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TUMORIGENESIS AND NEOPLASTIC PROGRESSION

Recurrence of Cervical Cancer in Mice after Selective Estrogen Receptor Modulator Therapy

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Estrogen and its nuclear receptor, estrogen receptor α, are necessary cofactors in the initiation and multistage progression of carcinogenesis in the *K14E6/E7* transgenic mouse model of human papillomavirus–associated cervical cancer. Recently, our laboratory reported that raloxifene, a selective estrogen receptor modulator, promoted regression of high-grade dysplasia and cancer that arose in the cervix of *K14E6/E7* transgenic mice treated long-term with estrogen. Herein, we evaluated the recurrence of cervical cancer after raloxifene therapy in our preclinical model of human papillomavirus–associated cervical carcinogenesis. We observed recurrence of cervical cancer in mice re-exposed to estrogen after raloxifene treatment, despite evidence suggesting the antagonistic effects of raloxifene persisted in the reproductive tract after treatment had ceased. We also observed recurrence of neoplastic disease in mice that were not retreated with exogenous estrogen, although the severity of disease was less. Recurrent neoplastic disease and cancers retained functional estrogen receptor α and responded to retreatment with raloxifene. Moreover, continuous treatment of mice with raloxifene prevented the emergence of recurrent disease seen in mice in which raloxifene was discontinued. These data suggest that cervical cancer cells are not completely eradicated by raloxifene and rapidly expand if raloxifene treatment is ceased. These findings indicate that a prolonged treatment period with raloxifene might be required to prevent recurrence of neoplastic disease and lower reproductive tract cancers. *(Am J Pathol 2014, 184: 530–540; http://dx.doi.org/10.1016/j.ajpath.2013.10.013)*

Human papillomaviruses (HPVs), particularly those classified as high-risk mucosal subtypes, are associated with nearly all invasive cervical cancers.[1] One high-risk genotype, HPV-16, is the most prevalent mucosal papillomavirus and accounts for more than half of all cervical cancer cases.[2] Cervical cancer incidence has decreased in developed countries because of effective screening methods, and a further decrease is expected after the introduction of prophylactic vaccines, yet cervical cancer still accounts for 8% of the global female cancer burden and remains the second most common cancer in women.[3] A lack of effective treatment options, coupled with disproportionate access to vaccination and screening worldwide, generates an ongoing need for new cervical cancer therapeutics.

Although persistent HPV infections are necessary for the development of HPV-associated cancers, they are not sufficient because only a fraction of patients with persistent infections develop cervical cancer or even the high-grade precancerous lesions from which cervical cancer arises.[4] Epidemiological data support a role for other factors that range from smoking to insufficient screening, but the two most significantly associated factors are multiparity and long-term oral contraceptive use.[5–8] Both of these variables are associated with elevated levels of the female hormone, estrogen, and their link to cervical cancer risk supports a growing body of research from *in vivo* animal models that identifies a requirement for estrogen in HPV-induced cervical carcinogenesis.[9] A recent study found that patients with HPV-associated cervical neoplasia have significantly heightened levels of one active form of estrogen, 16α-hydroxyestrone, in their blood.[10] In this same study, a

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positive, but not statically significant, association also was seen with circulating levels of estradiol. A positive association of circulating estradiol levels with cervical cancer was also reported in the larger and more recent European Prospective Investigation into Cancer and Nutrition study.[11] Estrogen was first identified as a cofactor necessary for squamous carcinogenesis in the *K14-HPV16* transgenic mouse model,[12] and subsequent research revealed that the hormone promotes a multistage process of neoplastic progression in the cervical transformation zone.[13] The two main HPV-16 oncogenes have individual functions in this estrogen-induced neoplastic progression to cervical squamous cell carcinoma, with E7 being the more potent oncogene and E6 acting to augment the malignant phenotype of E7-induced tumors.[14] Cervical cancers that arise in the murine reproductive tract in response to long-term estrogen treatment are dependent on the continuous expression of E7[15] and require continuous exposure to exogenous estrogen for growth and persistence.[16,17] The requirement for estrogen is reinforced by studies demonstrating that cervical cancer development in HPV-transgenic mice is dependent on estrogen receptor α (ERα), with its expression in the underlying stroma being critical.[18,19] Taken together, the epidemiological data and results from transgenic mouse model studies provide compelling evidence for a synergistic relationship between the HPV-16 E6 and E7 oncogenes and estrogen in cervical cancer biogenesis, growth, and persistence.

The identification of estrogen as a cocarcinogen in murine models of HPV-associated cervical carcinogenesis previously prompted our laboratory to determine the efficacy of estrogen receptor antagonists as a means of treatment for cancers that arise after long-term estrogen exposure in HPV transgenic animals. Indeed, treatment of established squamous cell carcinomas of the lower reproductive tract with either the complete ERα antagonist, fulvestrant (ICI 182,780), or the selective estrogen receptor modulator (SERM), raloxifene, was highly efficient in promoting cancer regression.[17] Furthermore, treatment of precancerous lesions with fulvestrant prevented further disease progression and inhibited the onset of lower reproductive tract cancers. Considering the significant implications to human health, we were further motivated to investigate if the cancer recurs after drug treatment. In this study, we examined the recurrence of lower reproductive tract cancers after treatment with raloxifene. We found that neoplastic disease recurs after cessation of treatment with raloxifene, regardless of exogenous estrogen, although it did increase the severity of the recurrent neoplastic disease. All recurrent cancers retained an active estrogen/ERα signaling pathway and were responsive to retreatment with raloxifene. Maintaining mice on raloxifene prevented the high recurrence rates of cancer. We hypothesize that residual cancer cells drive cancer recurrence in mice upon release from treatment with raloxifene. This study supports the premise that SERMs, such as raloxifene, may be effective in treating HPV-associated human cervical cancers, but that their effectiveness may require long-term treatment.

Materials and Methods

Transgenic Mice and Hormone Treatment

The *K14E7* and *K14E6* transgenic mouse lines were maintained on the FVB/n inbred genetic background and have been described previously.[20,21] At 4 to 6 weeks of age, *K14E6/K14E7* (referred to as *K14E6/E7* herein) double transgenic virgin females were anesthetized with 5% isoflurane, and a continuous-release estrogen (E2) tablet (17β-estradiol; 0.05 mg/60 days; Innovative Research of America, Sarasota, FL) was inserted s.c. in the shoulder fat pads of the dorsal skin. A new tablet was inserted every 2 months as needed. Mice were housed in the American Association of Laboratory Animal Care–approved McArdle Laboratory Animal Care Unit of the University of Wisconsin School of Medicine and Public Health (Madison, WI). All procedures were performed according to a protocol approved by the University of Wisconsin Institutional Animal Care and Use Committee.

Drug Treatment

Treatment of mice with raloxifene was performed as previously described.[17] Briefly, the human formulation of raloxifene hydrochloride (60 mg tablets; EVISTA; Eli Lilly, Indianapolis, IN) was purchased from the University of Wisconsin Hospital Pharmacy. Tablets were resuspended in PBS at a final concentration of 10 mg/mL. Mice were administered a 150-μL drug suspension (equivalent to 1.5 mg) by i.p. injection at each treatment. The duration of treatment was 4 weeks (5 days a week) for a total of 20 treatments. Treatment with raloxifene did not increase morbidity or mortality.

