Dysregulated Estrogen Receptor Signaling in the Hypothalamic-Pituitary-Ovarian Axis Leads to Ovarian Epithelial Tumorigenesis in Mice

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Abstract

The etiology of ovarian epithelial cancer is poorly understood, mainly due to the lack of an appropriate experimental model for studying the onset and progression of this disease. We have created a mutant mouse model in which aberrant estrogen receptor alpha (ERα) signaling in the hypothalamic-pituitary-ovarian axis leads to ovarian epithelial tumorigenesis. In these mice, termed ERα−/−, the ERα gene was conditionally deleted in the anterior pituitary, but remained intact in the hypothalamus and the ovary. The loss of negative-feedback regulation by estrogen (E) at the level of the pituitary led to increased production of luteinizing hormone (LH) by this tissue. Hyperstimulation of the ovarian cells by LH resulted in elevated steroidogenesis, producing high circulating levels of steroid hormones, including E. The ERα−/− mice exhibited formation of palpable ovarian epithelial tumors starting at 5 months of age with 100% penetrance. By 15 months of age, 80% of ERα−/− mice die. Besides proliferating epithelial cells, these tumors also contained an expanded population of luteinized stromal cells, which acquire the ability to express P450 aromatase and synthesize E locally. In response to the elevated levels of E, the ERα signaling was accentuated in the ovarian epithelial cells of ERα−/− mice, triggering increased ERα-dependent gene expression, abnormal cell proliferation, and tumorigenesis. Consistent with these findings, treatment of ERα−/− mice with letrozole, an aromatase inhibitor, markedly reduced circulating E and ovarian tumor volume. We have, therefore, developed a unique animal model, which serves as a useful tool for exploring the involvement of E-dependent signaling pathways in ovarian epithelial tumorigenesis.

Introduction

Ovarian cancer is the most lethal malignancy of the female reproductive system and the fifth leading cause of cancer-related death among women [1]. Approximately 90% of malignant ovarian tumors are derived from either the ovarian surface epithelium (OSE) or fallopian tube epithelium (FTE) [2]. Due to the absence of specific symptoms and the lack of strategies for early detection of ovarian cancer, the majority (70%) of women with this disease are diagnosed at a late stage when the cancer has spread beyond the confines of the ovary [1]. Despite its clinical significance, the etiology of ovarian cancer is poorly understood, mainly due to the lack of an appropriate experimental model for studying the onset and progression of this disease.

Multiple theories regarding the etiology of ovarian cancer have been proposed, but the precise molecular defects underlying the development of this disease remain elusive [3]. The “gonadotropin hypothesis” proposes that high gonadotropin levels can have a stimulatory effect on OSE cells, promoting their neoplastic transformation [4,5]. It was reported that the addition of gonadotropins to rodents in which ovarian cancer was induced upon treatment with the chemical carcinogen, 7,12-dimethylbenz[a]anthracene (DMBA) led to increased lesion severity, suggesting that gonadotropins play a role in tumor progression [6]. In humans, epidemiologic evidence, indirectly supporting this hypothesis, includes the well-documented protective effects of oral contraceptives and multiparity, which suppress gonadotropin secretion by the pituitary gland [5,7]. The majority of women with epithelial ovarian cancer present the disease at a postmenopausal stage where circulating follicle stimulating hormone (FSH) and luteinizing hormone (LH) levels are elevated, indicating a causal relationship between chronically elevated gonadotropin levels and ovarian cancer development [5,8].

Besides gonadotropins, epidemiologic studies have reported altered ovarian cancer risk associated with the use of steroid hormones to ease menopausal symptoms. Estrogen (E) is a well-known mitogenic factor associated with the genesis of many cancers. It has been reported previously that the risk of developing ovarian cancer increases in women who use hormone replacement therapy (HRT) for more than five years or use E-only regimens.
Ovarian Summary

Ovarian cancer is currently the most lethal gynecological cancer in the United States. Multiple epidemiological studies indicate that women who take hormone replacement therapy, estrogen or estrogen with progesterone, peri- or postmenopause will have an increased chance of developing ovarian cancer. Unfortunately, the five-year survival rate after diagnosis is very low indicating that better tools are needed to diagnose and treat ovarian cancer. The models that would allow investigation of this disease are severely limited. In this article we introduce a mouse model that develops epithelial ovarian tumors, and by employing inhibitors of estrogen synthesis, we show that ovarian tumorigenesis in this model is dependent on estrogen production within the ovarian tumor. These studies suggest that estrogen may play a role in promoting ovarian tumor growth.

