder obstruction (data not shown). There was no significant difference in mortality between transgenic and nontransgenic mice, and none of the mice treated with 0.01 mg/60-day 17β-estradiol died (Table 1).

To determine the effect of each estrogen dose on blood hormone levels, serum was obtained from transgenic and nontransgenic mice treated for 7 weeks with 60-day release hormone or placebo pellets (Fig. 2). Previous studies from the pellet manufacturer demonstrated constant hormone release without “peak and trough” effects at the beginning and end of the 60-day dosing interval (18). Compared with placebo, the 0.25 and the 0.10 mg/60-day doses elevated serum estrogen 11–21-fold and 6–15-fold, respectively. Although there was no statistically significant elevation of serum estrogen levels produced by the 0.05 mg/60-day 17β-estradiol dose compared with placebo-treated mice in diestrus, the 50% elevation of serum hormone levels in this group was most likely biologically significant (Fig. 2). Moreover, cumulative reproductive tract histopathological analysis of all transgenic and nontransgenic mice treated with 0.05 mg/60-day 17β-estradiol revealed that this dose prevented estrus cycling, whereas progesterone effects were seen in mice treated with 0.01 mg/60-day 17β-estradiol.

Time Course of Transformation Zone Carcinogenesis. The remainder of the study focused on elucidation of the biology of multistage transformation zone carcinogenesis induced by treatment of transgenic and nontransgenic mice with 0.05 mg/60-day 17β-estradiol. Here, cancer incidence was determined by serial sacrifice of transgenic and concurrent nontransgenic control mice at intermediate time points of neoplastic progression—1, 3, 4, 6 –15-fold, respectively. Although there was no statistically significant elevation of serum estrogen levels produced by the 0.05 mg/60-day 17β-estradiol dose compared with placebo-treated mice in diestrus, the 50% elevation of serum hormone levels in this group was most likely biologically significant (Fig. 2). Moreover, cumulative reproductive tract histopathological analysis of all transgenic and nontransgenic mice treated with 0.05 mg/60-day 17β-estradiol revealed that this dose prevented estrus cycling, whereas progesterone effects were seen in mice treated with 0.01 mg/60-day 17β-estradiol.

Fig. 2. Scatter diagram of the distribution of serum estrogen levels in mice at the beginning and in the middle of the estrus cycle or treated with different continuous doses of 17β-estradiol. Transgenic and nontransgenic mice were treated with 17β-estradiol for 7 weeks prior to sample collection for serum estrogen levels. Mice treated with 0.25 and 0.10 mg 17β-estradiol released over 60 days displayed 11–21- or 6–15-fold elevations in serum estrogen levels, respectively. Although serum estrogen levels in mice treated with 0.05 mg/60-day 17β-estradiol were not statistically significantly elevated compared with untreated mice in the beginning of the estrus cycle (diestrus), the mean 50% elevation in serum hormone levels in this group was sufficient to place treated mice in persistent estrus. a, mg of 17β-estradiol per 60-day dose.
and 5 months of hormone treatment—inclusive of the 6-month hormone treatment group discussed above (Fig. 3). Transformation zone cancers were first detected after 4 months of treatment in 30% of transgenic mice, and increased to 60% after 5 months and 91% after 6 months of hormone treatment (Fig. 3a, also see “Discussion”). Fig. 3b illustrates the reproductive tract histopathology of 17β-estradiol-treated nontransgenic (panels 1, 3, 5, and 7) and transgenic (panels 2, 4, 6, and 8) mice. After 1 and 4 months of treatment, there was a progressive increase in the thickness and proximal extension of the squamous epithelium into the lower uterus in the transgenic compared with the nontransgenic mice (Fig. 3b, panels 1–4). In addition, squamous metaplasia was evident in lower uterine glands, particularly in the reproductive tracts of transgenic mice (see arrowhead in Fig. 3b, panel 4). Metaplastic lower uterine glands were separated from the anatomical squamo-columnar junction by columnar epithelium (Fig. 3b, panels 4, 6, and 8). After 5 months of 17β-estradiol treatment, the transformation zone of both nontransgenic and transgenic mice displayed a profusion of lower uterine glands. These glands were hyperplastic, with extensive branches penetrating into the stroma (Fig. 3b, panels 5 and 6, and Fig. 3c, lower panel). In the transgenic mice, extensive squamous metaplasia was detectable in these hyperplastic glands and was the source for high-grade dysplasia and multifocal squamous carcinomas (Fig. 3b, panel 6, and Fig. 3c, lower panel). After 6 months of hormone treatment, there were further increases in the number and stromal extension of transformation zone glands in nontransgenic mice (Fig. 3b, panel 7) and more extensive glandular squamous metaplasia and stromal invasion by multifocal squamous carcinomas in transgenic mice (Fig. 3b, panel 8).