Tissue Procurement and Disease Scoring

One hour before sacrifice, mice were treated with 100 mg/kg 5-bromodeoxyuridine (BrdU) by i.p. injection. Reproductive tracts were harvested, fixed in 4% paraformaldehyde, and embedded in paraffin. Serial sections (5 μm thick) were cut and every 10th section was stained with H&E. H&E-stained sections were evaluated for histopathological features and scored for worst disease, according to the histopathological grading system outlined by Riley et al.[14] Images of H&E-stained cervical tumors and epithelia were captured using a Zeiss AxioImager M2 microscope and AxioVision software version 4.8.2 (Jena, Germany).

IHC Data

Sections were deparaffinized and rehydrated with xylenes and graded ethanol, respectively. Endogenous peroxidase activity was quenched with 3% H_2O_2 in methanol and followed with heat-induced antigen retrieval in 10 mmol/L citrate buffer (pH 6.0). Antibodies used include anti-BrdU (Calbiochem, Billerica, MA), anti–mini-chromosome maintenance protein 7 (MCM7) (ThermoScientific, Fremont, CA), anti-progesterone

receptor (PR; Santa Cruz Biotechnology, Santa Cruz, CA), anti-ERα (Santa Cruz Biotechnology), and biotinylated horse anti-mouse/rabbit IgGs (Vector Laboratories, Burlingame, CA). Proteins were visualized with 3,3′-diaminobenzidine (Vector Laboratories), and tissues were counterstained with hematoxylin. All images were taken with a Zeiss AxioImager M2 microscope using the AxioVision software version 4.8.2.

Tumor Measurement and BrdU Quantitation

Tissues were first H&E stained and then tumor images were captured with a Zeiss AxioImager M2 microscope fitted with a 2.5× objective. By using the AxioVision software version 4.8.2, the total area of each tumor was measured by tracing the tumor boundaries. Manual pixel-to-micrometer conversions were not necessary, because this conversion is automatically configured for each microscope objective using the AxioVision software version 4.8.2. For tumors spanning more than one field of view, the sum of tumor areas from contiguous images was calculated.

For BrdU quantitation, representative slides were processed for immunohistochemistry (IHC), as previously described. For each group, one slide from three individual mice was analyzed by microscopy and multiple 20× images (5 to 10 per mouse) of the cervical epithelium or cervical cancers were captured. The total number of cells and the number of BrdU-positive cells were quantified with an automated cell counting program [David Ornelles (Wake Forest University School of Medicine, Winston-Salem, NC)] using ImageJ software version 1.47 (NIH, Bethesda, MD; unpublished data). The percentage of BrdU-positive cells was calculated using the two resulting values.

Statistical Analysis

The two-sided Fisher's exact test was used for analyses comparing cancer incidence, incidence of multiple tumors, and incidence of large tumors. For statistical analyses comparing disease severity, tumor multiplicity, tumor size, and BrdU quantitation, the two-sided Wilcoxon rank sum test was used. When comparing overall disease severity using the Wilcoxon rank sum test, each lesion was given an arbitrary score (rank) before analysis: no disease, 0; cervical intraepithelial neoplasia (CIN)/vaginal intraepithelial neoplasia (VIN) 1, 1; CIN/VIN2, 2; CIN/VIN3, 3; and cancer, 4. All statistical analyses were performed using MSTAT statistical software version 5.5.7 (*http://www.mcardle.wisc.edu/mstat*, last accessed October 5, 2013).

Results

Selection of Raloxifene for Recurrence Studies and Clearance from the Murine Reproductive Tract

Prior attempts were made to experimentally address the issue of cancer recurrence after cessation of fulvestrant treatment.[17] However, these experiments evaluated for recurrence only 6 weeks after termination of ERα antagonist treatment. Although no tumors recurred in these mice, this time frame was not sufficient to allow for clearance of fulvestrant effects in the reproductive tract, preventing our ability to assess cancer recurrence in the absence of effects of the drug. In the study reported herein, we evaluated cancer recurrence after cessation of treatment with raloxifene, an SERM that was equally effective at promoting cancer regression, in which we extended the recurrence evaluation to 12 weeks after termination of SERM treatment.

To our knowledge, the pharmacokinetics of raloxifene in the lower reproductive tract of mice have not been systematically evaluated. Therefore, we sought to determine the length of time required for clearance of overt anti-estrogenic phenotypes of raloxifene in the murine reproductive tract. Female, non-transgenic FVB/n mice were separated into two groups: one group received a 1-month raloxifene treatment identical to that used in the recurrence study, and the other group received no treatment. Both groups of mice were then treated with exogenous estrogen, and a contingent of mice was sacrificed, reproductive tracts were collected, and wet weights were measured every 2 weeks for a total of 3 months (Supplemental Figure S1). In mice that were not treated with raloxifene, the reproductive tract wet weight increased within 2 weeks and remained elevated for the remainder of the 3-month estrogen treatment period (Supplemental Figure S1A). In contrast, the increase in the reproductive tract wet weight in mice treated with raloxifene before exogenous estrogen exposure was substantially delayed and did not appear until 10 weeks after cessation of raloxifene treatment (Supplemental Figure S1B). The cervical epithelium in those mice never treated with raloxifene displayed a physiological hyperplasia by 2 weeks of estrogen treatment, yet the same was not seen in those mice previously treated with raloxifene until approximately 10 weeks (Supplemental Figure S1C). The cervical epithelium of mice treated with raloxifene was hypoplastic and displayed a loss of differentiation consistent with previous observations of ERα inhibition,[17,19] and this phenotype persisted for 10 weeks. Indeed, the appearance of cells positive for an ERα transcriptional target, PR, was concomitant with the increase in reproductive tract wet weight and the appearance of hyperplastic epithelium in both treatment groups (Supplemental Figure S1C), suggesting these results are a legitimate reflection of raloxifene persistence in the murine reproductive tract.

Cervical Cancers Recur after Raloxifene Treatment

Because the effects of raloxifene disappeared somewhere between 8 and 10 weeks after cessation of treatment, we chose to evaluate cancer recurrence 3 months after cessation of raloxifene therapy. Mice are approximately 1 year old at this end point, making it difficult to extend these studies much longer because of morbidity issues associated with the HPV16 E7 transgene that are unrelated to the female reproductive tract (eg, apnea arising from thymic hyperplasia and bladder distention by exogenous estrogen).

