

# Oestrogen and benign prostatic hyperplasia: effects on stromal cell proliferation and local formation from androgen

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## Abstract

Oestrogens have been implicated as a cause of benign prostatic hyperplasia (BPH). Previous animal studies led to the hypothesis that oestrogens can stimulate prostate growth, resulting in hyperplasia of the gland. In humans, the precise role of oestrogens in BPH pathogenesis is currently unclear. We investigated the direct effects of oestradiol on the proliferation of BPH-derived prostate cells in culture. Oestradiol ( $10^{-7}$  and  $10^{-6}$  M) moderately increased the proliferation of stromal cells in culture; this stimulation was antagonised by anti-oestrogen ICI 182 780, indicating an oestrogen receptor (ER)-mediated mechanism. By contrast, oestradiol had no effects on the proliferation of epithelial cells in culture. Parameters that can determine the response of stromal cells to oestrogens, including expression of the two

ER subtypes and aromatase activity, were investigated. ER $\beta$  expression in stromal cells in culture was demonstrated by immunohistochemistry and western blot analysis, and was confirmed by semi-quantitative RT-PCR showing higher expression of ER $\beta$  than ER $\alpha$  mRNA in stromal cells. Aromatase, the enzyme that converts androgen precursors to oestrogens, was also examined. Aromatase mRNA and activity were detected in stromal, but not epithelial cells in culture, suggesting a mechanism whereby oestrogen concentrations can be regulated in the BPH stroma. Taken together, these findings support the hypothesis that oestrogens play a role in the pathogenesis of BPH, a disease characterised predominantly by stromal overgrowth.

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## Introduction

Benign prostatic hyperplasia (BPH) is a common urological problem in ageing men; patients usually present with symptoms of lower urinary tract obstruction (Uson *et al.* 1991, Verhamme *et al.* 2002). The precise aetiology of this condition is unknown. Two factors are generally considered essential for the development of BPH: ageing and the presence of a testis. Although androgens are believed to be important in the pathogenesis of BPH, it is paradoxical that the disease typically manifests at a stage in life when androgen levels are declining (Gray *et al.* 1991, Harman *et al.* 2001). With advancing age, plasma androgen levels decrease gradually while oestrogen levels remain constant or decrease slightly, resulting in increasing ratios of oestrogen to androgen levels in plasma (Gray *et al.* 1991, Harman *et al.* 2001, Vermeulen *et al.* 2002).

Although oestrogens have long been implicated in causing BPH, their exact role in the pathophysiology of this condition is unclear (Walsh & Wilson 1976, Collins *et al.* 1994). An animal model of BPH showed that oestradiol exerted a synergistic effect with androgens in inducing glandular prostatic hyperplasia in castrated dogs (Walsh & Wilson

1976, DeKlerk *et al.* 1979), whereas castrated dogs treated with oestradiol alone developed squamous prostatic epithelial metaplasia (DeKlerk *et al.* 1979). These early studies led to the hypothesis that oestrogens might also exert a synergistic effect with androgens in inducing prostatic hyperplasia in man. In a model using prostate tissues isolated from aborted human fetuses and grown in athymic male mice, diethylstilbestrol (a synthetic oestrogen) induced squamous metaplasia of the prostatic epithelium (Sugimura *et al.* 1988). However, none of the above models generated the histopathology of prostatic hyperplasia commonly seen in patients, which consists of a mixture of stromal and epithelial hyperplasia. Moreover, previous studies using animal models to investigate a role for oestrogens in BPH pathogenesis have been problematic because multiple endocrine factors including plasma androgen levels were altered in these animals. Oestrogens can exert both direct and indirect effects on the prostate. On the one hand, high levels of circulating oestrogens can indirectly cause regressive changes in the prostate, which are believed to be mediated by the inhibition of pituitary gonadotrophin secretion, leading to decreased testicular output of androgens and lowered plasma androgen levels (Jin *et al.* 1996). On the

other hand, oestrogens may exert their effects directly on prostate cells, mediated by their cognate receptors expressed in the prostate, or via further metabolism to catechol or quinine intermediates (Ho 2004).

Oestrogens exert their effects on target gene expression by binding to specific intracellular oestrogen receptors (ERs) that function as hormone-inducible transcription factors. To date, two ER subtypes have been identified in humans: ER $\alpha$  (Greene *et al.* 1986) and ER $\beta$  (Mosselman *et al.* 1996). There is currently no consensus on the localisation of the two ER subtypes in human prostate tissues (Ehara *et al.* 1995, Bonkhoff *et al.* 1999, Pasquali *et al.* 2001b, Royuela *et al.* 2001, Tsurusaki *et al.* 2003), and their expression in BPH-derived cells in primary culture is unclear.

