
Changes in gene expression following androgen receptor blockade is not equivalent to androgen ablation by castration in the rat ventral prostate

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Involvement of the rat ventral prostate and concomitant modulation of gene expression post-castration is a well-documented phenomenon. While the rat castration model has been extensively used to study androgen regulation of gene expression in the ventral prostate, it is not clear whether all the gene expression changes post-castration are due to androgen depletion alone. To obtain insights into this, we performed differential display reverse transcriptase polymerase chain reaction (DD-RT-PCR) which resulted in the identification of castration and/or flutamide-regulated genes in the rat ventral prostate. These include clusterin, methionine adenosyl transferase II α , and prostate-specific transcripts such as PBPC1BS, S100RVP and A7. While clusterin, PBPC1BS and methionine adenosyl transferase II α are regulated by both castration and flutamide, S100 RVP and A7 are regulated by castration alone. Interestingly, we show that flutamide, unlike castration, does not induce apoptosis in the rat ventral prostate epithelium, which could be an underlying cause for the differential effects of castration and flutamide treatment. We propose that castration leads to enrichment and depletion of stromal and epithelial cell types, respectively, resulting in erroneous conclusions on some of the cell type-specific transcripts as being androgen regulated.

[Limaye A M, Asangani I, Kalyani T and Kondaiah P 2008 Changes in gene expression following androgen receptor blockade is not equivalent to androgen ablation by castration in the rat ventral prostate; *J. Biosci.* **33** 209–220]

1. Introduction

Androgens play an important role in the development, growth and maintenance of differentiated functions of the prostate. Much of the current understanding of the role of androgens in the tissue homeostasis of the prostate is based on studies on the rat ventral prostate (RVP) following castration. Lee *et al* (1990) demonstrated that the adult RVP is a dynamic system in which cell death in the proximal epithelium balances out cell proliferation in the distal epithelium. Castration of adult male rats leads to a rapid reduction in serum androgens, and a collapse of this dynamic equilibrium results in rapid involution and loss of secretory activity of the ventral prostate. The process of involution is an active one involving a cascade of biochemical steps leading to programmed cell

death of the glandular epithelial cells (Kerr and Searle 1973; Sandford *et al* 1984; Kyprianou and Isaacs 1988; English *et al* 1989). The glandular epithelial cells constitute about 85% of the RVP. By day 7 post-castration, loss of 70% of the glandular epithelial cells has been demonstrated (Kyprianou and Isaacs 1988; English *et al* 1989). These effects of castration on the RVP can be reversed by androgen supplementation, which leads to regrowth of the prostate and restoration of secretory activity (Coffey *et al* 1968; Wright *et al* 1999). Castration-induced programmed cell death in the RVP is accompanied by modulation of gene expression, which is also reversed by androgen supplementation. Thus, castration has been extensively used as a tool to study *in vivo* gene expression changes in the context of regression/regrowth of the prostate and identification of androgen-

Keywords. Castration; DD-RT-PCR; flutamide; gene expression; rat ventral prostate

Abbreviations used: DD-RT-PCR, differential display reverse transcriptase polymerase chain reaction; DHT, dihydrotestosterone; MATII α , methionine adenosyl transferase II α ; PBS, phosphate buffered saline; PKC, protein kinase C; RVP, rat ventral prostate

regulated genes, which are of relevance for understanding (i) androgen-dependent and -independent growth of prostate cancer, and (ii) identification of diagnostic/prognostic and therapeutic targets for prostate cancer.

Till date, a plethora of androgen-regulated genes have been identified in the RVP using the castration model. However, very few studies have used flutamide as a tool to probe into androgen-regulated gene expression in the prostate. Although it is expected that castration and flutamide will exert similar effects on gene expression and physiology of the prostate, a few reports contrary to this have been published recently. Flutamide does not induce TRPM-2 mRNA (a testosterone-repressed transcript) in the RVP (Russo *et al* 1994). Conversely, Turner *et al* (2001) reported the inability of flutamide to block suppression of TRPM-2 by testosterone. However, in an independent study, Leger *et al* (1988) demonstrated induction of TRPM-2 following flutamide treatment in castrated rats receiving a maintenance dose of androgen. Castration and flutamide administration have been shown to have opposite effects on the expression of protein kinase C (PKC) isoforms in the RVP. While castration leads to reduced expression, flutamide has been shown to increase the levels of PKC isoforms (Montalvo *et al* 2002). In our laboratory, we had previously identified a few flutamide-regulated transcripts in the RVP. Interestingly, we found that castration had a similar or no effect on the expression of the flutamide-regulated transcripts (Limaye *et al* 2007), thus reiterating the disparity in the effects of castration and flutamide treatment as reported by other investigators. These intriguing observations warrant further investigation into the effects of castration and flutamide on gene expression, as they may provide useful insights into the androgen regulation of gene expression in the RVP.

Here, we present further insights into the differential effects of castration and flutamide treatment in the RVP. We analysed the effect of castration and flutamide treatment on expression levels of a few known and unknown transcripts identified by differential display reverse transcriptase polymerase chain reaction (DD-RT-PCR). Furthermore, we show that flutamide does not induce apoptosis of the RVP epithelium. Our data suggest that the observed regulation of genes following castration could be due in part to depletion of the epithelial compartment and not due to androgen regulation. This study clearly highlights the caveat in using the castration model as a means to identify androgen-regulated genes.

2. Methods

2.1 Animals

Wistar male rats used in this study were obtained from the Central Animal Facility at the Indian Institute of Science.

The animals were 60–70 days old and maintained according to the guidelines of the institute with access to rat chow and water *ad libitum*.

2.2 Hormonal manipulations

Castrations were performed via the scrotal route under ether anaesthesia and the animals were maintained for 1 day (day 1 post-castration), 3 days (day 3 post-castration) and 5 days (day 5 post-castration). Sham-castrated animals maintained for 5 days (day 5 sham-castrated) served as controls. The castrated animals receiving testosterone replacement were given three intraperitoneal injections of testosterone propionate (1 mg/kg body weight) after day 2, day 3 and day 4 post-castration. Flutamide was administered intraperitoneally in doses of 50 mg/kg body weight every 12 h for 5 days (day 5 flutamide). In experiments to study the effect of flutamide treatment on testosterone replacement, 5 doses of flutamide as mentioned above were administered to testosterone-replaced rats, each given every 12 h, starting from 12 h after the first testosterone propionate injection (testosterone-replaced + flutamide-treated). In these experiments, castrated rats also received similar doses of flutamide at identical time points (day 5 castrated + flutamide-treated).

