Regulation of Estrogen Receptor α and β Expression by Testosterone in the Rat Prostate Gland

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Abstract. Although ERβ is known to be expressed at high levels in the rat prostate gland, its regulation is not well understood. Here we examined ER mRNA expression and the effects of testosterone administration in male rats at 1, 4 and 9 weeks of age who were castrated and/or treated with testosterone for a week, and then sacrificed. ERα was the major type of ER expressed in 2 week-old animals while dominant expression of ERβ mRNA was apparent in older age groups. Interestingly while ERβ expression was diminished and ERα mRNA increased in the castrated group, testosterone administration reversed this effect. A time-course study indicated that induction of ERβ mRNA increased within 9 hr and ERα decreased in 2 days after an injection (i.p.) of testosterone. Our results suggested that 1) testosterone up-regulates ER β mRNA expression while ER α is down-regulated; and that 2) great changes in ERα and β expression in the prostate gland during development from the newborn to adult may be due to the influence of testosterone.

Key words: Estrogen receptor α and β, Testosterone, Rat prostate, Competitive RT-PCR

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IT has been demonstrated that appreciable numbers of estrogen binding sites are present in rat prostate tissue, although their significance is not clearly understood [1–3]. In the human prostate tissue, despite conflicting early results, immunohistochemical studies as well as radioligand-binding assays have also demonstrated the presence of ER, albeit at low concentrations [4–6]. While some involvement in tumorigenesis might be expected, studies of ER levels in prostate cancer have shown no correlation with pathologic features [7–9].

Interest in ER and its roles in the prostate gland was recently revived by the cloning and characterization of a second ER, ERβ [10, 11]. Subsequent studies revealed that ERα is localized in the stromal tissue and ERβ is present in epithelial cells in both primates and rodents [12–14]. Furthermore, in the rat prostate, expression of ERβ mRNA is developmentally regulated and a significant increase takes place during the initial stages of epithelial cytodifferentiation. It is assumed that androgen plays the major role in this developmental change since castration has been known to decrease ER expression in the gland. However, the effects of testosterone on ERα and β expression have not been quantitatively addressed in the prostate gland [15]. In the present study, ERα and ERβ mRNA levels were therefore measured with a competitive RT-PCR technique in tissue of rats castrated and treated with testosterone (T).

Materials and Methods

Hormones

17β-estradiol and diethylstilbestrol (DES) were purchased from Sigma Chemicals, St. Louis, MO, U.S.A., and testosterone (T) was from Wako Junyaku KK,

Osaka, Japan.

Animal experimentation

Male F344 rats were purchased from Charles River Co., Kanagawa, Japan, and maintained under constant condition with free access to basal diet and tap water. All experiments were conducted under the guidelines of the Guide for the Care and Use of Laboratory Animals of Hiroshima University. Animals were sacrificed under anesthesia and ventral prostate tissues were dissected and frozen. Blood samples were collected from the abdominal artery and separated sera were stored at –20°C until assayed.

Experiment 1. One, 2, 4 and 9 week-old rats were maintained for a week and sacrificed to examine the prostate gland.

Experiment 2A. Four and 9 week-old animals were castrated and/or treated with 5 mg of T in a silicone tube subcutaneously for a week. For 2 week-old rats, 0.5 mg of T in silicone tubes was given instead of 5 mg for a week and 0.5 mg of DES was administered instead of surgical castration.

Experiment 2B. For the cytosolic ER binding assay of the prostate tissue, 9 week-old animals were castrated and treated with 5 mg of T for a week.

Experiment 3. Four week-old male rats were castrated. One week later, 10 mg of T was administered i.p. They were sacrificed at hours 0, 3, 9, 24 and 48 after the injection.

PCR primers

RER-a1u and RER-a1d with the sequences 5'-AAT TCTGACA ATCGACGCCA G (473–493) and 5'-GT GCTTCAAC ATTCTCCCTC CTC (794–816) were employed for rERα detection (344bp), and RER-b1u and RER-b1d with the sequences 5'-TTCTTGGCAG CACCAGTAAC C (38–58) and 5'-TCCCTCTTTG CGTTTGGACT A (279–299) for rERβ (262bp). For construction of an ER α competitor, RER-a2u, RER-a2d, RER-a12u (RER-a1u linked to RER-a2u) and RER-a12d (RER-a1d linked to RER-a2d) with the sequences 5'-GAGACTCTCC AGCAGCAGCG AG (515–536), 5'-AAAGCCTTGC AGCCTTCACA GG (605–626), 5'-AATTCTGACA ATCGACGCCA GGAGACTCTC CAGCAGCAGC GAG and 5'-GT GCTTCAAC ATTCTCCCTC CTCAAAGCCT TG CAGCCTTC ACAGG were used. For the ERβ

competitor, RER-b2d and RER-b12d with the sequences 5'-GAGGTTCTGC ATAGAGGAGC G (152–172) and 5'-TCCCTCTTTG CGTTTGGACT AGAG GTTCTG CATAGAGGAG CG were applied.

