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Estrogen action and prostate cancer

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Abstract

Early work on the hormonal basis of prostate cancer focused on the role of androgens, but more recently estrogens have been implicated as potential agents in the development and progression of prostate cancer. In this article, we review the epidemiological, laboratory and clinical evidence that estrogen may play a causative role in human prostate cancer, as well as rodent and grafted *in vivo* models. We then review recent literature highlighting potential mechanisms by which estrogen may contribute to prostate cancer, including estrogenic imprinting and epigenetic modifications, direct genotoxicity, hyperprolactinemia, inflammation and immunologic changes, and receptor-mediated actions. We discuss the work performed so far separating the actions of the different known estrogen receptors (ERs), ERα and ERβ, as well as G-protein-coupled receptor 30 and their specific roles in prostate disease. Finally, we predict that future work in this field will involve more investigations into epigenetic changes, experiments using new models of hormonal dysregulation in developing human prostate tissue, and continued delineation of the roles of the different ER subtypes, as well as their downstream signaling pathways that may serve as therapeutic targets.

Keywords

estradiol; estrogen; prostate cancer; prostate gland

As the most common noncutaneous form of cancer in men, prostate cancer is the subject of over US$300 million in research funding annually from both the NIH and the National Cancer Institute in the USA, with comparable funding levels in other nations worldwide [201,202]. Despite these significant efforts, the exact mechanism of carcinogenesis is unknown, although it is believed to involve a combination of dietary, environmental, genetic, lifestyle and hormonal causes. While the hormonal regulation of prostate cancer has been studied for over 75 years, the focus has largely been on androgen action. More recent research increasingly suggests a role of estrogen in the etiology and progression of prostate cancer and this article will highlight the evidence on this topic.

Huggins first demonstrated the androgenic dependence of prostate cancer as a potential cause, as well as a point of intervention and therapy, ultimately leading to a Nobel prize in 1966 [1,2]. The finding that castration caused regression of metastatic prostate cancer has

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led to decades of research on the role of androgens in prostate cancer, as well as anti-androgen therapy being a mainstay treatment for meta-static disease [3,4] and neoadjuvant therapy prior to treatment in locally advanced disease [5]. In the early era of prostate cancer research, the role of estrogen was primarily seen as an indirect anti-androgen action mediated through feedback inhibition of hypothalamic luteinizing hormone (LHRH) and pituitary luteinizing hormone (LH) release, resulting in decreased testicular androgen synthesis and release. Consequently, administration of exogenous estrogens such as the nonmetabolized diethylstilbestrol (DES) was used to reduce circulating androgens to castrate levels in patients with advanced prostate cancer [4]. Moreover, this method of effective 'chemical castration' was noted to lower the incidence of bone mineral density loss, a known complication of other forms of androgen deprivation therapy that also reduce the level of circulating endogenous estrogen [6,3]. This highlights the fact that estrogens and estrogen analogs play important physiologic roles in normal, healthy adult males [3]. The ability of estrogens to treat or prevent prostate cancer is under continued investigation. It was recently demonstrated that DES may be able to directly affect prostate cancer cell division by inhibiting telemorase [7]. In a xenograft model with human prostate cancer cells injected into castrated severe combined immune-deficient mice, estrogen appears to suppress tissue androgen levels as well as tumor growth, independent of estrogen receptor (ER) blockade [8]. As discussed later, the effects of phytoestrogens have also been investigated for several years as potential agents to prevent prostate cancer.

Estrogens & prostate disease

In contrast to the early work on estrogen as a treatment for castration-resistant cancer or as a cancer preventive in the case of phytoestrogens, an ample body of evidence suggests that estrogens may play a critical role in predisposing, or even causing, prostate cancer. In this aspect, it is noteworthy that estradiol-17β has been classified as a carcinogen by the International Agency for Research on Cancer [9–11], primarily based on its association with endometrial and breast carcinoma in women [12–15].

There is correlative evidence in humans that indicates an effect of estrogen on the prostate gland that is conducive to cancer onset. Although circulating estrogen levels may not correlate well with intraprostatic levels, particularly in light of recent evidence of intraprostatic estrogen production, serum values offer an easily accessible surrogate marker to investigate associations between estrogen and prostate cancer risk. Epidemiological data from adult men have shown mixed results, with one prospective study finding an association of elevated plasma estrogens with an increased risk of prostate cancer [16], and another correlating risk with chronically elevated estrogens [17]. However, the Physician's Health Study suggested the opposite finding, with an increasing prostate cancer risk associated with decreasing estradiol levels after adjusting for sex hormone-binding globulin [18]. Additional studies and meta-analyses have failed to show any association between circulating estrogen levels and risk of prostate cancer [19–21]. This divergence in outcomes related to serum steroid levels and prostate cancer risk may, in part, be understood by recent revelations that prostatic tissue has the capacity to produce its own steroids, including androgens and estrogens. While initially discussed in the 1970s and 1980s, technical advances have revived research on intracrine steroid synthesis and metabolism with new findings revealing that local steroid production by prostate tumors, rather than circulating steroids, may be a major player in driving prostate cancer growth in men following androgen ablative therapy [22]. While the current focus of research on the role of intracrine steroids has been on tumoral androgens, it is important to note that aromatase (CYP19), the enzyme that catalyzes estradiol production from testosterone, is altered in prostate cancer tissues [23] and CYP19A1 expression is elevated 30-fold in prostate cancer metastatic tissue, as compared with primary tumors [24]. Elegant studies performed in aromatase-knockout mice

demonstrated that these animals have a reduced incidence of prostate cancer following exposure to testosterone plus estrogen when compared with wild-type mice. This suggests that *in situ* production of estrogen, either locally or systemically, remains a factor in this rodent model, even in the presence of exogenously administered estrogen [25]. In this context, it is possible that intraprostatic estradiol synthesis may contribute to prostate cancer onset in addition to playing a role in tumor progression. Finally, there is evidence that local metabolism of estrogen may also play a role. As discussed later, some metabolites of estrogens have been linked to free radical generation and direct genomic damage. A recent analysis of 1983 French men identified an association between polymorphisms in genes related to estrogen metabolism (*CYP1B1* and *CYP19*) and prostate cancer risk [26]. Real-time PCR and histopathological studies have demonstrated that CYP1B1 is more highly expressed in the peripheral zone of the prostate than the transitional or central zones [27,28]. This mirrors the predilection of prostate cancer for the peripheral zone, raising the possibility that local estrogen metabolites produced by the CYP1B1 pathway (e.g., 4-OH-estradiol) play an important role in prostate carcinogenesis.

