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ORIGINAL PAPER

Requirement for Stromal Estrogen Receptor Alpha in Cervical Neoplasia

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Abstract The major etiological factor for cervical cancer is the high-risk human papillomavirus (HPV), which encodes *E6* and *E7* oncogenes. However, HPV is not sufficient, and estrogen has been proposed as an etiological cofactor for the disease. Its requirement has been demonstrated in mouse models for HPV-associated cervical cancer (e.g., *K14E7* transgenic mice). Although germline knockout of estrogen receptor alpha (ERα) renders mice resistant to cervical cancer, the cell-type-specific requirement for ERα is not known. In this study, we demonstrate that temporal deletion of stromal ERα induced complete regression of cervical dysplasia in *K14E7* mice. Our results strongly support the hypothesis that stromal ERα is necessary for HPV-induced cervical carcinogenesis and implicate paracrine mechanisms involving ERα signaling in the development of estrogen-dependent cervical cancers. This is the first evidence to support the importance of stromal ERα in estrogen-dependent neoplastic disease of the female reproductive tract.

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Introduction

Cervical cancer is the second most frequent cancer and the second leading cause of death by cancer in women worldwide [1, 2]. The vast majority of cervical cancer is associated with specific types of human papillomavirus (HPV), the so-called high-risk HPVs. Specifically, the high-risk HPV16 and HPV18 genotypes are found in approximately 60 and 20 % of all cervical cancers, respectively [3]. The tumorigenic potential of these viruses stems mainly from two viral oncogenes, E6 and E7, which are best known for their ability to inactivate p53 and pRb tumor suppressor protein, respectively [2–4]. These oncogenes are necessary for the progression of cervical disease (CIN1, CIN2, CIN3, and invasive cancer) and the continued growth of cervical cancer. It is estimated that approximately 75 % of sexually active women are infected with HPVs, yet only a minor fraction of such women develops cervical cancer [5]. This observation has suggested that HPV infection alone is not sufficient for cervical cancer and that other cofactors are also necessary. Long-term use of oral contraceptives (OCs) or high parity is associated with higher risk for cervical cancer in HPV-infected women [6, 7]. These results implicate estrogen and/or progesterone in HPV-induced cervical cancer because they are the factors common to both variables (OCs and pregnancy). Complications in looking at a specific association of estrogen in human cervical cancer are discussed in a recent review [8], and the role of estrogen in human cervical cancer therefore remains unclear.

An essential role of estrogen in cervical cancer, however, has been clearly defined in HPV transgenic mouse models. HPV16 transgenic mice express the E6 (*K14E6*), E7 (*K14E7*), or both (*K14E6*/*K14E7*) oncogenes under the control of human keratin 14 (K14) promoter, which drives transgene expression in stratified squamous epithelia, the natural host cell type for HPV infection. An HPV oncogene in conjunction with physiological levels of exogenous

estrogen promotes the development of cervical cancer, whereas either one of the two factors alone does not [9–12]. Using this validated hormone/oncogene codependence mouse model, we previously determined that estrogen receptor α (ERα) is necessary for estrogen to cooperate with HPV in the development and continued growth of cervical cancer [13, 14].

Stromal cells play a pivotal role in development. For example, recombination of uterine stroma with vaginal epithelium results in the development of uterine epithelium in vivo [15]. More recently, an in vivo uterine epithelial specific ERα knockout shows estrogen-induced proliferation dependent on uterine stroma [16]. Stromal microenvironment also contributes to the development of carcinomas. For instance, cancer-cell-derived transforming growth factor beta (TGF-β) promotes transdifferentiation of fibroblasts to myofibroblasts, which in turn support and/or promote cancer cell invasion and metastasis [17]. Stromal p53 mutation is associated with nodal metastasis in sporadic breast cancers [18], and deletion of the APC tumor suppressor in the stroma promotes the development of endometrial cancer in mice [19]. Such signaling pathways in stroma that support carcinogenesis are attractive targets for cancer therapy.

ERα is crucial for the estrogenic responses (e.g., epithelial cell proliferation) of hormone-responsive tissues such as mammary glands and female reproductive tracts [20]. It is also critical for various cancers including breast cancer [21]. Although the role of stromal ERα in tissue homeostasis and organogenesis has been extensively evaluated [16, 22, 23], it is poorly understood in the context of cancer. In the present study, we utilized conditional *ERα* knockout ($ER\alpha^{f/f}$) mice to assess whether stromal ERα is important for cervical carcinogenesis in the *K14E7* transgenic mouse model. Our results show for the first time that ERα expressed in stromal cells is required for estrogen-dependent cervical cancer in the HPV transgenic mouse model.