Fig. 3. Cancer incidence and histopathology of multistage transformation zone carcinogenesis. a, incidence and onset of transformation zone cancers in transgenic mice treated at 1 month of age with 0.05 mg/60-day 17β-estradiol for indicated intervals. b, histopathology of multistage transformation zone carcinogenesis in transgenic (panels 2, 4, 6, and 8) and nontransgenic (panels 1, 3, 5, and 7) mice. After 1 month of hormone treatment, there is squamous epithelial hyperplasia in both nontransgenic (panel 1) and transgenic (panel 2) mice. Following 4 months of treatment, increased squamous hyperplasia, epithelial papillomatosis, dysplasia, and squamous metaplasia (arrowhead) are evident in the transgenic (panel 4) compared with nontransgenic (panel 3) mice. After 5 months of treatment, lower uterine glands in nontransgenic mice were hyperplastic (panel 5, arrowhead; region magnified in c, upper panel), whereas metaplasia, dysplasia, and squamous cancer were present in the corresponding region in transgenic mice (panel 6, arrowhead; region magnified in c, lower panel). Following 6 months of hormone treatment, lower uterine glandular hyperplasia and hypertrophy further increase in nontransgenic mice (panel 7), whereas multifocal squamous cancer replaces nearly the entire transformation zone in transgenic mice (panel 8). c, hyperplastic lower uterine glands in a nontransgenic mouse (upper panel), and multifocal in situ glandular squamous metaplasia, dysplasia, and cancer in a transgenic mouse (lower panel). Bars = 100 μm.
Transformation Zone Metaplasia Appears to Arise from Sub-columnar Glandular Reserve Cells. To further investigate the origins of metaplasia during transformation zone carcinogenesis, expression of keratin-14, a squamous epithelial cell marker, was determined (Fig. 4). After 1 month of 17β-estradiol treatment, transformation zones in both transgenic and nontransgenic mice demonstrated focal keratin-14 expression within two to three lower uterine glands (Fig. 4, panels 1 and 2). Metaplastic lower uterine glands were separated from the cervical squamous epithelium by a considerable length of intervening columnar epithelium (Fig. 4, panels 1 and 2). After 6 months of hormone treatment, the transformation zones in transgenic mice displayed an increase in the frequency of metaplastic lower uterine glands (Fig. 4, panel 4).

Higher magnification of the transgenic transformation zone revealed multifocal squamous metaplasia in several different lower uterine glands as well as within individual lower uterine glands (Fig. 4, panel 5). Metaplastic foci appeared to arise from single cells located at the epithelial-stromal interface (Fig. 4, panel 5 inset). In contrast, squamous metaplasia was decreased at the transformation zone of nontransgenic mice (Fig. 4, panel 3).

Proliferation and Transgene Expression during Transformation Zone Carcinogenesis. One explanation for localization of transformation zone carcinogenesis would be a focal increase of proliferation. Squamous epithelial proliferation was quantified by counting transformation zone BrdUrd-labeled nuclei compared with a separate enumeration of BrdUrd-positive cells within the cervical canal. Enumeration

