

Changes in mitotic reorientation and Wnt/AR signaling in rat prostate epithelial cells exposed to subchronic testosterone

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Abstract. The aim of the present study was to investigate the changes in mitotic reorientation and relative differential gene expression in rat prostate epithelial cells following long-term exposure to testosterone propionate (TP). Sprague-Dawley rats were randomly divided into two groups as follows: TP group, which received 3.7 mg/kg/day TP for 30 days (n=10); and control group, in which rats were injected with olive oil (n=10). Microscopic analysis of the prostate tissue was performed by immunohistochemical analysis and hematoxylin and eosin staining. Differential gene expression analysis was performed via gene microarray, and a total of five genes (*Dkk3*, *Ran*, *Fas*, *Tgm4* and *Wnt2*) were selected and their expression levels were verified using reverse transcription-polymerase chain reaction. For rats treated with TP, mitosis was significantly reoriented, becoming parallel to the basement membrane. By contrast, in the control group cells mitotic orientation remained perpendicular to the basement membrane. Genes such as *Ran* and *Tgm4* in the androgen receptor (AR) signaling pathway and *Wnt2* in the Wnt signaling pathway, were upregulated following treatment with TP. Conversely, the *Dkk3* and *Fas*

genes were downregulated following treatment with TP. In conclusion, mitotic orientation of prostate epithelial cells was altered following long-term administration of TP. Wnt and AR signaling pathways influenced cell proliferation and may have participated in the mitotic orientation change.

Introduction

Testosterone propionate (TP) is known to serve a crucial function in benign prostatic hyperplasia (BPH) and prostatic carcinoma (Pca). Pca is common in the developed world and is the most prevalent cancer among men in the USA. BPH is also a common disease in men >50 years of age (1). However, the mechanisms through which TP affects these processes are not clear. Mitotic orientation is generally perpendicular to the basement membrane in the glandular epithelium of control castrated rats; however, a recent study observed that mitotic orientation was parallel to the basement membrane in the glandular epithelium of castrated rats treated with TP (2).

TP is a sex hormone that primarily activates the androgen receptor (AR) (3), a key regulatory protein that is a critical intermediate in the intracellular androgen pathway (4). Chronic androgen stimulation may promote the expression and secretion of AR from epithelial cells. Additionally, prostate basal tissue may contain stem cells, and the aging and molecular injury mechanisms in stem cells are crucially involved in the development of age-associated diseases, such as BPH and cancer (5). Prostate basal cells are not dependent on androgen; however, the presence of androgen is able to induce the proliferation and differentiation of prostate basal cells (6), which is a key mechanism for the induction of prostate cell proliferation.

In order to elucidate the details of the mechanism(s) involved in changes in mitotic orientation in the prostatic glandular epithelium of castrated rats exposed to subchronic TP and the associated differential gene expression, an animal model was established in Sprague-Dawley (SD) rats (2). The aim of the present study was to determine the changes in mitotic orientation in SD rats that received long-term TP exposure and to compare the differences in gene expression between TP-treated and control rats using gene microarrays.

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Abbreviations: AR, androgen receptor; TP, testosterone propionate; BPH, benign prostate hyperplasia; H&E, hematoxylin and eosin; Pca, prostatic carcinoma; RT-PCR, reverse transcription-polymerase chain reaction; SD, Sprague-Dawley

Key words: prostate, mitotic orientation, testosterone propionate, differential gene expression

Table I. Primers used in reverse transcription-polymerase chain reaction.

Gene	Bidirectional primer sequence	Annealing temperature (°C)	Product length (bp)
<i>β-actin</i>	F: 5'-CCTCTATGCCAACACAGTGC-3' R: 5'-GTAATCCTGCTTGCTGATCC-3'	58	211
<i>Ran</i>	F: 5'-CGTAGACGGTAAAGCAATGG-3' R: 5'-AGTCAAAGAAGCAGCAAACAC-3'	59	149
<i>Dkk3</i>	F: 5'-ACACCCAACAAGAAGCAACC-3' R: 5'-AGGGAAAGAGGGAGAAAGC-3'	59	174
<i>Fas</i>	F: 5'-GACAACAAGTCTCAGAAGG-3' R: 5'-TCAGAATAGTGTTCCTGTCC-3'	59	105
<i>Tgm4</i>	F: 5'-CGAGAACAGGCGTCAAGATA-3' R: 5'-GTCTGTAGGTCAAGGGAGCAT-3'	59	246
<i>Wnt2</i>	F: 5'-GTGCCGACTGGACAACCTCT-3' R: 5'-ACCTCAGAATGCCCAAGACA-3'	59	224

F, forward; R, reverse.

