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Epithelial ERα Is Dispensable for the Development of Estrogen–Induced Cervical Neoplastic Diseases

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Abstract

Human papillomavirus (HPV) is required but not sufficient for cervical carcinoma (CxCa). Estradiol (E_2) promotes CxCa development in *K14E7* transgenic mice expressing the HPV16 E7 oncoprotein under the control of the keratin 14 (K14) promoter. E_2 mainly works through estrogen receptor α (ERα). However, the role of ERα in human CxCa has been underappreciated largely because it is not expressed in carcinoma cells. We have shown that deletion of *Esr1* (the ERα-coding gene) in the cervical stroma of *K14E7* mice promotes regression of cervical intraepithelial neoplasia (CIN), the precursor lesion of CxCa. Here, we deleted *Esr1* in the cervical epithelium but not stroma. We found that E_2 induced cervical epithelial cell proliferation in epithelial ERα-deficient mice. We also found that E_2 promoted the development of CIN and CxCa in epithelial ERα-deficient *K14E7* mice, and all neoplastic epithelial cells were negative for ERα. In addition, proliferation indices were similar between $ERα^-$ and $ERα^+$ CxCa. These results indicate that epithelial ERα is not necessary for E_2-induced CIN and CxCa. Taken together, we conclude that stromal ERα, rather than epithelial ERα, mediates oncogenic E_2 signaling in CxCa. Our results support stromal ERα signaling as a therapeutic target for the disease.

Keywords

cervical cancer; ERα; human papillomavirus (HPV); mouse model

Introduction

Cervical carcinoma (CxCa) caused by high-risk human papillomavirus (HPV) is the fourth leading cause of cancer death in women worldwide [1]. Among more than a dozen high-risk HPVs, HPV16 is responsible for 50% of CxCa. The HPV E6 and E7 oncoproteins are best known for their ability to inactivate p53 and pRb, respectively. Expression of E6 and E7 are not sufficient to cause CxCa in mice or to transform human cervical keratinocytes *in vitro* [2,3].

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Author Contributions

Conception and design: JS and SHC; development of methodology: JS, YP and SHC; acquisition, analysis and interpretation of data: JS, YP and SHC; writing, review and/or revision of the manuscript: JS, YP and SHC; study supervision: SHC.

Long-term use of oral contraceptives and high parity are associated with increased risk of CxCa in HPV-infected women [4,5]. Exposure to diethylstilbestrol (synthetic estrogen) increases the risk of cervical intraepithelial neoplasia (CIN), the precursor lesion for CxCa [6]. Breast cancer patients who have used aromatase inhibitors are at a reduced risk of CIN compared to the nonuser group [7]. Aromatase is required for the biosynthesis of estradiol (E_2), the most potent estrogen. Whilst these observations support estrogen as a risk factor, its mechanism in the pathogenesis of CxCa has been underexplored. In a transgenic mouse model expressing HPV16 E7 (*K14E7*), E_2 promotes the development of CxCa preceded by CIN1, CIN2 and CIN3, which recapitulates human multistage cervical carcinogenesis [3]. Germline knockout of *Esr1* (the murine ERα-coding gene) abrogates cervical carcinogenesis in *K14E7* mice, demonstrating the requirement of estrogen receptor α (ERα), a ligand-dependent transcription factor [8]. However, its mechanism in human CxCa has been underappreciated. ERα is expressed in cancer-associated stroma, but not in cancer epithelial cells, suggesting that ERα in the stroma, rather than tumor cells, promotes CxCa [9]. In the present study, we show that specific deletion of epithelial *Esr1* does not abrogate the development of CIN and CxCa. Our results support the notion that epithelial ERα plays little role in the cooperation of HPV with E_2 in promoting CxCa.

Materials and Methods

Mouse strains are described in Table S1. Hormone treatment and procedures are described in Supplementary Information. All procedures for mice were carried out according to an animal protocol approved by the University of Houston Institutional Animal Care and Use Committee. Detailed procedures for tissue processing and histopathological analyses are described in Supplementary Information. Antibodies and immunohistochemistry (IHC) conditions are described in Table S2. One-sided Fisher's exact tests were used for disease incidence and one-sided Wilcoxon rank sum test for epithelium thickness, cell proliferation, apoptosis and disease severity.