Fig. 4. Keratin-14 expression patterns during multistage transformation zone carcinogenesis. Following 1 month of estrogen treatment, there are scattered metaplastic lower uterine glands expressing keratin-14 in both nontransgenic (panel 1) and transgenic (panel 2) mice. After 6 months of estrogen treatment, the extent of lower uterine and upper cervical keratin-14 expression decreases in nontransgenic mice (panel 3) compared with their counterparts treated for 1 month. Transformation zone squamous metaplasia is extensive in the transgenic mice (panel 4, arrowheads; indicate region magnified in panel 5). Multifocal squamous metaplasia occurs in several glands and at multiple sites within each gland (panel 5, arrowhead; gland magnified in inset). Keratin-14 expression in single basal cells immediately adjacent to the stroma (inset, panel 5) is consistent with reserve cell squamous metaplasia. Bars: panels 1–4 = 200 μm; panel 5 = 100 μm; inset = 20 μm.
rather than determination of an incorporation index was performed because the architecture of the transformation zone was complex, encompassing convoluted glands and multiple cell types. From 1 to 6 months of hormone treatment, there was a statistically significant increase in transformation zone S-phase nuclei of transgenic mice compared with nontransgenic mice (data not shown, and Fig. 5). Representative immunohistochemical tissue sections demonstrated that estrogen induced an increase in BrdUrd-positive cells within both the squamous epithelium and the stroma in transgenic mice compared with nontransgenic mice. Subcoluminar cells resembling reserve-like cells or keratinocytes were more frequently in S-phase in estrogen-treated transgenic compared with nontransgenic mice (Fig. 5, arrowheads).

Increases in proliferation could be due to up-regulation of transgene expression during estrogen-induced transformation zone carcinogenesis. However, similar to our previous study (14), HPV E6/E7 transgene expression remained at a threshold level of detection at each time point of cervical neoplastic progression, without a focal increase at the transformation zone compared with other parts of the reproductive tract (data not shown). Hybridization to a keratin-14 riboprobe (14) and selected other nuclear transcription factors demonstrated that the reproductive tract mRNA was intact (data not shown).

**Estrogen Receptor Expression Patterns during Transformation Zone Carcinogenesis.** Because estrogen receptor-α is known to stimulate reproductive tract proliferation (19, 20), we investigated the distribution of receptor expression immunohistochemically. Estrogen receptor-α protein was detected in both basal squamous epithelial and underlying stromal cells in both nontransgenic and transgenic mice after 1 month of 17β-estradiol treatment (Fig. 6, panels 1 and 2, respectively). After 6 months of hormone treatment, the populations of basal and basaloid cells expressing estrogen receptor-α were markedly expanded in the transgenic mice compared with the nontransgenic mice, particularly in dysplastic squamous epithelium (Fig. 6, panels 2 and 4 compared with panel 5). In metaplastic and dysplastic lower uterine glands of transgenic mice in which columnar epithelium was juxtaposed to squamous epithelium, estrogen receptor-α expression appeared to be greater in the former compared with the latter. Estrogen receptor-α expression was also evident in the squamous epithelial component of transformation zone cancers (Fig. 6, panels 6 and 7).

**DISCUSSION**

Using hormone titration, we refined our previous study, creating a model of cervical carcinogenesis originating from and within squa-
uous metaplasia at the transformation zone of K14-HPV16 transgenic mice. Although this model closely emulates human disease, there are features, unique to its creation, that are specific to the system. In particular, cervical carcinogenesis is induced by continuous exposure to estrogen. Although the 17β-estradiol dose did not result in a several-fold elevation in serum hormone levels, the 30–40% elevation in serum hormone levels was sufficient to place these mice in persistent estrus (data not shown). As such, our analysis of serum hormone levels has motivated future experiments to test whether spontaneous cervical carcinogenesis can be produced in adult transgenic mice by induction of persistent estrus, using the model of neonatal estrogen treatment (21). Urinary obstruction secondary to bladder dilation also is a concomitant of continuous estrogen treatment. Indeed, the 30–45% mortality in mice treated with 0.25 and 0.10 mg/60-day 17β-estradiol was unexpectedly higher in than our previous study (14). One explanation for this result is that our present study was performed in transgenic and nontransgenic littermates backcrossed into the FVB/n strain for 24–25 generations, whereas our previous study used mice at n = 13–14 into FVB/n (14). Although 10 backcross generations is sufficient to generate congenic strains (22), it