Materials and Methods

Mice and Treatments *K14E7* transgenic mice and conditional *ERα* knockout ($ER\alpha^{f/f}$) mice were described previously [24, 25]. *CAGGCre-ER*^{TM} (referred to as *CMVCreER* herein) transgenic mouse was purchased from the Jackson Laboratory [26]. This mouse was generated to drive expression of tamoxifen-inducible cre recombinase ubiquitously in all tissues and cell types. Experimental mice were generated by crossing *K14E7/*$ER\alpha^{f/f}$ and *CMVCreER/*$ER\alpha^{f/f}$, which were obtained by intercrossing F1 generations of *K14E7* (FVB) and $ER\alpha^{f/f}$ (albino C57BL/6) mating and *CMVCreER* (C57BL/6 x CBA x SWR) and $ER\alpha^{f/f}$ mating, respectively. Female progenies were genotyped by PCR. A slow-releasing 17β-estradiol tablet (0.05 mg/60 days) (Innovative Research of America, Sarasota, FL) was inserted subcutaneously under the dorsal skin every 2 months beginning at 4–6 weeks of age. Groups of mice were injected intraperitoneally (i.p.) with tamoxifen (4 mg/day) for 5 days after 6-month estrogen treatment to activate cre [26]. Mice were injected i.p. with 0.3 ml of bromo-deoxyuridine (BrdU) (12.5 mg/ml) 1 hr prior to euthanasia to measure cellular proliferation. All procedures were carried out according to an animal protocol approved by the University of Wisconsin Medical School Institutional Animal Care and Use Committee.

Tissue Processing and Histological Analyses Female reproductive tracts were fixed in 4 % paraformaldehyde and embedded in paraffin. Serial sections were made throughout cervices at 5-μm thickness. Every tenth slide was stained with hematoxylin and eosin (H&E), and the worst disease in each mouse was determined as described previously [11].

Immunohistochemistry Antibodies were purchased from Santa Cruz [PR (H190) and ERα (MC20)], Calbiochem (BrdU), Rockland (biotinylated anti-rabbit/mouse IgG), and Invitrogen (anti-rabbit IgG conjugated with Alexa 488). Immunohistochemical stainings for PR, ERα, and BrdU were performed as described previously [13, 27, 28].

Statistical Analyses Two-sided Fisher's exact test and Wilcoxon rank sum test were carried out with MSTAT software version 5.4.[1] Fisher's exact test was used for cancer incidence and number of disease-free mice, and Wilcoxon rank sum test for disease severity and number of $ER\alpha^{+}$ or $BrdU^{+}$ cells.

Results

Tamoxifen Treatment Induces Deletion of ERα in the Cervical Stroma but not in the Epithelium of CMVCreER/$ER\alpha^{f/f}$ *Mice* The initial goal of this study was to evaluate the temporal requirements for ERα in all cells within the cervix during different stages in cervical carcinogenesis. To accomplish this, we made use of the $ER\alpha^{f/f}$ mice carrying a conditional (floxed) allele of ERα, crossed to the *CMVCreER* mice, which were chosen because they were expected to drive cre expression ubiquitously in all tissues and cell types of the mouse reproductive tract and other organs. We tested various tamoxifen treatment regimens (daily i.p. injections, 0.5, 1, 2, 3, 4, 5 mg/day for 1, 3, or 5 days) based on prior studies [26, 29]. The effect of each dosing schedule was initially evaluated by monitoring for gross changes in the reproductive tracts and measuring their wet weight after 2 weeks of the first dose. We observed that

[1] http://www.mcardle.wisc.edu/

treatment with 4 mg of tamoxifen for 5 days resulted in most dramatic morphological changes without morbidity (Fig. 1a). Tamoxifen-treated mice had hemorrhagic ovaries and atrophic reproductive tracts, which is reminiscent of ERα knockout mice [30]. Although treatment with 5 mg of tamoxifen for 3 days resulted in similar effects in surviving animals, this dose incurred morbidity and mortality in two of five mice (40 %). We also evaluated ERα expression by immunohistochemistry (IHC). To our surprise, ERα expression was not affected in the cervical epithelium, yet absent in the cervical stroma (Fig. 1b, top panel). In contrast, ERα expression was abrogated in both epithelium and stroma of the uterus (Fig. 1b, bottom panel). We did not observe epithelial ERα deletion in cervices of *CMVCreER/ERα$^{f/f}$* mice treated with 4 mg of tamoxifen for 1, 3, or 5 days and killed 24 h after the final injection (Online Resource 1). ERα expression was also retained in the cervical epithelium of *K14Cre/ERα$^{f/f}$* mice of which ovaries are removed (Online Resource 1), despite the fact that *K14Cre* efficiently deletes other floxed genes in the cervical epithelia [31, 32]. This raises the possibility that the floxed ERα allele in cervical epithelial cells is resistant to cre-mediated recombination. Regardless of why the ERα allele was not deleted in the cervical epithelia, this fact provided us the opportunity to evaluate the individual role of stromal ERα in cervical carcinogenesis.

Cervical Disease is Absent in CMVCreER/K14E7/ERα$^{f/f}$ Mice Treated with Tamoxifen for 5 Days To address whether stromal ERα is crucial for cervical carcinogenesis in the mouse model, we generated *CMVCreER/K14E7/ERα$^{f/f}$* and *K14E7/ERα$^{f/f}$* mice, and each genotype was divided into three treatment groups (Fig. 2a). Female reproductive tracts were harvested after treatment with 17β-estradiol (E2) for 6 months (6 mE2 group), which is sufficient to promote cervical cancer in *K14E7* mice at varying penetrance depending on experimental conditions and genetic background [11, 13, 32, 33]. The other groups were further treated with E2 for two more months and given oil vehicle [8 mE2 (–Tam) group] or tamoxifen [8 mE2 (+Tam) group] for 5 days at 6-month treatment with E2. These treatment regimens were designed to evaluate importance of stromal ERα in continued growth of cervical cancer and progression of CIN to invasive cancer. Female reproductive tracts were isolated at each end point as depicted in Fig. 2a. Each mouse was histopathologically evaluated for the worst cervical and vaginal disease as previously described (ERα-dependent vaginal cancer also arises in our mouse model) [10, 13].

