Estrogen receptor beta in prostate cancer: friend or foe?

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Abstract

Prostate cancer is the commonest, non-cutaneous cancer in men. At present, there is no cure for the advanced, castration-resistant form of the disease. Estrogen has been shown to be important in prostate carcinogenesis, with evidence resulting from epidemiological, cancer cell line, human tissue and animal studies. The prostate expresses both estrogen receptor alpha (ERA) and estrogen receptor beta (ERB). Most evidence suggests that ERA mediates the harmful effects of estrogen in the prostate, whereas ERB is tumour suppressive, but trials of ERB-selective agents have not translated into improved clinical outcomes. The role of ERB in the prostate remains unclear and there is increasing evidence that isoforms of ERB may be oncogenic. Detailed study of ERB and ERB isoforms in the prostate is required to establish their cell-specific roles, in order to determine if therapies can be directed towards ERB-dependent pathways. In this review, we summarise evidence on the role of ERB in prostate cancer and highlight areas for future research.

Key Words

- ▶ estrogen receptor beta
- ▶ prostate
- ▶ cancer
- ▶ androgen
- ▶ estrogen

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Introduction

Prostate cancer is the commonest, non-cutaneous cancer in men, affecting 214 per 1000 European men. It is the second commonest cause of cancer death, accounting for 15% of all male cancers in developed countries (Heidenreich *et al.* 2011, Mottet *et al.* 2011). Ever since the landmark research of Huggins and Hodges (Huggins 1943, Huggins & Hodges 1972) demonstrating the importance of steroid hormones in the development and treatment of prostate cancer, there has been interest in the effects of estrogen on the prostate gland. Initially, hormone treatment for prostate cancer involved manipulation of systemic hormone levels with exogenous estrogen therapy (in the form of high-dose diethylstilbestrol) to suppress androgen production indirectly via the hypothalamo–pituitary–gonadal axis (Huggins & Hodges 1972). However, the unacceptably

high rates of cardiovascular side effects associated with systemic estrogen therapy, coupled with the advent of alternative treatment options, resulted in reduced use of this therapy (Morales & Pujari 1975). Consequently, for a period of time, interest in understanding the effects of estrogen in the prostate also declined. Hormonal suppression of prostate cancer is now primarily achieved with androgen deprivation therapy (ADT) involving synthetic leutinising-hormone-releasing-hormone (LHRH) analogues, which suppress androgen production via negative feedback inhibition of the hypothalamic–pituitary–gonadal axis (Heidenreich *et al.* 2011). Although most prostate tumours initially respond well to ADT, after a period of time prostate cancer inevitably ceases to respond to androgen deprivation. Disease progression with ADT is

termed castration-resistant prostate cancer (CRPC) (Scher *et al.* 2004), and is characterised by altered androgen receptor (AR) signalling. Possible mechanisms for this include amplification (Bubendorf *et al.* 1999) or mutation of the AR gene, stabilisation of AR protein (Holzbeierlein *et al.* 2004), altered expression of AR-coregulators (Chmelar *et al.* 2007), generation of constitutively active AR splice variants (Dehm *et al.* 2008, Hu *et al.* 2012) and increased intratumoural androgen biosynthesis (Cai & Balk 2011, Ishizaki *et al.* 2013), all of which contribute to the maintenance of AR-dependent transcription in a castrate environment (Knudsen & Penning 2010). Genome-wide mapping of AR DNA-binding has shown that in CRPC AR binds to new sites on the DNA, resulting in an alternative transcriptional programme to that seen in primary disease (Sharma *et al.* 2013). CRPC carries a poor prognosis and a median survival of 18 months from diagnosis (Wu *et al.* 2007). New generation chemotherapeutics agents targeting AR signalling such as abiraterone and enzalutamide have led to modest improvement in prostate cancer survival, but they are not curative (Attard *et al.* 2011, Lamb *et al.* 2013).

In recent years, with advances in the understanding of AR function in CRPC and the cross-talk that occurs between AR and estrogen receptor alpha (ERA) in prostate cancer (Grubisha & Defranco 2013) and specific subtypes of breast cancer (Robinson *et al.* 2011), there has been renewed interest in understanding ER biology in the prostate and its role in prostate cancer. Further interest has arisen as a result of recent phase 2 clinic trial evidence supporting the use of transdermal estrogen therapy in prostate cancer, which avoids first-pass liver metabolism and has an improved side-effect profile over both parenteral estrogens and LHRH analogues (Langley *et al.* 2013). It is now known that the prostate gland expresses both ERA and estrogen receptor beta (ERB) (Horvath *et al.* 2001, Celhay *et al.* 2010). ERB is expressed in a wide range of reproductive and non-reproductive tissues including the CNS, cardiovascular system, gastrointestinal tract, urogenital tract (male and female) and skeleton (Bottner *et al.* 2014). The physiological role of ERB in each of these tissues has not been fully elucidated, but it has been implicated in the regulation of glucose homeostasis and insulin signalling and may also modulate immunologically mediated inflammatory pathways (Harris *et al.* 2003, Foryst-Ludwig *et al.* 2008). In addition, ERB is considered a negative regulator of ERA, acting to modulate transcriptional responses to estrogen in a tissue- and cell-context dependent manner (Bottner *et al.* 2014).