Group sizes were rather small because of these reasons. Groups of female, virgin *K14E6/E7* transgenic mice were administered various regimens of exogenous estrogen and raloxifene treatment (Figure 1A). A histopathological analysis was performed on reproductive tract tissue sections from these mice to score for disease severity (Table 1) and cancer incidence (Figure 1B). Disease severity was determined by comparing the overall incidence of precancerous (CIN1 to CIN3) and cancerous lesions in each group. Treating mice with estrogen for 6 months ($E2^{6m}$) induces the development of high-grade dysplasia and squamous cell carcinoma in the lower reproductive tract.[13,14,16,17] Archival data from our recent publication are consistent with these findings, such that squamous cell carcinomas developed in the cervix of all *K14E6/E7* transgenic mice (six of six) and in the vagina of half of the mice (three of six).[17] Any pathological feature that arises in this group of mice is henceforth considered initial disease. Another group of mice was treated with exogenous estrogen for 6 months to induce initial disease and subsequently treated with raloxifene for 1 month ($E2^{6m}/Ral^{1m}$). Both cancer incidence ($P = 0.0006$) and disease severity ($P = 0.0004$) were significantly reduced in the cervix; similar results were observed in the vagina (incidence $P = 0.029$, severity $P = 0.0008$). This level of cancer regression in response to raloxifene was nearly identical to our prior study.[17]

To monitor recurrence of cancer after cessation of raloxifene treatment, another group of mice ($n = 11$) was treated with exogenous estrogen for 6 months and raloxifene for 1 month to induce initial carcinogenesis and cancer regression, respectively, and then retreated with exogenous estrogen for an additional 3 months ($E2^{6m}/Ral^{1m}/E2^{3m}$). In these animals, a high incidence of lower reproductive tract cancers was observed: 8 (73%) of 11 mice had cervical cancer and 7 (64%) of 11 mice had vaginal cancer, representing a significant increase in both cancer incidence and disease severity compared with $E2^{6m}/Ral^{1m}$ mice (cervix: incidence $P = 0.008$ and severity $P = 0.0001$; vagina: incidence $P = 0.004$ and severity $P = 0.0002$). The incidence of lower reproductive tract cancers and disease severity in the $E2^{6m}/Ral^{1m}/E2^{3m}$ mice were comparable to those observed in the $E2^{6m}$ mice (incidence $P = 0.27$ and severity $P = 0.17$).

To determine whether the cancers found in the $E2^{6m}/Ral^{1m}/E2^{3m}$ mice originated from initial tumors that had developed after the initial 6 months of estrogen treatment, and were not

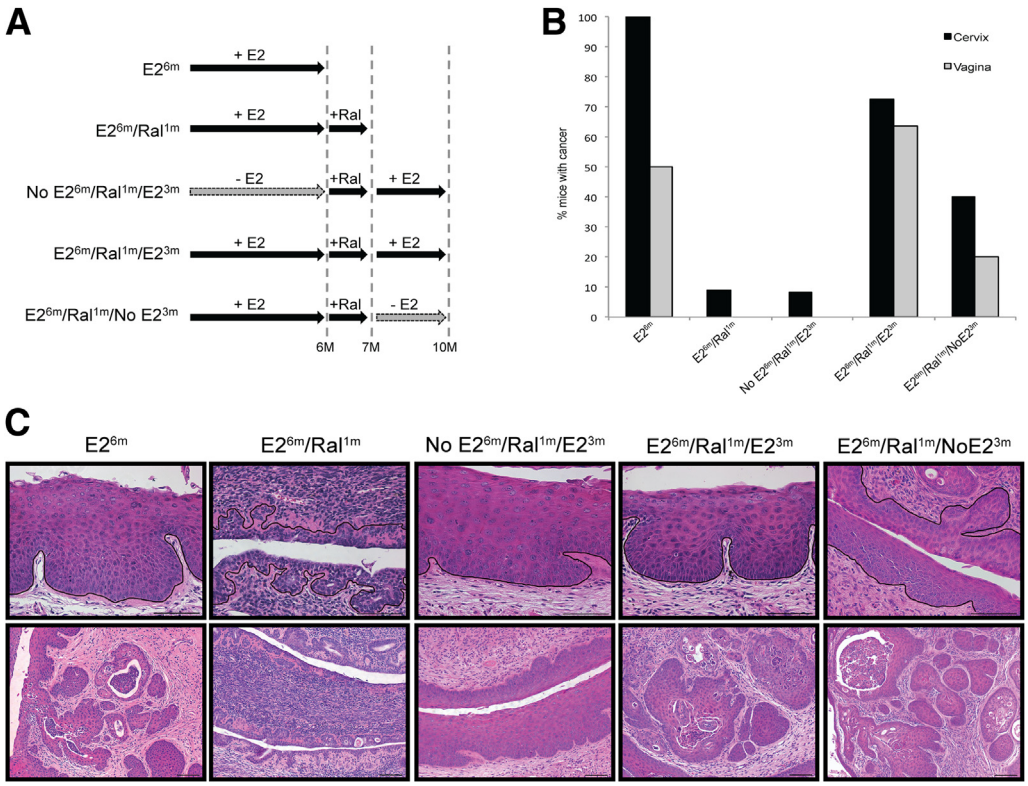

Figure 1 Cancers of the lower reproductive tract recur after raloxifene treatment and do not require exogenous estrogen. **A:** Schematic of treatment regimens used to determine disease recurrence after raloxifene therapy. Black arrows indicate treatment with estrogen or raloxifene; gray arrows, absence of treatment. Vertical dashed lines indicate treatment duration. **B:** End point cancer incidence in groups of mice administered different E2/raloxifene treatment regimens. Statistical comparisons between groups are noted in the text. For numbers of mice per group and disease severity, see Table 1. **C:** Representative images of H&E-stained endocervical sections from the indicated treatment groups. High-magnification images of the cervical squamous epithelium (**top row**) and low-magnification images of the cervical region and cervical cancers, where applicable (**bottom row**), are shown. A **black line** is used to highlight the basement membrane in the high-magnification images. Scale bars: 100 μm.

Table 1 Summary of Disease Recurrence in *K14E6/E7* Transgenic Mice after Raloxifene Treatment

Treatment group	Group size (*n*)	No disease, cervix (vagina)	Dysplasia only			Cancer and dysplasia, cervix (vagina)
			CIN1 (VIN1)	CIN2 (VIN2)	CIN3 (VIN3)	
$E2^{6m}$*	6	0 (0)	0 (0)	0 (1)	0 (2)	6 (3)
$E2^{6m}/Ral^{1m}$	11	10 (9)	0 (0)	0 (1)	0 (1)	1 (0)
No $E2^{6m}/Ral^{1m}/E2^{3m}$	12	1 (0)	4 (3)	6 (9)	0 (0)	1 (0)
$E2^{6m}/Ral^{1m}/E2^{3m}$	11	0 (0)	1 (1)	1 (3)	1 (0)	8 (7)
$E2^{6m}/Ral^{1m}/NoE2^{3m}$	10	3 (2)	3 (4)	0 (2)	0 (0)	4 (2)

Mice were scored histopathologically for the worst disease present in the cervix and vagina (in parentheses). For statistical comparisons between groups, please refer to the text in the *Results* sections *Cervical Cancers Recur after Raloxifene Treatment* and *Role of Exogenous Estrogen in Re-Emergence of Neoplastic Disease*.