Aromatase is a member of the cytochrome P450 family of enzymes and catalyses the conversion of C-19 androgen precursors to C-18 oestrogens (Simpson *et al.* 1994). Kinetic studies have demonstrated that this enzyme has high affinity for the weak androgen androstenedione and converts it to oestrone (Schweikert 1979, Stone *et al.* 1986). Although expression and function of aromatase in the human prostate is not entirely clear (Brodie *et al.* 1989, Matzkin & Soloway 1992, Tsugaya *et al.* 1996, Hiramatsu *et al.* 1997), recent studies reported associations between aromatase polymorphisms and altered risks of prostatic hyperplasia (Azzouzi *et al.* 2002, Roberts *et al.* 2006). Expression of aromatase in oestrogen target tissues is likely to increase oestrogenic actions by the local conversion of androgens to oestrogens and may also represent a mechanism whereby the effects of oestrogens are regulated.

In this study, the hypothesis that oestrogens play a role in BPH pathogenesis was tested by investigating the effects of oestradiol on the proliferation of BPH-derived prostate cells in primary culture. Parameters that can influence the response of prostate cells to oestrogens were also examined; these include the expression of aromatase and ER subtypes.

## Materials and Methods

### Prostate tissue and cell culture

Prostate specimens were obtained from patients undergoing transurethral resection for the treatment of BPH with informed consent and approval of the local hospital ethics committee. Prostate tissue chips were placed in ice-cold RPMI 1640 medium supplemented with 5% fetal calf serum (FCS, v/v), penicillin (100 U/ml) and streptomycin (100 µg/ml) for cell separation and culture. Prostate stromal and epithelial cells were isolated from BPH tissues and cultured as described previously (Tsugaya *et al.* 1996, Bayne *et al.* 1998). Stromal and epithelial cells were maintained in fibroblast growth medium (FGM) and epithelial cell growth medium (EGM) respectively. FGM was prepared by supplementing RPMI 1640 medium with 10% FCS, 2 mM L-glutamine, 100 units/ml penicillin and 100 µg/ml streptomycin. Each litre of EGM consisted of 11 g WAJC 404

medium powder, 6.7 g HEPES, 1.2 g sodium hydrogen carbonate, 0.5 mg zinc stabilised insulin, 20 µg cholera toxin, 392 µg dexamethasone, 10 µg epidermal growth factor, 0.5% FCS, 100 000 units penicillin and 100 mg streptomycin. Stromal and epithelial cell identities were established by phase contrast microscopy and immunocytochemical staining as described in previous reports (Tsugaya *et al.* 1996, Bayne *et al.* 1998). MCF-7 human mammary adenocarcinoma cells were obtained from ATCC (Teddington, UK) and maintained in minimal essential medium supplemented with 5% FCS. All cell culture reagents were purchased from Life Technologies.

### Immunocytochemical localisation of ER subtypes

ER $\alpha$  antibody used is a mouse monoclonal antibody raised against N-terminal amino acids 2–185 of human ER $\alpha$  (Santa Cruz Biotechnology, Santa Cruz, CA, USA). This region of ER $\alpha$  corresponds mainly to the A/B functional domain of the receptor and shares little homology with ER $\beta$  (Katzenellenbogen *et al.* 2000). ER $\beta$  antibody is a mouse monoclonal antibody against the C-terminal amino acids CSPAESKSKS-KEGSQNPQSQ, specific for the ER $\beta$ 1 protein (Serotec, Oxford, UK) (Moore *et al.* 1998, Saunders *et al.* 2000). Immunostaining of prostate cells in primary culture derived from six BPH patients was performed as described previously (Tsugaya *et al.* 1996). In brief, the cells were fixed with 1% formaldehyde in PBS, followed by treatment with 5% acetic acid in ethanol for antigen retrieval. Antibody dilutions used were 1:100 and 1:20 for ER $\alpha$  and ER $\beta$  respectively. MCF-7 cells, previously reported to express both ER $\alpha$  and ER $\beta$  (Boyan *et al.* 2003), were used as a positive control. Primary antibody was omitted in negative controls. Secondary antibody used was a biotinylated goat anti-mouse antibody (Autogen Bioclear, Calne, UK) and colour development was achieved using diaminobenzidine as chromogen (Vectastain ABC System; Vector Laboratories, Peterborough, UK).

### Western blot analyses

Western blot analyses were carried out as described previously (Habib *et al.* 2005). In brief, MCF-7 and prostate stromal and epithelial cells were washed with PBS and lysed in loading buffer (100 µM Tris-HCl (pH 6.8), 200 µM dithiothreitol, 4% SDS, 0.4% bromophenol blue and 20% glycerol). Protein lysates (60 µl containing 50 µg protein) were electrophoresed on 8% SDS polyacrylamide gels. The gels were then electroblotted onto nitrocellulose and probed sequentially with either the ER $\alpha$  antibody used in immunohistochemistry (diluted 1:100) or a rabbit polyclonal antibody raised against amino acids 1–150 of human ER $\beta$  (diluted 1:200; Santa Cruz Biotechnology), and a monoclonal mouse anti-human actin antibody (diluted 1:200; Dako, Ely, UK). Chemiluminescence detection of antibody binding was carried out using the ECL system (Amersham).