2.3 RNA extraction

RNA was extracted from frozen tissues using TRI Reagent (Sigma-Aldrich, USA) according to the manufacturer's instructions. The total RNA was further purified using the RNeasy Kit (Qiagen, GmbH, Germany). RNA concentration was estimated by measuring the absorbance at 260 nm and the quality was assessed by agarose–formaldehyde gel electrophoresis.

2.4 DD-RT-PCR

The DD-RT-PCR protocol described by Wan and Erlander (1997) was used with minor modifications. Briefly, 500 ng of total RNA was reverse transcribed with anchored primer (table 1). The resultant cDNAs were diluted 20 times in water and 1 μ l was used for PCR using 500 ng of anchored primer (corresponding primer used for cDNA synthesis) and 500 ng of arbitrary primer (table 2) in the presence of α -³²P dCTP. The PCR conditions were 94°C for 20 s, 42°C for 20 s and 72°C for 30 s for 40 cycles. Of the PCR product, 1 μ l was mixed with 1 μ l of sequencing loading dye (95% formamide, 10 mM EDTA, 0.1% xylene cyanol and 0.1% bromophenol blue), heated at 80°C for 5 min, chilled on ice and resolved on a 6% polyacrylamide gel containing 7 M urea, transferred onto 3 M Whatman paper (3 MM CHR),

Table 1. Sequences of the anchored primers

Name	Primer sequence
E1T12MA	5'- CG GAATTC GGTTTTTTTTTTTTVA -3'
E1T12MC	5'- CG GAATTC GGTTTTTTTTTTTTVC -3'
E1T12MG	5'- CG GAATTC GGTTTTTTTTTTTTVG -3'
E1T12MT	5'- CG GAATTC GGTTTTTTTTTTTTVT -3'

V = A, G or C; sequence in bold italics is the *EcoRI* restriction site.

Table 2. Sequences of the arbitrary primers

Name	Primer sequence
AP1	5'- CGT GAATTC GAGCCAGCGAA -3'
AP2	5'- CGT GAATTC GGACCGCTTGT -3'
AP3	5'- CGT GAATTC GAGGTGACCGT -3'
AP4	5'- CGT GAATTC GGGTACTCCAC -3'
AP5	5'- CGT GAATTC GGTTGCGATCC -3'
AP6	5'- CGT GAATTC GGCAATCGATG -3'
AP7	5'- CGT GAATTC GCCGAAGGAAT -3'

Sequence in bold italics is the *EcoRI* restriction site.

dried under vacuum at 80°C and exposed to an X-ray film. Differentially expressed cDNAs were excised from the gel and eluted in 100 μ l of water by boiling for 15 min. Following centrifugation at 10 000 rpm for 5 min, the cDNA in the supernatant was amplified using the appropriate combination of anchored and arbitrary primers. The amplified cDNAs were cloned in pBluescript or pGEMT-Easy vector prior to sequencing or sequenced directly using arbitrary primers with an automated sequencer (ABI).

2.5 Northern blot analysis

cDNAs were labelled with α -³²P dCTP (specific activity 3000 Ci/mmol, Perkin Elmer) using the Random Primer Labeling Kit (Bangalore Genei, India) and purified using Sephadex-G50 spin columns. Fifteen to 20 μ g of RNA was resolved on 1% agarose-formaldehyde gel and blotted onto a nylon membrane (Biodyne® B Membrane, Pall Corporation). The prehybridization, hybridization and washing steps were performed as described previously (Desai and Kondaiah 2000). For quantification of northern blots, the band intensities obtained for the various cDNAs were normalized against those obtained for 18S rRNA. The normalized values obtained for day 5 sham-castrated controls were assigned the value of 1 or 100, and those obtained for other treatments were plotted as fold induction or percentage of control. The quantifications have been graphically represented where bars represent mean \pm SD. Data obtained from two animals,

each from independent experiments, were used to calculate the mean and SD.

2.6 Tissue fixation and sectioning

Ventral prostates were fixed in 4% paraformaldehyde for a period of 48 h. The fixed tissues were washed thoroughly with water and gradually dehydrated, cleared and paraffin embedded using the standard protocol in an automated tissue processor TP1020 (Leica, Nussloch, Germany). Sections of 5–7 μ m were cut and placed on glass slides. These were stored at 4°C until used for TUNEL assay and *in situ* hybridization analysis.

2.7 TUNEL assay

Sections were deparaffinized and rehydrated using a standard procedure. Endogenous peroxidase activity was blocked by incubating the slides in 0.6% hydrogen peroxide followed by treatment with 20 μ g/ml proteinase K for 30 min at 37°C. The slides were then incubated in 100 mM glycine in phosphate buffered saline (PBS) for 10 min followed by PBS washes (3 times for 5 min each). Sections were then equilibrated with TdT reaction buffer for 20 min at 37°C followed by incubation for 90 min in TdT reaction buffer containing 10 μ M dATP, 5 μ M of biotin-16-dUTP and 8 units of TdT per section. The slides were washed in 1X SSC at room temperature for 15 min followed by washes in PBS (3 times for 5 min each). The sections were blocked with 0.2% blocking reagent (Boehringer Mannheim) and 0.05% Tween-20 in PBS for 30 min. Sections were then treated with streptavidin-peroxidase conjugate (Bangalore Genei, 1:250 dilution) in blocking reagent for 2 h. The excess conjugate was washed with PBS (3 times for 5 min each). Subsequently, the sections were exposed to 0.05% diaminobenzidine and 0.1% hydrogen peroxide in PBS until brown staining appeared. The sections were counterstained with methylene blue and mounted with 50% glycerol and observed under the microscope for the presence of TUNEL-positive cells.

2.8 In situ hybridization

S100RVP cDNA was cloned in pBluescript (Stratagene) such that T7 and T3 polymerases generated the sense and antisense RNA, respectively. The plasmid was linearized with *HindIII* or *XbaI*, purified and used as a template in separate reactions for *in vitro* transcription with T7 and T3 RNA polymerases, respectively, using DIG-RNA labelling mix (Roche).

Deparaffinized and hydrated tissue sections were treated with 0.2 M HCl for 10 min and washed thrice in PBS for

3 min each. The tissue was made permeable with 0.3% Triton X-100 in PBS and subjected to proteinase K digestion and glycine treatment, as described in the previous section. The tissue sections were post-fixed in neutral buffered formalin and acetylated with 0.1 M triethanolamine and 0.25% acetic anhydride for 10 min. The slides were then dipped in ice-cold 20% glacial acetic acid for 10 s and immediately washed with deionized water for 5 min. The sections were dehydrated in increasing grades of ethanol (50%, 70% and absolute ethanol).