Results and Discussions

The cervical epithelium of *Esr1*$^{ed/ed}$ displays partial responses to E_2

To study the role of epithelial ERα in the cervix we employed *Wnt7a-Cre/Esr1*$^{f/f}$ mice (referred to as *Esr1*$^{ed/ed}$ hereafter). ERα was specifically ablated in the cervical epithelia of *Esr1*$^{ed/ed}$, confirmed by the absence of epithelial progesterone receptor (PR) (Figure 1A), a marker for ERα function [12]. E_2 increased cervical epithelium thickness in *Esr1*$^{f/f}$ (P = 0.02) and *Esr1*$^{ed/ed}$ mice (P = 0.03) (Fig. 1B). Consistently, E_2 increased BrdU incorporation (i.e., proliferation) in the basal layer of *Esr1*$^{f/f}$ and *Esr1*$^{ed/ed}$ cervical epithelium (Fig. 1C). However, thickness (P = 0.03) and basal cell proliferation (P = 0.04) decreased in E_2-treated *Esr1*$^{ed/ed}$ compared to *Esr1*$^{f/f}$ mice(Figure 1B,1C). While proliferation indices of the suprabasal layer were much lower than the basal layer, E_2 decreased BrdU incorporation in suprabasal cells in both genotypes (Figure 1C). Expression of K10 increased in E_2-treated *Esr1*$^{f/f}$, but not *Esr1*$^{ed/ed}$ mice (Figure 1D), indicating the requirement of epithelial ERα for differentiation of the cervical squamous epithelium. E_2 increased neither epithelial thickness nor proliferation in the *Esr1*$^{-/-}$ cervix (Figure S1A,S1B). These results indicate that, while

epithelial ERα is necessary for full E_2 responses, stromal ERα also mediates E_2-induced proliferation of cervical epithelial cells, consistent with previous results that stromal ERα is necessary and sufficient for E_2-induced epithelial cell proliferation in the uterus and vagina [18].

ERα⁻ cervical neoplastic diseases develop in *K14E7/Esr1$^{ed/ed}$* mice

To determine whether epithelial ERα was required for E_2-induced cervical neoplasia (CIN and CxCa) we treated *K14E7/Esr1$^{f/f}$*, *K14E7/Esr1$^{ed/ed}$*, nontransgenic (*NTG)/Esr1$^{f/f}$* and *NTG/Esr1$^{ed/ed}$* mice with E_2 for 6 months [3]. All of fourteen *K14E7/Esr1$^{f/f}$*, but none of six *NTG/Esr1$^{f/f}$*, had CIN or CxCa (Table 1; $P = 2.58\times10^{-5}$). All of thirteen epithelial ERα-deficient *K14E7/Esr1$^{ed/ed}$*, but none of ten *NTG/Esr1$^{ed/ed}$*, displayed CIN or CxCa (Table 1; $P = 8.74\times10^{-7}$). Vaginal neoplastic diseases also developed in *K14E7/Esr1$^{f/f}$* (100%) and *K14E7/Esr1$^{ed/ed}$* mice (84.6%) (Table S3), which were not significantly different ($P = 0.22$). ERα was expressed in the stroma, but not cancerous and dysplastic epithelial cells in *K14E7/Esr1$^{ed/ed}$* unlike *K14E7/Esr1$^{f/f}$* mice (Figure 2A). PR was specifically undetectable in dysplastic and cancerous epithelium of *K14E7/Esr1$^{ed/ed}$* (Figure 2B), confirming no expression of ERα. These results rule out the possibility that some epithelial cells escaped *Esr1* deletion and became neoplastic. We concluded that epithelial ERα is not required for the development of CIN and CxCa. This is the first time to mimic ERα status of human CxCa, in which cancer cells are ERα $^{-}$ and stroma is ERα^{+} [9]. Expression of ERβ, the other nuclear ER, was undetectable in CIN and CxCa in *K14E7/Esr1$^{ed/ed}$* mice, suggesting no compensatory overexpression of ERβ. ERβ was detected in the ovary of *Esr2$^{+/+}$* but not *Esr2$^{-/-}$* mice, verifying specificity of the antibody (Figure S2A,S2B). We have demonstrated that deletion of stromal *Esr1* promotes CIN regression in *K14E7* mice [19]. Taken together, we conclude that stromal ERα is the major player in CIN and CxCa. *FGF7, FGF9, HBEGF, IGF1, IL1A, CXCL1* and *CXCL5* are upregulated by ERα in the cervical stroma [19–21]. It is plausible that secretory factors encoded by these stromal ERα target genes activate oncogenic signaling pathways in the epithelium through their cognate receptors on the plasma membrane of epithelial cells.