The vast majority of *K14E7/ERα$^{f/f}$* 6mE2 (14 of 14) and *CMVCreER/K14E7/ERα$^{f/f}$* 6mE2 (12 of 14) mice had high-grade dysplasia, CIN2/3, indicative of neoplastic progression, though none had developed cervical cancer (Table 1). This was surprising because E2 treatment for 6 months is sufficient to promote cervical cancers in the majority of *K14E7* transgenic mice on FVB background [11, 32]. By 8-month E2 treatment, cervical cancers were beginning to arise in both the *K14E7/ERα$^{f/f}$* and *CMVCreER/K14E7/ERα$^{f/f}$* mice (Table 1). Considering that mice used in this study are on a mixed genetic background from four strains, these data indicate that the rate of progression of cervical carcinogenesis likely depends on the genetic background of mice. The high penetrance of high-grade dysplasia at the 6-

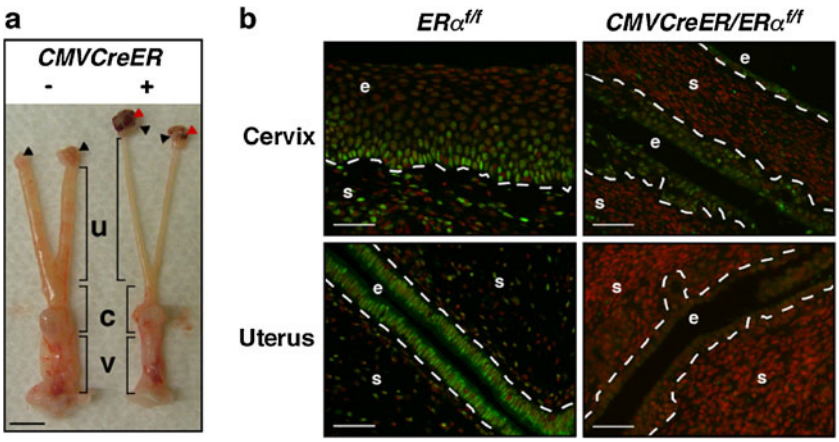

Fig. 1 Tamoxifen induces efficient deletion of ERα only in cervical stroma of *CMVCreER/ERα$^{f/f}$* mice. **a** Tamoxifen treatment induces atrophic female reproductive tracts in *CMVCreER/ERα$^{f/f}$* mice. *ERα$^{f/f}$* (CMVCreER−, *left*) and *CMVCreER/ERα$^{f/f}$* (CMVCreER+, *right*) mice were i.p. injected with tamoxifen (4 mg/day for 5 days). The female reproductive tracts were harvested 2 weeks after the first dose. *Black* and *red arrowheads* indicate ovaries and hemorrhagic cysts, respectively. *u* uterus, *c* cervix, *v* vagina. Scale bar, 5 mm. **b** ERα expression is retained in the cervical epithelium of *CMVCreER/ERα$^{f/f}$* mice treated with tamoxifen. Mice were treated as in **a** and paraffin sections were stained for ERα (*green*). DAPI-stained nuclei are pseudocolored red. Note that ERα is readily detected in cervical epithelium (*e*) but not in cervical stroma (*s*) (*upper panel*) and both compartments of the uterus (*bottom panel*) in *CMVCreER/ERα$^{f/f}$* mice. *Dotted lines* indicate basement membrane separating epithelium from stroma. Scale bar, 50 μm