Results

ERα conditional knockout mice (ERαΔ/d) were generated by crossing progesterone receptor cre recombinase (PR-Cre) knock-in mice with ERα floxed (ERαΔ/f) [17,18]. By five months of age, the ERαΔ/d mice developed palpable ovarian tumors with 100% penetrance. In contrast, the ERαΔ/f and the global ERα knockout mice did not develop any tumor (Fig. 1A). The ovarian tumors of ERαΔ/d mice grew progressively with age and became as large as 11 mm in size with an average weight of 300 mg by eight months of age (Fig. S1A). Because of this large tumor burden, 80% of the ERαΔ/d mice die by 68 weeks of age (Fig. S1C). Histological analyses of the ERαΔ/d ovaries showed cystic hemorrhagic follicles at 3 months of age. By 6 months, there was evidence of neoplastic epithelial cells migrating into the ovarian stroma, and by 11 months, extensive cellular proliferation occurred, resulting in the formation of a large tumor mass (Fig. S1B). Immunohistochemical analysis of ovaries of ERαΔ/d mice at 6 months of age, using cell proliferation markers, revealed that the follicular granulosa cells were proliferative but the OSE cells were quiescent (Fig. 1B, panel a,c). In sharp contrast, both OSE and the tumor cells within ERαΔ/d ovaries exhibited pronounced proliferative activity (panels b, d; proliferative cells indicated by arrow) (Fig. 1B).

Next we assessed the expression of ERα in the key tissues of the hypothalamic-pituitary-ovarian (HPO) axis. As shown in Fig. 2A, ERα expression was detected near the third ventricle of the hypothalamus in ERαΔ/d mice, and this expression remained intact in ERαΔ/d mice. Widespread expression of ERα was also observed in the anterior pituitary of ERαΔ/d mice. However, the pituitary expression of ERα was absent in ERαΔ/d mice. The ERα expression was evident in OSE of ERαΔ/d mice and remained intact in ERαΔ/d OSE (panels e,f). In addition theca cell expression of ERα also remained intact in the ERαΔ/d ovaries (panel h). Most notably, ERα was present in the tumor cells of ERαΔ/d ovaries.

Figure 1. The ERαΔ/d mice form proliferative ovarian tumors. (A) Gross morphology of ERαΔ/f, ERαΔ/d, and ERα global KO mouse ovaries at 3, 5, and 8 months of age. (B) Immunohistochemistry of ERαΔ/d ovary (panels a, c) and ERαΔ/d ovaries (panels b, d) with tumors at 6 months of age using anti-PCNA antibody. Red staining indicates proliferating PCNA positive cells. Arrows point to hyperproliferative OSE (b) and tumor cells (d) in ERαΔ/d ovarian tumors. F indicates follicle. doi:10.1371/journal.pgen.1004230.g001
tumors are not found in the ER
are unable to receive an implanting embryo. Furthermore, uterine
mouse phenotype, ER
reports, the levels of LH, progesterone, testosterone, and E are also
different between ER
(Table 1). In contrast, the level of FSH was not statistically
different between ER
(Fig. 3B, panel f).

In agreement with this view, we observed marked up-regulation of a transcriptionally active form of ERα, phosphorylated at serine 118, in OSE of ERαα/d mice (Fig. 2B, b). We also examined the status of the phosphoinositide 3-kinase [PI3K]/AKT pathway, which is reported to be activated in response to E treatment of ovarian cancer cell lines [21–23]. We noted that the level of AKT phosphorylated at Ser 473 (p-AKT) is elevated in the OSE and tumor cells of ERαα/d ovaries, while p-AKT level is maintained at a low level in ERαf/f ovaries (Fig. 2B, c,d). It is likely that the increased level of phosphorylated AKT is linked to the elevated E signaling in ERαα/d ovaries.

To further characterize the nature of the ovarian tumor in ERαα/d mice, we performed immunohistochemical analyses using epithelial and granulosa cell biomarkers. Anti-mullerian hormone (AMH) is a well-known marker for normal granulosa cells and granulosa cell tumors [24]. While both ERαα/d and ERαf/f ovaries expressed AMH exclusively in the granulosa cells of follicles, ERαα/d ovaries did not express AMH in the tumor cells, indicating that these tumors are not of granulosa cell origin (Fig. 3A).