Construction of competitor DNAs

A rER α plasmid was mixed with 20 pmol each of primers RER-a2u and RER-a2d in a total volume of 50 μl containing 1.25 U of Ex-Taq DNase polymerase (Takara Shuzo Co., Otsu, Japan), 0.2 mM dNTP, 10 mM Tris-HCl (pH 8.3), 2 mM MgCl$_2$ and 50 mM KCl. and a 112-bp DNA fragment was isolated with a GFX Gel Band Purification Kit (Amersham pharmacia biotech, Uppsala, Sweden). Using this fragment as a template, a further PCR amplification with primers RER-a12u and RER-a12d and gel purification were performed to obtain a 126-bp competitor DNA for ERα. The same procedures with RER-b1u and RER-b2d followed by amplification of RER-b1u and RER-b12d was performed to obtain the ERβ competitor (156 bp). The sequences of the resulting DNA fragments were confirmed with a capillary sequencer, ABI PRISM 310 (Applied Biosystems, Foster City, CA, U.S.A.).

Reverse transcription (RT) and competitive PCR

These assays were performed as described previously [16]. Briefly, total RNAs from tissues were prepared by a modified acid guanidium thiocyanate-phenol-chloroform extraction method and treated with RQ1 DNase (Promega, Madison, WI, USA). One μg of total RNA was reverse-transcribed with 100 U of MMLV-RT (Invitrogen Corp., Carlsbad, CA, USA) and 1.25 pmol of oligo-dT primers.

Sample cDNA (equivalent of 0.2 μg of total RNA) and competitor DNA at various concentrations (0–20 fg) were co-amplified by PCR with Ex-Taq using the RER-a1u and RER-a1d primer set. The amplification conditions were as described above in a final volume of 20 μl. Each PCR product was electrophoretically separated on a 1.5% agarose gel containing ethidium bromide at 0.2 μg/ml. The image was digitized with a video capturing device, PrintGraph (Atto Co., Tokyo, Japan) and intensities of the blots were quantified using Scion Image software (Scion Corp., Frederick, MD, U.S.A.). The log ratio of the blot intensities of sample cDNA over the competitor in each lane was plotted against amounts of the competi-

tor. Quantity of rER mRNAs was determined where the ratio was equal to 1.

ER assay

Cytosol fractions of prostate tissues were prepared in TEDMG buffer (10 mM Tris-HCL, 1 mM disodium EDTA, 1 mM dithiothreitol (DTT), 10 mM sodium molybdate and 10% (v/v) glycerol, pH 7.4) and incubated in 0.05–50 nM of [17β-2, 4, 6, 7-^{3}H] - estradiol (NEN Life Science Products, Boston, MA, U.S.A.), with or without a 100-fold molar excess of unlabelled estradiol (10 nM), at 30°C for 40 min. Bound [^{3}H]-estradiol was separated by the hydroxyl apatite method and binding data were analyzed by Scatchard plotting.

Serum T levels

Serum T was measured with a RIA kit for rat serum E_2, purchased from Immunotech Inc. (Marseille Cedex, France).

Statistical analysis

Statistical comparisons were made using the Student's t-test.

Results

Age dependent changes in ER α and ER β mRNA levels in rat prostate

ERα mRNA was the major type of ER expressed in the 2 week-old rat prostate. Its expression was decreased at later time points and ERβ mRNA expression became apparent after 3 weeks, coming to predominant in 5 and 10 week-old rats (Fig. 1).