In a recent cross-sectional analysis, serum steroid levels were assayed in 1413 matched men of various races and while testosterone levels did not differ, African–American men had significantly higher serum estradiol levels than Caucasian or Mexican–American men, a difference that was most pronounced in early and mid-adulthood [29]. This is highly significant in that African–American men have a twofold higher rate of prostate cancer than Caucasian–American men, as well as a greater prostate cancer risk than Hispanic males. Studies on pre- and peri-natal exposure to estrogens have also raised concerns about their carcinogenic potential. Some authors have suggested that the higher incidence of prostate cancer among African–American men is partly due to *in utero* exposure to maternal estrogens since African–American women have higher circulating estrogen concentrations during pregnancy than Caucasian women [30,31], although gestational androgen levels are also elevated.

Animal models of prostate cancer have demonstrated that, at least in rats, estrogen is a necessary, if not sufficient, condition for prostate carcinogenesis. Although rats do not naturally develop prostate cancer, it can be induced in Noble rats by combined treatment with estradiol and testosterone, with testosterone playing a supportive role since androgen supplementation alone is insufficient to drive carcinogenesis [32–34]. A recent review by Bosland reinforced this notion, citing an incidence of prostate cancer induction in Noble rats of 100% with testosterone and estrogen, but only 40% with testosterone alone. Dihydrotestosterone, which is not aromatized to estrogen as testosterone is, could induce cancer in only 4% of rats [35]. In addition, rodent studies have consistently demonstrated increased susceptibility to prostate cancer in mice or rats exposed to DES or pharmacologic levels of estradiol-17β *in utero* or neonatally [36–39]. While rodent models offer a chance to study the developing reproductive system following estrogenic exposures, the possibility of species-specific findings limits their applicability to humans. New emerging models offer the opportunity to study the effects of estrogen on undifferentiated human prostate tissue. The first such model is the isolation of adult prostate stem or progenitor cells [40–44]. Although only present at a low frequency in the epithelial cell population, these cells have the ability to differentiate into three different epithelial cell types [45] and to survive and form spheroids in 3D matrigel culture [46–48], permitting their isolation from adult epithelial cell populations. These ‘prostaspheres’ can begin to differentiate on their own with extended culture and form branched-like structures with epithelial bilayers and lumens [49]. Importantly, recent results from our laboratory have found that undifferentiated prostasphere cells express robust levels of ERs, including ERα, ERβ and G-protein-coupled receptor (GPR)30, indicating that prostate progenitor cells may be direct estrogen targets [49]. Furthermore, the normal prostate progenitor cells exhibit a proliferative response to 1 nM

estradiol-17β, suggesting that estrogen may regulate stem/progenitor cell self-renewal in the prostate gland. This new model thus provides a novel opportunity to investigate the effects of estrogenic exposure during human prostate differentiation.

The second emerging model is human chimeric tissue grafts. Taylor *et al.* first reported the formation of human prostate tissue by combining human embryonic stem cells with rat urogenital mesenchyme (UGM) and implanting it as a tissue graft under a mouse renal capsule with the formation of a normal prostate-like structure after 1–2 months [50]. We have recently reported a similar model using prostate stem/progenitor cells isolated from normal human organ donors, which generate normal human prostate-like tissue when combined with rat UGM as renal grafts in nude mice. Exposure of these engrafted mice to elevated testosterone and estrogen for 2–4 months resulted in the induction of prostate cancer over time, progressing from normal histology to epithelial hyperplasia, prostate intra-epithelial neoplasia (PIN) and prostate cancer with local renal invasion [49]. An example of a locally invasive adenocarcinoma seen in a xenograft after explantation is shown in Figure 1. The tumor was induced using estrogen plus testosterone supplementation after implanting human epithelial progenitor cells and rat UGM under the renal capsule of a nude mouse. This is the first direct evidence with normal human prostate epithelial cells that estrogens in an androgen-supported milieu are carcinogens for human prostate epithelium, capable of driving prostate cancer initiation and progression to adenocarcinoma. This new model allows the study of normal human prostate tissue development and differentiation, as well as providing a format to interrogate pathways involved in prostate hormonal carcinogeneis with the aim of uncovering therapeutic interventions.

For all the observations on the changes in prostate tissues affected by estrogens as well as the associated risk of cancer, relatively little is known about the exact mechanisms by which these compounds predispose to or directly cause prostate cancer. Proposed mechanisms include epigenetic modifications, direct genotoxicity, hyper-prolactinemia, immunotoxic or inflammatory changes, and prostatic ER-mediated changes. While these mechanisms are probably interrelated and should by no means be considered mutually exclusive, the following sections will examine evidence for estrogen's role in prostate cancer by focusing on each of these potential mechanisms individually. Their interrelationships are depicted graphically in Figure 2, with selected references supporting each pathway.