The sections were prehybridized for 30 min in hybridization buffer (6X SSC, 3% SDS and 50% deionized formamide containing 100 µg/ml yeast t-RNA) in a humidified chamber equilibrated with 50% formamide and maintained at 55°C. The hybridization mixture containing DIG-labelled riboprobe in hybridization buffer was heated to 80 °C for 5 min, placed on the sections (2–5 ng of riboprobe per section), covered with glass cover slips and the slides were immediately transferred to the humidified chamber maintained at 55°C. Hybridization was carried out for 18 h.

After the hybridization, the cover slips were removed in 2X SSC and the slides were washed with 1X SSC/0.1% SDS twice for 5 min each. This was followed by two high-stringency washes at 55°C for 10 min each. The non-specifically bound RNA probe was digested with 10 µg/ml RNase A in 4X SSC at 37°C for 30 min. The slides were again rinsed in 2X SSC followed by high-stringent washes with 0.2X SSC/0.1% SDS, twice for 10 min each. The sections were blocked with blocking solution containing 0.2% blocking reagent (Boehringer Mannheim) and 0.05% Tween-20 in PBS for a minimum of 2 h. The blocking solution was removed and 50 µl of alkaline phosphatase-conjugated anti-DIG Fab fragments (diluted 1:1000 in blocking solution) was applied to each section and incubated at room temperature for 2 h. Excess antibody was

washed with PBS and the sections were exposed to NBT/BCIP substrate until specific staining was observed with minimum background. The reaction was stopped by dipping the slides in 10 mM Tris and 1 mM EDTA (pH 7.6), rinsed in water and counterstained with methyl green. The sections were mounted with 50% glycerol, and observed under the microscope and photographed.

3. Results

3.1 DD-RT-PCR

We performed DD-RT-PCR analysis of the total RNA isolated from the ventral prostates of day 5 sham-castrated, day 5 castrated and day 5 flutamide-treated rats. Using a combination of anchored and arbitrary primers (tables 1 and 2) several differentially expressed transcripts were observed. Figure 1 shows differentially expressed transcripts along with two transcripts (A4 and A8; figure 1), which were not differentially expressed in castrated and flutamide-treated RVPs. These transcripts were re-amplified and either directly sequenced using the arbitrary primers or cloned into pBluescript or pGEMT-Easy vector before sequencing. Table 3 shows the identities of only the differentially regulated transcripts obtained using BLAST (Altschul *et al* 1990). We could not clone the intact A7 fragment but, surprisingly, after ligation of A7 and screening for recombinants, we obtained two clones with inserts of unexpected sizes designated as A7.2 and A7.3. These were sequenced and found to contain inserts corresponding to sialic acid synthase and S100RVP, respectively. However, sequencing of the A7 PCR product and a search for identical sequences in the existing database with BLAST (Altschul *et al* 1990) revealed its identity to a human sequence containing

Table 3. Identities and nature of regulation of differentially regulated fragments obtained by differential display reverse transcriptase polymerase chain reaction (DD-RT-PCR)

Fragment	Regulation	Sequenced clone/fragment	Acc. No.	Gene name (% identity)	Gene name (% identity)
A1	DOWN	ND	Clone A1.2	BK000198	Rat PBPC1BS mRNA (98%)
A3	UP	UP	A3 PCR pdt	AK049455	Mouse 7-day embryo whole body cDNA
A5	UP	UR	A5 PCR pdt	BC062394	Rat methionine adenosyl transferase II α mRNA
A6	UP	UP	Clone A6.3	BC061534	Rat clusterin mRNA (100%)
A7	DOWN	DOWN	A7 PCR pdt	AL031073	HS142F18 human DNA sequence from clone RP1142F18 on Xq26.3 27.2 chromosome, contains MAGEE1 melanoma antigen (99%)
			Clone A7.2	XM_216398	Rat sialic acid synthase mRNA (100%)
			Clone A7.3	NM_176076	Rat S100 calcium-binding protein ventral prostate mRNA (98%)

ND, not determined.