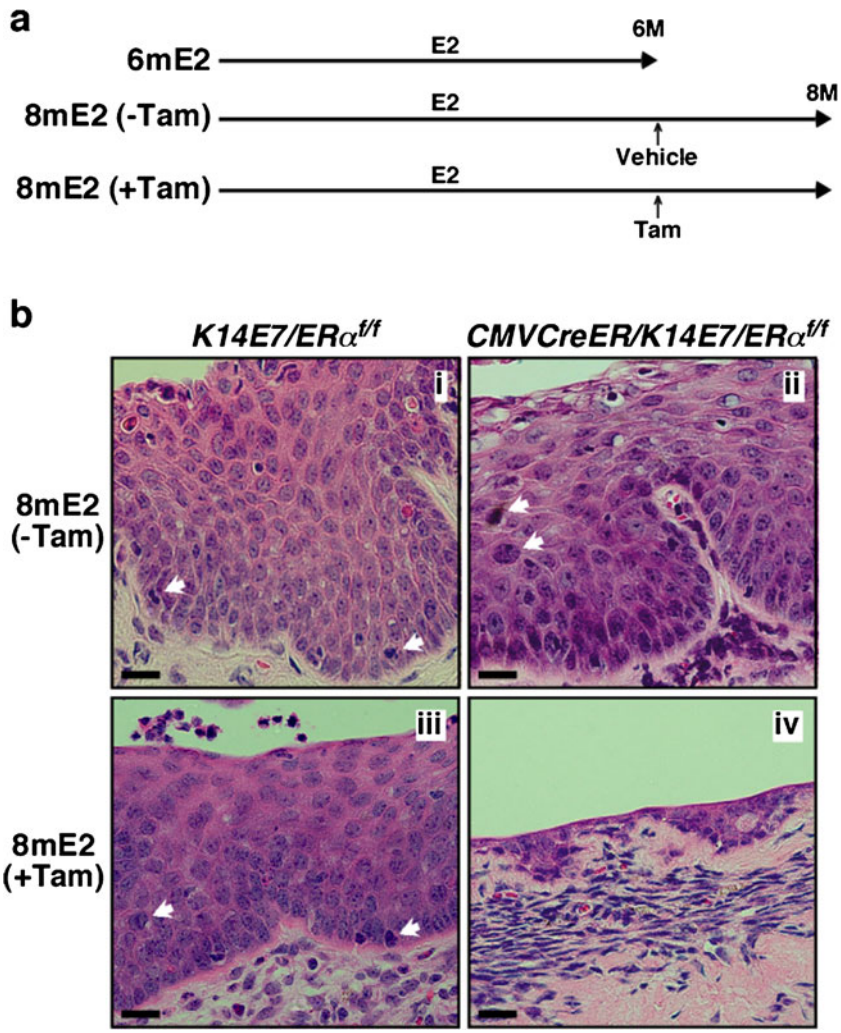

Fig. 2 Cervical disease is absent in *CMVCreER/K14E7/ERα$^{f/f}$* mice treated with tamoxifen. **a** Treatment regimen is depicted. E2 and Tam indicate estrogen and tamoxifen, respectively. **b** Shown are high-magnification images of representative H&E-stained endocervical sections from indicated groups of mice. *Arrows* point to atypia manifested as dark and enlarged nuclei. Note that cervical intraepithelial neoplasia (*CIN*) is evident in *K14E7/ERα$^{f/f}$* 8mE2 (−Tam), *CMVCreER/K14E7/ERα$^{f/f}$* 8mE2 (−Tam), and *K14E7/ERα$^{f/f}$* 8mE2 (+Tam) mice (*panels i–iii*) but absent in *CMVCreER/K14E7/ERα$^{f/f}$* 8mE2 (+Tam) mice (*panel iv*). Scale bar, 20 μm

month E2 treatment endpoint did provide us the ability to ask what is the importance of stromal ERα in this stage of cervical neoplasia. That the overall disease severity (p=0.07) and number of cervical disease-free mice (p=0.48) were not significantly different between the *K14E7/ERα$^{f/f}$* 6mE2 and *CMVCreER/K14E7/ERα$^{f/f}$* 6mE2 (not treated with tamoxifen) confirmed that *CMVCreER* transgene itself had no influence on cervical carcinogenesis. As mentioned before, cervical cancers were observed when both genotypes were treated with E2 for 8 months [2 of 15 *K14E7/ERα$^{f/f}$*

Table 1 State of lower reproductive tract disease

Group name (genotype and treatment)	ERα status		Group size (n)	No disease	Dysplasia only			Cancer & dysplasia
	Stroma	Epithelia		Cervix (Vagina)	CIN1 (VIN1)	CIN2 (VIN2)	CIN3 (VIN3)	Cervix (Vagina)
K14E7/ERα$^{f/f}$ 6mE2	+	+	14	0 (0)	0 (0)	0 (5)	14 (9)	0 (0)
CMVCreER/K14E7/ERα$^{f/f}$ 6mE2	+	+	14	2 (2)	0 (0)	1 (3)	11 (9)	0 (0)
K14E7/ERα$^{f/f}$ 8mE2 (+Tam)	+	+	18	4 (3)	0 (0)	0 (2)	13 (12)	1 (0)
K14E7/ERα$^{f/f}$ 8mE2 (-Tam)	+	+	15	0 (0)	0 (0)	0 (1)	13 (13)	2 (0)
CMVCreER/K14E7/ERα$^{f/f}$ 8mE2 (+Tam)	−	+	18	16 (16)	0 (0)	0 (1)	2 (1)	0 (0)
CMVCreER/K14E7/ERα$^{f/f}$ 8mE2 (−Tam)	+	+	4	0 (0)	0 (0)	0 (1)	3 (3)	1 (0)

Mice were scored histopathologically for the worst disease present in the cervix or, in parentheses, the vagina. For Wilcoxon rank sum test (see text), each lesion was given the following arbitrary score; no disease=1; CIN1 (VIN1)=2; CIN2 (VIN2)=3; CIN3 (VIN3)=4; cancer=5