The traditional paradigm regarding the roles of the two ERs in the prostate is that ERB is predominantly protective, being anti-carcinogenic and pro-apoptotic (Chang & Prins 1999, Horvath *et al.* 2001, Zhu *et al.* 2004, Ellem & Risbridger 2007, McPherson *et al.* 2010, Muthusamy *et al.* 2011, Nakajima *et al.* 2011, Attia & Ederveen 2012), whereas ERA is oncogenic and promotes cell proliferation and survival (Ellem & Risbridger 2007, Risbridger *et al.* 2007, McPherson *et al.* 2008, Bonkhoff & Berges 2009, Celhay *et al.* 2010, Attia & Ederveen 2012). This view is based on a range of observations including epidemiological and *in vivo* studies, preclinical drug trials and expression profiles of the two ERs in human prostate cancer. However, much of the published data regarding the role and function of ERB appear to be conflicting, with studies conducted in cancer cell lines (McPherson *et al.* 2010, Dey *et al.* 2012, Yang *et al.* 2012), rodent models (Ricke *et al.* 2008, Attia & Ederveen 2012) or human tissues (Horvath *et al.* 2001, Celhay *et al.* 2010, Leung *et al.* 2010, Hussain *et al.* 2012) generating apparently contradictory results. Thus the precise actions of ERB in the prostate remain to be completely elucidated (Shaaban *et al.* 2003, Risbridger *et al.* 2007, Zhao *et al.* 2007, Celhay *et al.* 2010, Nelles *et al.* 2011, Dey *et al.* 2012, Yang *et al.* 2012). In this article, we review recent research in the area of ERB biology, with particular attention to its relevance in clinical aspects of human prostate cancer, and highlight areas for future research.

Evidence for the role of estrogen in prostate cancer: epidemiological

It is well established that European men have a lower risk of developing prostate cancer than African-American men, and that for Japanese men the lifetime risk of developing prostate cancer is lower still (de Jong *et al.* 1991, Ross *et al.* 1992, Ellem & Risbridger 2007). Two interesting observations are noteworthy in this respect. First, there are no significant differences in levels of circulating testosterone between these three ethnic groups (Ross *et al.* 1992), whereas levels of serum estrogens are higher in black men as compared with Caucasian men (Rohrmann *et al.* 2007, Abd Elmageed *et al.* 2013). However, a direct correlation between serum estrogen levels and prostate cancer risk has not been demonstrated (Yao *et al.* 2011, Bosland 2013). Secondly, the age of peak prostate cancer incidence occurs at a time when serum testosterone levels are in decline, but estrogen levels remain constant (Vermeulen *et al.* 2002). This has led to the hypothesis that it is the ratio of serum estrogen to

testosterone, rather than the absolute values of each, which gives rise to the observed differences in prostate cancer risk between the various ethnic groups (Ellem & Risbridger 2007, Bosland 2013). However, recent technological advances, which now permit more accurate measurement of both ultra-low and intra-tissue hormone levels, mean that a contemporary reassessment of this subject is needed (Barth *et al.* 2010, Hickey & Norman 2010, Stener-Victorin *et al.* 2010). Determining the actual levels of estrogen and androgen metabolites in the prostate will be essential for understanding ERA, ERB and AR behaviour. Racial differences in levels of prostatic ERB expression as measured by immunohistochemistry have been demonstrated by Abd Elmageed *et al.* (2013) who showed that the frequency of ERB immunostaining in prostate tumours was significantly higher in black men than in Caucasian men, which in turn, correlated with poorer clinical outcome.

Estrogen-related prostate cancer risk has been linked to dietary factors (Hori *et al.* 2011). The traditional Japanese diet contains high levels of dietary phytoestrogens, which have been shown in prostate cancer cell lines to upregulate ERB activity resulting in decreased expression of AR (Thelen *et al.* 2005, 2007, Stettner *et al.* 2007) and induction of G1-cell cycle block (Shen *et al.* 2000). In rat models, phytoestrogens can induce prostate epithelial cell apoptosis (Attia & Ederveen 2012), thereby demonstrating protective effects against prostate cancer. Phytoestrogens, along with other dietary estrogens such as lignans, flavonoids and lipoflavonoid are known to have up to 30-fold greater affinity for ERB than ERA, and are thought to promote the beneficial, protective effects of estrogen in the prostate (Kuiper *et al.* 1998, Ellem & Risbridger 2007, Thelen *et al.* 2014). A large population-based case-control study from Sweden demonstrated a reduced incidence of prostate cancer in those with a diet rich in phytoestrogens (Hedelin *et al.* 2006). Incidence of prostate cancer in Japan has been rising since the 1940s, coinciding with increased 'westernisation' of the Japanese diet. Specifically, it has been proposed that the 20-fold increase in the consumption of milk and animal fat, both of which contain estrogens with a high affinity for ERA (mediating the adverse effects of estrogen) may explain some of the recent rise in prostate cancer incidence among Japanese men (Ganmaa *et al.* 2003, Carruba 2007). There are, however, conflicting results in the literature regarding the effects of phytoestrogens in prostate cancer, as genistein (a highly ERB-specific isoflavone (Jiang *et al.* 2013)) has been shown in a study of prostate cancer xenograft-bearing mice to promote the development of

metastatic disease progression in an ERB-dependent manner (Nakamura *et al.* 2013) (discussed further in section 'Evidence for the role of estrogen in prostate cancer: drug trials').

Evidence for the role of estrogen in prostate cancer: animal studies

Some of the most compelling evidence for the importance of estrogen in prostate carcinogenesis comes from a series of animal studies (Ricke *et al.* 2008) (Fig. 1). Ricke *et al.* demonstrated in mice that androgen, estrogen, aromatase and ERA are all required for prostate carcinogenesis. Aromatase is a highly substrate-specific cytochrome p450 enzyme, found in the membrane of the endoplasmic reticulum, which has the unique function in vertebrates of being able to convert androgens to estrogens (Ghosh *et al.* 2009). In normal prostate, aromatase is expressed in the stromal cells and is responsible for local paracrine conversion of androgen to estrogen (Risbridger *et al.* 2007).