Analysis using anti-cytokeratin 8 (CK8) antibody revealed that ERαf/f mice express this epithelial marker exclusively in a single layer of OSE at 3, 6, and 11 months of age (Fig. 3B, panels a,c,e). In contrast, the OSE of ERαα/d mice at 3 months of age exhibited multiple layers of cytokeratin-positive cells (panel b). At 6 months of age, we observed pronounced cytokeratin 8 expression within the ovaries of ERαα/d mice, indicating the presence of epithelial cells within the tumor mass (panel d). By 11 months of age, widespread cytokeratin 8 immunostaining was observed within the ovarian tumor, highlighting its remarkable epithelial component (Fig. 3B, panel f).

Current literature suggests that the human ovarian epithelial tumors are derived from either OSE or FTE [2,25]. Although these epithelia are derived from a common embryologic precursor, OSE is thought to retain mesothelial characteristics, while FTE is terminally differentiated [25–27]. Recent studies on the serous subtype of ovarian cancer have suggested that either OSE differentiates to resemble FTE or the cancer originates in the fallopian tube and spreads to the ovary [27]. To investigate further the origin of epithelial ovarian tumor cells in ERαα/d mice, we removed the oviducts of these mice prior to tumor formation. Interestingly, removal of the oviducts from pre-pubertal ERαα/d mice did not prevent the onset of ovarian tumor growth in these animals, indicating that the tumor cells originate from the OSE rather than the ovotubal epithelium (Fig. 4A). We also examined the epithelia of ERαf/f and ERαα/d ovaries by monitoring the expression of biomarkers specific for either OSE or FTE. As shown in Fig. 4B, we detected prominent expression of calretinin, a mesothelial marker [2,23], in OSE of ERαf/f ovaries but not in OSE of ERαα/d ovaries. We also noted marked up regulation of tubal-specific makers, including PAX8, WT1, and Ber-EP4 in the ovaries of ERαα/d mice, while the ovaries of ERαf/f mice lacked their expression. Since PAX8, WT1, and Ber-EP4 are normally expressed in FTE and are present in serous epithelial ovarian tumors [2,28–30], it is likely that the OSE cells of ERαα/d ovaries have undergone differentiation to resemble FTE. Furthermore, it has been reported previously that PAX8 is expressed in serous, endometrioid, and mucinous ovarian cancer while expression of WT1 is restricted to the serous subtype of ovarian cancer [29].

Currently there are no available biomarkers that can differentiate between high and low-grade serous ovarian carcinoma. It is clear that the ERαα/d tumors do not grow aggressively.

To investigate the molecular pathways underlying ovarian tumorigenesis in ERαα/d mice, we next performed gene expression profiling, using RNA isolated from the ovaries of ERαf/f and ERαα/d mice. We identified more than 2500 genes that were differentially expressed in the tumor tissue compared to the normal ovaries. The GEO accession number for the microarray data is GSE39402. When we compared the differentially regulated genes to three different datasets of differential gene expression profiles of human serous adenocarcinoma versus control human ovaries that exist in the Oncomine database, we noted that a large number of genes, which are differentially expressed in human serous ovarian cancer specimens, are also present in ERαα/d ovarian tumors (Fig. S2A). Remarkably, the identity of genes expressed in ERαα/d ovaries and human serous ovarian cancer ranged from 25–40%. Prominent among these genes were those encoding platelet derived growth factor receptor alpha (PDGFRα), vascular cell adhesion molecule (VCAM), clusterin, intercellular adhesion molecule 1 (ICAM-1), and serine/threonine phosphatase 1 (Wip1), which are overexpressed in human serous ovarian cancer [31–36]. We observed that the levels of PDGFRα, VCAM, ICAM1, and clusterin were markedly elevated in the ovaries of ERαα/d mice compared to those of ERαf/f mice (Fig. S2B). Collectively, the presence of these cancer biomarkers in ERαα/d ovarian tumors underscored the importance of this model in deciphering the pathways involved in genesis and progression of epithelial ovarian tumorigenesis.