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

8mE2 (−Tam) mice and one of four *CMVCreER/K14E7/ERα$^{f/f}$* 8mE2 (−Tam) mice]. Overall disease severity in *CMVCreER/K14E7/ERα$^{f/f}$* 8mE2 (−Tam) and *K14E7/ERα$^{f/f}$* 8mE2 (−Tam) was similar (p=0.29), confirming no significant effect of *CMVCreER* transgene in the absence of tamoxifen treatment. Consistently, cervical epithelia of *K14E7/ERα$^{f/f}$* 8mE2 (−Tam) and *CMVCreER/K14E7/ERα$^{f/f}$* 8mE2 (−Tam) were histologically indistinguishable (Fig. 2b, subpanels i and ii). Next, we compared cervical disease phenotypes between *K14E7/ERα$^{f/f}$* 8 mE2 (−Tam) and *K14E7/ERα$^{f/f}$* 8mE2 (+Tam). The number of cervical disease-free mice (p=0.11) and overall disease severity (p=0.06) were not significantly different between these two control groups (Table 1). Their epithelia also were similar to each other at the histological level (Fig. 2b, panels i and iii). These control comparisons indicate that the 5-day-long tamoxifen treatment itself has no significant effect on cervical carcinogenesis in our mouse model. Strikingly, only 2 of 18 (11.1 %) *CMVCreER/K14E7/ERα$^{f/f}$* 8mE2 (+Tam) mice had CIN3, and the rest were disease-free, whereas 14 of 18 *K14E7/ERα$^{f/f}$* 8 mE2 (+Tam) mice had CIN3 or cervical cancer (Table 1). Differences in the overall disease severity ($p=3.7\times10^{-5}$) and the frequency of disease-free mice ($p=1.3\times10^{-4}$) between the two groups were highly significant. The cervical epithelia of *CMVCreER/K14E7/ERα$^{f/f}$* 8mE2 (+Tam) mice were hypoplastic compared to those of *K14E7/ERα$^{f/f}$* 8mE2 (+Tam) mice (Fig. 2b, panels iii and iv). Similar differences in disease phenotypes between these two groups were observed in vaginal tissues (Table 1).

Cervical Disease States Correlate with ERα Status in the Cervical Stroma In order to confirm that the absence of cervical disease in *CMVCreER/K14E7/ERα$^{f/f}$* 8mE2 (+Tam) mice was due to lack of ERα expression in the stroma, we evaluated cervical tissues for ERα expression by IHC. As expected, ERα expression was readily detected in stroma and epithelia of *K14E7/ERα$^{f/f}$* 8mE2 (−Tam), *K14E7/ERα$^{f/f}$* 8mE2 (+Tam), and *CMVCreER/K14E7/ERα$^{f/f}$* 8mE2 (−Tam) mice (Fig. 3a, panels i–iii). In contrast and similar to that shown in Fig. 1b, ERα-positive stromal cells were rarely found in *CMVCreER/K14E7/ERα$^{f/f}$* 8mE2 (+Tam) mice, while ERα expression in the epithelia remained highly penetrant (Fig. 3a, panel iv). Quantitative analyses showed that only 1.2 % of cervical stromal cells in disease-free *CMVCreER/K14E7/ERα$^{f/f}$* 8mE2 (+Tam) mice expressed ERα, whereas 77.2 % in *K14E7/ERα$^{f/f}$* 8mE2 (+Tam) mice did (Fig. 3b). This difference was highly significant (p=0.005). We also investigated ERα status in the cervices of two *CMVCreER/K14E7/ERα$^{f/f}$* 8mE2 (+Tam) mice that had CIN3 (see Table 1). We found that 79.0 % of cervical stromal cells expressed ERα (Figs. 3b, c), which is comparable to *K14E7/ERα$^{f/f}$* 8mE2 (+Tam) mice (compare Fig. 3a, panel iii, and Fig. 3c; p=0.22). It is unclear why tamoxifen treatment was not efficient in activating cre activity in these two mice. Nonetheless, these results point further to the correlation between the retention of cervical neoplastic disease and ERα expression in the stroma. Female reproductive tracts were isolated from a subset of *CMVCreER/K14E7/ERα$^{f/f}$* 8mE2 (+Tam) mice a day after tamoxifen treatment for 3 or 5 days to confirm the absence of ERα deletion in cervical epithelia. While stromal ERα was deleted, expression of epithelial ERα was not affected (Fig. 3d), further supporting that absence of cervical diseases is due to loss of ERα in the stroma but not in the epithelium. Expression of progesterone receptor (PR) in the epithelium and stroma of female lower reproductive tracts is dependent upon ERα in the epithelium and stroma, respectively [15, 27]. We found that PR was expressed in cervical epithelial cells in *CMVCreER/K14E7/ERα$^{f/f}$* 8mE2 (+Tam) mice as well as *K14E7/ERα$^{f/f}$* 8mE2 (+Tam) mice (Fig. 3e). In contrast, PR expression was barely detectable in the *ERα*-deleted cervical stroma of the *CMVCreER/K14E7/ERα$^{f/f}$* 8mE2 (+Tam) mice, unlike *ERα*-intact stroma of *K14E7/ERα$^{f/f}$* 8mE2 (+Tam) mice. This result indicates that ERα is functional specifically in the epithelium, but not the stroma of *CMVCreER/K14E7/ERα$^{f/f}$* 8mE2 (+Tam) mice. Taken together, we conclude that stromal ERα is necessary for cervical carcinogenesis in HPV transgenic mouse model.