Estrogen imprinting & epigenetic modifications

There is reason to believe that some of the effects of estrogen on the prostate gland are the result of developmental exposures that predispose to prostate disease later in life, fitting into the emerging paradigm on the fetal basis of adult disease. A classic example is *in utero* exposure to DES, which has been linked to increases in breast and vaginal cancer of exposed offspring, although adult DES exposure presents limited risk for these cancers [51–55]. The prostate gland is similarly dependent upon steroid hormones, and imbalances in these hormones during development can result in abnormalities in growth and differentiation [56]. For example, *in utero* DES exposure has been associated with an increased rate of structural abnormalities of the prostatic utricle in newborn sons [56], while elevated maternal estrogen levels during pregnancy have been associated with increased prostate cancer risk in humans [57]. In rodents, DES exposure during the perinatal period predisposes the offspring to prostate cancer [36]. While it remains to be investigated if the overall risk of developing prostate cancer is increased in humans after *in utero* exposure to DES as exposed sons are just now entering the typical age of prostate cancer risk, the earlier onset of disease as seen with breast cancer in DES-exposed daughters has not been demonstrated with prostate cancer.

Rodent models are useful for studying estrogenic imprinting since their prostate gland undergoes morphogenesis and differentiation postnatally [58], as opposed to the human gland, which is fully formed and differentiated at birth. Studies from our laboratory using the rat model have shown that brief perinatal exposure to high doses of estrogens results in permanent changes, including alterations in gene expression and cell signaling [58,59], a process known as estrogenization or estrogen imprinting. These animals go on to develop inflammation, prostatic hyperplasia and PIN, considered the precursor lesion for prostate cancer [60]. The ability of this early estrogenic exposure to predispose, rather than cause, cancer is best demonstrated by a 'two-hit' model of prostate carcinogenesis in Sprague Dawley rats. Neonatal rats were briefly exposed to 17β-estradiol or the environmental estrogen bisphenol A (BPA) at serum concentration levels similar to those measured in humans – considered the first hit – followed by a second exposure to estradiol in adulthood to mimic increased estrogen levels observed in aging men [33,61]. While the low-dose estrogen or BPA exposures alone had no effect on the rate of premalignant PIN lesions in the prostate lobes, they markedly increased the susceptibility of the adult prostate to high-grade PIN lesions induced by adult estradiol, increasing the incidence from 40% in control rats to 100% in those exposed neonatally to estrogens [62,63]. Thus, the developmental exposure appears to 'prime' the prostate, resulting in increased carcinogenesis with subsequent exposures later in life.

Since early-life estrogenic exposure does not appear to drive carcinogenesis by itself, it is possible that it increases susceptibility by silencing tumor-suppressor genes, augmenting expression of tumor-promoter genes or otherwise reducing the tissue's resistance to malignant transformation. Evidence from our rodent model of prostate neonatal estrogenization has shown lifelong alterations in DNA methylation of specific genes, suggesting an epigenetic basis for estrogen imprinting [62,64]. DNA and histone methylation are mechanisms of epigenetic gene silencing or activation that can permanently alter gene expression since they are mitotically heritable and can be maintained over the lifespan. Epigenetic alterations have been associated with the initiation of multiple cancers [65,66], including prostate cancer, in which promoter CpG island or histone modifications have been identified that result in silencing of tumor-suppressor genes [62,67–71]. Of interest, these epigenetic alterations are reversible through DNA demethylating agents such as 5-azacytidine and histone modifiers, causing transcription of these genes to resume, with some identifiable phenotypic changes [69,70]. In this context, it is noteworthy that alterations in DNA methylation have been reported in human reproductive tissues developmentally exposed to DES [72–74]. Thus, hypermethylation of the promoter regions of tumor-suppressor genes or hypomethylation of tumor-promoter genes is one potential mechanism by which developmental estrogens may predispose to prostate cancer later in life.

Direct genotoxicity

Although initial studies of estrogen at pharmacologic concentrations suggested that estrogens only have weak, if any, direct mutagenic properties [75–79], more recent studies using lower, physiologic concentrations have shown multiple direct genetic lesions that appear to be inducible by estrogens or their metabolites. Specifically, estrogen-induced changes have been identified in animal or human tissue studies consisting of aneuploidy [80–87], chromosomal aberrations [84,88,89], point mutations [79,90,91] or microsatellite instability [91]. In one study in the human mammary carcinoma cell line MCF-7, concentrations of estrone and β-estradiol in the nanomolar range were able to induce single-stranded DNA breaks by the Comet assay and micronuclei after only 24 h of exposure [92]. Although these studies were primarily performed in nonprostatic tissue, they highlight potential mechanisms of genotoxicity. In rat prostates, single-stranded DNA breaks and lipid

peroxidation have been identified in *in vivo* rat prostate models [32] following adult exposure to estrogens, which supports the proposal that estrogen genotoxicity may play a role in prostate carcinogenesis.

Considerable research on the carcinogenic actions of estrogens in breast tissue as well as other female reproductive tract organs has identified catechol-estrogen formation as a significant factor in carcinogenesis [93]. The phase I enzymes CYP1A1 and CYP1B1 catalyze the conversion of estradiol to 2-hydroxyestradiol and 4-hydroxyestradiol, respectively, in end organs that express these enzymes. Subsequently, the 2- and 4-hydroxyestradiols can be rapidly oxidized to 2,3- and 3,4-semiquinones and quinones, respectively, which are capable of forming DNA adducts, leading to DNA damage including single- and double-stranded DNA breaks. In general, 2-hydroxyestradiol is considered less damaging, while 4-hydroxyestradiol and its respective quinone have greater genotoxicity. To maintain intracellular homeostasis, the phase II enzyme, catechol-*O*-methyl transferase converts these metabolites to 2- and 4-methoxyestrone, respectively, which are rapidly excreted. Importantly, while the human prostate expresses these enzymes [94], estrogens are capable of markedly increasing CYP1B1 expression in a variety of tissues. Work in our laboratory determined that the adult prostates of rats treated neonatally with high-dose estradiol expressed sixfold higher levels of CYP1B1 and twofold more catechol-*O*-methyl transferase than oil-treated controls, indicating permanent estrogen metabolic stress [Prings GS, Unpublished Data]. As previously described, CYP1B1 is also differentially expressed in the zones of the prostate, with higher levels of both protein and mRNA in the more cancer-prone peripheral zone [27,28]. It is noteworthy that polymorphisms have been identified in the *CYP1B1* gene that affect its catalytic activity, and African–Americans have the more potent Leu432Val at a 0.75 frequency compared with 0.43 in Caucasians and 0.17 in the Chinese population [95]. Furthermore, in a small case-controlled study, 34% of Caucasian men with prostate cancer (n = 50) were homozygous for the *Leu432Val* polymorphism, while only 12% of matched control subjects (n = 50) had this genotype [95]. Together, these limited findings support the notion that genotoxic estrogen metabolites may contribute to prostate carcinogenesis.