### Cell proliferation experiments

Prostate cell proliferation in primary culture was determined using the MTS [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulphophenyl)-2H-tetrazolium] assay, according to the manufacturer's protocol (Promega). MTS tetrazolium compound is bioreduced by mitochondrial dehydrogenase activity in viable cells to a coloured formazan product that is soluble in tissue culture medium. This assay provides a colorimetric method for determining the number of viable cells in culture and has the advantage that nonviable cells do not form a coloured product (Maghni *et al.* 1999). To determine the linear range of the assay, epithelial and stromal cells were plated in triplicate at a density of  $10^3$ – $10^4$  cells per well in 96-well plates. The MTS assay was performed 4 h after seeding, when the cells had attached to the bottom of wells. Absorbance at 490 nm of 100  $\mu$ l MTS solution in 96-well plates was measured using a Microplate reader (Bio-Rad Laboratories) and was found in preliminary experiments to be proportional to the number of epithelial and stromal cells determined using a haemocytometer and seeded onto each well up to  $\sim 8 \times 10^3$  cells/well (data not shown).

To investigate the effects of oestradiol (Sigma) and anti-oestrogen ICI 182 780 (Faslodex; Tochril, Bristol, UK), epithelial cells (3000 cells/well, 6 wells per treatment/control group) were treated with physiological to pharmacological concentrations of oestradiol ( $10^{-9}$ – $10^{-5}$  M) and viable cell numbers were determined by the MTS assay. Similarly, stromal cells (1800 cells/well) in FGM supplemented with either 2% or 10% dextran-coated charcoal-stripped FCS (DCC-FCS) were treated with various concentrations of oestradiol, with or without ICI 182 780. ICI 182 780 is an oestrogen antagonist that competitively inhibits oestradiol binding to ERs and exhibits no agonist actions *in vivo* or *in vitro* (MacGregor & Jordan 1998). Three wells per plate containing no cells served as blanks. Culture medium with or without steroids was changed every 24 h and contained no phenol red because of previously reported oestrogenic effect of this compound (Berthois *et al.* 1986). Following 2–6 days incubation at 37 °C, culture medium was carefully aspirated and viable cell numbers determined using the MTS assay as described above.

### Detection of ER and aromatase gene expression

Total RNA was extracted from prostate stromal and epithelial cells derived from eight BPH patients using the TRI RNA isolation reagent (Sigma), according to the manufacturer's protocol. For RT-PCR, 2  $\mu$ g total RNA was reverse transcribed with 15 U avian myeloblastosis virus reverse transcriptase in a 20  $\mu$ l reaction mix containing 5 mM MgCl<sub>2</sub>, 1 mM dNTPs, 20 U RNasin and 0.5  $\mu$ g oligo(dT)<sub>15</sub> primer at 42 °C for 60 min. Hundred microlitres of water were then added and the RT reactions from four patients were further diluted 1:10 and 1:100 with water. Ten microlitres of the diluted RT reaction mix were used in a 50  $\mu$ l PCR containing 1.25 U *Taq* DNA polymerase (Promega), 0.4  $\mu$ M

each of forward and reverse primers, 2 mM MgCl<sub>2</sub> and 200  $\mu$ M dNTPs. PCR was carried out for 40 cycles (denaturing at 94 °C for 1 min, annealing at 56 °C for 1 min and extension at 72 °C for 1 min, followed by a final extension step of 10 min at 72 °C). All RT-PCR reagents were obtained from Promega. Oligonucleotide primers (Eurogentec, Southampton, UK) corresponding to exon 2 and complementary to exon 3 of the aromatase gene were 5'-CCTCTGAGGT-CAAGGAACAC-3' and 5'-CAGAGATCCAGACTCG-CATG-3' respectively. PCR primers used for ER subtypes were as follows: ER $\alpha$ , 5'-TACTGCATCAGATCCAAGGG-3' and 5'-ATCAATGGTGCCTGTTG-3'; ER $\beta$ , 5'-GTTGCGCCAGCCCTGTTAC-3' and 5'-CTCGTC-GGCACTTCTCTGTCTC-3'. Negative controls were carried out for each pair of primers in which cDNA was omitted from the PCR or reverse transcriptase was omitted from the reverse transcription reaction. PCR products were size fractionated on a 1.5% agarose gel and visualised by ethidium bromide staining under u.v. illumination. The authenticity of the PCR products was confirmed by restriction digestion using *Av*II (aromatase), *Pvu*II (ER $\alpha$ ) or *Hinf*I (ER $\beta$ ).