*Archival data from Chung and Lambert.[17]

CIN, cervical intraepithelial neoplasia; VIN, vaginal intraepithelial neoplasia.

the product of *de novo* tumorigenesis, an additional control group of mice was analyzed. These mice received no estrogen for the initial 6 months but did receive raloxifene treatment, followed by 3 months of exogenous estrogen ($NoE2^{6m}/Ral^{1m}/E2^{3m}$). Three months of exogenous estrogen exposure is rarely sufficient to promote tumor development in the cervix of *K14E6/E7* mice.[12,13,17] Correspondingly, the cervical epithelium in mice from this $NoE2^{6m}/Ral^{1m}/E2^{3m}$ treatment group primarily contained low to moderate dysplasia, and only 1 (8%) of 12 mice developed a cervical tumor. The disease severity and the incidence of cancer observed in the $E2^{6m}/Ral^{1m}/E2^{3m}$ mice were significantly higher than those observed in the $NoE2^{6m}/Ral^{1m}/E2^{3m}$ mice (all comparisons $P < 0.004$). Together, these observations indicate that lower reproductive tract cancers recur in *K14E6/E7* transgenic mice administered exogenous estrogen again after cessation of raloxifene therapy. These results also imply that initial tumor cells were not completely eliminated by the 1-month treatment with raloxifene.

Role of Exogenous Estrogen in Re-Emergence of Neoplastic Disease

To determine whether recurrence of neoplastic disease requires exogenous estrogen, an additional group of mice ($n = 10$) treated for 6 months with estrogen, followed by 1-month treatment with raloxifene, was held for 3 months without retreatment with exogenous estrogen ($E2^{6m}/Ral^{1m}/NoE2^{3m}$) (Figure 1A). Neoplastic disease recurred in the lower reproductive tracts of these mice (Table 1 and Figure 1B). Cervical and vaginal cancers were present in 40% (4 of 10) and 20% (2 of 10) of mice, respectively. The incidence of cancer was not significantly different from that observed in the $E2^{6m}/Ral^{1m}$ mice ($P > 0.15$), which could suggest that the cancers found in the $E2^{6m}/Ral^{1m}/NoE2^{3m}$ mice reflect tumors that did not respond to raloxifene. However, the overall severity of disease, which accounts for the frequency of precancerous and cancerous lesions, was significantly worse in both the cervix and vagina ($P < 0.018$). This finding indicates that at least precancerous lesions recur upon cessation of raloxifene treatment and in the absence of re-exposure to exogenous estrogen. The incidence of cancer and disease severity within the cervix of the $E2^{6m}/Ral^{1m}/NoE2^{3m}$ mice remained significantly lower than initial disease observed in the $E2^{6m}$ mice ($P = 0.034$ and $P = 0.024$, respectively), whereas in the vagina, the incidence of cancer was not significantly different but overall disease severity was significantly lower ($P = 0.026$). To assess further the influence of exogenous estrogen on recurrence of lower reproductive tract neoplastic disease, we compared the cancer incidence and disease severity in the $E2^{6m}/Ral^{1m}/E2^{3m}$ and $E2^{6m}/Ral^{1m}/NoE2^{3m}$ cohorts. There was no difference in cancer incidence (cervix $P = 0.198$, vagina $P = 0.08$); however, the mice retreated with exogenous estrogen exhibited more severe disease (cervix $P = 0.045$, vagina $P = 0.014$). Based on these initial comparisons, we conclude that re-emergence of neoplastic disease within the lower female reproductive tract after release from raloxifene treatment is not absolutely dependent on exogenous estrogen, but the severity of the recurrent neoplasia is clearly greater when mice are re-exposed to estrogen.

Histopathological Characteristics of Recurrent Cervical Disease and Cancers

We next sought to evaluate and compare the cervical histopathological characteristics in mice from the various treatment groups (Figure 1C). In mice retreated with exogenous estrogen after cessation of raloxifene treatment ($E2^{6m}/Ral^{1m}/E2^{3m}$), the stratified squamous cervical epithelium reverted from a hypoplastic, undifferentiated phenotype associated with SERM treatment ($E2^{6m}/Ral^{1m}$) to a thick, hyperplastic epithelium with papillomatosis similar to that observed in the cervices of mice in the $E2^{6m}$ treatment group. On the other hand, although the cervical epithelium in mice not retreated with exogenous estrogen ($E2^{6m}/Ral^{1m}/NoE2^{3m}$) was hyperplastic compared with the epithelium after cessation of raloxifene treatment ($E2^{6m}/Ral^{1m}$ group), it did not revert to the $E2^{6m}$ phenotype to the same extent as seen in the $E2^{6m}/Ral^{1m}/E2^{3m}$ mice. A moderate portion of the cervical epithelium in the $E2^{6m}/Ral^{1m}/NoE2^{3m}$ cohort retained histological features seen in the $E2^{6m}/Ral^{1m}$ mice, such as loss of differentiation and a generally thinner

squamous epithelium, and this was particularly evident in the endocervix (data not shown). However, the increased thickness compared with the $E2^{6m}/Ral^{1m}$ mice suggests that these mice did return to normal estrus cycling by the end point. These histopathological traits of the cervical epithelium in mice from the $E2^{6m}/Ral^{1m}/NoE2^{3m}$ treatment group may help explain the reduced disease severity observed compared with initial disease and disease that recurs in the presence of exogenous estrogen (Table 1). Although these minor differences existed in the stratified squamous epithelium, the histological characteristics of initial and recurrent squamous cell carcinomas were largely comparable. Most recurrent cancers were reasonably well organized and developed in the outer cervix or at the cervicovaginal junction, regardless of whether the mice were retreated with exogenous estrogen (data not shown). Therefore, any major differences between initial and recurrent tumors, or between tumors arising in the presence and absence of exogenous estrogen after cessation of raloxifene treatment, were not evident at the level of tissue histopathological features.

Recurrent Cancers Are More Proliferative in Mice Retreated with Exogenous Estrogen after Cessation of Raloxifene Treatment, and Proliferation of the Cervical Epithelium Is a Predictor of Cancer Recurrence

After cessation of raloxifene treatment, we observed cervical cancer in the presence and absence of estrogen (Table 1). However, cervical cancers re-emerged with an incidence similar to initial cancer, and disease was generally more severe in mice administered exogenous estrogen again (Figure 1B). To determine whether the cervical tumors and epithelium in mice retreated with exogenous estrogen were more proliferative than in non-treated mice, the proliferative indices of individual cancers were scored by counting the number of cells that incorporated BrdU into their DNA. Recurrent cervical tumors in mice retreated with exogenous estrogen had a significantly higher average percentage of BrdU-positive cells than the cervical tumors in mice not retreated with exogenous estrogen (11.9% versus 7.9%; $P = 0.05$) (Figure 2A). However, this difference in recurrent tumor proliferation could not be explained by an inherent increase in the proliferative index of the cervical epithelium in mice retreated with exogenous estrogen ($P = 0.13$) (Figure 2B).

We further evaluated if the proliferative index of the epithelium was predictive of cancer recurrence. For this analysis, we compared BrdU incorporation in the cervical epithelia of mice within each treatment group between those animals that developed recurrent cervical cancers and those that did not. Regions of epithelium both proximal and distal to tumors were included in the analysis of mice with recurrent tumors to accurately measure proliferation throughout the entire cervix. The cervical epithelium in mice that developed recurrent cervical tumors had a significantly higher average percentage of BrdU-positive cells than the cervical epithelium in mice that were tumor-free in both the $E2^{6m}/Ral^{1m}/E2^{3m}$ group (12.2% versus 6.6%; $P = 0.05$) and the $E2^{6m}/Ral^{1m}/NoE2^{3m}$ group (9.6% versus 3.5%; $P = 0.05$) (Figure 2B). Overall, these data indicate that cell proliferation in the cervical epithelium positively correlates with the presence of recurrent cervical tumors after cessation of raloxifene therapy.