Effects of DES and T administration on ER mRNA levels in the prostate gland in 3 week-old rats

ERα mRNA in the prostate of 3 week-old rats, which was relatively high, was increased by DES and decreased by T (Fig. 2). ERβ, on the other hand, was decreased by DES treatment. There were no significant differences in ventral prostate weight among treatments.
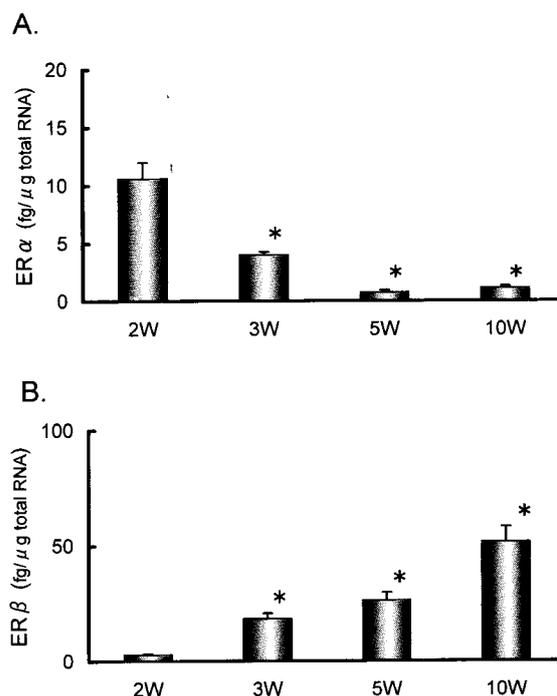

Fig. 1. Expression of ERα (A) and ERβ (B) mRNAs in the normal prostate glands of 2, 3, 5 and 10 week-old rats. Bars indicate mean ± SEM (n = 3–6, each point). * indicates significant differences from the values at 2 weeks old at p<0.05.

Effects of castration and T administration on ER mRNA levels in the rat prostate gland at 5 and 10 weeks old

At ages of 5 and 10 weeks, the ERβ mRNA levels were decreased by castration and recovered on T administration (Fig. 2). Although the control ERα mRNA level was low, it was increased by castration at 5 weeks old. At 10 weeks old, however, castration did not alter the ERα mRNA level and T administration decreased it. The weight of the ventral prostate was decreased by castration and increased by T administration.

Time dependent changes in ER mRNA levels in the prostate gland after T injection in 5 week old rats