Hyperprolactinemia

Chronic estrogen exposure increases circulating prolactin levels in adult humans, and has been used as a model for inducing hyper-prolactinemia in rodents [96]. This effect on prolactin levels holds true for both endogenous estrogens as well as xenoestrogens [97]. Although prolactin levels had been considered to be of little consequence in oncology for many years, recent evidence of the role of prolactin in the development of breast and prostate cancer has changed this view. The proliferative effects of prolactin are well known, and these effects have been cited as a factor that both supports tumor growth and induces chemoresistance in multiple cancers [98]. Two recently identified mechanisms – locally produced prolactin and genetic variants of the prolactin receptor – have been championed as potential factors in prolactin's role in prostate cancer [99]. Specifically in human prostate epithelial cells, autocrine prolactin appears to be responsible for activation of Stat5 [100], an important survivability factor for prostate cancer cells associated with hormone resistance, higher histological grade and earlier recurrence, possibly through interaction with the androgen receptor [101]. Whether this locally produced prolactin is under estrogen regulation, as is the case in the posterior pituitary, is presently unclear and requires further investigation.

Evidence also exists for the role of circulating endogenous prolactin, which is under estrogenic control, in driving prostate cancer. It was identified as a survival factor for androgen-deprived prostate tissue in rats [102], and in the presence of androgen can induce

enlargement and inflammation of the lateral rat prostate without any histological changes on ventral and dorsal lobes [103]. These histological changes are accompanied by over-expression of Bcl-2, which may inhibit apoptosis [104]. As will be discussed in the next section, inflammation of the prostate has been associated with the development of prostate cancer in numerous epidemiological and ontological studies. Importantly, several, but not all, of the gene-expression and inflammatory changes induced by estrogen in the prostate appear to be mediated by prolactin produced by the pituitary [105]. In one recent study, 2504 genes were identified as having their expression significantly altered in rat lateral prostate by treatment with testosterone and estradiol. Of these, 80% were blocked by cotreatment with the antiestrogen ICI and bromocriptine, which blocks production of prolactin in the pituitary gland [106]. However, only 10% of these changes could be blocked by ICI alone, suggesting that not only does prolactin play an important role in mediating these changes, but also that centrally produced, rather than local, prolactin is involved. These findings have led some to suggest that blockade of the prolactin receptor is a potential therapy for both breast and prostate cancer [98]. Indeed, in a transgenic mouse model, prolactin antagonism has been shown to block prostate tumorigenesis [107].

Inflammation & immunogenic changes

One of the histopathologic hallmarks of estrogen-treated prostate tissue is chronic inflammation. Exogenously administered estrogen has resulted in numerous prostatic changes including loss of basal/apical orientation, and relative increase in stromal elements and inflammatory cell infiltrates [38,108,109]. Chronic immune cell infiltrates also play a role in prostate dysplasia of adult rat prostates after neonatal estrogen exposure, with an increased prevalence of $CD4^+$ and $CD8^+$ T cells as well as macrophages in the adult tissues long after estrogen exposure, suggesting an estrogenic priming of the immune system [105]. These inflammatory effects can be partially, but not completely, reduced using bromocriptine to block hyperprolactinemia, suggesting estrogen-induced prolactin-dependent and -independent components [105]. Using a murine model with aromatase gene overexpression to increase endogenous estrogen levels, Ellem *et al.* described these inflammation-related prostatic changes in detail [110]. Mice with overexpressed aromatase had smaller prostates than controls at puberty, with a significant increase in mast cell numbers. By 40 weeks of age, inflammatory changes were present, accompanied by increases in mast cell, macrophage, neutrophil and T-lymphocyte numbers. In another study using adult Wistar rats, inflammatory signaling began soon after estrogen exposure, with upregulation of IL-1β, IL-6, macrophage inflammatory protein 2 and inducible nitric oxide synthase transcripts after only 4 days treatment with injected estradiol [111]. The significance of estrogen-induced inflammation is the association of immune cells and cytokines with cancer. Chronic inflammation has been associated with a number of malignancies, including prostate cancer. By some measures, infection and inflammation have been linked to as many as 15–20% of all cancers in humans [112–114]. Several studies have now indicated that prostate cancer may fall into that category, with clear associations drawn between prostate cancer and prostatitis [111,115,116]. Evidence of this in humans includes the finding of activated inflammatory cells next to areas of prostate cancer and premalignant lesions [112] and an increase in the incidence of prostate cancer in men with a history of prostatitis [117]. Prostate inflammation often displays epithelium which is atrophic but paradoxically expresses increased proliferation and decreased apoptosis [118]. These findings have been termed ‘proliferative inflammatory atrophy’ and are considered by some to be a potential precursor lesion for high-grade PIN (HGPIN) and prostate cancer based on the proliferation and tendency to be found in the peripheral rather than transition zone of the prostate, as is the case with prostate cancer [119].

Proposed mechanisms of inflammation-induced cancer include oxidative stress with free radical formation and subsequent DNA damage [120–122]. In addition, several inflammatory cytokines and prostaglandins, including IL-1α and IL-8, are associated with angiogenesis [123–125], a necessary process in the growth of most tumors. These multiple pathways by which inflammation can lead to cancer formation make it an attractive target for research in cancer prevention. Indeed, multiple recent publications have investigated the association of aspirin or other nonsteroidal anti-inflammatory drugs with prostate cancer risk or treatment. A meta-analysis of studies conducted prior to June 2008, including 24 studies and 24,230 patients, revealed a small but statistically significant reduction in the risk of developing prostate cancer with a pooled odds ratio of 0.83 [126]. In the past year, at least five large studies have been published on this topic, with conflicting results. One large prospective cohort with a total of 51,529 patients revealed a decrease in the risk of developing prostate cancer of nearly 10% with chronic aspirin use [127], while another cohort study with 34,132 patients showed no significant difference [128].