### Metabolism of androstenedione by stromal cells

On day 1 of each steroid metabolism experiment, stromal cells (passage 2 or 3) in FGM were seeded onto 6-well plates at a density of  $3 \times 10^5$  cells/well. The medium was replaced with phenol red-free RPMI medium supplemented with 10% DCC-FCS the following day. Twenty-four hours later, the cells were incubated with 2 ml of the same medium containing 20 nM [<sup>3</sup>H]androstenedione (S.A. 100 Ci/mmol, Amersham Life Science) with or without aromatase inhibitor letrozole (10 or 100 nM). Letrozole (up to 100 nM) did not alter the growth of stromal cells maintained for up to 4 days. Preliminary experiments demonstrated that conversion of androstenedione to oestrone and testosterone was linear within 24 h. No oestradiol formation was detectable within this incubation period. When testosterone was used as a substrate, the vast majority was rapidly metabolised to androstenedione in stromal cells due to high 17 $\beta$ -hydroxysteroid dehydrogenase activity, with no oestradiol detected (data not shown). After incubation at 37 °C, the culture medium was collected and <sup>14</sup>C-labelled steroids (50.3–53.3 mCi/mmol; NEN Life Science Products, Hounslow, UK) added for determination of recovery. Recovery of steroids was more than 75%. Steroids were extracted with 5 vol ethyl acetate and dried at 45 °C under a nitrogen gas stream. The cells were harvested in lysis buffer (25 mM Tris phosphate (pH 7.8), 2 mM DTT, 1% Triton X-100 and 10% glycerol) for protein assay (Bio-Rad Laboratories). Thin layer chromatography (TLC) was employed to separate the steroid metabolites. For the first TLC run, the residue was reconstituted in 60  $\mu$ l ethanol:ethyl acetate (50:50, v/v) and developed in dichloromethane:diethyl ether (9:1, v/v) on polysilica acid gel TLC plates impregnated with glass fibre (Gelman Sciences, Ann Arbor, MI, USA), in parallel to steroid standards. To further

separate steroid metabolites from one another, areas of TLC plate corresponding to oestrone and  $5\alpha$ -androstenedione were scraped off, eluted with ethyl acetate, dried and reconstituted as above. The second TLC run consisted of a mobile phase of benzene:ethanol (40:1, v/v). Steroids were visualised by spraying with 10% phosphomolybdic acid. Steroid metabolites were scraped off and quantified using a liquid scintillation counter as described previously (Bayne *et al.* 1998).

### Statistical analysis

All cell proliferation experiments were carried out at least three times. Data are expressed as mean  $\pm$  S.E.M. Dunnett's test was used to compare viable cell numbers in ICI-182 780 treatment group with those of controls, and also steroid metabolites in letrozole-treated cells with those in controls. The Tukey-Kramer test was used to detect any difference in cell numbers between controls and oestradiol-supplemented groups, with or without ICI-182 780. Statistical analysis was performed using Prism version 4 (GraphPad Software, San Diego, CA, USA).  $P < 0.05$  is considered statistically significant.