Fig. 6. Pattern and distribution of estrogen receptor-α expression during transformation zone carcinogenesis. After 1 month of estrogen treatment, estrogen receptor-α expression is detected in basal and occasional suprabasal cells in the squamous epithelium of nontransgenic and transgenic mice (1 and 3). Following 6 months of estrogen treatment, the population of estrogen receptor-α-positive basal and suprabasal squamous epithelial cells is increased in high-grade dysplastic lesions in the transgenic (2 and 4) compared with the nontransgenic mice (5). Within individual dysplastic glands, columnar cells with high level estrogen receptor-α expression are juxtaposed to squamous epithelial cells that appear to have lower levels of hormone receptor expression (2). Estrogen receptor-α expression persists in invasive squamous cancers (6 and 7). Dashed lines in 1–4 indicate epithelial-stromal junctions; solid lines in 6 indicate nests of malignant cells. Estrogen receptor-α expression is also evident in stromal nuclei at all of the time points. Bars = 20 μm.
is possible that a modifier locus closely linked to the transgene was responsible for increased sensitivity to 17β-estradiol. Indeed, polymorphisms such as differential methylation of estradiol have been described in humans and are hypothesized to be a susceptibility factor in hormonal carcinogenesis (23, 24). Moreover, the degree and location of estrogen hydroxylation have also been reported to potentially play roles in either direct genotoxicity (9, 25) or cytotoxicity and inhibition of angiogenesis (26). As such, the incidence of bladder obstruction was a prime motivation to test further dose reduction for both carcinogenic induction and concomitant reduction in this complication. We are confident that the 21% mortality in mice treated with 0.05 mg/60-day 17β-estradiol is a true decrease in mortality because further study with this model, using larger cohorts of transgenic and nontransgenic mice, demonstrated that 6 months of hormone treatment with this dose can be accomplished with 0–10% mortality.4 Moreover, we have preliminary data from interspecific F1 hybrids suggesting that the FVB/n background may be more permissive for estrogen-induced cervical carcinogenesis compared with other inbred strains, which is reminiscent of sensitivity of FVB/n to epidermal carcinogenesis (11, 27). Further study has also revealed a 30–80% (wide range due to small numbers) incidence of invasive cervical cancer after 4.5 months of hormone treatment with no mortality.4 These emerging data suggest that studies of shorter duration can be designed to test for either genetic complementation or chemoprevention of cervical carcinogenesis.

Squamous metaplasia appears to be the first stage of transformation zone carcinogenesis in both our model and in clinical disease. Although the location of the transformation zone in mice is at the junction of the upper cervix and lower uterus, in contrast to the portio of the cervix in humans, there are data implicating the glandular reserve cells in human disease (28–30), as we suspect in our transgenic mouse model. For example, both ectocervical and endocervical cells can be cultured from the human cervix (31). Although endocervical cells are derived from endocervical glands lined by columnar epithelium, they also produce colonies that simultaneously express both the squamous marker keratin-14 and the columnar marker keratin-18 (31). Transplantation of endocervical cells into immuno-deficient hosts elaborates monolayers resembling immature squamous metaplasia, whereas transplantation of ectocervical cells produces well-differentiated, stratified squamous epithelium (31). The more commonly held view of the origins of glandular metaplasia is migration and replacement of glandular columnar epithelium by adjacent squamous cells. Recent data suggest that enhanced cell motility, possibly facilitated by HPV E6 binding to paxillin (32), may contribute to this phenomenon. However, development of multifocal squamous metaplasia at a distance from the squamo-columnar junction in our model is consistent with the possibility that cervical carcinogenesis may originate from individual glandular reserve cells (29, 31).

Squamous metaplasia is also more extensive in estrogen-treated transgenic versus nontransgenic mice. Squamous metaplasia appears to be induced in the vagina and cervix by a decrease in pH (1). Epithelial acidification occurs during adolescence as a result of increased estrogen production, vaginal bacterial flora alterations, and epithelial wounding (1, 33). Yeast two-hybrid screens have demonstrated binding of M2 pyruvate kinase to HPV16 E7 (34). HPV E7 stabilizes the dimeric compared with the tetrameric form of M2 pyruvate kinase (34). Substrate utilization by dimeric M2 pyruvate kinase shifts intracellular glucose metabolism to aerobic glycolysis rather than tricarboxylic acid cycle oxidation (34). One hypothesis suggested by these biochemical data is that E7-mediated intracellular acidosis due to lactate accumulation may be a signal that contributes to a squamous rather than a columnar fate decision in glandular reserve cells in estrogen-treated transgenic mice.