Receptor-mediated changes & hormonal dysregulation

Estrogen's primary hormonal functions are mediated through estrogen-specific receptors, of which there are several types with possibly opposing functions. These different receptors are distributed in varying expression levels in human tissues with the highest expression observed in estrogen-sensitive tissues [129]. Importantly, multiple ERs are localized in prostate tissues and their activation has been associated with a number of phenotypic changes *in vitro* and *in vivo*. A significant amount of evidence has accumulated demonstrating that direct estrogen signaling pathways within prostate cells play an important role in the development of the prostate gland and possibly in the development of cancer [130–135].

Most hormonally regulated effects of estrogens have been attributed to the nuclear steroid receptors ERα and ERβ [136]. Recent evidence has shown that rapid membrane-initiated signaling of estrogens can also occur through liganding of ERα or ERβ localized to the cell membrane that in turn initiates downstream kinase cascades [137], although these signaling pathways have not yet been well characterized in prostate cells. In addition, another binding moiety localized to cell membranes and activated by estrogens has been identified and named GPR30 [138]. These three proteins have tissue-dependent distribution and different effects on cell phenotype and activity. As discussed later, the activity of endogenous estrogens or dysregulation by exogenous estrogens on any or all of these receptors may play a role in prostate cancer development and/or progression. The demonstrated effects of each receptor on prostate tissue and disease are summarized in Table 1.

Estrogen receptor-α was the first ER to be described and is sometimes referred to as the 'classical' ER. In the prostate gland, it is primarily localized to the stroma and basal cell layer, which contains prostatic stem cells capable of proliferation [135,139]. In the common rodent model of prostate cancer utilizing testosterone plus estrogen to induce PIN and cancer, the ERα is clearly essential, as knockout mice that lack the ERα do not develop high-grade PIN or prostate cancer, while ERβ-knockout mice do, as do wild-type mice [25]. Similarly, neonatal exposure to DES requires ERα since ERα-knockout mice lack the estrogenized phenotype while ERβ-knockout mice exhibit full estrogenization [140]. In human tissue, ERα mRNA and protein have both been detected in HGPIN lesions [135]. Moreover, the activity of these receptors can be inferred from the presence of progester-one receptors in the lesions and the estrogen-inducible protein pS2. The progesterone receptor is inducible by estrogen and is an important marker for a functional ER. It can be found in 33–58% of primary prostate tumors, with an expression level correlated with ERα mRNA and stronger, more consistent expression in metastatic and androgen-insensitive lesions [141].

PS2 was expressed in HGPIN lesions as well as benign acini adjacent to cancerous lesions in radical prostatectomy specimens, but was consistently lacking in tissue obtained from individuals without histologic evidence of disease [142], suggesting ERα activity in a 'field effect' around these lesions. In addition, much of the inflammation induced in mouse prostates – which may play a role in prostate cancer as previously described – appears to be mediated by ERα [140,131]. In clinical studies, blocking ERα with toremifene caused a nearly 50% reduction in the rate of biopsy-detected prostate cancer in men with high-grade PIN on biopsy 12 months earlier [143]. However, actual ERα protein expression is typically only detectable in high-grade (Gleason 4 or 5) cancerous lesions [135]. Overall, this evidence appears to suggest that ERα is essential for at least one model of prostate cancer formation in rodents, and that in humans, it is at a minimum circumstantially associated with disease formation and progression, and may possibly be a target for chemoprevention.

The second nuclear ER, ERβ, was first cloned in 1996 from a rat prostate cDNA library [144]. Using different antibodies, this receptor is primarily localized to prostatic epithelium, although some stromal cells can express ERβ, albeit at lower levels than the epithelium [139,145,146]. In the developing human prostate, it appears to be the predominant receptor in both stromal and epithelial cells [146]. In contrast to ERα, ERβ appears to be antiproliferative and possibly even tumor suppressive. Its relatively high affinity for phytoestrogens compared with ERα [144] may in part explain the anticancer properties attributed to genistein and other soy isoflavones. These properties and their interaction with the ERβ receptor were highlighted in a case-control study conducted in 2100 Swedish participants, which found a significant multiplicative interaction between dietary phytoestrogen intake and specific single-nucleotide polymorphisms of the *ERβ* gene in the reduction in risk of prostate cancer [147]. In this study, subjects with the identified single-nucleotide polymorphism and the highest quartile in total phytoestrogen intake showed a 0.43 odds ratio for developing prostate cancer when compared with the lowest quartile. Subjects who were homozygous for the wild-type receptor showed no significant dependence on phytoestrogens. The association of ERβ with anti-tumor signaling is indicated from immunohistochemical studies revealing that although ERβ is present in the majority of localized prostate cancer, it is reduced or absent in 40% of hormone-refractory disease and HGPIN [145]. Other studies have confirmed an inverse correlation between ERβ expression and Gleason score, with decreasing ERβ expression attributed to hypermethylation of the gene promoter [148,149]. It has recently been reported that high Gleason grade cancers exhibit mesenchymal characteristics, including increased hypoxia-inducible factor-1α, vimentin and VEGF expression, a phenomenon known as epithelial–mesenchymal transition (EMT) [150]. These changes can be induced in androgen-sensitive or -resistant cells by exposure to hypoxic conditions or TGF-β, which also diminishes ERβ levels without affecting ERα. Conversely, silencing of ERβ with short hairpin RNA was sufficient to induce EMT. Moreover, EMT could be prevented and epithelial phenotype preserved using 3β-androstandiol, a purported ERβ ligand [150]. Thus, ERβ appears to play a role in preventing some of the changes associated with more aggressive local prostate cancer. Furthermore, a recent study using murine models, as well as human prostate cancer xenografts and cell lines, demonstrated that ERβ-specific agonists could drive prostate stromal and epithelial cell apoptosis, an event that was dependent on TNF-α signaling [151]. Importantly, this action was androgen independent, thus indicating potential therapeutic benefit in castration-resistant prostate cancers.