Exogenous Estrogen Increases the Multiplicity, But Not Size, of Recurrent Cervical Tumors in Mice after Raloxifene Treatment

To learn more about cervical cancer recurrence in the presence and absence of exogenous estrogen, we compared the multiplicity and size of the tumors in the $E2^{6m}/Ral^{1m}/E2^{3m}$ and $E2^{6m}/Ral^{1m}/NoE2^{3m}$ treatment groups, as well as other cancer development characteristics. In the presence of exogenous estrogen, recurrent cervical tumors developed

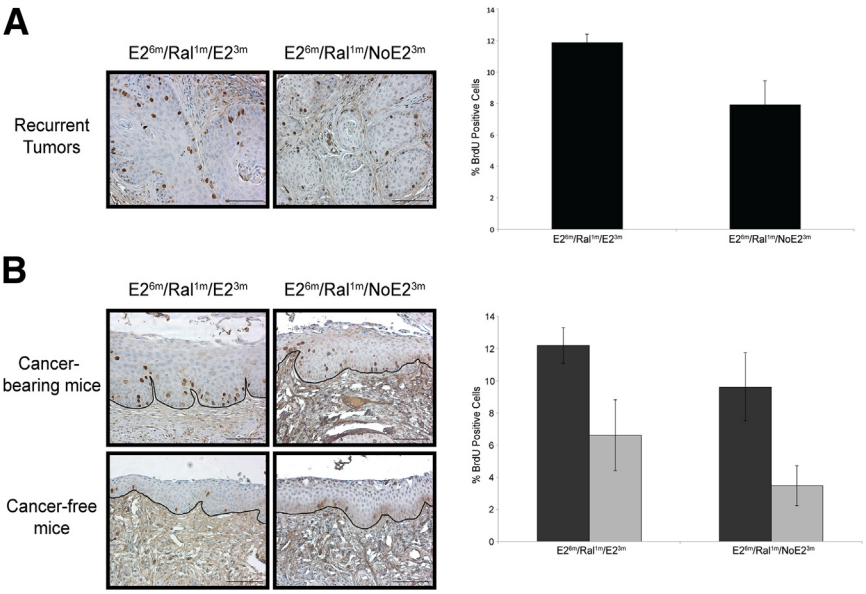

Figure 2 Proliferation of the cervical epithelium is a predictor of cancer recurrence. **A**: Representative images showing BrdU IHC results in recurrent cervical tumors and quantitation of the average percentage of BrdU-positive cells in the cervical epithelium of tumors. For each treatment group, three mice from each condition were included for analysis. Positive cells were quantified from 5 to 10 image fields captured from all areas of the cervix using an automated counting program designed for ImageJ software version 1.47 (see *Materials and Methods*). Statistical comparisons are discussed in the text. Error bars = ±SD. **B**: Representative images of BrdU IHC showing the cervical epithelium of mice with recurrent tumors (black bars) or free of recurrent tumors (gray bars). A **black line** is used to highlight the basement membrane. Quantification was performed and displayed as described in **A**. Scale bars: 100 μm (**A** and **B**).

with an approximately threefold greater multiplicity than in the absence of estrogen treatment (1.36 ± 1.12 versus 0.4 ± 0.52; $P = 0.036$) (Figure 3A). Similar results were also observed for recurrent vaginal cancers (1.09 ± 1.04 versus 0.3 ± 0.67; $P = 0.049$) (Figure 3A). Despite these differences, retreatment with exogenous estrogen did not significantly affect cervical tumor size (Figure 3B). Tumors in the $E2^{6m}/Ral^{1m}/E2^{3m}$ mice had a mean size of 1.29 ± 1.34 mm^2 compared with 1.45 ± 1.44 mm^2 in the $E2^{6m}/Ral^{1m}/NoE2^{3m}$ mice ($P = 0.689$). The largest recurrent cervical tumor measured in mice from the $E2^{6m}/Ral^{1m}/E2^{3m}$ treatment group was 4.39 mm^2, and 3.50 mm^2 in the $E2^{6m}/Ral^{1m}/NoE2^{3m}$ group. Similar results were seen in the vagina of the $E2^{6m}/Ral^{1m}/E2^{3m}$ and $E2^{6m}/Ral^{1m}/NoE2^{3m}$ mice (2.46 ± 2.38 mm^2 versus 1.17 ± 1.14 mm^2; $P = 0.67$), in which the largest tumors were 6.791 and 2.48 mm^2, respectively. Representative images of recurrent cervical tumors from H&E-stained endocervical sections showed the presence of large, recurrent tumors in both groups of mice after cessation of raloxifene therapy (Figure 3C), corroborating the finding that large tumors can re-emerge even in the absence of exogenous estrogen.

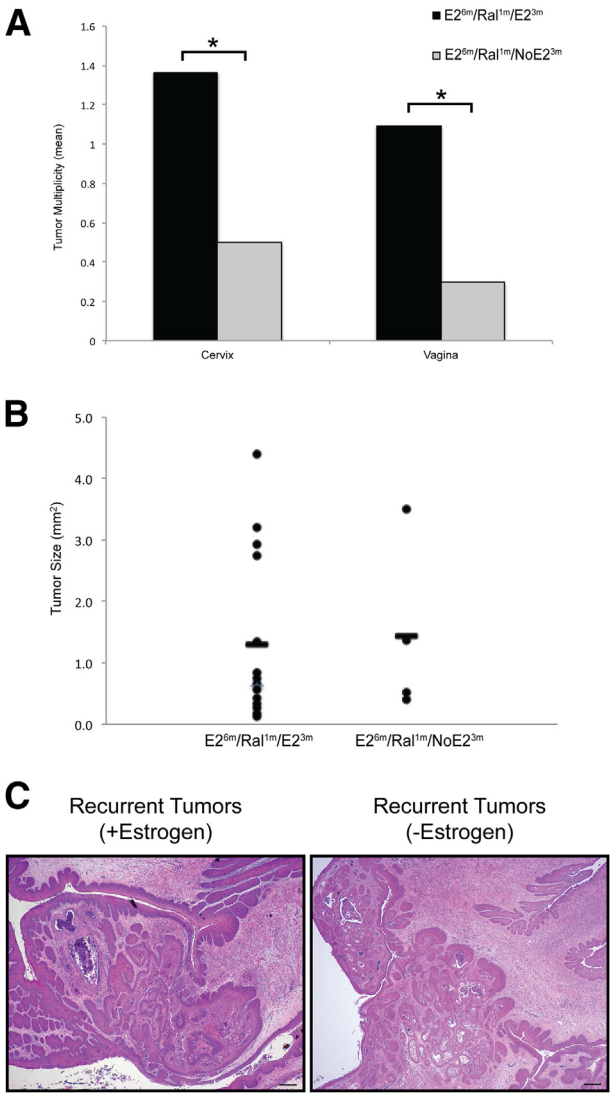

Figure 3 Exogenous estrogen increases the multiplicity of recurrent cervical tumors after raloxifene treatment. **A**: The multiplicity of recurrent tumors in the cervix and vagina is shown. Results are shown as means ± SD. $P < 0.05$ as determined by a two-sided Wilcoxon rank sum test. **B**: Distribution of recurrent cervical tumor sizes is shown. Individual tumor values are shown as black circles, and the average tumor size for the group of mice is shown as a horizontal black line. **C**: Low-magnification images of representative H&E-stained tissue sections highlighting recurrent tumors in the outer cervix and cervicovaginal region. Scale bars: 200 μm.