Deletion of Stromal ERα Abrogates Cell Proliferation in the Cervical Epithelia We also investigated if estrogen-dependent epithelial cell proliferation in the cervices of *CMVCreER/K14E7/ERα$^{f/f}$* 8mE2 (+Tam) mice was compromised. We found that proliferation indices of *K14E7/ERα$^{f/f}$* 8mE2 (−Tam), *K14E7/ERα$^{f/f}$* 8mE2 (+Tam), and *CMVCreER/K14E7/ERα$^{f/f}$* 8mE2 (−Tam) were similar in both basal (13.8–15.2 %) and suprabasal layer (5.7–6.1 %) of the cervical epithelia (Figs. 4a, b). These results demonstrate that tamoxifen or *CMVCreER* transgene, individually, had no effect on cervical epithelial cell proliferation, consistent with cervical disease phenotypes shown in Table 1. In contrast, proliferation indices of basal and suprabasal layer of the cervical epithelia of *CMVCreER/K14E7/ERα$^{f/f}$* 8mE2 (+Tam) mice were 1.6 and 0.1 %, respectively (Figs. 4a, b). These proliferation indices were significantly lower than that observed in *K14E7/ERα$^{f/f}$* 8mE2 (+Tam) mice (p=0.03) demonstrating that stromal ERα is necessary for proliferation of basal and suprabasal cells in the cervical epithelium.

Discussion

ERα plays a pivotal role in the development of various cancers including, but not limited to, breast cancers [20]. Estrogen cooperates with HPV oncogenes in a mouse model for HPV-associated cervical cancer [10–12, 34], and ERα is

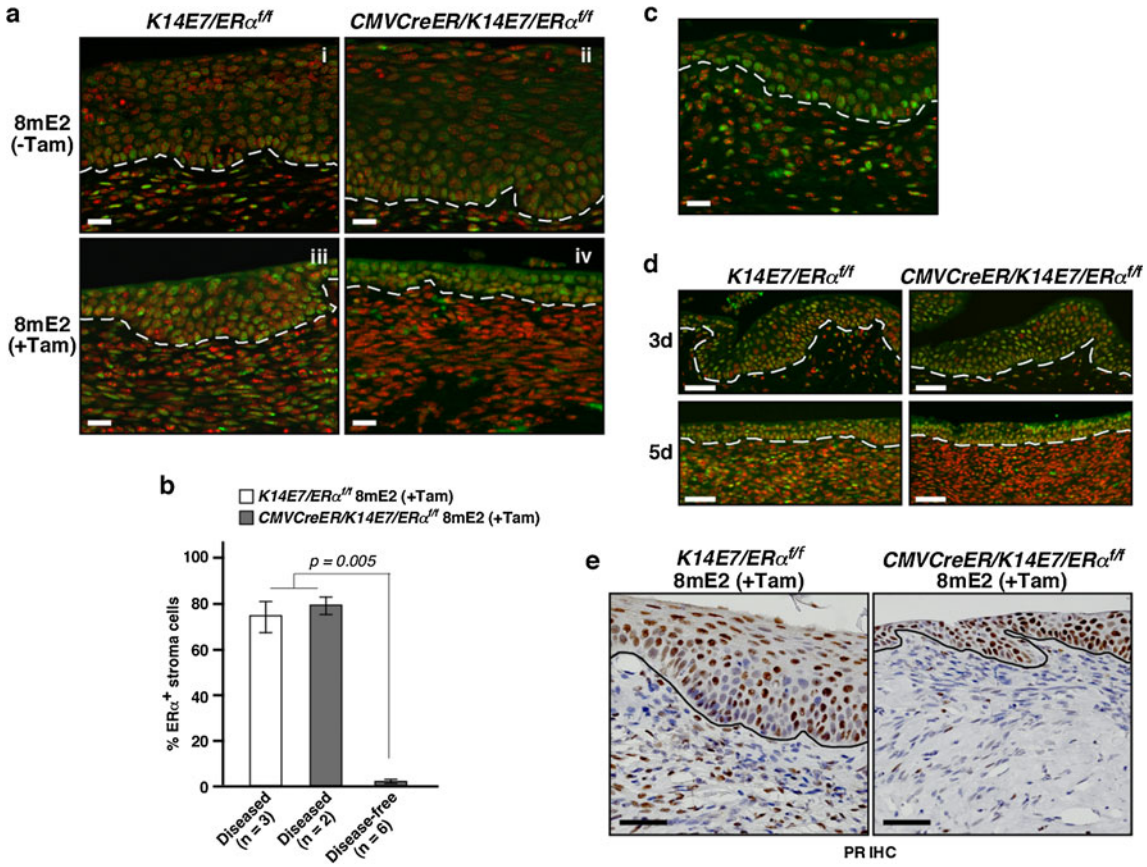

Fig. 3 Cervical disease states correlates with the ERα status in the cervical stroma. **a** ERα expression is ablated in the cervical stroma of *CMVCreER/K14E7/ERα$^{f/f}$* 8mE2 (+Tam) mice without cervical disease. Paraffin sections of female reproductive tracts from indicated groups of mice were stained for ERα (*green*). All diseased mice expressed ERα in both epithelium and stroma of the cervix (*panels i–iii*), yet ERα was barely detectable in the cervical stroma of disease-free *CMVCreER/K14E7/ERα$^{f/f}$* 8mE2 (+Tam) mice (*panel iv*). DAPI-stained nuclei are *pseudocolored red*. Scale bar, 20 μm. **b** Results shown in **a** and **c** were quantified for number of ERα^{+} cells. At least 1,000 cervical stromal cells in four random fields of each female reproductive tract were analyzed. Data are shown as mean±SEM. *p* value for two-sided Wilcoxon rank sum test is shown. **c** ERα expression is retained in cervical stroma of *CMVCreER/K14E7/ERα$^{f/f}$* 8mE2 (+Tam) mice with CIN. Paraffin sections from female reproductive tracts of the two mice that had cervical disease in the *CMVCreER/K14E7/ERα$^{f/f}$* 8mE2 (+Tam) group were stained for ERα (*green*). DAPI-stained nuclei are *pseudocolored red*. Scale bar, 20 μm. **d** Epithelial ERα is not deleted shortly after tamoxifen treatment. Mice were treated with E2 for 6 months, treated with tamoxifen (4 mg) for 3 days (*top panel*) or 5 days (*bottom panel*), and killed a day later. Paraffin sections were subjected to ERα IHC (*green*). DAPI-stained nuclei are *pseudocolored red*. Scale bar, 50 μm. **e** PR is expressed in the cervical epithelium of *CMVCreER/K14E7/ERα$^{f/f}$* 8mE2 (+Tam) mice. Cervical tissues from indicated study groups were subjected to PR IHC (brown nuclei). Nuclei were counterstained with hematoxylin. Representative images from three mice in each group are shown. The *black lines* point to basement membrane. Scale bar, 50 μm