Several features of our present model suggest that a combination of cell autonomous and non-cell autonomous factors conspire to induce transformation zone cervical carcinogenesis. Expression of estrogen receptor-α and the HPV oncoproteins (14) in the same basal cell population suggest a cell autonomous contribution to squamous epithelial dysplasia and subsequent invasive cancer. There are a number of molecular scenarios, predominantly involving parallel pathways, for estrogen receptor-α signaling to synergize with the cellular effects of the HPV16 oncoproteins. For example, increases in E2F activity secondary to pRB destabilization by HPV E7 up-regulates a collection of genes, including thymidylate kinase and dihydrofolate reductase, increasing nucleotide pools required for DNA synthesis by estrogen receptor-α target genes (35). Synergism between estrogen receptor-α and HPV could also occur at the level of the epithelial growth factor receptor, whose cell surface recycling is increased by HPV E5 oncoprotein (36–38). Up-regulation of estrogen receptor-α transcriptional activity can be produced by epithelial growth factor receptor-mediated activation of the MAP kinase pathway, which can directly phosphorylate the hormone receptor (39). Indirect interaction between the HPV oncoproteins and estrogen receptor-α transactivation may also occur at the level of chromatin remodeling. Ligand-bound nuclear receptors recruit coactivator(s) that possess histone acetylase activity to DNA (40). Chromatin histone acetylation increases transcription machinery access to DNA (40). Conversely, retinoblastoma protein transcriptional repression is mediated by recruitment of histone deacetylase to DNA (41). HPV E7 disrupts complex formation between histone deacetylase and the retinoblastoma protein (42). Thus, HPV E7 may further increase chromosomal DNA access at estrogen receptor response elements. Non-cell autonomous cooperation between ligand-activated estrogen receptor-α and the HPV oncoproteins may also occur. Metaplastic squamous epithelium emerges from highly branched, hyperplastic, lower uterine glands induced by chronic low-dose estrogen treatment. These structures likely form as a result of stromal-epithelial interactions at the transformation zone of estrogen-treated mice, possibly

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4 Unpublished data.
coordinated by transcription and release of growth factors from the underlying stroma in response to activation of estrogen receptor-a by ligand. Induction of dysplasia in the overlying neoplastic squamous epithelium of transgenic mice could be mediated by squamous epithelial cell cycle dysregulation and altered genomic stability mediated by HPV E7 and E6 (43).

Thus, similar to human disease, transformation zone carcinogenesis in estrogen-treated K14-HPV16 transgenic mice cannot be explained by a single molecular feature; rather, it is multifactorial (Fig. 7). Chronic estrogen treatment stimulates development of hyperplastic lower uterine glands. These glands become “fertile soil,” fostering squamous metaplasia and neoplastic progression. Glandular stem-like reserve cells can express low levels of keratin-14 (31) and, in transgenic mice, the HPV oncogenes. Interaction of HPV16 E7 oncoprotein with M2 pyruvate kinase could produce decreased intracellular pH (34), potentially biasing glandular reserve cell fate decision(s) toward metaplastic squamous rather than columnar epithelium (Ref. 1 and Fig. 7). Continuous HPV E6 and E7 oncogene expression can cause persistent cell cycle dysregulation (44) and facilitate genetic instability (45). Estrogen receptor-a signaling within squamous epithelial cells, between glandular columnar and metaplastic squamous cells, and elaborated from the underlying stroma may further contribute to carcinogenesis. This study sets the stage to elucidate cell autonomous and non-cell autonomous contributions to transformation zone carcinogenesis, using either genetic complementation or pharmacological treatments, either antineoplastic or chemopreventive, that target each element of this model.

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Sensitivity of the Cervical Transformation Zone to Estrogen-induced Squamous Carcinogenesis

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