The necessity for estrogen in prostate carcinogenesis was demonstrated in experiments where either androgen alone, or androgen and estrogen were administered to aromatase knockout (ArKO) mice (Ricke *et al.* 2008). ArKO mice given androgen and estrogen developed prostatic intraepithelial neoplasia (PIN – a premalignant histological phenotype which, especially when high grade or multifocal, is a recognised risk factor for the development of invasive prostate cancer (Nelson *et al.* 2003, Merrimen *et al.* 2009)), whereas ArKO mice given androgen alone had no such change (Ricke *et al.* 2008). From this, the authors concluded that local production of estrogen within the prostate, facilitated by aromatase-mediated conversion of androgen to estrogen, was likely to be

Figure 1

Summary of animal studies conducted by Ricke *et al.* (2008), demonstrating: (A) in order for prostate intraepithelial neoplasia (PIN) to arise it is necessary for androgen, estrogen and functional aromatase all to be present; (B) this is an ERA-mediated process, which is suppressed by ERB. ArKO, aromatase knockout; aERKO, ERA knockout; bERKO, ERB knockout.

a significant factor in prostate carcinogenesis. Epithelial expression of aromatase is upregulated in prostate cancer (Ellem *et al.* 2004, Celhay *et al.* 2010), a process that is driven by gene promoters (I.3, I.4 and PII) responsive to inflammatory cytokines (Santen *et al.* 1997, Zhao *et al.* 1997, Shozu *et al.* 2000). The implication of this is twofold; first, increased local production of estrogens with resulting carcinogenesis and, secondly, the establishment of a 'positive feedback loop' between aromatase, estrogen and tissue inflammation (Ellem & Risbridger 2007). Indeed, high expression levels of aromatase and aromatase gene polymorphisms in early onset primary human prostate cancer have been found to correlate with decreased time to disease relapse, further underlining its importance in prostate carcinogenesis (Cussenot *et al.* 2007, Celhay *et al.* 2010).

In order to determine which of the ERs is responsible for mediating adverse effects of estrogen, Ricke *et al.* (2008) administered testosterone and estrogen to ERB knockout (bERKO) or ERA knockout (aERKO) mice. There was no difference between wild type (WT) and bERKO mice receiving hormone treatment, whereas aERKO mice did not develop PIN, suggesting that ERA mediated this particular adverse effect of estrogen in the prostate (Ricke *et al.* 2008). Similar studies in intact rats demonstrated that testosterone alone is insufficient for the development of PIN; it was only with the addition of the selective ERA agonist, ERA-45, that PIN developed (ERA-45 is reported to have a 286-fold greater affinity for ERA than ERB (Attia & Ederveen 2012)). However, with the administration of testosterone, ERA-45 and an ERB-selective agonist (ERB-26), the onset of PIN was prevented (Attia & Ederveen 2012), demonstrating the differential function of the two ERs in prostate carcinogenesis. These findings must be interpreted with some caution due to the ongoing debate in the field regarding the phenotypic variability and validity of the bERKO mouse models used in previous studies (Harris 2007).

Evidence for the role of estrogen in prostate cancer: drug trials

ERs are attractive targets for prostate cancer treatment as therapeutic agents are already in existence and are widely used in hormone-dependent breast cancer (Lumachi *et al.* 2011). Raloxifene, a selective ER modulator (SERM), has been shown to induce cellular apoptosis and nuclear fragmentation in both androgen-sensitive and androgen-independent prostate cancer cell lines through activation of ERB, suppression of ERA and subsequent induction of the caspase-8 and -9 pathways (Kim *et al.* 2002a,b, Rossi *et al.*

2011). Studies of selective ERB agonists on prostate cancer cell lines have also been encouraging. Several investigators have demonstrated that selective ERB agonists will induce cystic atrophy in basal cells of the prostatic epithelium (McPherson *et al.* 2010, Hussain *et al.* 2012). These basal cells do not express AR (Ruizeveld de Winter *et al.* 1991), and therefore, are unaffected by conventional ADT. Thus, once ADT is withdrawn, the prostatic epithelium is able to regenerate from this basal cell population. Administration of an ERB agonist, however, perturbs this regenerative process resulting in cellular apoptosis via the extrinsic pathway, mediated by tumour necrosis factor α (TNFA). This, importantly, is an androgen-independent process and may therefore be relevant to the treatment of CRPC (McPherson *et al.* 2010, Hussain *et al.* 2012). Recent research has shown that ERB-mediated cellular apoptosis may also occur through the intrinsic pathway, via upregulation of the Forkhead protein, FOXO3a, which itself is regulated by PTEN. Deletion mutations of PTEN result in inhibition of the apoptotic mechanism of FoxO3a, providing a possible ERB-mediated mechanism by which PTEN mutations in prostate cancer contribute to cancer growth (Dey *et al.* 2013b).

In vitro studies of the effects of dietary phytoestrogens on prostate cancer cell lines have shown conflicting results. On the one hand it has been shown that administration of ERB-selective phytoestrogens in CRPC will revert cancer cells to a less malignant phenotype (Wuttke *et al.* 2002, Messina 2010, Andres *et al.* 2011, Reiter *et al.* 2011). This may be due to a number of mechanisms. It is known that mutated forms of AR are upregulated in CRPC allowing AR to continue driving prostate cancer growth and progression in the absence of androgen (Scher *et al.* 2004, Waltering *et al.* 2012). Upregulation of ERB in response to phytoestrogens results in down-regulation of AR, with a subsequent decline in serum levels of prostate-specific antigen (PSA) and other AR-dependent genes (Thelen *et al.* 2005, 2007). ERB may therefore function as a negative regulator of AR, as well as ERA. However, as previously mentioned, in a study conducted using a patient-derived prostate cancer tissue line mouse xenograft model (maintained by serial transplantation of sub-renal capsule xenografts) (Andersen *et al.* 2010), the ERB-selective phytoestrogen genistein has been shown to promote development of metastatic disease (Nakamura *et al.* 2013). The explanation for these conflicting results is not presently clear; however it has been hypothesised that in the mouse xenograft tumours, increased expression of metallothionein proteins in response to genistein-induced ERB activity

may contribute to tumour invasion and metastasis (Nakamura *et al.* 2013); an interaction, which may not be reflected in cell-line studies where the tumour microenvironment is absent.