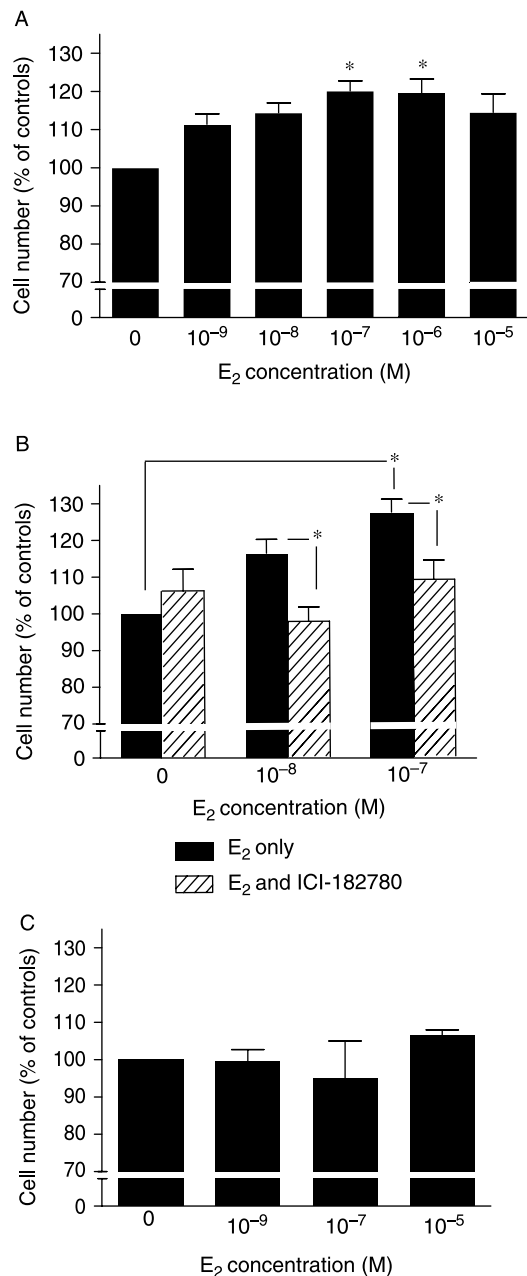
## Results

### Effects of oestradiol on prostate cell growth

To test the hypothesis that oestrogens play a role in the pathogenesis of BPH, we examined the effects of oestradiol on the growth of prostate cells in culture. Proliferation of stromal cells grown in FGM supplemented with 10% DCC-FCS was not altered by  $10^{-9}$ – $10^{-5}$  M oestradiol treatment up to 6 days (data not shown). However, oestradiol ( $10^{-7}$  and  $10^{-6}$  M) stimulated the proliferation of stromal cells when their growth medium was supplemented with 2% DCC-FCS (the minimum concentration required to maintain cell growth;  $P < 0.05$ , Fig. 1A). To determine whether these effects were mediated by the ER, stromal cells were treated with oestradiol, ICI 182 780 (anti-oestrogen) or both together. At concentrations ranging from  $10^{-10}$  to  $10^{-7}$  M, ICI 182 780 displayed no cytotoxic effects on stromal cells maintained in FGM with 2% DCC-FCS (data not shown). Whereas ICI 182 780 ( $5 \times 10^{-8}$  M) alone had no effect on stromal cell proliferation, it antagonised the stimulation of stromal cell proliferation by oestradiol (Fig. 1B). The latter observation suggests that oestradiol increased stromal cell proliferation via an ER-mediated mechanism. By contrast, oestradiol ( $10^{-9}$ – $10^{-5}$  M) treatment for 2–6 days had no effect on the proliferation of epithelial cells derived from three different BPH patients and maintained in EGM supplemented with 0.5% FCS (Fig. 1C).

### Expression of ER subtypes in human prostate cells

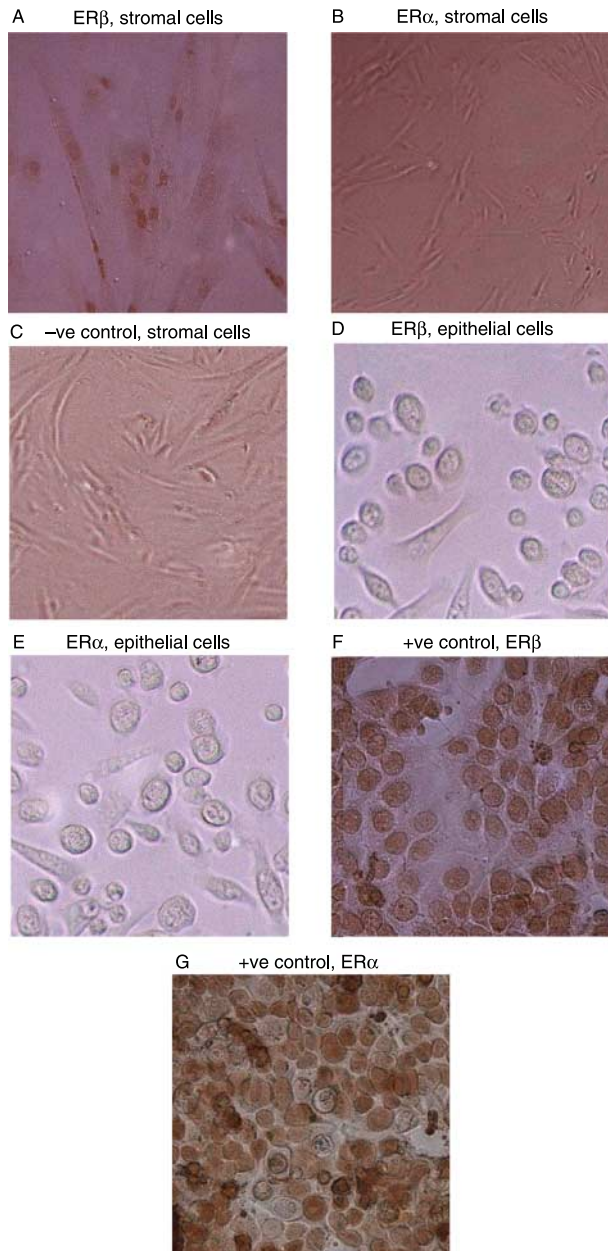
To examine whether the ER subtypes are expressed by human prostate cells in primary culture, we performed