Although the elevated systemic levels of E in ERαα/d mice likely contribute to the initiation of ovarian tumors by stimulating ERα signaling in OSE, we considered the possibility that, as the follicles are depleted with tumor progression, intratumoral E biosynthesis becomes a major regulator of tumorigenesis. Studies in postmenopausal women reported significantly increased expression and activity of P450 aromatase in serous ovarian carcinomas, but not in benign adenomas, supporting the view that intratumoral E derived from in situ aromatization could function as an autocrine growth regulator for cancer cells [14,37]. Previous studies have also reported elevated aromatase activity in tumors and ovarian cancer cell lines [38–41]. We observed that ovarian tumors of ERαα/d mice do indeed express high levels of P450 aromatase
mRNA (Fig. S3A). To localize aromatase expression we digested ERα+/d d/d mice into single-cell suspension, plated both fibroblast stromal and epithelial cells, and completed immunocytochemistry co-localizing both aromatase and a marker indicating the cell type. We observed that ovarian tumor cells isolated from ERα+/d mice express high levels of P450 aromatase protein in luteinized stromal cells of the tumor, suggesting that these cells acquired the ability to synthesize E (Figs. S3B). Furthermore, the activated form of ERα, phosphorylated at Ser-118, is abundantly expressed in the OSC, while aromatase is expressed in ovarian stroma of ERα+/d mice as early as 3 months (Fig. S3C). We postulated that the epithelial ERα signaling remains elevated in response to this locally produced E in ERα+/d ovarian tumors, supporting increased ERα-dependent gene expression, abnormal cell proliferation, and tumorigenesis.

To examine whether E plays a critical role in ovarian tumor progression in ERα+/d mice, we chronically treated these mice at 3 months of age with letrozole, a specific inhibitor of P450 aromatase, by implanting silastic capsules containing this drug. Following three months of letrozole treatment, ovarian tumors of 6-month-old ERα+/d mice displayed a remarkable reduction, up to 60%, in tumor volume when compared to sham-treated ERα+/d mice (Fig. 3A). It is important to note that while ERα+/d mice treated with letrozole exhibited significantly lower levels of serum E compared to sham-treated ERα+/d mice, their serum LH levels were not altered in response to this treatment (Fig. 3A). Interestingly, ERα+/d mice treated with the letrozole exhibited significantly lower levels of ovarian expression of PDGFRα and VCAM transcripts compared to sham-treated ERα+/d mice (Fig. 5B). Similarly, the levels of Wip1 mRNA and protein were markedly decreased in ovarian tumors of ERα+/d mice upon letrozole treatment (Fig. 5, B and C). Taken together, these results confirmed that elevated E signaling in the ovarian tumors of ERα+/d mice leads to dysregulated expression of a subset of genes with known links to ovarian epithelial cancer.

**Discussion**

Genetically engineered mouse models are considered to be among the most powerful and promising tools presently available for studying the biology of various forms of cancer and for developing therapeutics. Although the creation of mouse models of ovarian cancer has lagged behind models for many other neoplastic diseases, significant advances have been made in the last decade. Orsolic et al have shown that p53-deficient ovarian cells engineered to overexpress multiple oncopgenes, e.g. myc, Kras, and Akt, develop ovarian tumors when injected in mice [42]. Similar mouse models for ovarian epithelial tumors were developed via inactivation of various tumor suppressors, such as Pten, APC, p53 and Rh, through intrabursal administration of adenoviral vectors [43,44]. Conditional inactivation of multiple genes, such as Pten and Kras or PTEN and Dicer, by expression of Cre recombinase driven by the Amhr2 promoter also led to ovarian cancer in mice [45,46]. These mouse models have provided compelling evidence that OSE or FTE can be transformed by altering the expression of a variety of oncogenic factors or tumor suppressors. Some of these models display tumor histotypes similar to ovarian cancer subtypes seen in women. However, it is clear that these models typically require multiple genetic changes and are limited by very rapid tumor onset, which limits their usefulness for studying early modulators of ovarian tumorigenesis. In the present study, we report the development of a unique animal model, in which inactivation of ovarian tumorigenesis is independent of any oncogenic insult but dependent on elevated E signaling in the ovary. Since the onset and progression of tumorigenesis is relatively slow in ERα+/d mice, this model is potentially useful in providing insights into the factors involved in the initiation and early phases of ovarian epithelial tumorigenesis.