ERα⁺ and ERα⁻ CxCa display similar differentiation status, biomarker expression and growth

CINs and CxCa arising in *K14E7/Esr1$^{ed/ed}$* and *K14E7/Esr1$^{f/f}$* mice showed similar histology (Figure 2C). It was notable that non-diseased epithelium in *K14E7/Esr1$^{ed/ed}$* mice was as hypoplastic as the entire epithelium of *NTG/Esr1$^{ed/ed}$* mice (Figure 2C, *inset*). Expression of K10 in CIN and CxCa was similar between *K14E7/Esr1$^{ed/ed}$* and *K14E7/Esr1$^{f/f}$* (Figure 2D). Differentiation of cervical epithelial cells depends on epithelial ERα (see Figure 1D). These results indicate that neoplastic cells in *K14E7/Esr1$^{ed/ed}$* mice acquired an ERα-independent differentiation program. Expression of p16 and MCM7, biomarkers for E7 function [22], was similarly upregulated in CIN and CxCa in *K14E7/Esr1$^{ed/ed}$* and *K14E7/Esr1$^{f/f}$* compared to *NTG* control (Figure 3A,3B). Rare MCM7^{+} and Ki67^{+} basal cells in *NTG/Esr1$^{ed/ed}$* mice suggest that most epithelial cells are quiescent and epithelial ERα is required for proliferation of normal epithelial cells in response to long-term E_2 treatment (Figure 3B, Figure S3). Proliferation of cancer cells was similar between *K14E7/Esr1$^{f/f}$* and *K14E7/Esr1$^{ed/ed}$* mice (Figure 3C). Apoptotic indices of carcinomas were

also similar between the two genotypes (Figure 3D), indicating that ERα in cancer epithelial cells is not required for proliferation and survival of CxCa cells.

Epithelial ERα may play a role in an early stage of cervical carcinogenesis

CxCa burden (incidence, multiplicity and cancer size) was lower in *K14E7/Esr1*$^{ed/ed}$ than *K14E7/Esr1*$^{f/f}$ mice (Table 1). It is a caveat that ERα is not expressed in the normal cervical epithelium in *Esr1*$^{ed/ed}$ unlike *Esr1*$^{f/f}$ mice (see Figure 1A and 2A) and human cervix [9]. The entire epithelium was hypoplastic in *NTG/Esr1*$^{ed/ed}$, but hyperplastic in *NTG/Esr1*$^{f/f}$ mice (see Figure 2C). This difference in baseline state of the epithelium may have contributed to the reduced cancer burden. An improved system allowing temporal deletion of epithelial *Esr1* is required for better recapitulating the epithelial ERα status in human. Although most non-diseased cervical epithelia in *K14E7/Esr1*$^{ed/ed}$ mice were hypoplastic and undifferentiated (see Figure 2C,2D), CIN and CxCa in these mice were as differentiated and proliferative as those in *K14E7/Esr1*$^{f/f}$ (see Figures 2D and 3C). These results suggest that some ERα $^{-}$ epithelial cells acquired molecular changes mimicking the ERα pathway and were selected for during carcinogenesis. It appeared that E7 provided a selection advantage because a hyperplastic epithelium was absent in *NTG/Esr1*$^{ed/ed}$ mice (Figure 2C). It appeared that selection was a long-term process, because there was no hyperplastic epithelium in *K14E7/Esr1*$^{ed/ed}$ mice treated with E_2 for 2 months (data not shown). Gene expression profiling of CxCa infers overactivation of ERα even if tumor cells do not express the receptor [23], suggesting transcriptional activation of ERα target genes through an ERα-independent mechanism. This transcriptome change may be due to genetic and/or epigenetic alterations caused by E7. HPV16 E7 inhibits DNA damage repair and induces genomic instability [24]. It also causes epigenetic reprogramming by inducing expression of KDM6A and KDM6B histone demethylases [25]. Taken together, we argue that epithelial ERα plays a positive role, if any, in the development of CIN1 but not its progression to higher grade disease.