To evaluate the effect of exogenous estrogen on overall cancer phenotype within the entire cervicovaginal region, data from the cervix and vagina were combined (Table 2). Although the cancer incidence in the lower reproductive tract was higher in the $E2^{6m}/Ral^{1m}/E2^{3m}$ group of mice compared with the $E2^{6m}/Ral^{1m}/NoE2^{3m}$ group (82% versus 40%), it was not statistically significant ($P = 0.08$). Nonetheless, the proportion of mice that developed multiple recurrent tumors in the cervicovaginal region increased 3.7-fold in the presence of exogenous estrogen compared with untreated mice after cessation of raloxifene therapy (73% versus 20%; $P = 0.03$). On average, mice that were re-exposed to exogenous estrogen also had 3.5-fold more tumors in the lower reproductive tract than their untreated counterparts (2.45 ± 1.57 versus 0.7 ± 1.06; $P = 0.01$). These findings are consistent with data obtained from the cervix and vagina separately (Figure 3A). Finally, treatment with exogenous estrogen did not significantly increase the proportion of mice with recurrent tumors >2 mm^2 when compared with untreated mice after cessation of raloxifene therapy (44% versus 25%; $P = 1.0$). This finding is similar to data that showed exogenous estrogen does not significantly affect recurrent tumor size (Figure 3B). Taken together, we conclude that recurrent cancer burden is higher in mice retreated with exogenous estrogen after cessation of raloxifene therapy compared with mice not retreated with estrogen.

Recurrent Tumors Exhibit a Similar Pattern of Biomarker Expression as Initial Tumors and Retain ERα Expression and Function

In addition to cancer characteristics, we also sought to evaluate the stratified squamous epithelium and tumors in the cervices of mice with recurrent cancers for expression of pertinent biomarkers. The MCM7 protein is an E2F-induced cellular protein involved in DNA replication that was previously identified as a biomarker of cervical cancer.[22] Strong IHC positivity for MCM7 throughout all layers of stratified squamous epithelium is associated with high-grade dysplastic lesions and tumors of the cervix. The cervical epithelia of mice in the $E2^{6m}$, $E2^{6m}/Ral^{1m}/E2^{3m}$, and $E2^{6m}/Ral^{1m}/NoE2^{3m}$ treatment groups all had strong MCM7 staining throughout the basal and suprabasal layers (Figure 4A). Interestingly, mice from the $E2^{6m}/Ral^{1m}/NoE2^{3m}$ treatment group had a low, but consistent, level of

Table 2 Summary of Recurrent Cancer Characteristics in the Lower Reproductive Tracts of *K14E6/E7* Transgenic Mice after Raloxifene Treatment

Treatment group	Group size (*n*)	No. (%) of mice with cancer*	No. (%) of mice with multiple cancers†	Mean No. of tumors†	No. (%) of mice with cancers >2 $mm^{2‡}$
$E2^{6m}/Ral^{1m}/E2^{3m}$	11	9 (82)	8 (73)	2.45 ± 1.57	4 (44)
$E2^{6m}/Ral^{1m}/NoE2^{3m}$	10	4 (40)	2 (20)§	0.70 ± 1.06¶	1 (25)

For statistical comparisons between groups, please refer to the text in the *Results* section *Exogenous Estrogen Increases the Multiplicity, But Not Size, of Recurrent Cervical Tumors in Mice after Raloxifene Treatment*.

*Determined for cervix and vagina combined.

†Per total number of mice in group.

‡Per mice with cancer.

§$P < 0.05$ compared with $E2^{6m}/Ral^{1m}/E2^{3m}$ using a two-sided Fisher's exact test.

¶$P < 0.05$ compared with $E2^{6m}/Ral^{1m}/E2^{3m}$ using a two-sided Wilcoxon rank sum test.

deviation from this staining pattern; MCM7-positive cells were restricted to the basal layer within patches of the cervical epithelium (data not shown). No other mice displayed this pattern of MCM7 expression, and it is not understood why this phenotype was only evident in mice from the $E2^{6m}/Ral^{1m}/NoE2^{3m}$ treatment group. Nevertheless, all tumors that re-emerged after cessation of raloxifene therapy stained positively for MCM7 (Figure 4B). The pattern of MCM7 staining in the cervical epithelium and tumors of mice with recurrent cancer was similar to that seen in mice with initial tumors ($E2^{6m}$).

It was important to verify the expression and function of ERα, because this receptor was previously found to be necessary for cervical cancer development in the *K14E7* transgenic mouse model.[19] Therefore, IHC analysis was performed for ERα and one of its transcriptional target genes, PR, in the cervical epithelium and cancer of mice from each treatment group. As shown in Figure 4A, ERα was expressed throughout the stratified squamous epithelium and underlying stroma of mice from all groups analyzed. Regarding the functional capacity of ERα, the cervical epithelium of mice from all treatment groups