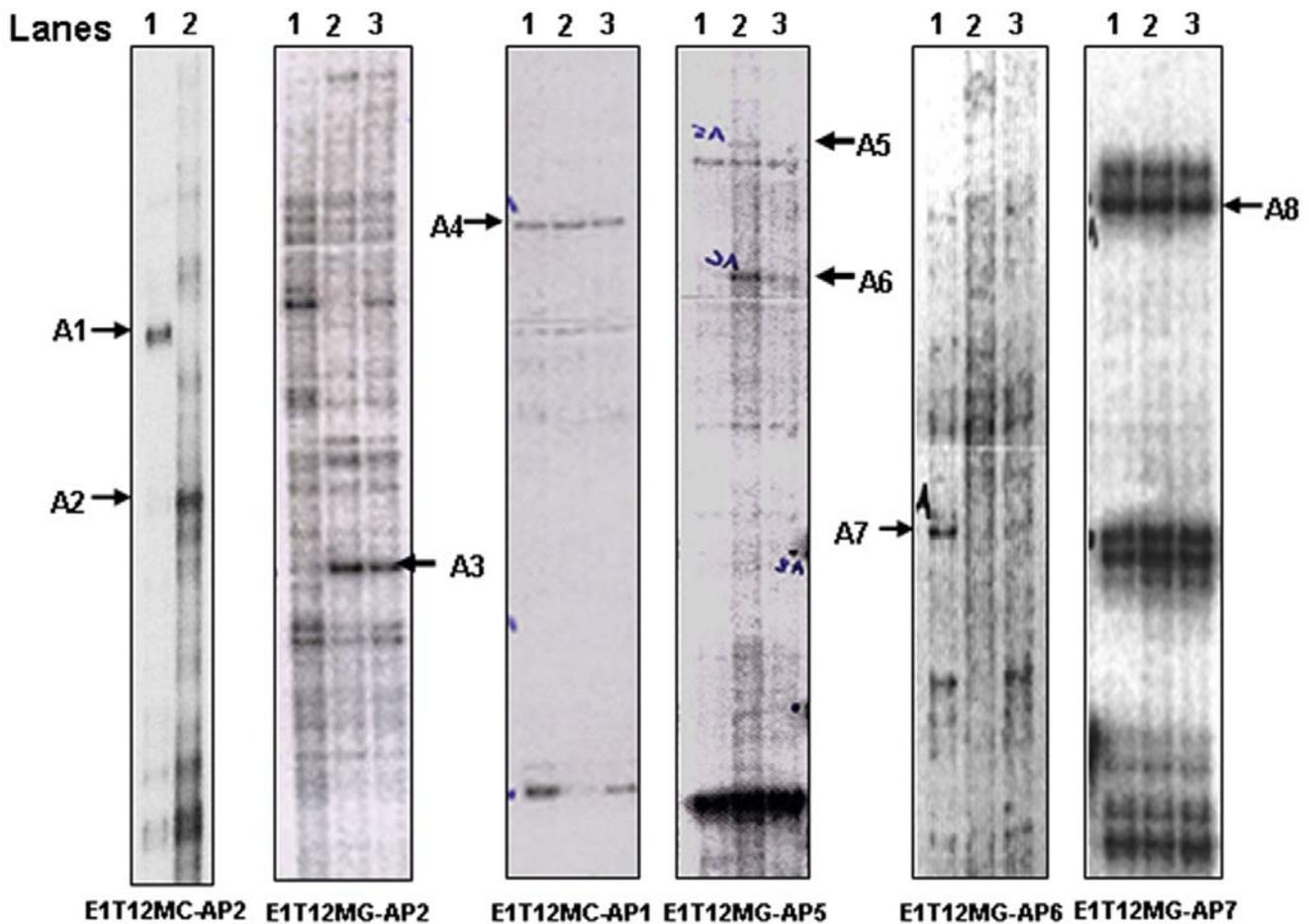


Figure 1. DD-RT-PCR analysis of the differentially expressed transcripts in the ventral prostates of sham-operated, castrated and flutamide-treated rats. The panels show differentially expressed transcripts obtained with combinations of anchored and arbitrary primers indicated at the bottom of each panel. The primer sequences are shown in tables 1 and 2. The differentially expressed transcripts (A1, A2, A3, A5, A6 and A7) and two transcripts whose expressions remain unchanged (A4 and A8) are indicated by arrows. Lanes: 1. day 5 sham-castrated, 2. day 5 castrated, 3. day 5 flutamide-treated.

MAGEE1 melanoma antigen and no similarity was found with the existing sequences in the rat database. Here we refer to this transcript as A7 mRNA. Among several novel genes, we identified clusterin (a known androgen-repressed gene in RVP), which was induced upon castration and flutamide treatment (table 3).

3.2 Effect of castration and flutamide treatment on clusterin and methionine adenosyl transferase II α mRNAs

The expression of clusterin mRNA was enhanced in the RVP at day 1, day 3 and day 5 post-castration. This effect of androgen withdrawal was reversed following supplementation with testosterone propionate. Flutamide treatment also caused a substantial increase in the clusterin mRNA levels, albeit at much lower levels than with castration. A 27.43 ± 3.9 -fold

induction was observed on castration as compared with 10.96 ± 4.0 on flutamide treatment (mean \pm SD, $N = 2$, figure 2). In similar experiments the regulation of methionine adenosyl transferase II α (MATII α) was also studied. Contrary to only one transcript of MATII α reported in the literature (Huang *et al* 1998), we observed two transcripts (approximately 3.4 and 3.0 kb). The level of the higher molecular weight transcript was increased by day 3 post-castration and appeared to fall at day 5 post-castration to levels seen in sham-operated and testosterone-replaced animals. On the other hand, the low molecular weight transcript showed a gradual decrease from day 1 to day 5 post-castration, which was partially restored on testosterone replacement. Flutamide treatment enhanced the levels of the higher molecular weight transcript and moderately decreased the levels of the lower molecular weight transcript (figure 2).

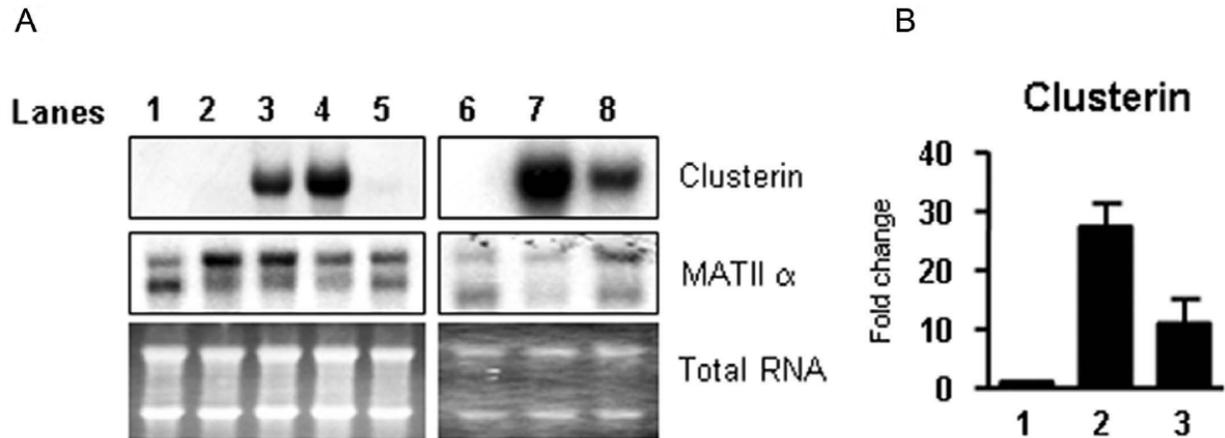


Figure 2. Regulation of clusterin and MATII α mRNA. **(A)** Time course of the modulation of gene expression following castration (lanes 1–5) and comparative analysis of the effects of castration and flutamide treatment (lanes 6–8). Lanes: 1. day 5 sham-castrated, 2. day 1 castrated, 3. day 3 castrated, 4. day 5 castrated, 5. testosterone-replaced, 6. day 5 sham-castrated, 7. day 5 castrated, 8. day 5 flutamide-treated. **(B)** Graphical representation showing the fold change of clusterin mRNA expression following castration and flutamide treatment. Bars represent mean \pm SD ($N = 2$). Bars: 1. day 5 sham-castrated, 2. day 5 castrated, 3. day 5 flutamide-treated.