Our results demonstrate that mouse cervical neoplastic diseases occur in the absence of epithelial ERα, recapitulating the human disease. The data described herein and published observations support the idea that oncogenic E_2 signaling is mediated mainly by stromal ERα in the cervix [19,20]. Further studies are warranted to determine molecular mechanisms of stromal ERα and signaling pathways complementing the loss of epithelial ERα in CxCa.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Abbreviations

HPV	human papillomavirus
E_2	estradiol
ERα/β	estrogen receptor α/β
Esr1/2	*estrogen receptor 1/2*
CIN	cervical intraepithelial neoplasia
CxCa	cervical cancer

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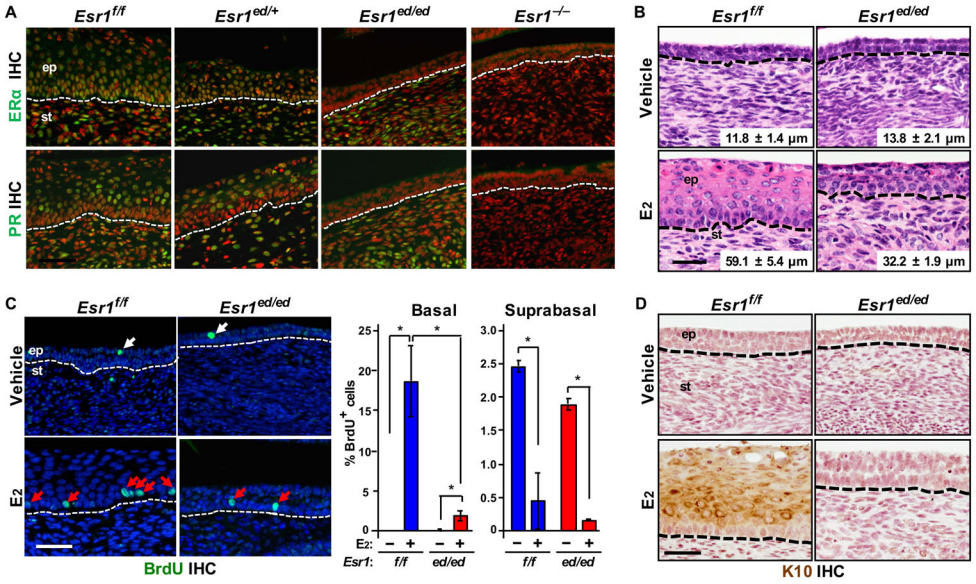

Figure 1.

Epithelial ERα-deficient cervical epithelium partially responds to E_2. (A) Specific ablation of ERα expression in the cervical epithelia. The female reproductive tracts were harvested from *Esr1*$^{f/f}$ and *Esr1*$^{ed/ed}$ mice at 8–10 weeks of age. Cervical sections were stained for ERα (green, *upper panel*) and PR (green, *lower panel*). Nuclei were stained with Hoechst 33258 (pseudocolored red). *Esr1* null cervix (*Esr1*$^{-/-}$) was used as negative control. Dotted lines separate epithelium (ep) from stroma (st). (B) Ovariectomized *Esr1*$^{ed/ed}$ mice were treated with vehicle or E_2 for 7 days. Shown are representative images of H&E-stained cervical tissues. The thickness of epithelium is shown as mean ± SEM (n = 3–5). (C) *Left panel*: cervical sections described in (B) were stained for BrdU (green). Nuclei were stained with Hoechst 33258 (blue). BrdU$^+$ basal and suprabasal cells are indicated by red and white arrows, respectively. *Right panel*: More than 200 cells per basal and suprabasal layer were counted. Results are shown as mean ± SEM (n = 3). $^*P < 0.05$ (one-sided Wilcoxon rank sum test). (D) Staining for K10 (brown). Nuclei were counterstained with hematoxylin. Scale bar = 50 μm for (A), (C) and (D); 30 μm for (B)