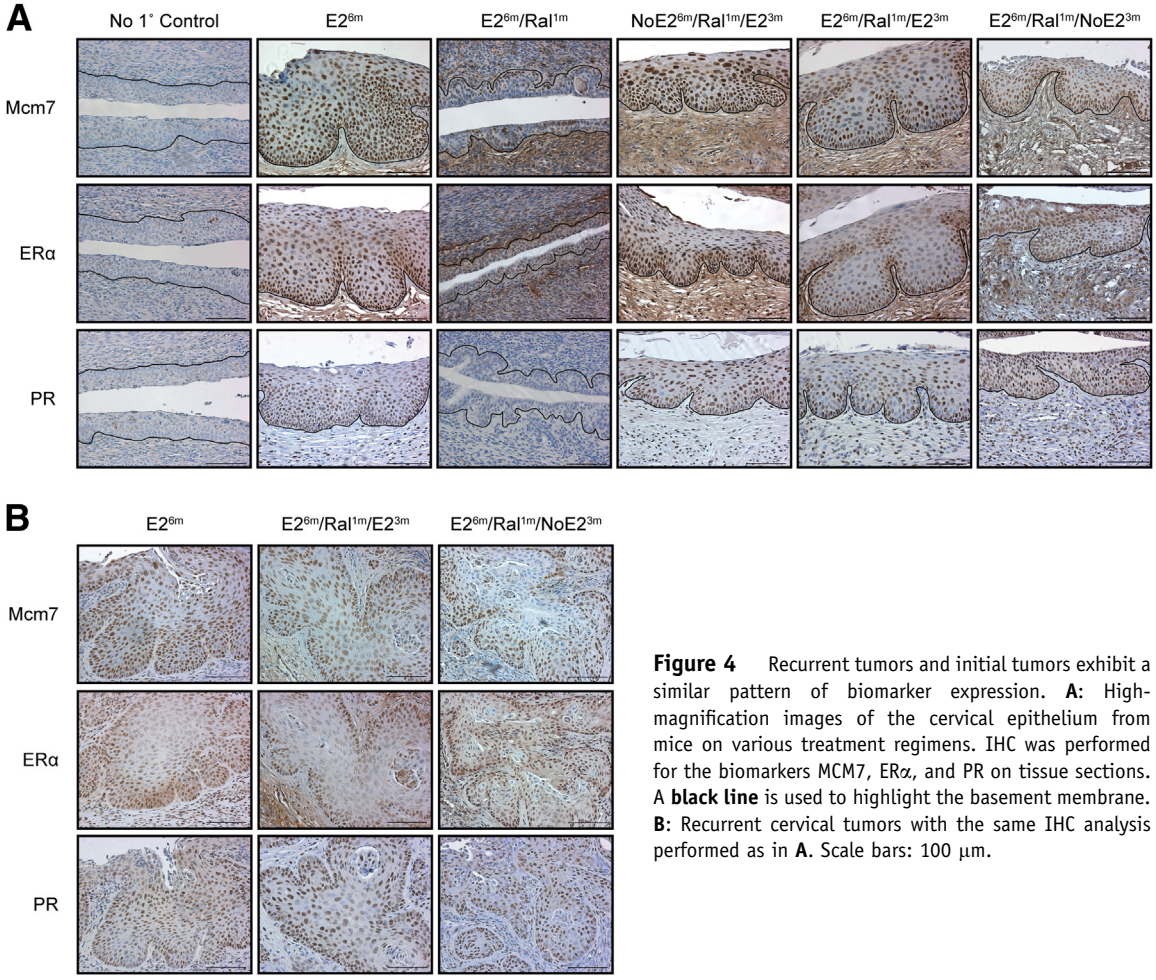

Figure 4 Recurrent tumors and initial tumors exhibit a similar pattern of biomarker expression. **A**: High-magnification images of the cervical epithelium from mice on various treatment regimens. IHC was performed for the biomarkers MCM7, ERα, and PR on tissue sections. A **black line** is used to highlight the basement membrane. **B**: Recurrent cervical tumors with the same IHC analysis performed as in **A**. Scale bars: 100 μm.

Table 3 Summary of Disease in Additional Treatment Groups Testing Models of Cervical Cancer Recurrence

Treatment group	Group size (*n*)	No disease, cervix (vagina)	Dysplasia only			Cancer and dysplasia, cervix (vagina)	No. of mice with cervical cancer (cervical cancer incidence)
			CIN1 (VIN1)	CIN2 (VIN2)	CIN3 (VIN3)		
$E2^{6m}/Ral^{1m}/E2^{3m}/Ral^{1m}$	6	6 (6)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
$E2^{6m}/Ral^{1m}/NoE2^{3m}/Ral^{1m}$	3	3 (3)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
$E2^{6m}/Ral^{4m}$	8	6 (6)	1 (1)	0 (0)	0 (0)	1 (1)	1 (12.5)

Mice were scored histopathologically for the worst disease present in the cervix and vagina (in parentheses). Cervical cancer incidence is compared with values of cervical cancer incidence in treatment groups shown in Table 1. All statistical comparisons noted were obtained using a two-sided Fisher's exact test. For statistical comparisons between groups, please refer to the text in *Results* sections *Recurrent Neoplastic Disease and Cancers Remain Responsive to Raloxifene* and *Sustained Initial Treatment with Raloxifene Prevents Tumor Recurrence*.

CIN, cervical intraepithelial neoplasia; VIN, vaginal intraepithelial neoplasia.

stained positive for the estrogen-responsive gene, PR, except for those from the $E2^{6m}/Ral^{1m}$ group (Figure 4A). These results verify that treatment with raloxifene efficiently suppressed ERα function during our experiments and demonstrate that mice regained ERα function by 3 months after cessation of raloxifene treatment. The latter observation correlates with the histopathological characteristics of the cervical epithelium for mice in the $NoE2^{6m}/Ral^{1m}/E2^{3m}$, $E2^{6m}/Ral^{1m}/E2^{3m}$, and $E2^{6m}/Ral^{1m}/NoE2^{3m}$ treatment groups (Figure 1C), all of which regain hyperplastic epithelia. Similarly, all recurrent tumors stained positive by IHC for ERα and PR (Figure 4B), regardless of whether the tumors re-emerged in mice that did or did not receive additional exogenous estrogen after raloxifene treatment. We also evaluated expression of ERα and PR in the cervical stroma because stromal ERα is required for cervical carcinogenesis.[18] ERα and PR were stained positive in the stroma associated with cancer (Figure 4B) and that distant from cancer (data not shown). Taken together, these data indicate that the ERα is both present and functional in the cervical epithelium, tumors, and tumor-associated stroma of mice that develop recurrent cancers after cessation of treatment with raloxifene.

Recurrent Neoplastic Disease and Cancers Remain Responsive to Raloxifene

The fact that recurrent cancers retained functional ERα led us to assess whether they remained responsive to the ERα antagonist, raloxifene. Two groups of HPV16 transgenic mice were studied ($E2^{6m}/Ral^{1m}/E2^{3m}/Ral^{1m}$ and $E2^{6m}/Ral^{1m}/NoE2^{3m}/Ral^{1m}$). Both were initially treated with exogenous estrogen for 6 months to induce cervical cancers, and then treated with raloxifene for 1 month to cause regression. One group ($E2^{6m}/Ral^{1m}/E2^{3m}/Ral^{1m}$; $n = 6$) was then retreated with estrogen for 3 additional months, whereas the other group ($E2^{6m}/Ral^{1m}/NoE2^{3m}/Ral^{1m}$; $n = 3$) was not re-exposed to estrogen for 3 additional months, to allow cancers to recur. Both groups of mice were then retreated with raloxifene for 1 month. At the end of this retreatment with raloxifene, female reproductive tracts were harvested and scored for disease (Table 3). None of the mice from either group developed cancers. The histopathological features of the cervix and vagina were indistinguishable from those seen in the $E2^{6m}/Ral^{1m}$ mice, such that there was an absence of any neoplastic lesions of any grade, and the cervical/vaginal epithelia were hypoplastic (data not shown). In the case of the $E2^{6m}/Ral^{1m}/E2^{3m}/Ral^{1m}$ group, the reduction in both cancer incidence and severity of disease compared with $E2^{6m}/Ral^{1m}/E2^{3m}$ mice was significant for both the cervix ($P = 0.009$ and $P = 0.0003$, respectively) and the vagina ($P = 0.009$ and $P = 0.0004$, respectively). The lack of cancers in the smaller $E2^{6m}/Ral^{1m}/NoE2^{3m}/Ral^{1m}$ group did not reach statistical significance compared with the $E2^{6m}/Ral^{1m}/NoE2^{3m}$ mice, although the severity of disease was significantly less in the vagina ($P = 0.03$) and nearly significant in the cervix ($P = 0.06$) in the raloxifene-retreated group. Although the sizes of these study groups were small compared with other study groups because of the previously mentioned age-related morbidity issues, the complete absence of neoplastic disease in all nine mice retreated with raloxifene is strongly suggestive that recurrent tumors and their precursor lesions remain responsive to anti-estrogen therapy.