**Figure 1** Effects of oestradiol on proliferation of prostate cells in primary culture. Stromal cells were plated in FGM supplemented with 2% DCC-FCS, and cell numbers were determined using the MTS assay 48 h after oestradiol (E<sub>2</sub>) treatment. (A) Oestradiol ( $10^{-7}$  and  $10^{-6}$  M) stimulated the proliferation of stromal cells. (B) Anti-oestrogen (ICI 182 780,  $5 \times 10^{-8}$  M) treatment abolished the stimulatory effect of E<sub>2</sub> on stromal cell proliferation. (C) Epithelial cells plated in EGM supplemented with 0.5% FCS were treated with E<sub>2</sub> for 48 h, and cell numbers were determined using the MTS assay. All results are expressed as mean  $\pm$  S.E.M. of at least three separate experiments. Cell numbers in controls are set as 100%. \* $P < 0.05$  compared with untreated control or ICI 182 780 treatment group.



immunocytochemical staining on stromal and epithelial cells derived from six BPH patients (Fig. 2). Stromal cells in culture displayed only ER $\beta$  immunostaining, which was largely localised to the nuclei (Fig. 2A). No ER $\alpha$  immunoreactivity



**Figure 2** Immunocytochemical detection of ER subtypes in prostate cells in culture. ER $\beta$ , but not ER $\alpha$ , was detected in prostate stromal cells in primary culture by immunocytochemical staining. (A) Strong staining of ER $\beta$  was present in the nuclei of stromal cells, whereas (B) ER $\alpha$  immunostaining was not detectable. (C) Primary antibody was omitted from control cells. (D) ER $\beta$  and (E) ER $\alpha$  immunostaining was not detected in prostate epithelial cells. MCF-7 cells were used as positive controls for both (F) ER $\beta$  and (G) ER $\alpha$  (400 $\times$ ).

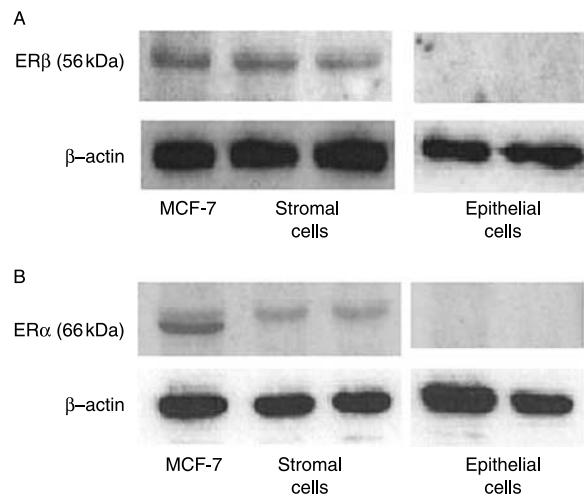
was detectable in stromal cells in culture (Fig. 2B). No staining was detected in stromal cells when the primary antibody was omitted (Fig. 2C). In comparison, ER $\beta$  and ER $\alpha$  immunoreactivity was not detected in epithelial cells in culture (Fig. 2D and E). MCF-7 cells that express both ER isoforms (Boyan *et al.* 2003) were used as positive controls and showed strong staining with both ER $\beta$  and ER $\alpha$  antisera (Fig. 2F and G).

The expression of the ER subtypes in prostate cells was further examined by western analyses. Stromal, but not epithelial, cells from three BPH patients expressed ER $\beta$  protein detectable by western blotting (Fig. 3A). In contrast to the above immunocytochemical findings, protein lysates prepared from stromal cells displayed weak ER $\alpha$  immunoreactivity by western analysis compared with MCF-7 cells, whereas ER $\alpha$  was not detected in protein lysates from epithelial cells (Fig. 3B).

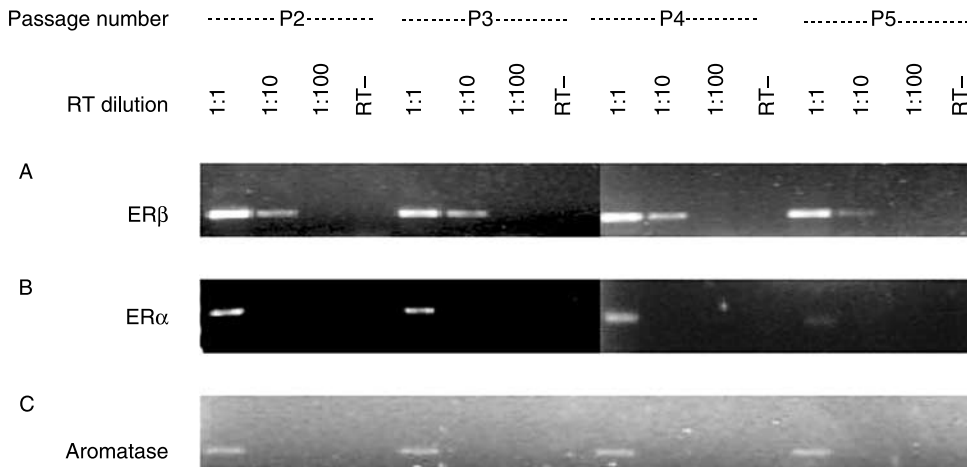
Using RT-PCR, mRNA expression of both ER $\beta$  and ER $\alpha$  was detected in prostate stromal cells in culture (passages 2–5) from eight BPH patients. To investigate the expression levels of the two ER subtypes in stromal cells, semi-quantitative RT-PCR was performed in four RNA samples. ER $\beta$  RT-PCR product was detected in the undiluted RT reactions and 10-fold diluted RT reactions, but was not detected in the 100-fold diluted RT reactions (Fig. 4A). By contrast, ER $\alpha$  RT-PCR product was only detectable in undiluted RT reactions (Fig. 4B), suggesting that levels of mRNA encoding ER $\alpha$  were lower than ER $\beta$  mRNA in these cells.