required for this synergistic effect of estrogen and HPV oncogenes [13]. In this study, we investigated cell-type-specific requirement of ERα in HPV-mediated cervical carcinogenesis and learned that deletion of ERα in cervical stroma results in regression of CIN3 and dramatic reduction in cervical epithelial cell proliferation in *K14E7* transgenic mice (Table 1 and Figs. 3 and 4). Epithelial ERα was intact immediately after tamoxifen treatment and was functional as demonstrated by expression of PR in cervical epithelium of *CMVCreER/K14E7/ERα$^{f/f}$* 8mE2 (+Tam) mice (Fig. 3e). These results indicate that epithelial ERα is not sufficient, and stromal ERα is necessary for cervical carcinogenesis. These findings provide direct evidence that a paracrine mechanism mediated by stromal ERα is necessary for the maintenance of neoplastic state in the mouse cervix. It is, however, unclear if stromal ERα is required for continued growth of cervical cancer as well because we did not observe frank cancer in the control mice (*CMVCreER/K14E7/ERα$^{f/f}$* 6mE2). Nonetheless, this is the first study to show the requirement of stromal ERα for estrogen-dependent cervical carcinogenesis in vivo. This finding is consistent with prior observations that ERα expression is retained in the stroma surrounding cervical cancer in women [35, 36]. Most breast cancer cells require ERα for continued growth and epithelial ERα is required for proliferation of mammary epithelial cells in mice [37]. Although a role of stromal ERα in the development of ERα-positive breast cancer has not been elucidated, ERα expressed in Tie2-positive stromal cells

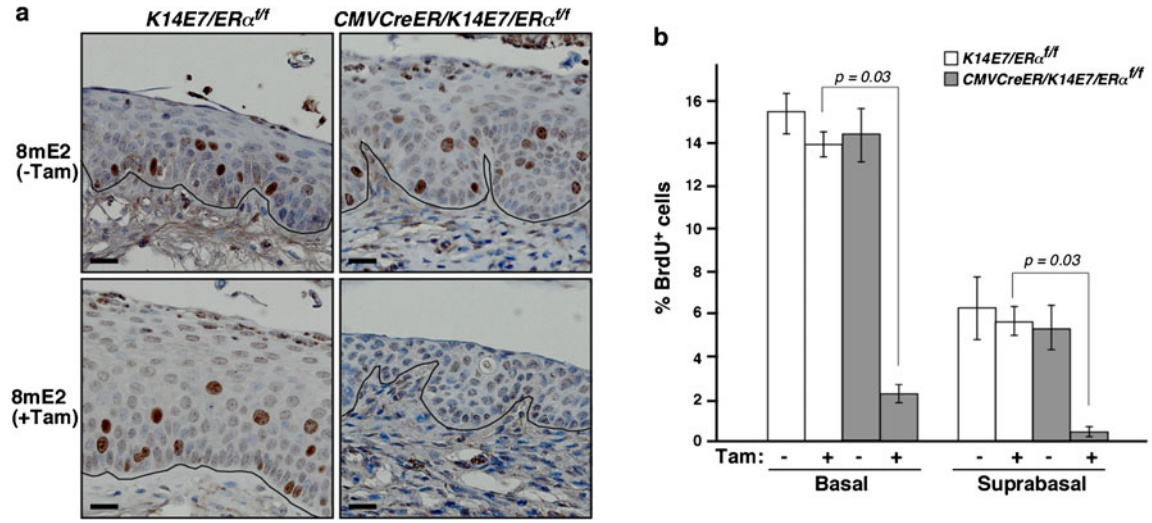

Fig. 4 Cervical epithelial cell proliferation is significantly reduced when ERα expression is ablated in the stromal cells. **a** BrdU incorporation is reduced in the cervical epithelia of *CMVCreER/K14E7/ERα*$^{f/f}$ 8mE2 (+Tam) mice. Paraffin sections from indicated study groups were stained for BrdU to measure cell proliferation (brown nuclei). Nuclei were counterstained with hematoxylin. Representative images from three mice for each group are shown. Scale bar, 20 μm. **b** BrdU^{+} cells shown in **a** were quantified. Data are shown as mean±SEM (n= 3). p values for two-sided Wilcoxon rank sum test are shown

(e.g., endothelial cells) promotes growth of ERα-negative cancers by mediating adaptation of tumor angiogenesis [38]. ERα expressed in prostate stromal cells promotes expression of MMP2 via induction of TGF-β1, which enhances invasion of prostate cancer cells into Matrigel in vitro [39]. These results support the idea that stromal ERα may exert distinct functions depending on cancers.