G-protein-coupled receptor 30 was first identified as a novel membrane-bound protein in 1998 [138], but has only recently been investigated primarily as a protein that mediates estrogen signaling in multiple cell types [152–160]. GPR30 displays similar affinity to estradiol-17β with ERα and ERβ in the 3–6 nM range, with almost no affinity for estradiol-17α corticosteroids or testosterone [161]. It is distinct from the two known ERs,

ERα and ERβ, not only in its structure, which is not a modular transcription factor, but also in that it is unaffected by standard anti-estrogens such as ICI [162]. In breast cancer, GPR30 overexpression has been positively associated with tumor size, the presence of distant metastases, increased human EGFR-2/neu expression [163] and aggressive inflammatory disease [164]. In many cell types, there is evidence that GPR30 collaborates with membrane ERα and requires the latter receptor to generate downstream signaling cascades [137]. Part of the mechanism by which GPR30 stimulates disease progression appears to be through stimulation of cancer-associated fibroblast (CAF) proliferation and migration [165]. CAFs are associated with disease progression [166–168] and are stimulated by physiologic doses of estradiol. These fibroblasts express GPR30, but not ERα or ERβ, and silencing GPR30 with short hairpin RNA blunts the stimulatory effect of estradiol on CAFs [165]. GPR30 has also been shown to promote proliferation in thyroid [161], endometrial [157] and ovarian [160] cancers. In contrast to these findings, GPR30 appears to cause growth inhibition in prostate cancer cells *in vitro* and in xenografts *in vivo* [169]. Using siRNA and the GPR30 agonist G-1, the authors demonstrated that GPR30 activation resulted in cell-cycle arrest of PC-3 cells at the G2 phase, which occurred via sustained activation of Erk1/2 and ultimately upregulation of p21. Interestingly, GPR30 has also been shown to induce Erk1/2 activation in breast cancer cells via activation by BPA [162]. The fact that this protein can mediate growth in one cell type and inhibition in another through pathways that are at least initially identical highlights the complexity of the estrogen signaling system.

Further differentiation of the roles of each receptor type, and possibly their splice variants, will depend on our ability to activate or inhibit individual receptor types without affecting the state of the other signaling mediators. Significant effort has been made in the development and modification of compounds that are selective agonists or antagonists for specific ER molecules and are referred to as selective estrogen receptor modulators (SERMs). Given the known functions of the receptors, there is particular interest is the development of ERα-selective antagonists [170] and ERβ selective agonists [171]. Some anti-estrogen SERMs have been evaluated for prostatic responses in animal and cell culture models and as a few clinical trials and were recently summarized in a review by Bonkhoff and Berges [130]. Briefly, the antiestrogens tamoxifen, raloxifene, trioxifene, ICI and toremifene inhibited cancer cell growth and/or induced cancer cell apoptosis *in vitro* or *in vivo* [172–178]. One recent study of particular note demonstrated that ER-selective agonists exhibited differential regulation of the oncogenic *TMPRSS2–ERG* fusion gene in prostate cells, with ERα agonists stimulating expression and ERβ agonists inhibiting the oncogene expression [179]. In clinical trials, however, the results have been less promising. Tamoxifen, which is a mixed agonist/antagonist and has significant activity at both ERα and ERβ, was well tolerated but demonstrated limited activity in 30 patients with hormone-refractory prostate cancer [180]. The pure anti-estrogen ICI, which antagonizes both ERα and ERβ, also produced no response in 20 patients with recurrence after androgen-deprivation therapy [177]. Only toremifene, an ERα antagonist, has shown real promise to date. As described previously, it decreases the incidence of prostate cancer detected in men on biopsy 12 months following a previous biopsy with high-grade PIN [143]. In the transgenic adenocarcinoma of mouse prostate (TRAMP) model, toremifine appeared to be a powerful chemopreventive agent for prostate cancer. Untreated TRAMP mice develop HGPIN by 17 weeks of age, whereas none of the toremifine-treated mice exhibited these lesions. At 30 weeks of age, only 28% of TRAMP mice treated with toremifene had histologic evidence of prostate cancer, compared with 100% of placebo-treated mice; at 33 weeks, only 43% had tumors, whereas all of the placebo group died [178]. Although toremifine has not yet been proven to delay or prevent disease progression in humans, it appears to improve bone mineral density and lipid profiles in men on androgen-deprivation therapy [181,182]. The fact that these positive results come from an ERα selective antagonist as opposed to a ligand that affects both ERα and ERβ highlights the need for the

continued development of next-generation SERMs to differentiate the function of receptor subtypes. The picture is further complicated by the existence of multiple splice variants of each receptor. Although most studies use primers and ligands that do not distinguish splice variants from full-length ERs, evidence is growing that these variants play important roles in both normal estrogen signaling and disease susceptibility [183]. In particular, the ERα splice variant ERαΔ5 demonstrated increased levels of expression by reverse-transcriptase PCR in tissue adjacent to tumor when compared with benign tissue [184].

Phytoestrogens & prostate health

In addition to their ability to influence testosterone levels and bone mineral homeostasis, it has been proposed that natural estrogens and dietary estrogen analogs may be protective against the development of prostate cancer, rather than simply being a treatment for prostate disease. This has resulted in extensive research into the anticancer properties of phytoestrogens, and in particular soy isoflavones such as genistein. The initial evidence for this was epidemiological, with the age-standardized incidence of prostate cancer in Japan, where soy consumption is high, being 12.6 per 100,000 men as compared with 119.9 in the USA [185]. However, among second- and later-generation Japanese populations living in the USA, the incidence of prostate cancer is much closer to that of the general US population, suggesting an environmental or dietary cause for the difference observed. Subsequent studies focusing on soy and its associated proteins investigated the dietary impact on the risk of prostate cancer, with a large meta-analysis suggesting that both fermented and nonfermented soy were protective against cancer, with odds ratios of 0.69 and 0.75, respectively [186]. While tofu was the only individual food showing a protective effect, the phytoestrogens genistein and daidzein were also associated with a lower risk of prostate cancer.