#### Aromatase expression and activity in prostate cells

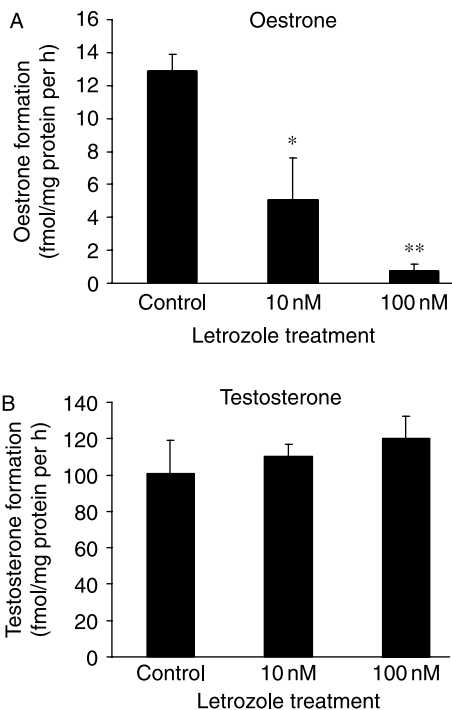
Aromatase mRNA was detected by RT-PCR in all stromal cell samples (passages 2–5) derived from eight BPH patients, using undiluted RT reactions (Fig. 4C). By contrast, no



**Figure 3** (A) ER $\beta$  was identified by western blot analysis of protein lysates from stromal, but not epithelial, cells. (B) Weak immunoreactivity was detected in stromal, but not epithelial, cell lysates using anti-ER $\alpha$  antisera. MCF-7 cells were used as positive controls for both ER $\beta$  and ER $\alpha$ .



**Figure 4** Expression of ER subtypes and aromatase mRNA in stromal cells in culture. Total RNA was prepared from stromal cells (passages 2–5) derived from three BPH patients. The same amount of RNA from each sample underwent RT under identical conditions. PCRs (40 cycles) were carried out on undiluted (1:1), 10-fold (1:10) and 100-fold (1:100) diluted RT reactions. Results shown are from one patient's stromal cells and representative of all BPH patients examined. RT–, negative control in which reverse transcriptase was omitted.



**Figure 5** Metabolism of androstenedione to oestrone and testosterone in prostate stromal cells in primary culture and the effects of letrozole (aromatase inhibitor). Stromal cells were incubated with 20 nM androstenedione for 24 h, with or without letrozole, before culture medium was collected and steroid metabolites analysed. Values are mean of triplicates  $\pm$  S.E.M. \* $P < 0.05$  and \*\* $P < 0.01$  compared with controls.

aromatase mRNA was detectable in epithelial cell RNA derived from any of the patients (data not shown). These observations suggest a differential distribution of aromatase in the two prostate cell compartments.

Given that expression of aromatase mRNA was restricted to stromal cells, we examined its enzyme activity in cell culture. The rate of oestrone formation from androstenedione in stromal cells was  $13 \pm 1$  fmol/mg protein per h (mean  $\pm$  S.E.M.). The aromatase inhibitor letrozole decreased androstenedione conversion to oestrone in a dose-dependent manner (Fig. 5A) but had no effect on testosterone formation (Fig. 5B), demonstrating that oestrone formation in stromal cells was due to aromatase activity.

## Discussion

The present study shows that the prostate stroma represents a target for oestrogen action. Stromal cells not only express the ER but, by the controlled expression of aromatase, an enzyme that converts androgen precursors to oestrogens, also regulate the response to oestrogen action.