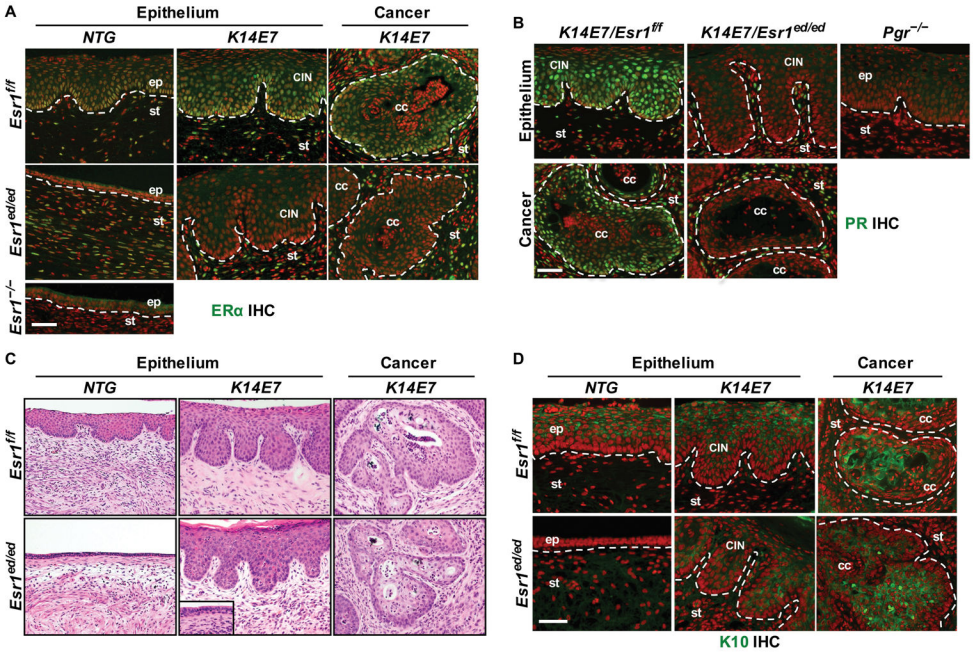

Figure 2.

E_2 promotes ERα⁻ CxCa in *K14E7/Esr1*$^{ed/ed}$ mice. (A) Cervical sections of CxCa arising in *K14E7/Esr1*$^{ed/ed}$ mice stained for ERα (green). An *Esr1*$^{-/-}$ tissue section was used as negative control. Dotted lines separate stroma (st) from normal epithelium (ep), dysplastic epithelium (CIN), and cancer epithelium (cc). (B) PR staining (green). A *Pgr*$^{-/-}$ tissue section was used as negative control. (C) Representative H&E staining. Note that the nondiseased epithelia in *K14E7/Esr1*$^{ed/ed}$ mice were hypoplastic (*inset*). (D) K10 staining (green). In (A), (B) and (D), nuclei were stained with Hoechst 33258 (pseoudocolored red). Scale bar = 50 μm.