3.3 Effect of castration and flutamide treatment on the prostate-specific PBPC1BS, A7 and S100RVP mRNA transcripts

Castration led to a dramatic fall in the steady-state expression of PBPC1BS mRNA to levels beyond detection at day 3 post-castration. Testosterone replacement restored PBPC1BS mRNA to a near-normal level (figure 3A). Using the A7 reamplified cDNA as a radiolabelled probe, we detected a transcript of approximately 1.7 kb, which was abundantly expressed in the intact RVP. Castration led to a dramatic fall in the steady-state level of the A7 mRNA transcript, which was reversed by testosterone replacement. S100RVP also exhibited a similar pattern of regulation by castration and testosterone replacement (figure 3A). The kinetics of downregulation of the A7 and PBPC1BS transcripts were determined. PBPC1BS mRNA appeared to decrease more rapidly than A7. At day 1 post-castration, PBPC1BS mRNA was reduced to $18.31 \pm 8.2\%$ of the control (mean \pm SD, $N = 2$) as opposed to $80.96 \pm 18.01\%$ in the case of A7 (mean \pm SD, $N = 2$) (figure 3B). The castration data suggest that PBPC1BS, S100RVP and A7 transcripts are androgen-regulated in the RVP. In order to probe further into the regulation of these genes by androgens, the effect of flutamide treatment on intact rats was studied. Flutamide administration caused a marked reduction in the steady-state level of PBPC1BS mRNA (figure 3C). Surprisingly, the expression of A7 and S100RVP mRNAs remained unchanged upon flutamide treatment (figure 3C, D). A survey of the published literature revealed that PBPC1BS

belongs to a large family of proteins called the secretoglobin family and is positioned close to RnPBPC1 and RnPBPC2 (coding for the C1 and C2 components of prostatic steroid-binding proteins, respectively) in a dendrogram showing the relatedness among members of this family (Reynolds *et al* 2002). These data suggested that the expression of PBPC1BS could be prostate-specific. The mRNA expression of PBPC1BS by northern blot using total RNA isolated from various adult rat tissues revealed its prostate-specific expression (figure 3E). The A7 transcript was also found to be prostate-specific (figure 3E).

3.4 Effect of flutamide on the RVP epithelium

In order to assess the apoptotic activity in the RVP following castration and flutamide treatment, *in situ* detection of apoptosis (TUNEL assay) was performed. Interestingly, there was almost complete absence of TUNEL-positive nuclei in ventral prostate sections of flutamide-treated rats (figure 4). In contrast, ventral prostate sections of castrated rats showed TUNEL-positive nuclei. This suggests that flutamide does not induce apoptosis in the RVP.

3.5 mRNA in situ hybridization analysis of S100RVP

Downregulation of the S100RVP and A7 transcripts by castration but not by flutamide, coupled with the absence of apoptotic activity following flutamide treatment, suggested that the regulation of these mRNAs could only be apparent

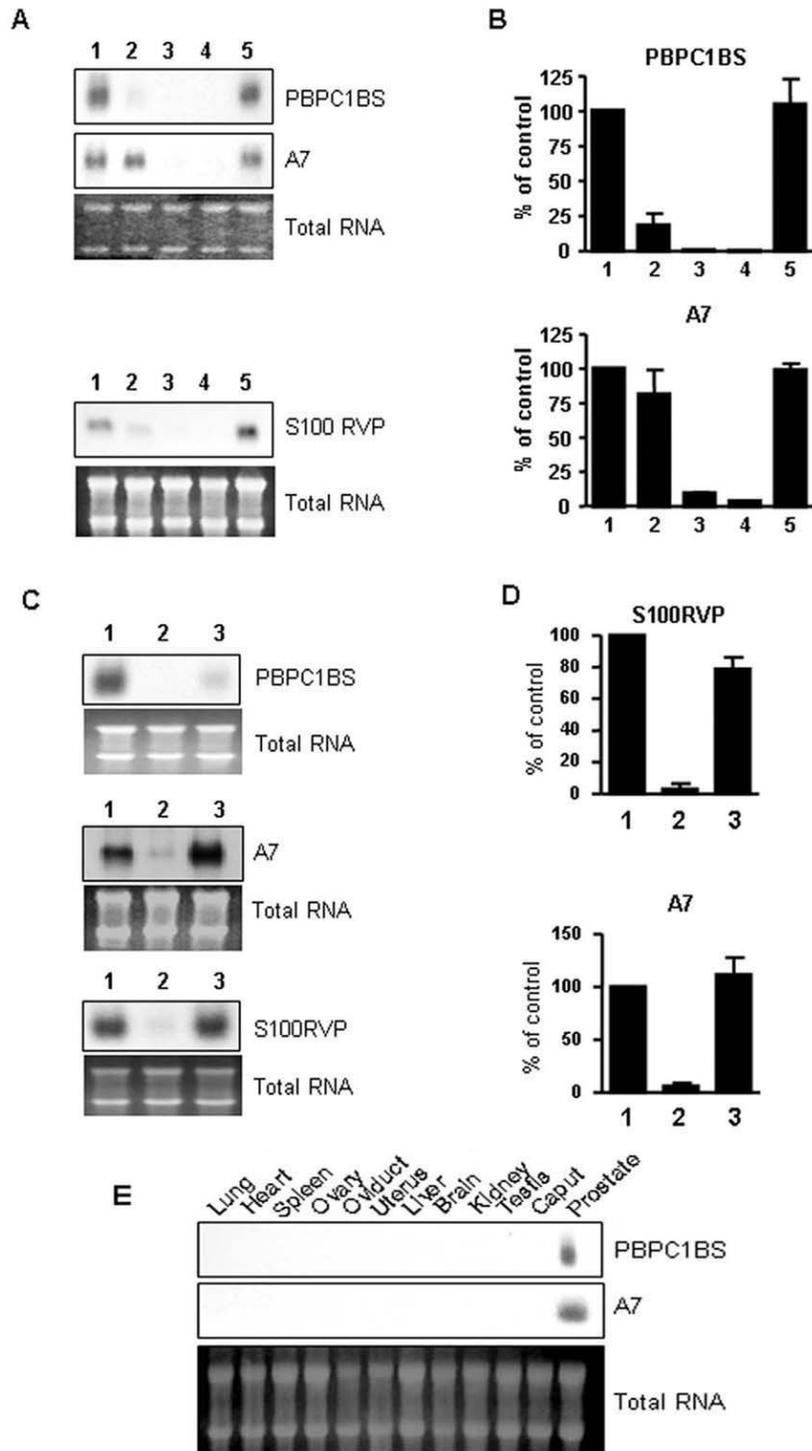


Figure 3. Effect of castration and flutamide treatment on the expression of PBPC1BS, A7 and S100RVP transcripts. **(A)** Time course of modulation of mRNA expression analysed by northern blotting. **(B)** Graphical representation of the change in expression of A7 and S100RVP post-castration. Bars represent mean \pm SD ($N = 2$). Lanes and bars for **A** and **B**: 1. day 5 sham-castrated, 2. day 1 castrated, 3. day 3 castrated, 4. day 5 castrated, 5. testosterone replaced. **(C)** Comparative analysis of the effect of castration and flutamide treatment by northern blot. **(D)** Graphical representation of S100RVP and A7 mRNA expressions following castration and flutamide treatment. The indicated genes are shown beside each panel of northern blots. Bars represent mean \pm SD ($N = 2$). Lanes and bars for **C** and **D**: 1. day 5 sham-castrated, 2. day 5 castrated, 3. day 5 flutamide-treated. **E.** Prostate-specific expression of PBPC1BS mRNA and A7 transcript.