Oestradiol stimulated the proliferation of stromal, but not epithelial, cells in culture. Two previous studies have examined the effect of oestradiol on the proliferation of BPH-derived stromal cells in culture. In one study, oestradiol ( $10^{-11}$ – $10^{-7}$  M) had no effect on prostate fibroblast proliferation in serum-free medium or when supplemented with 1% FCS (Levine *et al.* 1992). In another study, oestradiol increased fibroblast proliferation in the medium containing 0.5% FCS; this stimulatory effect was dose dependent, with maximal stimulation at  $10^{-9}$  M oestradiol, and was antagonised by the anti-oestrogen, tamoxifen (Collins *et al.* 1994). A recent study

reported that  $5 \times 10^{-12}$  M oestradiol stimulated proliferation of normal prostate stromal cells maintained in the medium containing insulin and 5% FCS. It is likely that growth factors present in FCS or culture medium could have determined the response of stromal cells to oestradiol stimulation in these previous studies. Indeed, our data show a cell proliferation response to oestradiol when stromal cells were supplemented with 2%, but not 10%, DCC-FCS, suggesting that the stimulatory effect of oestradiol on stromal cell proliferation may be masked by the potent mitogenic effect of high serum concentrations in the culture medium. In comparison, oestradiol had no effect on the proliferation of epithelial cells in our study. Recently, King *et al.* (2006) also showed that increasing ratios of oestradiol to dihydrotestosterone levels stimulated normal human prostate stromal, but not epithelial, cell growth in culture. Together, these findings indicate that oestrogens preferentially increase the proliferation of prostate stromal cells via an ER-mediated mechanism. The differential effects of oestrogens on the two main prostate cell types *in vitro* are in concordance with a previous *in vivo* study, which showed that oestradiol treatment increased the volume of the prostate stromal compartment but decreased that of the epithelial compartment in male rats that had been castrated and supplemented with testosterone (Daehlin *et al.* 1987).

In this study, we demonstrated aromatase activity (13 fmol/mg protein per h) in stromal cells comparable with activities previously reported in tissue homogenates prepared from normal and BPH tissues (Stone *et al.* 1986) and in cultured fibroblasts derived from BPH (Schweikert 1979). Moreover, aromatase mRNA was restricted to stromal cells and was not expressed in epithelial cells. The latter observation confirms findings from previous immunohistochemical studies, in which aromatase staining was localised to the stroma of normal and BPH prostate tissues (Matzkin & Soloway 1992, Hiramatsu *et al.* 1997). Given the higher oestrogen concentrations in BPH stroma than that in epithelium reported by Kozak *et al.* (1982), the differential distribution of aromatase between the stromal and epithelial compartments may represent a mechanism whereby BPH stromal cells regulate cell proliferation by providing an oestrogenic microenvironment through the aromatisation of androgen precursors to oestrogens. The latter notion is supported by a recent study designed to examine the importance of oestrogens locally synthesised in the prostate stroma; prostate stromal tissues derived from aromatase knockout mice, but not those from normal animals, induced hyperplasia of normal prostate epithelium derived from wild-type newborn mice when the recombinant tissues were grafted into immunodeficient male hosts (McPherson *et al.* 2007). Taken together, the above findings suggest a role for prostate stromal aromatase in regulating the proliferation of both stromal and epithelial cells.

Data from our immunocytochemical, western blotting and semi-quantitative RT-PCR experiments indicate higher expression levels of ER $\beta$  than ER $\alpha$  in stromal cells in culture. Both immunocytochemistry and western analysis

demonstrated the presence of ER $\beta$  protein in stromal cells in culture and RT-PCR confirmed the presence of the encoding mRNA. By contrast, weak ER $\alpha$  immunoreactivity was detected by western analysis in stromal cell lysates, but no ER $\alpha$  staining was found in stromal cells by immunocytochemistry. Although ER $\alpha$  mRNA was detected by RT-PCR in stromal cells, it was at lower levels than ER $\beta$  mRNA. The lack of ER $\alpha$  staining by immunocytochemistry in stromal cells was probably because of the low level of protein expression. To our knowledge, ER expression in human prostate stromal cells in primary culture has been reported in only one previous study; whereas mRNA encoding ER $\alpha$ , but not that encoding ER $\beta$ , was detected in fibroblasts isolated from normal prostate tissues, ER $\alpha$  protein expression was not examined in the fibroblasts (Pasquali *et al.* 2001a). These findings are in contrast to ours. To date, the distribution of the two ER subtypes in prostate cell cultures and prostate tissues remain controversial (Ehara *et al.* 1995, Bonkhoff *et al.* 1999, Lau *et al.* 2000, Pasquali *et al.* 2001a, Royuela *et al.* 2001, Tsurusaki *et al.* 2003).

In conclusion, our findings of 1) oestradiol stimulation of *in vitro* stromal cell proliferation and 2) the expression of ER and aromatase in stromal cells lend support to the hypothesis that oestrogens play a role in BPH pathogenesis. The response of prostate cell proliferation to oestrogens most likely depends on a number of factors including plasma and local levels of oestrogens and androgens, expression of aromatase and ER subtypes, and interaction between ERs and other intracellular regulators of oestrogen action.

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## Disclosure

The authors have no conflict of interest to declare.

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