A promising clinical study was published by Price *et al.* (2006). In a phase 2b clinical trial of 514 men with biopsy-proven high-grade PIN, toremifene, an ERA-selective antagonist (Kangas 1990), was shown to reduce the incidence of invasive prostate cancer at 12 months by 48% vs placebo, thus preventing 6.8% cancers per 100 men per year. However, the outcome of this trial was reported after only a 12-month study period and no long-term data on the use of toremifene in prostate cancer have since been generated to address potential long-term side effects or duration of treatment benefit.

Trials of other ER modulators have also been unsuccessful. Fulvestrant, an ERA antagonist, has been shown to be effective in preclinical models with growth inhibition of prostate cancer cell lines (Lau *et al.* 2000, Leung *et al.* 2006a). However, in a phase 2 study of 20 men with CRPC, fulvestrant failed to produce either a clinical or biochemical (PSA) response (Chadha *et al.* 2008). Similarly, tamoxifen, a mixed ERA agonist/antagonist has been shown to be ineffective in men with CRPC (Bergan *et al.* 1999) despite inhibiting the growth of prostate cancer cell lines in preclinical studies (Rohlf *et al.* 1998). The reasons underlying these observations are not presently clear; however, it is likely that prostate cancer cell lines used in the preclinical studies are not reflecting the complex cross-talk between AR, ERA and ERB, and other stromal–epithelial interactions known to occur *in vivo* (Hanahan & Weinberg 2011, Robinson *et al.* 2011, Grubisha & Defranco 2013, Madak-Erdogan *et al.* 2013). These studies highlight the critical need for improved preclinical models of prostate cancer, in which to test new therapeutic agents targeted to the ERs.

ERs in the prostate

ERB was first identified by Kuiper *et al.* (1996) in the rat prostate. In humans, it is a 55 kDa protein encoded by the *ESR2* gene located on chromosome 14 (Enmark *et al.* 1997). Expression of ERB is regulated epigenetically by a CpG island in the promotor region (Zhu *et al.* 2004) and ERB expression is silenced by DNA-hypermethylation of the promotor (Zhao *et al.* 2003, Rody *et al.* 2005). ERB is strongly expressed in the basal and secretory compartments of benign prostate epithelium in both rodents and humans (Horvath *et al.* 2001). The principle ligand of ERB in the prostate is 5 α -androstane-3 β ,17 β -diol (3 β -diol),

a metabolite of 5 α -dihydrotestosterone (DHT) (Oliveira *et al.* 2007). In prostate cell lines (benign and cancer) ERB has been shown to maintain differentiation of epithelial cells by regulation of epithelial–mesenchymal transition (EMT) genes such as Twist via hypoxia-inducible factor 1 alpha (HIF-1A) (Mak *et al.* 2013).

The gene coding human ERA (*ESR1*) is located on chromosome 6 (Menasce *et al.* 1993). In the prostate (rodent and human) ERA is predominantly expressed in the stroma (Celhay *et al.* 2010, Attia & Ederveen 2012). *In utero* studies of prostate development in rodents have shown that ERA expression appears before ERB, and excessive estrogenisation of the developing prostate (mediated via ERA) results in permanent changes in the prostate including squamous metaplasia, inflammation and epithelial dysplasia (Arai *et al.* 1978, Prins & Birch 1997). This ‘imprinting’ results in increased risk of a premalignant phenotype and prostate carcinogenesis (Prins *et al.* 2006, 2007, McPherson *et al.* 2008, Prins & Korach 2008). Although expressed from different genes, ERA and ERB share substantial sequence homology, in particular the DNA-binding domains (DBD) of the two receptors are 97% identical. This allows both ERs to recognise a consensus estrogen response element (ERE) on DNA with equal affinity (Le *et al.* 2013).

The advent of genome-wide transcription factor mapping by chromatin immunoprecipitation coupled with high-throughput sequencing (ChIP-seq) has enabled detailed study of how transcription factors such as steroid hormone receptors function, by revealing the locations of their DNA-binding sites (Carroll *et al.* 2006). We now know, for example, that in addition to proximal gene promoters, ERA and AR bind to distal enhancer elements, far from gene-transcription start sites, and by recruitment of co-regulatory factors initiate gene transcription by long-range chromatin interactions (Carroll *et al.* 2005, Wang *et al.* 2007, Massie *et al.* 2011). For ERA and AR, a number of these co-regulatory factors are now well-characterised and represent potential therapeutic targets (Carroll *et al.* 2005, Wang *et al.* 2007, 2009, 2011, Hurtado *et al.* 2011, Robinson *et al.* 2011, Sahu *et al.* 2011). Genome-wide mapping of both tagged (Zhao *et al.* 2010) and force-expressed recombinant ERB (Madak-Erdogan *et al.* 2013) DNA-binding in the MCF7 breast cancer cell line has demonstrated significant overlap between ERA and ERB DNA-binding sites, inferring complex cross-talk between the two receptors. In addition, there is evidence that ERB binds to distal enhancer elements in the same manner as ERA and AR to regulate gene expression (Carroll *et al.* 2005, Zhao *et al.* 2010, Massie *et al.* 2011). Despite these

significant insights, there is still very limited understanding of the mechanisms and co-regulators by which ERB activity may be modulated and thus the resulting effects on ERB transcription.