In ERα+/d mice, ERα is conditionally ablated in the pituitary but retained in the hypothalamus and ovary. The loss of negative-feedback regulation by E in the HPO axis led to elevated production of LH by the pituitary. Interestingly, high levels of gonadotropins in women in early postmenopause have been postulated to play a role in the development of epithelial ovarian neoplasms [4,5]. Consistent with this notion, it has been found that women with polycystic ovary syndrome, which is accompanied by high LH levels, have a greater risk of developing ovarian cancer [47]. Further supporting a role of gonadotropins in ovarian cancer development, gonadotropin levels in cysts and peritoneal fluid from ovarian cancer patients have been shown to be elevated [48]. However, not all studies have led to similar findings and it is clear that elevated gonadotropin levels alone do not cause ovarian cancer. In fact, our studies using the ERα+/d model suggested that hyperstimulation of ovarian cells by LH results in increased steroidogenesis, leading to high levels of circulating E as well as locally produced E in the ovarian tissue. High levels of testosterone coupled with increased expression of aromatase in the ovarian tissue would lead to increased synthesis of local E. We propose that this elevated E is an important factor in epithelial ovarian tumorigenesis as it stimulates signaling in the OSE, promoting its proliferation and phenotypic transformation. These results are supported by epidemiological and clinical studies, which indicate that postmenopausal women with elevated gonadotropin levels and receiving E replacement therapy exhibit an increased incidence of ovarian tumors [9–13]. Consistent with a role of E in the genesis of ovarian tumors, recent reports point to the clinical use of anti-estrogen drugs in stabilization of ovarian cancers [49,50].

Although many previous studies indicated that epithelial ovarian cancer arises from OSE, recent studies have revealed...
that the fimbriae of the fallopian tube is a possible site of origin of this malignancy, particularly high-grade serous carcinoma [51]. The common embryologic precursor of OSE and FTE is the coelomic epithelium, which gives rise to the epithelial linings of the fallopian tube and the ovary [27]. Unlike FTE, OSE retains mesothelial characteristics and is not terminally differentiated. It has been proposed that either OSE terminally differentiates to resemble FTE, or the cancer originates in fallopian tube and then spreads to the ovary. In support of the latter hypothesis, a recent study showed that conditional deletion of $\text{Pten}$ and $\text{Dicer}$, using the $\text{Amhr2-Cre}$, led to tumor development in the fallopian tube, which subsequently metastasized to the ovary [46]. Our studies, on the other hand, appear to indicate that ovarian tumorigenesis in $\text{ER}_a^{d/d}$ mice is associated with differentiation of OSE to FTE. We observed prominent expression of FTE marker proteins, such as PAX8, WT1, and Ber-EP4, which are not normally expressed in OSE, in the ovaries of $\text{ER}_a^{d/d}$ mice. Furthermore, removal of oviducts from $\text{ER}_a^{d/d}$ mice did not prevent the onset of ovarian tumorigenesis, indicating that FTE is not the precursor tissue for tumorigenesis in $\text{ER}_a^{d/d}$ mice.

Interestingly, we did not observe any intraperitoneal metastatic spread of the ovarian tumor in $\text{ER}_a^{d/d}$ mice. This could be partly due to the fact that the majority of the mutant mice died by 10 months of age due to the enlarged tumor, making it difficult to follow the progression of tumorigenesis beyond this point. The absence of overt malignancy in our model is not entirely surprising as several recent studies indicate that multiple genetic changes are necessary for metastatic transformation. It is conceivable that

Figure 3. Development of ovarian tumors in $\text{ER}_a^{d/d}$ mice. (A) Ovarian sections from $\text{ER}_a^{f/f}$ (a, c) and $\text{ER}_a^{d/d}$ (b, d) mice at 11 months of age were subjected to immunohistochemistry using an antibody against AMH. Red staining indicates AMH positive granulosa cells. (B) Ovarian sections from $\text{ER}_a^{f/f}$ mice at 3, 6, and 11 months of age (left panels a, c, e respectively) and $\text{ER}_a^{d/d}$ mice at 3, 6, and 11 months of age (right panels b, d, f respectively) were subjected to immunohistochemistry using anti-cytokeratin 8 antibody. Red staining indicates cytokeratin 8 positive epithelial cells. doi:10.1371/journal.pgen.1004230.g003