Changes in ER α and β mRNA levels in the ventral prostate in castrated rats after 10 mg T injection are summarized in Fig. 3. At time 0, higher ERα and lower ERβ mRNA levels were noted in the prostate gland as the result of castration. The level of the ERβ mes-
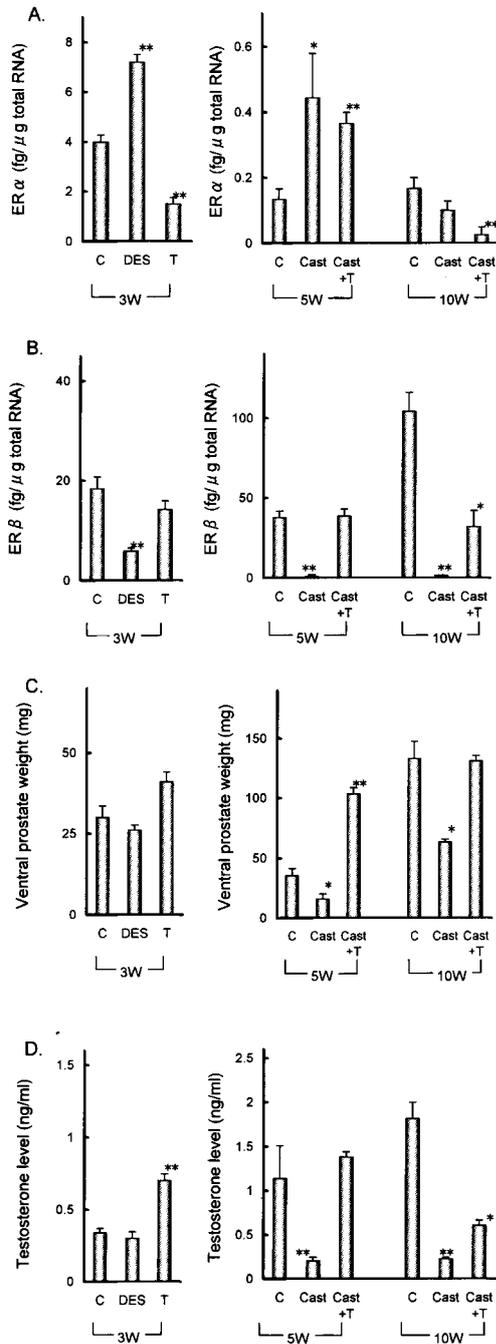
Fig. 2. Effects of castration and testosterone on ER mRNA levels (A, B), ventral prostate weight (C) and serum testosterone levels (D) in rats at different ages. F344 male rats were castrated (Cast) and treated with 5 mg of testosterone in a silicone tube subcutaneously (Cast+T) for a week. For 3 week-old rats, 0.5 mg of DES was administered (DES) and 0.5 mg of testosterone in a silicone tube was given (T). Intact animals were also examined (C). Bars indicate mean ± SEM (n = 6, each point). * and ** indicate significant difference from the control at p<0.05 and p<0.01, respectively.
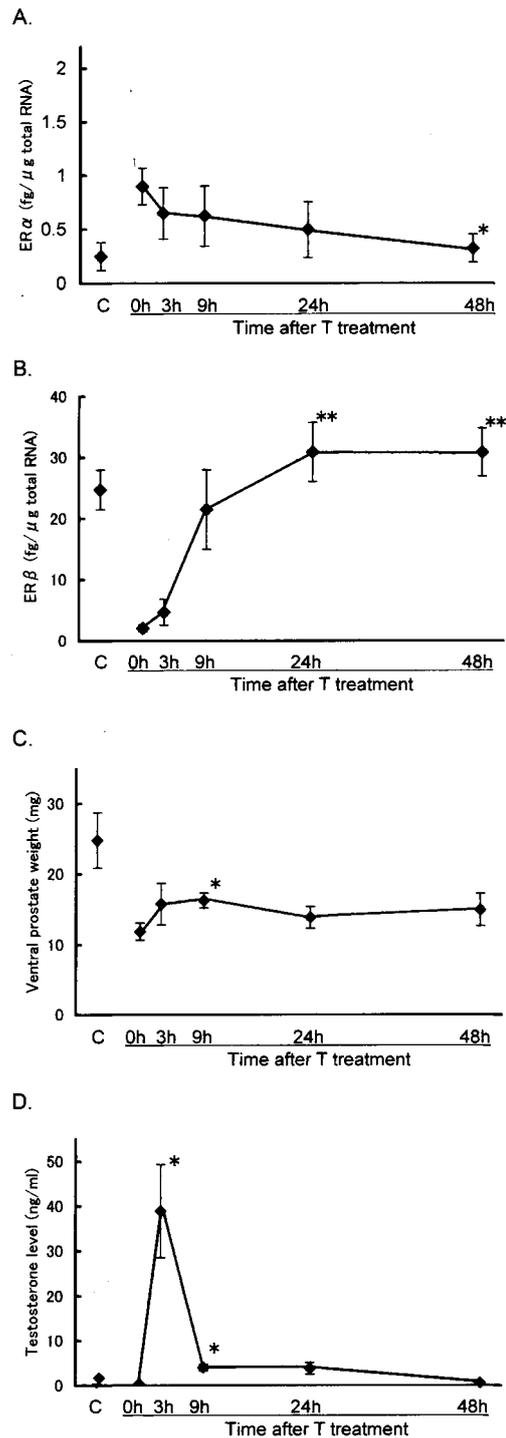
Fig. 3. Time dependent changes in ER mRNA levels (A, B), ventral prostate weight (C) and serum T levels (D) in 5 week-old rats after a T injection. 10 mg of T was administered i.p. to castrated F344 rats. The levels in intact animals are also noted (C). Bars indicate mean ± SEM (n = 6, each point). * and ** indicate significant differences from the values at 0 hr at p<0.05 and p<0.01, respectively.

Table 1. Cytosolic ER levels in the prostate determined by competitive binding assay

Prostate lobe	Treatment	Specific binding (fmol/mg prt)
Ventral	Control	15.9, 8.6
	Castration	0, 0
Dorsolateral	Control	24.6, 13.7
	Castration	0, 0

The number of ^{3}H-17β estradiol specific binding sites in prostate cytosol in 10 week-old F344 rats were determined. Each assay was conducted with combined tissues from two animals.

sage began to increase 3 hr after the injection and reached a maximum at 24 hours. The level of ERα mRNA decreased gradually. The weight of the ventral prostate remained unchanged over 48 hours. The serum T level reached a peak at 3 hr after the injection.

Number of specific estradiol binding sites in the prostate cytosol in normal and castrated rats

Table 1 shows ER levels in the prostate gland determined by competitive binding assay. No specific estradiol binding sites were detected in the prostate cytosol in castrated animals, while significant numbers were apparent in the controls.