ER expression in prostate cancer

In normal prostate, ERA expression is confined to the prostatic stroma (Tilley *et al.* 1985, Wernert *et al.* 1988, Leav *et al.* 2001). In contrast to ERB, ERA mRNA has been detected in high-grade PIN of the prostate, and ERA expression is upregulated in prostatic epithelium of intermediate- and high-grade tumours and in CRPC (Bonkhoff & Berges 2009, Celhay *et al.* 2010, Nelles *et al.* 2011). Stromal ERA expression and elevated expression of aromatase have been shown to be independent predictors of shorter time to relapse in CRPC (Celhay *et al.* 2010). Expression of the *TMPRSS2-ERG* fusion gene, which has been suggested to be a marker of an aggressive tumour phenotype found in up to 50% of prostate cancer (Qu *et al.* 2013, Razzak 2013), increased in the NCI-H660 prostate cancer cell line following treatment with an ERA agonist (Setlur *et al.* 2008). NCI-H660 is an AR-negative prostate cancer cell line expressing the *TMPRSS2-ERG* fusion gene (Mertz *et al.* 2007), derived from the lymph node metastasis of a small-cell prostate tumour with neuro-endocrine differentiation (Johnson *et al.* 1989, Lai *et al.* 1995). Expression of ERA and aromatase with the R264C polymorphism has been shown to result in shorter progression-free survival and an increased risk of developing CRPC in a study of 115 men treated with docetaxel (Sissung *et al.* 2011). Taken together, these observations support the hypothesis that ERA can act as an oncogene by mediating the adverse effects of estrogen in the prostate.

Declining levels of ERB have been observed with progression from benign prostatic hyperplasia to malignant disease (Horvath *et al.* 2001), with a further decrease associated with increasing Gleason grade of prostate cancer (Leav *et al.* 2001, Asgari & Morakabati 2011, Attia & Ederveen 2012, Dey *et al.* 2013b). ERB expression is low in high-grade PIN of the prostate (Risbridger *et al.* 2007), reflecting its pre-malignant phenotype. It has been shown that as ERB expression declines with the development of prostate cancer, levels of HIF-1A increase, resulting in epithelial de-differentiation and growth of high-grade, aggressive tumours (Mak *et al.* 2013).

Horvath *et al.* (2001) showed in a study of 159 prostates obtained by radical prostatectomy that over 75% of tumours in their cohort did not express ERB. However, in

low-grade (Gleason 3) tumours, ERB expression was maintained, and correlated positively with disease-free survival (Horvath *et al.* 2001). In an additional finding that seems to contradict these results, where ERB expression was maintained, there was a higher rate of disease relapse irrespective of tumour grade (Horvath *et al.* 2001). Other studies have demonstrated high ERB expression in bone and lymph node metastases (Zhu *et al.* 2004, Bouchal *et al.* 2011). A recent study has shown that the combination of ERB expression and AR phosphorylation in hormone-naïve prostate cancer correlates with poor clinical outcome (Zellweger *et al.* 2013). In that study, increased expression of WT ERB (ERB1) was associated with higher Gleason grade and greater proliferative activity. Fifty percent of the patients in the study cohort showed a significant increase in ERB expression with subsequent development of CRPC (Zellweger *et al.* 2013).

The variability of ERB expression in differing grades and stages of prostate cancer presents some difficulty in deciphering the underlying mechanisms and role of ERB in prostate carcinogenesis. If ERB is tumour-suppressive, then it is logical that its expression declines with advancing carcinogenesis. However, this does not explain why ERB expression is then high in lymph node or bone metastases (Zhu *et al.* 2004, Bouchal *et al.* 2011), or the observed correlation between high ERB expression and poor prostate cancer prognosis (Horvath *et al.* 2001, Zellweger *et al.* 2013). This may be due to varying levels of promotor methylation throughout the carcinogenic process introducing reversible, stage- and tissue-specific changes in ERB expression and altering its transcriptional role (Risbridger *et al.* 2007, Cotrim *et al.* 2013). In addition, it has been proposed that ERB expression may confer a selective advantage for subclones of prostate cancer cells to metastasise (Zhu *et al.* 2004), resulting in the maintenance of ERB expression in metastatic deposits. A further possible explanation for this discrepancy is variability in the specificity and sensitivity of commercially available ERB antibodies (Skliris *et al.* 2002, Hartman *et al.* 2012). Different ERB antibodies have been shown to only be suitable for particular experimental applications (Weitsman *et al.* 2006), creating some difficulty in the interpretation of results from different studies.

More recently, there is increasing evidence that expression of the ERB isoform, ERB2, is increased in high-grade and metastatic prostate cancer (Dey *et al.* 2012). ERB2 may act as an oncogene and has been implicated specifically in the process of cancer metastasis (Chen *et al.* 2009, Leung *et al.* 2010, Dey *et al.* 2012). If the antibodies used in the abovementioned studies (Horvath *et al.* 2001,

Zhu *et al.* 2004, Bouchal *et al.* 2011, Zellweger *et al.* 2013) are in fact detecting ERB2, rather than ERB1, some of the abovementioned contradictions may be explained. Further detailed study is required to answer this definitively.