Figure 4. OSE is the site of origin for ovarian tumorigenesis in $\text{ER}_a^{d/d}$ mice. (A) Ovarian tumor growth in $\text{ER}_a^{d/d}$ mice after surgical removal of oviducts. The right oviduct was surgically removed from $\text{ER}_a^{f/f}$ and $\text{ER}_a^{d/d}$ mice at four weeks of age, leaving the left oviduct intact as an internal control. Upper panel, Gross morphology of $\text{ER}_a^{f/f}$ and $\text{ER}_a^{d/d}$ ovaries at 5 months after oviduct removal surgery. Lower panel, The graph depicts ovarian volume comparing $\text{ER}_a^{d/d}$ tumors with and without the oviduct. Two-tailed t-test was performed for statistical analysis: $n = 5$; $p = 0.99$. (B) Immunohistochemistry of $\text{ER}_a^{f/f}$ (left panel) and $\text{ER}_a^{d/d}$ (right panel) ovaries from mice at 6 months of age using calretinin (a, b); WT1 (c, d); PAX8 (e, f); and Ber-EP4 (g, h) antibodies. Red staining indicates immunostaining. doi:10.1371/journal.pgen.1004230.g004
additional mutation(s) in tumor suppressor genes, such as p53, is required to drive the tumorigenic pathways in ER\textsuperscript{a/d} ovaries to rapidly progressing ovarian carcinoma, which will culminate in metastasis. Indeed, recent studies, utilizing genomic sequencing data from human high-grade serous ovarian cancer specimens, have shown that these cancers exhibit genomic instability and harbor genetic mutations in p53, Rb, BRCA1, and/or BRCA2 loci [52–55].

The ovarian tumors in ER\textsuperscript{a/d} mice are composed of cells of both epithelial and stromal origins. These tumors appear to be distinct from the tubular or tubulostromal adenomas, which are reported to occur spontaneously in a number of mutant mouse strains, including the W\textsuperscript{5}W\textsuperscript{5} mice [36,57]. The adenomas, composed of numerous tube-like structures plus abundant large luteinized stromal cells, arise due to a defect in primordial germ cell proliferation and rapid loss of oocytes at birth, resulting in destruction of graafian follicles [58,59]. They also arise when mice are irradiated and there is a rapid loss of oocytes after radiation exposure [60]. However, these adenomas are not lethal and administration of E prevents rather than promotes their development [61]. Furthermore, in contrast to these mutant mouse strains, the ER\textsuperscript{a/d} mice exhibit normal number of oocytes at 3 months of age, which then start to decline when ovarian epithelial and stromal cells expand and form the tumor mass at 6 months. Therefore, the initial stages of tumorigenesis in ER\textsuperscript{a/d} mice are independent of the oocyte loss. Most importantly, the growth of the ovarian tumors exhibited by the ER\textsuperscript{a/d} mice is inhibited by letrozole, indicating that these tumors, unlike adenomas, are E-dependent. The ovarian tumors in the ER\textsuperscript{a/d} mice are presumably dependent on pituitary LH production, which help luteinize the stromal cells. However, the local production of E by these tumors and the resulting estrogenic effects on ovarian surface epithelial expansion and transformation appear to be the two key features that distinguish these tumors from the endocrinologically inactive tubular adenomas or tubulostromal adenomas.

Although the ovarian neoplasm in ER\textsuperscript{a/d} mice did not show signs of overt malignancy, there was nevertheless clear evidence of tumorigenic transformation. Particularly striking is the finding that a large number of genes, associated with human serous ovarian cancer, are also expressed in ER\textsuperscript{a/d} ovarian tumors. Specifically, these tumors exhibit dysregulated expression of PDGFR\textbeta, VCAM, and Wip1, which were previously reported to be involved in human ovarian cancer. PDGFR\textbeta, a cell surface tyrosine kinase receptor for members of the platelet-derived growth factor family, is over-expressed in human serous ovarian tumors and is targeted in clinical trials to treat ovarian cancers [32,33]. VCAM, a vascular cell adhesion molecule, is found in the blood circulation of cancer patients and has recently been proposed as a marker to detect early stages of ovarian cancer [34,35]. Wip1, a p53-inducible phosphatase and an oncogene, is of particular interest. Under normal conditions, it restores cellular homeostasis following DNA-damage by cooperating with p53 to induce G2/M cell cycle arrest, thereby allowing ample time for repair of the damaged DNA [62]. However, amplification of Wip1 leads to sustained inhibition of DNA damage response and tumor suppressors, and consequently, its overexpression has been implicated in a variety of human malignancies, including ovarian carcinoma [37,63]. Recent studies have revealed that Wip1 is regulated by ER\textgreekalpha [64]. Consistent with this finding, administration of letrozole to ER\textsuperscript{a/d} mice, which decreased the ovarian tumor size, also markedly reduced the expression of Wip1 along with PDGFR\textbeta, and VCAM. These results are consistent with our hypothesis that accentuated E signaling in the ovarian tissue promotes aberrant expression of genes that participate in tumorigenesis.