Discussion

Several studies have indicated that ERβ in the rat prostate is developmentally regulated, probably by androgen [12, 15, 17, 18]. The present results clearly confirmed that testosterone controls the expression of ERα mRNA as well as ERβ in the rat prostate gland. More importantly, we showed that a significant amount of ERα mRNA to be expressed in younger rats when ERβ expression was still low.

It has been reported that ERβ is localized in prostatic epithelial cells, in contrast to the stromal localization noted for ERα. At birth, low levels of ERβ message are observed in the epithelial and mesenchymal cells followed by decreases as mesenchymal cells differentiate. Subsequent increase in ERβ begins around day 15 probably due to elevation in testosterone with maximal expression at day 90 when adult levels of the hormone are at their peak [17]. Our quantitative results for ERβ mRNA measurement con-

firmed this to be the case while ERα was found to be regulated in the opposite way. These development changes in ER expression might be considered indirect effects of morphological change in the glands, since epithelial differentiation occurs in a proximal to distal fashion during this period. However, our finding of relatively rapid induction of ERβ after an injection of testosterone suggested that the regulation of ER is closely linked to the androgen. In fact, the androgen receptor (AR) colocalizes with ERβ in the same tissue, i.e. strong expression is apparent in the epithelium and low levels in the stroma [19]. On the other hand, AR could be detected in the prostate of long-term castrated animals. Promoter regions of the ERα gene have been reported and well characterized [20, 21]. The human ERα gene is transcribed from three different promoters, while only two promoters are involved in the rat case. Although there seem to be no androgen responsive transcriptional motifs in the known promoter structures, investigations of ERβ promoters are still ongoing [22, 23].

It is interesting to note that competitive binding studies, measuring the number of binding sites for both ERα and ERβ, have clearly demonstrated androgen down-regulation of ER in humans and monkeys, whereas the ER level increases by androgen treatment in rats [2, 24, 25]. This might be explained by the opposite regulation of ERα and β expression rather than interspecies variation, since relative expression of ERα is high in the human prostate gland while the ERβ level is higher in the case of rat [16].

The roles of ERβ in the prostate gland are not clear, although the differential distribution patterns of both receptors in the tissue and the distinctive regulation of the transcripts, as we demonstrated here, indicate physiological roles. ERβ may function to restrain epithelial growth since the tissue in ERβ knockout mice contains multiple hyperplastic foci [26]. In a human cancer study, loss of ERβ expression was reported to be associated with progression from normal prostate epithelium to cancer [27].

Acknowledgements

References

1. Bacher M, Rausch U, Goebel HW, Polzar B, Mannherz HG, Aumuller G (1993) Stromal and epithelial cells from rat ventral prostate during androgen deprivation and estrogen treatment—regulation of transcription. *Exp Clin Endocrinol* 101: 78–86.

2. Zhao GQ, Bacher M, Friedrichs B, Schmidt W, Rausch U, Goebel HW, Tuohimaa P, Aumuller G (1993) Functional properties of isolated stroma and epithelium from rat ventral prostate during androgen deprivation and estrogen treatment. *Exp Clin Endocrinol* 101: 69–77.

3. Suzuki K, Ito K, Suzuki T, Honma S, Yamanaka H (1995) Synergistic effects of estrogen and androgen on the prostate: effects of estrogen on androgen- and estrogen-receptors, BrdU uptake, immunohistochemical study of AR, and responses to antiandrogens. *Prostate* 26: 151–163.

4. Ekman P, Brolin J (1991) Steroid receptor profile in human prostate cancer metastases as compared with primary prostatic carcinoma. *Prostate* 18: 147–153.

5. Mobbs BG, Johnson IE, Liu Y (1990) Quantitation of cytosolic and nuclear estrogen and progesterone receptor in benign, untreated, and treated malignant human prostatic tissue by radioligand binding and enzyme-immunoassays. *Prostate* 16: 235–244.

6. Wolf RM, Schneider SL, Pontes JE, Englander L, Karr JP, Murphy GP, Sandberg AA (1985) Estrogen and progestin receptors in human prostatic carcinoma. *Cancer* 55: 2477–2481.

7. Ehara H, Koji T, Deguchi T, Yoshii A, Nakano M, Nakane PK, Kawada Y (1995) Expression of estrogen receptor in diseased human prostate assessed by non-radioactive in situ hybridization and immunohisto-chemistry. *Prostate* 27: 304–313.

8. Kruithof-Dekker IG, Tetu B, Janssen PJ, Vander Kwast TH (1996) Elevated estrogen receptor expression in human prostatic stromal cells by androgen ablation therapy. *J Urol* 156: 1194–1197.