The role of ERB isoforms

At least five splice variants of ERB have been identified (Leung *et al.* 2006b) (Fig. 2). Expression of ERB3 is limited to the testis (Moore *et al.* 1998), but ERB1, ERB2, ERB4 and ERB5 are known to be expressed in the prostate, and there is increasing evidence indicating that ERB2 in particular acts as an oncogene in direct opposition to ERB1 (Chen *et al.* 2009). ERB1 is composed of eight exons, the first six of which are common to the five isoforms. The isoforms share the same first four functional domains with ERB1 (including the DBD), but the LBD differs (Moore *et al.* 1998, Hanstein *et al.* 1999, Leung *et al.* 2010). ERB2 and ERB5 have been studied in detail in prostate cancer and shown to correlate with poor prognosis (Leung *et al.* 2010, Dey *et al.* 2012). Specifically, co-expression of nuclear ERB2 and cytoplasmic ERB5 was shown in a study of 144 patients with long-term follow up to be an independent prognostic marker for biochemical relapse, postoperative metastasis and time to metastasis following radical prostatectomy for localised prostate cancer (Leung *et al.* 2010). While ERB2 is the dominant isoform in prostate cancer, its mechanism of action remains unclear as it lacks

the LBD. ERB2 seems to act as a transcriptional repressor of ERB1, thus disabling the usual, protective effect of ERB1 (Cotrim *et al.* 2013). One hypothesis proposed by Leung *et al.* (2006b) is that whilst ERB1 functions as a homodimer, ERB isoforms function only when heterodimerised with ERB1. These ERB heterodimers form preferentially under the influence of oestradiol (E2) and have higher transcriptional activity than the ERB1 homodimer. Interestingly, phytoestrogens such as genistein promote formation of the ERB1 homodimer. As ERB2 lacks a LBD, it is proposed that when it is heterodimerised with ERB1, transcription is inhibited. In this model, ERB2 may therefore function as a dominant-negative regulator of ERB1 activity. This may explain one way that ERB transcription can be modulated in cell- and tissue-specific contexts (Leung *et al.* 2006b, Cotrim *et al.* 2013).

In ovarian carcinoma, levels of ERB5 mRNA are elevated, compared with benign tissues, suggesting it has an oncogenic role in that particular context (Suzuki *et al.* 2008). A study of stable tetracycline-inducible ERB2-expressing MCF7 breast cancer cells has suggested that ERB2 can also heterodimerise with ERA to induce ERA degradation and inhibition of ERA transcription (Zhao *et al.* 2007).

The influence of ERB2 in both prostate and breast cancer metastasis is thought to result from regulation of genes responsible for EMT (Leung *et al.* 2010, Dey *et al.* 2012, Roy *et al.* 2012, Yang *et al.* 2012). EMT is a marker of early oncological change, which enables cancer cells to

Figure 2

Schematic showing the structure of ERB isoforms. ERB1 (WT) contains eight exons, the first six of which are common to the other isoforms. All five isoforms share common DNA-binding domains (DBD), but the

ligand-binding domains (LBD) differ. Data from Uniprot (<http://www.uniprot.org/>) and ClustalW2 (<http://www.ebi.ac.uk/Tools/msa/clustalw2/>).

invade surrounding tissues and eventually metastasise to distant sites (Hanahan & Weinberg 2011). Although well-characterised *in vitro*, there is still some controversy as to whether the EMT programme occurs *in vivo* or is an artefact of cell line studies, principally because of difficulties differentiating transformed epithelial cells from the surrounding tumour stromal tissue, and the fact that metastatic deposits often closely resemble the epithelial tissue of origin (Hollier et al. 2009). Nevertheless, evidence is emerging that EMT markers can be observed in circulating tumour cells, suggesting that it does occur *in vivo* (Li et al. 2013).

In a study conducted in prostate cancer cell lines, expression of ERB2 was shown to result in upregulation of the EMT genes *TWIST1* (which correlates with high-grade prostate cancer) and *RUNX2* (normally repressed by ERB1) (Dey et al. 2012). This interaction between ERB2 and EMT genes is facilitated by a proto-oncogene, PELP1, which interacts with a number of steroid hormone receptors including ERA (Vadlamudi et al. 2001), ERB and AR (Yang et al. 2012). In breast cancer, PELP1 has been shown to interact with histones to remodel chromatin and modulate expression of key EMT genes such as *TWIST1*, *SNAIL* and *ZEB* (Roy et al. 2012). It is therefore possible that PELP1 and ERB2 have a role in the promotion of

prostate cancer metastasis. If correct, this hypothesis could explain the previously discussed findings of high ERB expression in bone and lymph node metastases, and the correlation between persistent ERB expression in high-grade prostate cancer and greater risk of disease relapse (Horvath et al. 2001, Zhu et al. 2004, Bouchal et al. 2011, Zellweger et al. 2013). This is an important area for further investigation.

The role of ER-mediated inflammation in mechanisms of cancer progression

Inflammation is a well-established process in the development and progression of cancer (Hanahan & Weinberg 2011) (Fig. 3). Several inflammatory mechanisms, centering on ER function, have been implicated in the development and progression of prostate cancer. Loss of E-cadherin is a well-established marker of EMT, with associated loss of cell adhesion and a resulting increase in cell motility (Guarino et al. 2007, Grubisha & Defranco 2013). ERB has been shown to be a negative regulator of inflammatory processes (Harris et al. 2003) and in the prostate its expression is known to correlate with E-cadherin levels. One hypothesis is that as ERB expression declines during the progression from benign to low-grade,

Figure 3

Proinflammatory positive feedback loops in prostate cancer progression, centering on ERB and ERA function. In response to tissue inflammation, decreased ERB expression results in upregulation of EMT programme genes leading to prostate cancer metastasis. Tissue inflammation leads to

increased aromatase expression and increased inflammatory-cell epithelial infiltration. Decreased ERB transcription results in loss of direct repression of ERA. WBC, white blood cell; E2, oestradiol; NO, nitric oxide; H₂O₂, hydrogen peroxide; COX2, cyclo-oxygenase-2; PCa, prostate cancer.

to high-grade cancer (Leav *et al.* 2001, Celhay *et al.* 2010, Asgari & Morakabati 2011), the resulting decline in E-cadherin leads to an increased propensity to develop metastatic disease. ERB transcriptional activity is sensitive to oxidation resulting from tissue inflammatory processes and a local paracrine signalling network (Grubisha *et al.* 2012). When ERB is oxidised by H_2O_2 and other reactive oxygen species, DNA binding is lost and expression of E-cadherin is reduced. The pro-inflammatory enzyme COX2, expressed by prostatic stroma, generates sufficient H_2O_2 to inactivate ERB. As COX2 is overexpressed in prostate cancer, a pro-inflammatory positive feedback loop is established (Grubisha & Defranco 2013).