In summary, we describe a unique mouse model that allows us to identify hormonal effectors, particularly elevated E signaling, which play an important role in the development of ovarian epithelial tumorigenesis. In the future, the ER\textsuperscript{a/d} model will serve as a valuable tool for exploring the involvement of E-dependent signaling pathways in the onset and progression of this deadly disease.

Materials and Methods

Animals

Mice (C57BL/6; Jackson Laboratory) were maintained in the designated animal care facility at the University of Illinois College of Veterinary Medicine according to the institutional guidelines for the care and use of laboratory animals. We crossed mice harboring floxed ER\textgreekalpha gene (Esr1tm1.2Mma), termed ER\textsuperscript{a/d/f/f}, with PR-Cre mice expressing Cre recombinase under the control of progesterone receptor promoter (Pgr\textsuperscript{m22creer2}). This allowed development of mice of genotype Esr1tm1.2Mma/Er\textsuperscript{a/d}/Pgr\textsuperscript{m22creer2}/Pgr\textgreekalpha, which we termed ER\textsuperscript{a/d}. The PR-Cre knock-in mice expression of cre recombinase in pituitary, uterus, ovichute, mammary gland, and corpora lutea of the ovary have been described previously [18]. It has been used extensively to ablate floxed genes in tissues expressing PR [17,18].

Immunohistochemistry (IHC)

Paraffin-embedded ovarian tissue sectioned at 4 \mu m, mounted on slides and subjected to immunohistochemistry as described previously [65]. Sections were incubated at 4°C with polyclonal antibodies against PCNA (Santa Cruz sc-56), cytokeratin 8 (Developmental Studies Hybridoma Bank, TROMA I), ER\textgreekalpha (Novacastra Laboratories), p-Akt1/2/3 serine 473 (Santa Cruz D9E8), calretinin (Invitrogen 18-0291), Ber-EP4 (Dako), aromatase (Abcam ab5604), vimentin (Sigma Aldrich V5255). Biotinylated secondary antibodies were used followed by incubation with horse radish peroxidase-conjugated streptavidin (Invitrogen). Sections were stained in AEC Solution.

Real-time PCR analysis

Total RNA was isolated from ovaries by standard Trizol-based protocols and converted to cDNA. The cDNA was amplified by real-time PCR to quantify gene expression using gene-specific primers and SYBR Green (Applied Biosystems). As a loading control, the expression level of RPLP0 (36B4), which encodes a ribosomal protein, was determined. For each treatment, the mean threshold cycle (CT) and standard deviation were calculated from CT values obtained individually from 3 to 4 replicates of that
sample. Each sample was subjected to three independent real-time PCR trials. The fold change was derived from the mean Ct values. Primer sequences recognizing each gene are located in Table S1.

Measurement of serum hormones

Hormones were measured by radioimmunoassay (RIA) at the Ligand Core facility, University of Virginia, Charlottesville. Statistical significance was determined on SAS program using the Tukey procedure to control for comparison-wise error rate. Significance cutoff value of $p<.05$ was determined to be statistically significant.

Isolation of cells from ovaries with tumors

Ovarian tumors were removed from mice and digested with either 6 g/liter dispase (Invitrogen) and 25 g/liter pancreatin (Sigma Aldrich), or 0.5 g/liter collagenase (Sigma Aldrich) in Hank’s balanced salt solution (HBSS). After incubation for 1 h at 37°C, the tubes were vortexed for 10–12 s until the supernatant became turbid with dispersed cells. The contents were then passed through an 80-μm gauze filter (Millipore). Cells were re-suspended in Dulbecco’s modified Eagle’s F12 medium (DMEM-F12; with 100 unit/liter penicillin, 0.1 g/liter streptomycin, 1.25 mg/liter fungizone) containing 10% heat-inactivated fetal calf serum. Cell culture was continued for 48 h after addition of fresh medium.