A Model for Roles of ERα in Cervical Carcinogenesis Although HPV oncogenes (*i.e.*, *E6* and *E7*) are necessary for continued growth of cervical cancer cells [40, 41], their ability to promote cell proliferation is largely restricted to the suprabasal layer of the murine cervical epithelium [12, 33]. However, this latter activity is severely compromised when expression of wt ERα is abolished in the whole reproductive tract [13]. ERα is known to induce proliferation in basal layer of the cervical epithelium but not in suprabasal layer [13]. We learned in this study that stromal ERα is necessary for the proliferation of both the basal and suprabasal cells within the cervical epithelium of *K14E7* mice (Fig. 4). These results are similar to prior findings showing a requirement of stromal ERα for physiological proliferation of uterine columnar and vaginal squamous epithelial cells in response to estrogen [16, 22, 42]. Based on our and others' studies, we propose that stromal ERα provides a major mitogenic signal for basal cells in the cervical epithelium, which in turn supports suprabasal cell proliferation induced by HPV. HPV also inhibits apoptosis and induces chromosomal instability, which is known to promote cancers [4, 11, 43]. It has been proposed that epithelial ERα may also play a role in cervical carcinogenesis. Estrogen activates HPV promoter that drives E6/E7 expression in the cervical epithelium of *HPV18URR-lacZ* transgenic mice [44]. Enhanced expression of E6 and E7 provides selective growth advantage to cells [45]. We predict that ERα is responsible for this regulation because ERβ is not detectable in the cervix [13] and the HPV genome contains putative estrogen responsive elements, ER-binding sites [46]. A negative role of epithelial ERα has been also demonstrated. ERα expressed in cervical cancer cells or dysplastic cells inhibits their ability to invade chick chorioallantoic membrane [47], which is consistent with the observation that ERα inhibits migration and invasion of breast cancer cells [48–50]. It is plausible that epithelial ERα plays a positive role in early stages of carcinogenesis (i.e., development of CIN) and a preventive role in later stages (i.e., progression to invasive cancer and metastasis).

If this model were true, one would predict that deletion of ERα in cervical epithelia will enhance invasion of dysplastic cells, thereby increasing cancer burden in the context of our mouse model in which HPV oncogenes are under the control of K14 promoter unresponsive to estrogen [51]. Experiments to test this possibility were hampered by our inability to delete ERα in cervical epithelia (Online Resource 1 and Figs. 1 and 3). Use of *K14Cre* transgenic mice was also unsuccessful to induce efficient deletion of ERα in cervical epithelium even when ovaries were removed to block a potential selective pressure against ERα-deleted cells provided by estrogen (Online Resource 1). *K14Cre* transgenic mice have been used successfully to delete other floxed alleles (e.g., *p53*, *pRb*) in cervical epithelium [31, 32] and the floxed ERα allele was readily deleted in cervical

stroma and the whole uterus (Fig. 1). It is possible that *CMVCreER* is less active in cervical epithelia than in cervical stroma or whole uteri similar to mosaicism shown in *Chx10 BAC* transgenic mice [52, 53]. It is also possible that the absence of recombination in the cervical epithelia in *CMVCreER* and *K14Cre* mice reflects the fact that recombination efficiency varies depending on target alleles [53, 54].

Potential ERα Target Genes in Stromal Cells That are Crucial for Cervical Carcinogenesis It will be challenging to identify ERα target genes in cervical stromal cells that are necessary to support cervical carcinogenesis because (1) ERα is known to regulate (i.e., activation and repression) thousands of genes and (2) it is unclear if the same genes are regulated by ERα when mice are treated with estrogen for hours compared to months (6 months in the case of our mouse model). However, the fact that paracrine factors induced by ERα likely contribute to the development of neoplastic states (Table 1 and Fig. 3) narrows down the list of candidate genes. Among them, insulin-like growth factor I (IGF-1), keratinocyte growth factor (KGF), and Wnt ligands are of particular interest. IGF-1 is a direct target of ERα and necessary for estrogen-induced cell proliferation in uterine epithelium [55, 56] and higher serum levels of IGF-1 are associated with increased risk for CIN [57]. KGF receptor is expressed in cervical cancer cell lines and cancer specimens [58]. In HPV16-immortalized human cervical epithelial cells, KGF promotes proliferation and anchorage-independent growth as well as secretion of urokinase-type plasminogen activator that is known associated with invasiveness of cancer cells [59, 60]. Inhibition of canonical wnt signaling abrogates estrogen-dependent epithelial cell proliferation in mouse uterus and wnt signaling is aberrantly activated in cervical cancer cell lines due to loss of *Skt11* [61–63].

In summary, we demonstrate that deletion of stromal ERα promotes regression of cervical neoplasia and abrogates epithelial cell proliferation in the cervix. These results provide an incentive for the pursuit of studies investigating the role of stromal ERα in other estrogen-dependent cancers and developing strategies to target stromal ERα to treat such cancers.

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