ERA and aromatase also play a critical role in tissue inflammation in prostate cancer and expression of these two factors in tumour cells is an independent predictor of time to biochemical relapse (defined as two consecutive rises in serum PSA) in men treated with ADT (Celhay *et al.* 2010). In prostate cancer, expression of aromatase is increased, particularly in epithelial cells resulting in increased levels of intraprostatic estrogen, which acts via ERA to promote tissue inflammation via local generation of nitric oxide (Pinzone *et al.* 2004, Ellem & Risbridger 2007, Risbridger *et al.* 2007, Celhay *et al.* 2010, Nelles *et al.* 2011). Pro-inflammatory mediators such as TNFA and prostaglandin E_2 in turn upregulate *CYP19* expression, resulting in increased aromatase activity (Subbaramaiah *et al.* 2011). Thus an additional pro-inflammatory positive feedback loop centering on ERA function is established (Ellem & Risbridger 2007). Evidence of neutrophil and leucocyte migration from the stroma to the epithelium in mouse models confirms the presence of this inflammatory process (Bianco *et al.* 2002, 2006). Inflammatory cytokines released by the migrating immune cells result in abnormal proliferation of prostate epithelium and increase the risk of further premalignant change (Bianco *et al.* 2006).

A change in perspective: an oncogenic role for ERB?

ERB1 has been implicated directly as an oncogene (Yang *et al.* 2012). In that study, ERB1-mediated, non-androgenic AR signalling was demonstrated in several prostate cancer cell lines in hormone-deplete (castrate) conditions (Fig. 4). In the presence of DHT, AR binds to androgen-responsive elements (AREs) on DNA to initiate AR-dependent transcription. In these conditions, ERB1 and PELP1 form a complex in the nucleus. However, in the absence of DHT and with addition of E_2 , the ERB1–PELP1 complex binds

Figure 4

A hypothesis of non-androgenic, ERB-mediated transcription of AR-dependent genes via interaction with PELP1, as proposed by Yang *et al.* (2012). (A) In the presence of DHT ERB–PELP1 forms a complex, which does not bind to AR. (B) In the absence of DHT, and with oestradiol (E_2) treatment, the ERB–PELP1–AR complex binds to the androgen-responsive element (ARE) to initiate AR-dependent gene transcription. Figure adapted from Yang L, Ravindranathan P, Ramanan M, Kapur P, Hammes SR, Hsieh JT & Raj GV 2012 Central role for PELP1 in nonandrogenic activation of the androgen receptor in prostate cancer. *Molecular Endocrinology* **26** 550–561.

to AR (with PELP1 acting as a bridge between the two nuclear receptors) and is recruited to an ARE, resulting in the transcription of AR-dependent genes. This ERB1–PELP1–AR complex was shown to facilitate cellular proliferation in response to E_2 treatment, demonstrating a clear mechanism by which estrogens might continue to drive prostate cancer growth and progression in the castrate environment, thereby highlighting the potential oncogenic role of ERB1. Furthermore, it has been proposed that ERB1 may have a role in mediating the ‘switch’ from hormone-sensitive prostate cancer to CRPC (Zellweger *et al.* 2013). It may be that ERB is only tumour-suppressive in early stages of the disease until, by means of a currently unknown mechanism, it subsequently becomes an oncogene. This is an important question for detailed investigation, as therapeutic silencing of such a ‘switch’ could theoretically reduce the risk of developing CRPC.

Our understanding of the role of ERB in the development and progression of prostate cancer is evolving, but there are many unresolved issues. Given the divergent activity of ERB isoforms and the potential for an oncogenic role for ERB1 (Yang *et al.* 2012), we can no longer hold to the classical paradigm of estrogen signalling in the prostate, which surmises that ERA is tumour promoting and ERB is tumour suppressive. In order to progress our understanding of estrogen biology in cancer it is critical that the mechanisms underlying the differential functions of ERA, ERB and the various ERB isoforms are elucidated in detail (Madak-Erdogan *et al.* 2013). Recent advances in