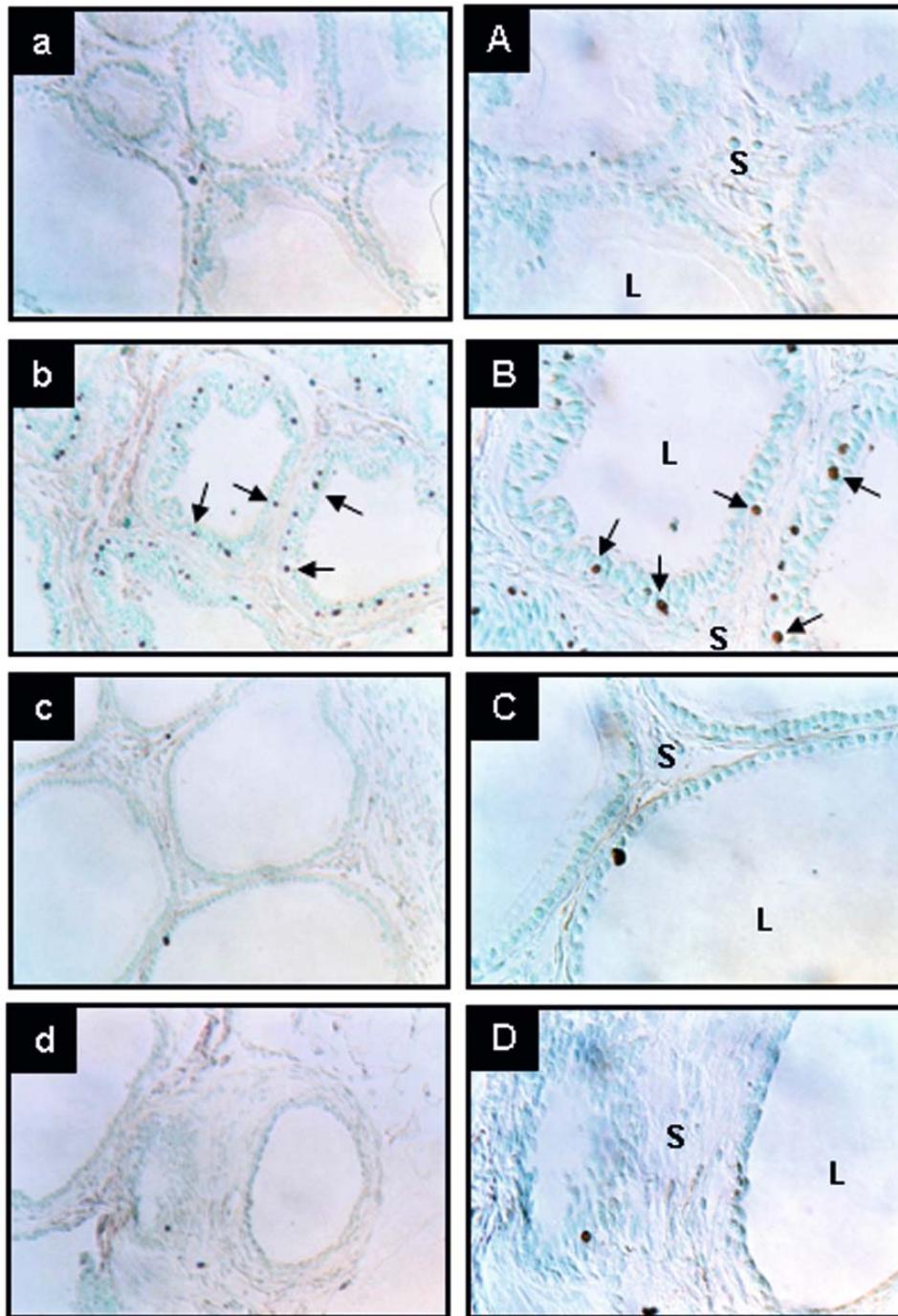


Figure 4. *In situ* detection of DNA fragmentation using TUNEL assay. (a,A) day 5 sham-castrated, (b,B) day 5 castrated, (c,C) day 5 flutamide-treated, (d,D) testosterone-replaced. Images are taken with 20X objective in a, b, c and d, and with 40X objective in A, B, C and D. Arrows indicate apoptotic nuclei. S, stroma; L, lumen.

due to depletion of the majority of prostate epithelial cells at day 5 post-castration. This would be possible if S100RVP was localized to the glandular epithelial cells of the prostate. To test this, mRNA *in situ* hybridization using a DIG-labelled S100RVP antisense riboprobe was carried out. As

shown in figure 5, S100RVP mRNA was detected only in the glandular epithelial cells, which was lost on castration and restored with testosterone supplementation. In agreement with the data of northern blot, flutamide treatment did not affect the epithelial expression of S100RVP.