Further evidence of the protective effect of genistein can be gleaned from studies using rodent models and human cell lines. In two separate studies, Mentor-Marcel and colleagues investigated the effects of genistein on the progression of prostate cancer in the TRAMP mouse model of prostate cancer [187,188]. When dietary genistein was used to elevate mouse serum genistein to levels comparable to that of Asian men, the rate of poorly differentiated adenocarcinoma decreased in a dose-dependent manner [187], while survival improved as a function of decreased tumor burden [188]. Recent studies using a rat hormonal carcinogenesis model have shown that a soy isoflavone mixture that includes genistein and diazein is able to protect against carcinogenesis in the dorsolateral and anterior prostate lobes [189]. *In vitro* studies revealed that genistein inhibited growth of two prostate cancer cell lines alone or in combination with selenium [190]. The treatment also induced apoptosis through caspase-dependent pathways, and reduced expression of matrix metalloproteinase 2, which has been associated with active invasion and metastases.

While the exact mechanisms of genistein's protective effects have not been elucidated, many current studies are focused on changes in DNA methylation and other mechanisms that alter gene and protein expression. The promoter CpG islands of many tumor-suppressor genes become methylated in several prostate cancer cell lines, resulting in silencing of their transcription. Treatment with genistein and other soy phytoestrogens resulted in demethylation of the glutathione-*S*-transferase P and epoxide hydrolase receptor B2 gene promoters, with parallel rises in their protein expression by immunohistochemistry [69]. This is significant since glutathione-*S*-transferase P, a π-class glutathione-*S*-transferase, is silenced by promoter hypermethylation in prostate basal cells and is one of the known early events in human carcinogenesis due to the loss of its protective antioxidant capacity [191]. A similar study in prostate cancer tissues as well as cell lines demonstrated that the tumor-suppressor B-cell translocation gene 3 is silenced in prostate cancer, with re-expression

induced by demethylation with either genistein or 5-azacytidine [70]. An alternative proposed mechanism of action is through changes in histone rather than promoter modification. Several tumor-suppressor genes (*PTEN*, *CYLD*, *p53*, *FOXO3a* and *SIRT1*) were identified that are silenced in prostate cancer cell lines, but have unmethylated promoter regions. Expression of these tumor-suppressors was restored after treatment with genistein, and this was found to be associated with either methylation or deacetylation of histone H3-lysine 9 [71]. Finally, there is ample evidence that genistein induces at least some of its changes through ER-mediated pathways, in particular ERβ, for which it has higher affinity. Apoptosis-inducing effects of genistein on T-cell leukemia cells can be blocked by the anti-estrogen ICI [192], and other observed effects of genistein appear to be blocked by silencing of the ERβ in gerbil neurons [193] or PC-3 prostate cancer cells [194]. As described previously, the ERβ-mediated effects of phytoestrogens may partly depend on specific *ERβ* gene polymorphisms [147].

Expert commentary

Our understanding of estrogen's function in the pathogenesis, prevention and treatment of prostate cancer is still evolving. Although androgens are clearly involved in the progression of prostate cancer and anti-androgen therapy will probably remain the treatment of choice for metastastic disease for the foreseeable future, it is equally clear that androgens are only one side of the story. At least in rats, testosterone alone is necessary, but not sufficient, for the development of prostate cancer. It is only with the addition of estrogen that cancer can be reliably induced. *In vivo* and *in vitro* studies have identified multiple mechanisms of potential carcinogenesis, including direct genotoxicity, epigenotoxicity, hyperproloctinemia, chronic inflammation and prostatic ER-mediated changes that are in addition subject to disruption by environmental estrogens. Moreover, ample evidence for estrogen's role in the development of human prostate cancer exists in the form of epidemiological data and associations between inflammation and cancer, which parallels findings in rodent prostates. The true role of estrogen in prostate cancer development and progression is probably complex and multifactorial, incorporating more than one of the mechanisms already described and with interplay between them.

Five-year view

Given the relatively low rate of mutations caused by estrogen at physiologic concentrations and the few consistent mutations identified in clinical prostate cancer, it is likely that future breakthroughs will be in the field of gene silencing or activation through epigenetic effects or hormonal dysregulation. The use of new models such as stem cells, prostaspheres and chimeric tissues will allow well-controlled investigations into the effects of estrogen on human prostate development and carcinogenesis. Finally, the development of new selective ER modulators offers exciting opportunities to examine the effect of individual ER subtype activation or antagonism with therapeutic potential. Further elucidation of the roles of the different receptors and their splice variants and polymorphisms may direct future laboratory research and ultimately clinical therapeutic intervention in the form of treatment or prevention by dietary modification or pharmacologic treatment.

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Key issues

- Estrogens have been used to treat prostate cancer; however, these hormones and related compounds have also been identified as potential causative agents of prostate cancer.
- Evidence that supports estrogen as a prostate cancer-causing agent includes association of elevated levels of estrogen with prostate cancer, changes in estrogen receptor status in advanced prostate cancer, and rodent models and chimeric human tissue graft models showing induction of prostate cancer using estrogen plus testosterone.
- Early exposure to estrogens appears to predispose individuals to later development of prostate cancer.
- Potential mechanisms of estrogen-driven carcinogenesis include prostatic estrogen receptor-mediated events, epigenetic changes, genotoxicity, hyperprolactinemia and chronic inflammation.
- Future work on estrogen's role in the development and progression of prostate cancer is likely to include investigations of epigenetic changes, further elucidation of the role of individual estrogen receptor types using selective estrogen receptor modulators, and new models of developing human prostate tissue, including prostate stem/progenitor cells and xenografts.