9. Konishi N, Nakaoka S, Hiasa Y, Kitahori Y, Ohshima M, Samma S, Okajima E (1993) Immunohistochemical evaluation of estrogen receptor status in benign prostatic hypertrophy and in prostate carcinoma and the relationship to efficacy of endocrine therapy. *Oncology* 50: 259–263.

10. Mosselman S, Polman J, Dijkema R (1996) ER β: identification and characterization of a novel human estrogen receptor. *FEBS Lett* 392: 49–53.

11. Kuiper GG, Enmark E, Pelto HM, Nilsson S, Gustafsson JA (1996) Cloning of a novel receptor expressed in rat prostate and ovary. *Proc Natl Acad Sci USA* 93: 5925–5930.

12. Nishihara E, Nagayama Y, Inoue S, Hiroi H, Muramatsu M, Yamashita S, Koji T (2000) Ontogenetic changes in the expression of estrogen receptor α and β in rat pituitary gland detected by immunohistochemistry. *Endocrinology* 141: 615–620.

13. Pelletier G, Labrie C, Labrie F (2000) Localization of oestrogen receptor α, oestrogen receptor β and androgen receptors in the rat reproductive organs. *J Endocrinol* 165: 359–370.

14. Pelletier G (2000) Localization of androgen and estrogen receptors in rat and primate tissues. *Histol Histopathol* 15: 1261–1270.

15. Prins GS, Birch L (1997) Neonatal estrogen exposure up-regulates estrogen receptor expression in the developing and adult rat prostate lobes. *Endocrinology* 138: 1801–1809.

16. Maruyama S, Fujimoto N, Asano K, Ito A, Usui T (2000) Expression of estrogen receptor α and β mRNAs in prostate cancers treated with leuprorelin acetate. *European Urology* 38: 635–639.

17. Prins GS, Marmer M, Woodham C, Chang W, Kuiper G, Gustafsson JA, Birch L (1998) Estrogen receptor-β messenger ribonucleic acid ontogeny in the prostate of normal and neonatally estrogenized rats. *Endocrinology* 139: 874–883.

18. Shughrue PJ, Lane MV, Scrimo PJ, Merchenthaler I (1998) Comparative distribution of estrogen receptor-α (ER-α) and β (ER-β) mRNA in the rat pituitary, gonad, and reproductive tract. *Steroids* 63: 498–504.

19. Sar M, Welsch F (2000) Oestrogen receptor α and β in rat prostate and epididymis. *Andrologia* 32: 295–301.

20. Grandien K, Berkenstam A, Gustafsson JA (1997) The estrogen receptor gene: promoter organization and expression. *Int J Biochem Cell Biol* 29: 1343–1369.

21. White R, Lees JA, Needham M, Ham J, Parker M (1987) Structural organization and expression of the mouse estrogen receptor. *Mol Endocrinol* 1: 735–744.

22. Ishibashi O, Kawashima H (2001) Cloning and characterization of the functional promoter of mouse estrogen receptor β gene. *Biochim Biophys Acta* 1519: 223–229.

23. Hirata S, Shoda T, Kato J, Hoshi K (2001) The multiple untranslated first exons system of the human estrogen receptor β (ER β) gene. *J Steroid Biochem Mol Biol* 78: 33–40.

24. Habenicht UF, Etreby MF (1988) The periurethral zone of the prostate of the cynomolgus monkey is the most sensitive prostate part for an estrogenic stimulus.

Prostate 13: 305–316.

25. West NB, Roselli CE, Resko JA, Greene GL, Brenner RM (1988) Estrogen and progestin receptors and aromatase activity in rhesus monkey prostate. *Endocrinol-* · *ogy* 123: 2312–2322.

26. Weihua Z, Makela S, Andersson LC, Salmi S, Saji S, Webster JI, Jensen EV, Nilsson S, Warner M, Gustafsson JA (2001) A role for estrogen receptor β

in the regulation of growth of the ventral prostate. *Proc Natl Acad Sci USA* 98: 6330–6335.

27. Horvath LG, Henshall SM, Lee CS, Head DR, Quinn DI, Makela S, Delprado W, Golovsky D, Brenner PC, O'Neill G, Kooner R, Stricker PD, Grygiel JJ, Gustafsson JA, Sutherland RL (2001) Frequent loss of estrogen receptor-β expression in prostate cancer. *Cancer Res* 61: 5331–5335.