Sustained Initial Treatment with Raloxifene Prevents Tumor Recurrence

Given that recurrent tumors remain responsive to raloxifene, we then asked if keeping mice on raloxifene could prevent recurrent disease from arising altogether. A group of *K14E6/E7* ($n = 8$) mice was treated with estrogen for a period of 6 months to induce dysplasia and cancer, and then these mice were treated for 4 months with raloxifene ($E2^{6m}/Ral^{4m}$). Female reproductive tracts were then harvested and histopathologically scored for disease (Table 3). Six of the eight mice in this group had no disease at all. One retained a cervical cancer, whereas another retained low-grade CIN. Similar results were seen in the vagina. These findings were similar to that observed in the $E2^{6m}/Ral^{1m}$ cohort (Table 1), both in terms of incidence of cancer and severity of disease in the cervix ($P = 1.0$ and $P = 0.4$, respectively) and the vagina ($P = 0.4$ and $P = 0.7$, respectively). These findings suggest that recurrent cancers and other precancerous lesions that arise when raloxifene is discontinued in mice (ie, that seen in the $E2^{6m}/Ral^{1m}/E2^{3m}$ and $E2^{6m}/Ral^{1m}/NoE2^{3m}$

groups) are not arising because they acquired resistance to the SERM. Further analysis of the data indicates that there were significant differences in the incidence of cancer and severity of diseases between the $E2^{6m}/Ral^{4m}$ and $E2^{6m}/Ral^{1m}/E2^{3m}$ groups of mice in both the cervix ($P = 0.02$ and $P = 0.001$, respectively) and the vagina ($P = 0.02$ and $P = 0.002$, respectively), but such differences between the $E2^{6m}/Ral^{4m}$ and $E2^{6m}/Ral^{1m}/NoE2^{3m}$ groups did not reach significance, except in the case of severity of disease in the vagina ($P = 0.05$). These results are similar to the comparisons made between the $E2^{6m}/Ral^{1m}$ group of mice and the two groups in which raloxifene was discontinued ($E2^{6m}/Ral^{1m}/E2^{3m}$ and $E2^{6m}/NoRal^{1m}/E2^{3m}$).

Discussion

Our study demonstrates that treatment of cervical cancers in *K14E6/E7* mice with the ERα antagonist, raloxifene, fails to completely eliminate neoplastic cells, because our findings indicate that cancers re-emerge once SERM treatment is ceased (Table 1 and Figure 1). That these re-emergent cancers and underlying precancerous lesions retain functional ERα (Figure 4) and respond to retreatment with raloxifene (Table 3) indicates that most of the recurrent neoplastic disease is not a consequence of acquired resistance to raloxifene. Consistent with this conclusion, maintaining mice on continuous raloxifene treatment prevented re-emergence of disease (Table 3). If applicable to women, these results provide insight into areas of optimization and improvement for the treatment of HPV-associated cervical cancers using SERMs, which may represent a potential breakthrough for a disease that has few treatment options.

The finding that lower reproductive tract cancers recur after cessation of raloxifene therapy was not completely unanticipated; however, the extent to which recurrent tumors re-emerged within 3 months was surprising because we could not histopathologically detect remnants of cervical cancer after the initial raloxifene treatment in the vast majority of mice (Table 1). We were able to discount the possibility that the cancers arising after cessation of raloxifene treatment arose through *de novo* tumorigenesis, given that cancer incidence was negligible in the $NoE2^{6m}/Ral^{1m}/E2^{3m}$ control group. Therefore, neoplastic cells that arose during the initial 6-month treatment with estrogen and before raloxifene treatment must have been preserved and were subsequently responsible for the re-emergence of neoplastic disease. This re-emergent neoplastic disease was as follows: i) more severe in mice retreated with exogenous estrogen (Table 1), ii) retained functional ERα (Figure 4), and iii) remained dependent on ERα function because retreatment with raloxifene caused the regression of this disease (Table 3). The latter indicates that the disease recurrence is not a consequence of acquired resistance to raloxifene. Consistent with this conclusion, re-emergence of neoplastic disease was prevented by sustained treatment with raloxifene (Table 3). Furthermore, our data indicate that the recurrent disease retains its dependence on estrogen signaling, even if it is only endogenous estrogen that is available. Yet, it appears that the neoplasia recurred rapidly when one considers that the time it took the female reproductive tract to regain responsiveness to estrogen after cessation of raloxifene treatment was substantial (8 to 10 weeks), therefore leaving only 2 to 4 weeks during which the estrogen was actively stimulating ERα function. Consequently, the question remains as to how recurrent neoplastic disease reappears so quickly when our experience with the *K14E6/E7* transgenic mouse model indicates that such a short period of estrogen exposure is unlikely to promote complete neoplastic progression.

One possibility is that the persisting neoplastic cells acquired as-yet-identified properties that make them highly aggressive in their growth once estrogen signaling in the tissue was restored. That those mice with recurrent tumors had more hyperproliferative cervical/vaginal epithelium than mice without recurrent tumors (Figure 2) might suggest that the remaining malignant cells that facilitate the recurrence of cancers are expressing paracrine factors that promote rapid growth. Alternatively, these mice may have some inherent difference (not genetic, because these are inbred mice) that predisposes them to the re-emergence of disease. This difference could be as simple as the time it took for raloxifene to wash out of their reproductive tracts.

The location of recurrent tumors may also reveal insight into what contributes to recurrent disease. Regardless of whether the mice were treated with exogenous estrogen, cervical tumors arose almost exclusively within the outer cervix and cervicovaginal region and rarely developed in the transformation zone. Interestingly, our laboratory found that cervical cancers in *K14E6/E7* mice were more likely to persist in the cervicovaginal junction and outer cervix than in the cervix proper when the initial 6-month exogenous estrogen treatment was followed with a 3-month period of estrogen withdrawal.[16] Although the molecular mechanisms for this finding remain poorly understood, one hypothesis is that this anatomical location of the reproductive tract is more permissive for tumor growth. Qualitative observations made throughout the study presented herein support this hypothesis. In our analysis of cellular proliferation in the cervical epithelium presented in Figure 2, we observed that epithelial cells in the outer cervix had a higher level of BrdU incorporation than those in the endocervix and transformation zone in all mice (data not shown). Moreover, when we analyzed the amount of time required for estrogen responsiveness to reappear after raloxifene treatment (Supplemental Figure S1), the outer cervix and vagina contained PR^+ cells several weeks before the endocervix and transformation zone (data not shown). Taken together, the BrdU and PR IHC data indicate that there are various levels of proliferation and estrogen sensitivity within different areas of the cervix. These qualitative assessments

suggest that the outer cervix and cervicovaginal region may be more proliferative and more sensitive to estrogen than the cervix proper and may help explain why we see a preponderance for recurrent tumor development in the outer cervix. Perhaps these observations help explain the apparently rapid onset of recurrent disease. For instance, it is possible that in those regions where tumors recurred, estrogen function was regained several weeks earlier, thus providing more time for the expansion of persisting neoplastic cells.

Whether the recurrent neoplastic disease differs from the original disease, the fact that maintaining the mice on raloxifene was highly effective at preventing frequent recurrence provides important insight into any application of this targeted therapy to the treatment of human cervical cancer.

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Supplemental Data

Supplemental material for this article can be found at *http://dx.doi.org/10.1016/j.ajpath.2013.10.013*.

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