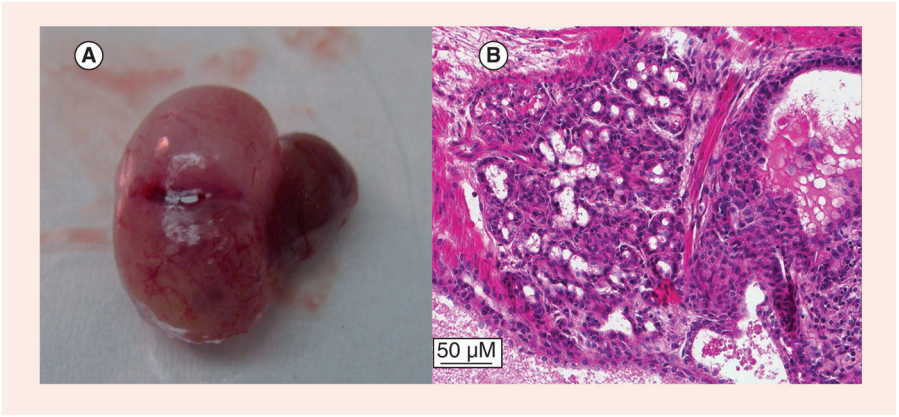

Figure 1. Renal graft of human prostate stem/progenitor cells recombined with rat urogenital mesenchyme following 3 months of elevated estradiol with testosterone support given to the host nude mouse

A locally invasive tumor can be seen in a prostate tissue xenograft after explantation **(A)**. The tumor was induced using estrogen plus testosterone supplementation after implanting human epithelial progenitor cells and rat urogenital mesenchyme under the renal capsule of a nude mouse. Histopathology reveals that this tumor is prostatic adenocarcinoma **(B)**. Images from [Prings GS et al., Unpublished Data].

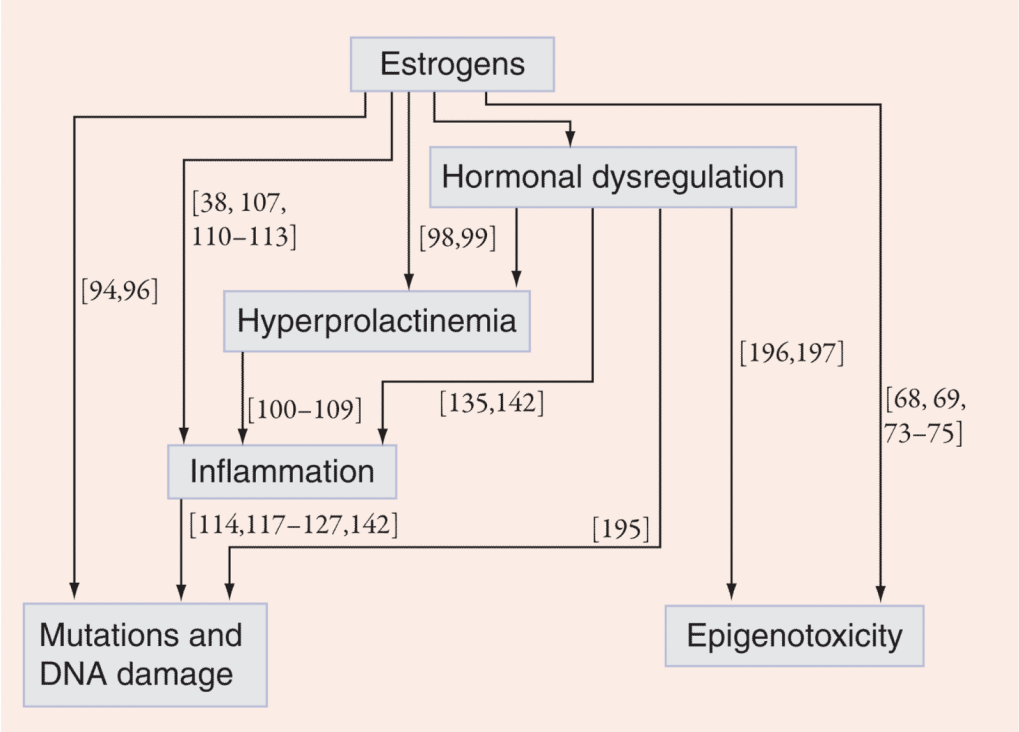

Figure 2. Diagram of proposed mechanisms of estrogenic carcinogenicity in the prostate
Numbers refer to references supporting the corresponding pathway of estrogenic action.

Table 1

Histopathologically identified locations of estrogen receptors, along with actions in prostate disease that have been identified.

Receptor	Localization	Significant findings in prostate disease	Agonists	Antagonists
ERα	Stroma and basal layer [126,130]	Mediates inflammation [133,140] Necessary for T + E model of rodent carcinogenesis [25] Increased expression in high-grade disease [135] Blockade appears to reduce detection of CaP on subsequent biopsy [143]	Estradiol PPT	ICI Toremifene
ERβ	Stroma and basal layer [135] Epithelial [130,134]	Predominant ER in developing human prostate [146] Increased affinity for phytoestrogens [144] Reduced or absent in hormone-refractory disease [146] Expression has inverse correlation with Gleason score [148,149] Inhibits epithelial–mesenchymal transformation [150]	Estradiol DPN 3β-adiol	ICI THC
GPR30	Cytoplasmic membrane [138]	Causes cell-cycle arrest in PC-3 cells [169]	Estradiol ICI G-1	G-15

CaP: Prostate cancer; DPN: Diarylpropionitrile; E: Estradiol 17β; ER: Estrogen receptor; GPR: G-protein-coupled receptor; MPP: Methyl-piperidino-pyrazole; PC: Prostate cancer; PPT: Propyl-pyrazole-triol; T: Testosterone; THC: *Cis*-tetrahydrochrysene.