Immunocytochemistry

Ovarian tumor cells were fixed with 10% formalin solution for 10 m. Cells were treated with 25% Triton X-100 (Sigma Aldrich), or 0.5 g/liter collagenase (Sigma Aldrich) in Hank’s balanced salt solution (HBSS). After incubation for 1 h at 37°C, the tubes were vortexed for 10–12 s until the supernatant became turbid with dispersed cells. The contents were then passed through an 80-μm gauze filter (Millipore). Cells were re-suspended in Dulbecco’s modified Eagle’s F12 medium (DMEM-F12; with 100 unit/liter penicillin, 0.1 g/liter streptomycin, 1.25 mg/liter fungizone) containing 10% heat-inactivated fetal calf serum. Cell culture was continued for 48 h after addition of fresh medium.

Statistical analysis

Statistical analysis was performed by ANOVA or two-tailed student’s t-test. Values of $P<.05$ were considered significant.

Supporting Information

Figure S1 (A) Average size and weight of ERαf/f ovaries and ERαd/d ovaries with tumors at 8–11 months of age (n = 62). * indicates $p<.05$ using two-tailed t-test to calculate statistical significance. (B) Histological analysis of ERαf/f ovary and ERαd/d ovarian tumors. Ovarian sections from ERαf/f mice at 3 months of age (a & b) and ovarian sections from ERαd/d mice at 3 months of age (c & d), 6 months of age (e & f), 11 months of age (g & h) are stained with hematoxylin and cosin. Panels b, d, f, and h are higher magnified images of a, c, e, and g, respectively. (C) Survival curve of ERαf/f and ERαd/d mice indicating probability of survival at different weeks of age. Data analyzed using GraphPad Prism. P<.0001 indicated that the survival curves between ERαf/f and ERαd/d mice are significantly different.

Figure S2 (A) Similarity between the genetic profiles of aberrantly regulated genes in ERαd/d ovarian tumors compared to the genetic profiles of aberrantly regulated genes in human serous adenocarcinoma from three independent microarray studies. Venn diagrams indicate similarity of aberrantly regulated genes in ERαd/d ovarian tumors compared to aberrantly regulated genes of human serous adenocarcinoma as assessed by microarray analysis published by Adib et al. (A), Hendrix et al. (B) and Lu et al. (C). (D) Table indicates the percentage of aberrantly regulated genes similar between ERαd/d ovarian tumors and human serous adenocarcinoma. Microarray lists indicating aberrantly regulated genes between human serous adenocarcinoma and normal human ovaries were exported from the Oncomine public database. Lists of aberrantly regulated genes between human serous adenocarcinoma and ERαd/d ovarian tumors were compared using Ingenuity software. (B) Real-time quantitative PCR was employed to measure mRNA levels of genes associated with ovarian carcinoma, PDGFRα, VCAM, clusterin, and ICAM-1, in ERαf/f and ERαd/d ovaries at 3, 6, and 11 months of age. * indicates significant difference of $p<.05$ using two-tailed t-test for comparison of gene expressions of ERαf/f and ERαd/d ovaries. (TIF)

Figure S3 Expression of P450 aromatase in ovarian tumor stromal cells of ERαd/d mice. (A) Real-time quantitative PCR was employed to measure mRNA levels of P450 aromatase in ERαf/f ovaries and whole ERαd/d ovarian tumors from mice at 11 months of age. (B) Localization of aromatase in cultured ERαd/d ovarian tumor cells is assessed by immunocytochemistry. Upper; Epithelial cells dually stained with cytokeratin 8 in red (a), P450 aromatase in green (b), and co-localized with blue dapi staining (c). Lower; Stromal cells are dually stained with vimentin in red (d), P450 aromatase in green (e) and co-localized with blue dapi staining (f). Yellow indicates co-localization of P450 aromatase and vimentin (f). (C) Localization of aromatase (a,b) in ERαd/d ovarian tumors compared to non-immune IgG (d). Localization of pERα in ERαd/d ovarian tumors (e,f) and in ERαf/f ovaries (g,h). Tissues are from mice at 3 months of age. Red color indicates localization of each protein. (TIF)

Table S1 Sequences of quantitative PCR Primer Sets. Primer sequences were designed to recognize coding regions of specific mRNA. (TIF)

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Author Contributions

Conceived and designed the experiments: MJL MKB ICB. Performed the experiments: MJL AK SP. Analyzed the data: MJL WMH MKB ICB. Wrote the paper: MJL WMH MKB ICB.

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