understanding the function of the two ERs at the genomic level have been beginning to provide insights into this complex area. Madak-Erdogan *et al.* (2013) used ChIP-seq to demonstrate the genome-wide chromatin binding profiles and differing transcriptional responses of ERA or ERB in the MCF7 breast cancer cell line. Their data confirmed the previous finding using the tagged ERB ChIP-seq approach (Zhao *et al.* 2010) demonstrating significant overlap between the binding sites of ERA and ERB. Specifically, when each of the receptors was present alone, there was a 40% overlap between ERA and ERB DNA-binding sites. However, when co-expressed, the number of binding sites available for each ER dropped by ~50%. This suggests a complex mechanism where each ER restricts the total number of binding sites available to the other, but when the activity of one ER is reduced, chromatin binding by the other is increased. While the functional consequences of altered ERA and ERB chromatin binding in these different contexts remain to be fully elucidated, the proliferative effects of estrogen acting via ERA were reduced with the co-expression of ERB. When expressed in isolation, ERA regulates cell cycle genes, helping push cells from growth-arrested states into DNA synthesis and subsequent mitosis. However, when ERB is co-expressed, ERA's ability to respond to ligand is reduced and cell proliferation decreases. ERB's anti-proliferative function was demonstrated to occur through direct binding to apoptosis and cell-cycle regulation genes (Madak-Erdogan *et al.* 2013). Similar results were demonstrated by Le *et al.* (2013) in ChIP-seq of MCF7/C4-12 cells (derivative of MCF7 with no ERA expression) transfected to stably express ERB. These data support the previously discussed hypothesis that ERB is tumour-suppressive and a negative regulator of ERA, functioning in a variable manner according to the particular cellular context (Zhao *et al.* 2010, Bottner *et al.* 2014, Cotrim *et al.* 2013). However, as these data were generated using breast cancer cell lines, it is important that the hypothesis is further tested in appropriate prostate cancer models. The isoform specificities of the antibodies used in the ChIP-seq study

(Madak-Erdogan *et al.* 2013) are unknown and therefore it is not clear how these findings in MCF7 cells are applicable to the previously discussed differential functions of ERB isoforms (Leung *et al.* 2010, Dey *et al.* 2012). Clearly, there is an urgent need to develop specific antibodies to ERB and its isoforms to address these questions (Haldosen *et al.* 2014).

Conclusions

Despite a number of promising preclinical studies showing efficacy of ERB-selective agents in prostate cancer (Kim *et al.* 2002a,b, McPherson *et al.* 2010), there is currently no evidence of clinical benefit from the use of these treatments in terms of disease-specific or overall survival. The underlying reasons for this necessitate further investigation. One possibility is insufficient dosing of the therapeutic agents in question (Chadha *et al.* 2008). Most of the preclinical studies of ERB-selective agents and much of our current knowledge of ERB biology results from studies conducted in various prostate cancer cell lines. The expression profiles of the nuclear receptors AR, ERA and ERB vary between each of the commonly used cell lines and different authors report contrasting results in individual cell lines (Table 1) (Veldscholte *et al.* 1990, Kim *et al.* 2002b, Holbeck *et al.* 2010, Nakajima *et al.* 2011). None of these commonly used cell lines are entirely representative of human tissue, as exemplified by the fact that in the human prostate ERA expression is predominantly stromal, whereas luminal epithelial cells express ERB and AR, and basal epithelial cells only express ERB (Ruizeveld de Winter *et al.* 1991, Bonkhoff & Berges 2009). Cell line models, therefore, cannot reproduce the stromal-epithelial interactions known to be important in cancer development and progression (Hanahan & Weinberg 2011), or the complex interplay that has been observed between ERA and ERB, and how transcription from activation of one receptor impacts the availability of DNA-binding sites to the other (Madak-Erdogan *et al.* 2013). Studies conducted in *ex vivo*

Table 1 Variability of reported nuclear receptor expression in commonly used prostate cancer cell lines

References	Cell line	AR	ERA	ERB
Veldscholte <i>et al.</i> (1990) and Kim <i>et al.</i> (2002a,b)	LNCaP	Positive (mutant)	Negative	Positive
Nakajima <i>et al.</i> (2011)	DU145	Negative	Negative	Positive
Holbeck <i>et al.</i> (2010)	DU145	Negative	Negative	Negative
Holbeck <i>et al.</i> (2010) and Nakajima <i>et al.</i> (2011)	PC3	Negative	Positive	Positive

primary human tissue culture (Centenera *et al.* 2012) or xenografts of human tumours (Lawrence *et al.* 2013) may be helpful in this regard.

Estrogen-related pathways are clearly of great importance in the development and progression of hormone-dependent cancers such as prostate cancer, but the role of ERB remains controversial, with numerous contradictions in the published literature. Our current understanding of ER biology in the prostate is insufficient to facilitate precise manipulation of the molecular machinery in a meaningful fashion (Abd Elmageed *et al.* 2013). Recent developments in the understanding of apparently opposing ERB isoforms (Leung *et al.* 2010) and the mechanisms governing ERB transcription are beginning to provide greater insights into ERB biology with implications not just for prostate cancer but also for colon, breast and ovarian cancers (Suzuki *et al.* 2008, Chantzi *et al.* 2013, Dey *et al.* 2013a). In order to determine whether ERB represents a useful therapeutic target in prostate cancer, and more specifically in CRPC, it is vital that these mechanisms are fully elucidated. Given that ERA and ERB can homo- or heterodimerise with ERB isoforms, the cross-reactivity between different estrogenic ligands, the differing effects of ERB in specific cellular contexts, and the fact that ERA and ERB can recognise the same DNA-binding sites and interact with common co-regulators, this is likely to be a difficult task (Shaaban *et al.* 2003, Zhao *et al.* 2010, Cotrim *et al.* 2013, Le *et al.* 2013, Madak-Erdogan *et al.* 2013). The challenge will be to identify and characterise the ERA- and ERB-unique DNA-binding sites, and furthermore, to define the ERB isoform-specific DNA binding sites in order to determine their respective functions. To improve outcomes for patients, there is an urgent need for detailed understanding of the mechanisms governing the differential functions of the two ERs in tissue- and disease-specific contexts as well as investigation of novel therapeutic agents that selectively target ERA- and ERB-dependent pathways.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the review.

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Author contribution statement

A W Nelson and W D Tilley conceptualised and designed the structure of the article. A W Nelson conducted the literature review. A W Nelson and W D Tilley co-wrote the manuscript. D E Neal and J S Carroll provided critical review and revision of the manuscript.

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