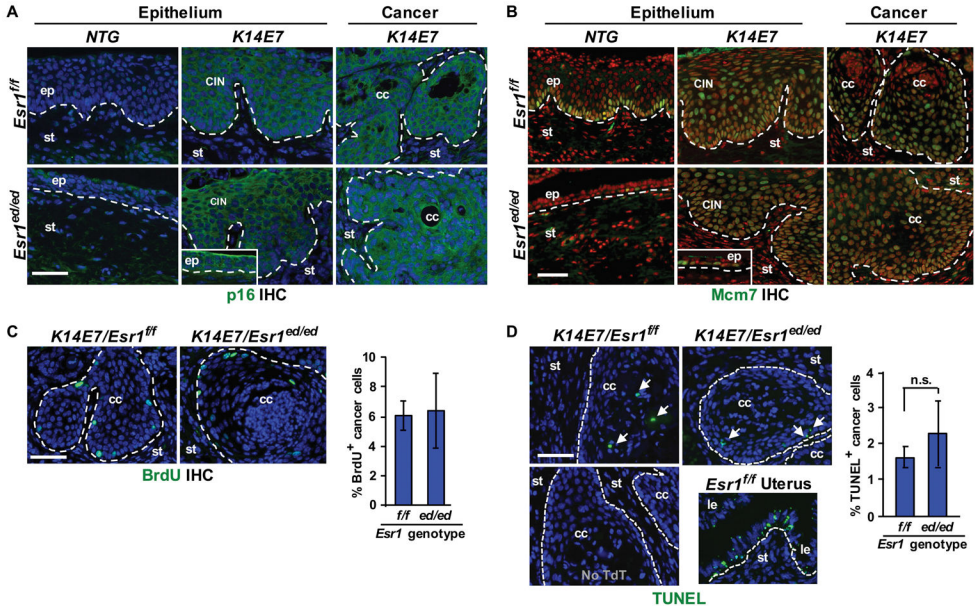

Figure 3.

Biomarker expression, proliferation, and apoptosis are similar between ERα$^+$ and ERα $^-$ CxCa. (A) CxCa biomarker p16 (green) in cervical neoplastic diseases arising in *K14E7/Esr1$^{ed/ed}$* mice. Nuclei were stained with Hoechst 33258 (blue). The inset shows p16 in the nondiseased epithelia of *K14E7/Esr1$^{ed/ed}$* mice. Dotted lines separate stroma (st) from normal epithelium (ep), dysplastic epithelium (CIN) and cancer epithelium (cc). (B) Cervical sections stained for Mcm7 (green). Nuclei were stained with Hoechst 33258 (pseoudocolored red). The inset shows Mcm7 in the nondiseased epithelia of *K14E7/Esr1$^{ed/ed}$* compared to *NTG/Esr1$^{ed/ed}$*. (C) *Left panel*: CxCa sections were stained for BrdU (green). Nuclei were stained with Hoechst 33258 (blue). *Right panel*: results were quantified by analyzing more than 200 cells per cancer and shown as mean ± SEM (n = 3). (D) Apoptotic indices. *Left panel*: CxCa sections were subjected to TUNEL assay (green). TUNEL$^+$ cells are indicated by arrows. Nuclei were stained with Hoechst 33258 (blue). A section that was not incubated with terminal deoxynucleotidyl transferase (TdT) was used as negative control (*lower left panel*). Uterus from E_2-treated wt mice was used as positive control (*lower right panel*). Le, luminal epithelium. *Right panel*: results were quantified and shown as mean ± SEM (n = 3). n.s., not significant. Scale bars = 50 μm.

Table 1

Summary of Worst Neoplastic Diseases in the Cervix[¶]

Genotypes	Group size, n	No disease	Dysplasia only			Cancer & dysplasia	Incidence(%)	Multiplicity[$]	Largest Cancer Size (mm^2)[$]	Total Invasion Area (mm^2)[$]
			CIN1	CIN2	CIN3					
NTG/Esr1$^{f/f}$	6	6	0	0	0	0	0	0	0	0
NTG/Esr1$^{ed/ed}$	10	10	0	0	0	0	0	0	0	0
K14E7/Esr1$^{f/f}$	14	0	0	1	2	11	78.6	2.79 ± 0.71	0.09 ± 0.03	0.17 ± 0.06
K14E7/Esr1$^{ed/ed}$	13	0	1	4	4	4	30.8 *	0.38 ± 0.18 **	0.03 ± 0.02 **	0.04 ± 0.02 **

[¶] Mice were scored histopathologically for the worst disease present in the cervix of each mouse.

[$] Mice without cancer are given '0' and data is shown as mean ± SEM.

* $P = 0.02$ compared to *K14E7/Esr1*$^{f/f}$ (Fisher's exact test).

** $P < 0.02$ compared to *K14E7/Esr1*$^{f/f}$ (Wilcoxon rank sum test).