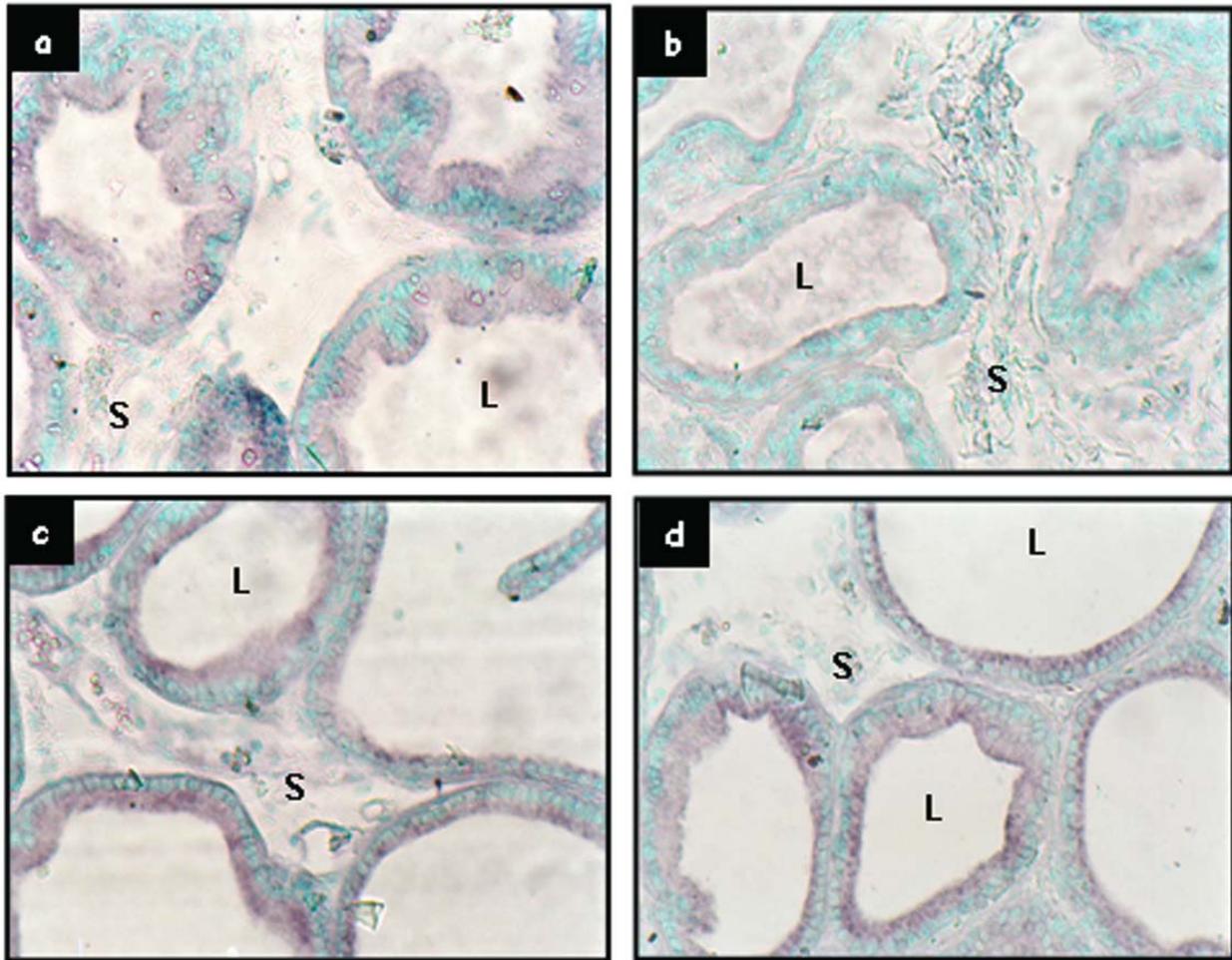


Figure 5. *In situ* hybridization analysis of the expression of S100RVP mRNA. **a**, day 5 sham-castrated; **b**, day 5 castrated; **c**, day 5 flutamide-treated; **d**, testosterone-replaced. S, stroma; L, lumen

3.6 Effect of flutamide on testosterone-mediated reversal of castration effects

Experiments discussed so far to study the effect of flutamide involved analysis of gene expression following administration of flutamide to intact animals. Testosterone supplementation to castrated rats not only causes repopulation of prostate epithelial cells but also induces and suppresses androgen-induced and androgen-repressed genes respectively. The ability of flutamide to block the testosterone-mediated reversal of castration effects on gene expression was also evaluated. For this purpose, animals were castrated and given intraperitoneal injections of flutamide, testosterone propionate or both. Castrated control animals received the vehicle alone. The induction of prostatein C1 mRNA (a known androgen-induced gene) and PBPC1BS mRNA by testosterone supplementation to

castrated animals was blocked by flutamide. Flutamide also blocked the suppression of clusterin by testosterone. Interestingly, although as shown previously, flutamide did not have any effect on A7 and S100RVP mRNA when injected into intact rats, it blocked the reappearance of A7 and S100RVP mRNAs by testosterone replacement (figure 6).

4. Discussion

Despite the widespread use of flutamide for the treatment of prostate cancer, its effect on the physiology of the prostate has hardly been studied. A few independent research groups have studied the effect of flutamide on gene expression in the RVP. These studies have revealed a differential effect of flutamide on gene expression compared with castration

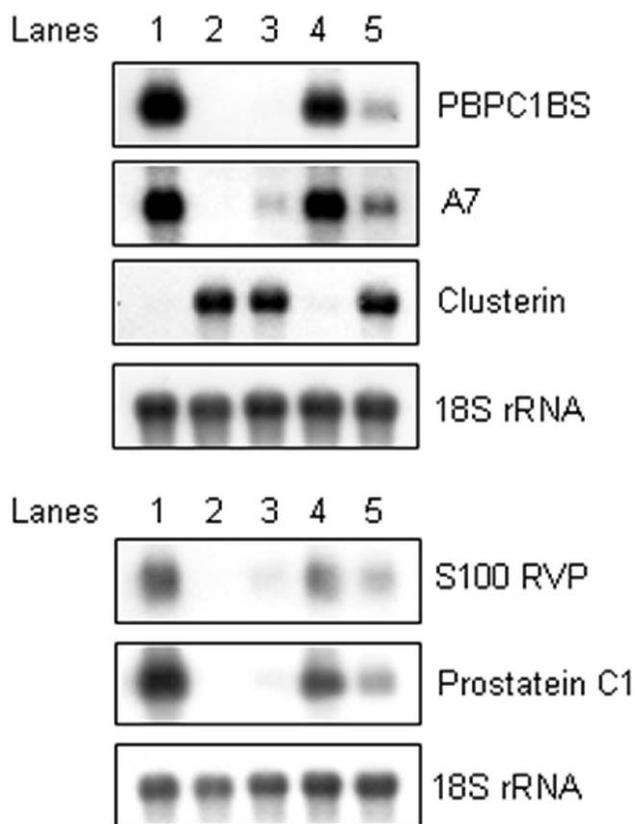


Figure 6. Effect of flutamide on reversal of castration effects by testosterone. Lanes: 1. control (intact rat ventral prostate), 2. day 5 castrated, 3. day 5 castrated + flutamide-treated, 4. testosterone-replaced, 5. testosterone-replaced + flutamide treated.

(Russo *et al* 1994; Turner *et al* 2001; Leger *et al* 1988; Montalvo *et al* 2002). Given the fact that flutamide is an androgen receptor antagonist, the question as to why castration and flutamide treatment should exert differential effects has not been addressed. The present study offers a possible explanation for the observed differential effects of castration and flutamide treatment in the RVP.

We have demonstrated differential expression patterns of transcripts identified by DD-RT-PCR following castration and flutamide treatment in the RVP. We have shown that the mRNA levels of clusterin, a known androgen-repressed gene, was increased by flutamide treatment, albeit to a much lesser extent compared with castration. The steady-state level of PBPC1BS mRNA, a prostate-specific transcript, was decreased by both castration and flutamide treatment. These findings suggest that the effects of androgen on PBPC1BS and clusterin are mediated via the androgen receptor. On the other hand, while expression of A7 and S100RVP was severely decreased to levels beyond detection

at day 5 post-castration, they were largely unaffected by flutamide treatment. To our knowledge, this is the first report on transcripts that are downregulated by castration but not by flutamide treatment. Such differential effects of castration are inconsistent with the well-established idea of flutamide as an androgen receptor antagonist.

Based on the published data on the effects of antiandrogens on androgen receptor function and localization, one may think of several possibilities for the differential effects of castration and flutamide treatment. Antiandrogens have been shown to inhibit androgen receptor-responsive gene activation despite promoting androgen receptor nuclear import and DNA binding (Kemppainen *et al* 1992; Jenster *et al* 1993; Warriar *et al* 1993; Masiello *et al* 2002). Interestingly, partial activation of the androgen receptor by androgen-receptor antagonists such as flutamide and cyproterone acetate has also been demonstrated. Recently, Nguyen *et al* (2007) showed agonistic activity of flutamide in neurons, which was androgen receptor dependent. Similar agonistic activities of flutamide and other antiandrogens have been reported in several studies (Peterziel *et al* 1999; Evangelou *et al* 2000; Zhu *et al* 1999). Both agonistic and antagonistic effects are also shown by selective oestrogen receptor modulators (Gustafsson 1998). Thus, it is possible that flutamide assembles a transcriptionally active receptor on some while assembling a transcriptionally inactive receptor on other promoters, depending perhaps on the coactivators/corepressors that these complexes recruit. In a previous report, we showed that flutamide increases the steady-state mRNA expression of pumilio 1 in the RVP while castration has no effect (Limaye *et al* 2007). Possibly, the androgen receptor-independent actions of flutamide may be the underlying cause of the observed differential effects of castration and flutamide treatment. In this study, we have clearly demonstrated that flutamide does not induce apoptosis of the RVP epithelium. We hypothesized that the differential effect of castration and flutamide on the integrity of the RVP epithelium could be the underlying cause for the observed differential effects. In the following sections we discuss the supporting evidence for such a hypothesis.

The glandular epithelium comprises 85% of the cells in the RVP. Castration leads to apoptosis of the glandular epithelial cells causing severe depletion of the epithelial cell compartment (Kyprianou and Isaacs 1988; English *et al* 1989). Hence, a gene represented only in the epithelial compartment would be observed as downregulated by castration merely due to its expression in the epithelium, irrespective of whether or not it is regulated by androgen. We observed a complete absence of TUNEL-positive nuclei in the ventral prostates of flutamide-treated rats compared with those who underwent castration. Hence, the observed differential regulation of PBPC1BS, S100RVP and A7 could be a manifestation of the differences in integrity of

the prostate epithelium following castration and flutamide treatment. We proposed that S100RVP and A7 transcripts are epithelial-specific genes, which may not truly represent androgen-regulated genes in the RVP and whose expression depends upon the integrity of the prostate epithelium. Evidence supporting this is the clear epithelial localization of S100RVP detectable in control, testosterone-replaced and flutamide-treated rats, but not in castrated rats. The other evidence for this is the kinetics of downregulation of the PBPC1BS and A7 mRNA transcripts following castration. Castration causes lowering of intraprostatic dihydrotestosterone (DHT) to levels below 5% of the control by 12–24 h post-castration (Kyprianou and Isaacs 1988). However, apoptotic activity at day 1 post-castration is very low. In these conditions of very low prostatic DHT and low apoptotic activity at day 1 post-castration, PBPC1BS mRNA levels are reduced to 20% of the control, whereas A7 transcripts are reduced to only 80% of the control. A marked reduction in the A7 transcript level is observed only when peak apoptotic activity has set in at around day 3 post-castration. This suggests that the reduction of PBPC1BS mRNA is associated with the loss of testosterone, whereas the reduction of A7 transcripts is associated with the loss of epithelial cells. PBPC1BS, whose expression is also reduced by flutamide treatment, may represent a gene that is truly androgen regulated.

Adult rats exposed to flutamide *in utero* have been shown to exhibit severely compromised prostate growth and differentiation in a dose-dependent manner (Imperato-McGinley *et al* 1992), indicating the importance of the androgen receptor in epithelial proliferation during development. The phenomenon of stroma-directed epithelial proliferation recurs during regrowth of the prostatic epithelium following testosterone supplementation of castrated rats. Testosterone-induced increase in ventral prostate weight in castrated rats was shown to be blocked by flutamide (Nellemann *et al* 2005), indicating the ability of flutamide to block re-proliferation of the epithelium. In the present study, the effect of flutamide on reversal by testosterone of the effects of castration was evaluated. Flutamide administered to intact rats did not have any effect on S100RVP and A7 transcript levels. However, the reappearance of these transcripts following testosterone supplementation of castrated animals was blocked by flutamide treatment. This can be explained by the fact that flutamide blocks re-proliferation of the epithelium induced by testosterone replacement, and that A7 and S100RVP are epithelial-specific genes. This in turn supports the view that the expression of S100RVP and A7 in the RVP depends on the presence of intact epithelium and that an epithelial-specific gene observed as downregulated by castration at the whole prostate level need not necessarily be an androgen-regulated gene.

The data presented in this paper clearly demonstrate that castration and flutamide treatment have differential effects on gene expression. This study highlights the caveat in the widely used models of castration and testosterone replacement in identification of androgen-regulated genes. We conclude that interpretation of genes as androgen regulated in the RVP based solely on the effects of castration and testosterone replacement could be misleading. This obviously has a lot of bearing with regard to the search for novel androgen-regulated genes as therapeutic targets or diagnostic markers for prostate carcinoma using the RVP model.

Acknowledgements

We thank Rajnish P Rao and Kalika Prasad for their help with *in situ* hybridization protocols. We thank the staff of the Central Animal Facility at the Indian Institute of Science for the supply of animals. This work was funded by the Department of Biotechnology, New Delhi. Infrastructure support from the Department of Science and Technology, Indian Council of Medical Research and University Grants Commission, New Delhi, is acknowledged.

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MS received 17 November 2007; accepted 14 April 2008

ePublication: 24 April 2008

Corresponding editor: PRAGNA I PATEL