In addition, the role of the Wnt and AR signal transduction pathways in prostate cell proliferation was evaluated by determining the expression of a number of associated genes.

Materials and methods

Experimental animals. A total of 20 male 4-month-old SD rats [certificate no. SCXK 2003-0002] were obtained from Shanghai Laboratory Animal Center of Chinese Academy Science (Shanghai, China) and housed in a specific pathogen-free room (temperature, 20-26°C; humidity, 40-70%), with free access to water and food. All procedures and animal experiments were approved by the Animal Ethical Committee of Shanghai Institute of Planned Parenthood Research (Shanghai, China) and complied with the Animal Management Rules of the Chinese Ministry of Health (document no. 55, 2001).

Establishment of a rat model for BPH. Castrated rats were treated with subcutaneous injection of TP for 30 days at a dose of 3.7 mg/kg/day (n=10). Rats in the control group (n=10) were treated with an equivalent volume of olive oil (100 µl). The prostate from each test and control rat was removed following the final treatment. All tissue specimens were removed under anesthesia (3% Nembutal, 0.2 ml/100 ml; Sigma-Aldrich, St. Louis, MO, USA), and the specimens were fixed in formaldehyde solution and processed in paraffin. Paraffin blocks were cut into 5-µm sections for hematoxylin and eosin (H&E) and immunohistochemical staining.

Immunohistochemical staining procedure. Expression of AR was detected in prostate cells using immunohistochemical staining. Following deparaffination and rehydration of the sections, slides were placed in sodium citrate solution (0.01 M, pH 6.0) and heated to 96-100°C for 25 min. After cooling, sections were incubated in 5% bovine serum albumin (Beijing Zhongshan Biological Technology Co., Ltd., Beijing, China) for 20 min. The sections were then incubated for 2 h with primary rabbit anti-mouse AR monoclonal antibody

(1:100; cat. no. BA0004; Wuhan Boster Biotechnology, Ltd., Wuhan, China) diluted to 1:100 in phosphate-buffered saline (PBS) at room temperature. Following primary incubation, the sections were washed in PBS three times and incubated with horseradish peroxidase-conjugated goat anti-rabbit secondary antibody (1:50; cat. no. BA1051; Wuhan Boster Biotechnology Co., Ltd.) for 30 min at 37°C. Subsequently, the sections were washed with PBS and subjected to treatment with 3,3'-diaminobenzidine color-substrate solution and placed under cover slips. AR-labeled cells were observed using an Eclipse 50i light microscope (Nikon Corporation, Tokyo, Japan) in bright field mode.

Orientation of mitosis. For prostate epithelial cells, a parallel orientation was defined as the alignment of the chromosome axis and basement membrane at a 0-45° angle. By contrast, a perpendicular orientation was defined as the alignment of the chromosome axis and the basement membrane at a 45-90° angle. The cells were observed and imaged using the Nikon Eclipse 50i optical microscope, as described in a previous study (2).

Evaluation of differential gene expression by rat genome microarray analysis. Evaluation of differential gene expression was conducted using the Rat Genome 230 2.0 Array (Affymetrix, Inc., Santa Clara, CA, USA), which provides comprehensive coverage of the transcribed rat genome: 31,000 probe sets analyze the expression level of >30,000 transcripts and variants from >28,000 known rat genes. Total RNA was extracted using TRIzol reagent (Ambion; Thermo Fisher Scientific, Inc., Foster City, CA, USA) and its quality was confirmed using agarose-formaldehyde (Beijing Zhongshan Biological Technology Co., Ltd.) (7). Subsequently, double-stranded cDNA was synthesized from 5 µg total RNA using the One-Cycle cDNA Synthesis kit (Affymetrix, Inc.), according to the manufacturer's instructions. Biotin-labeled cRNA was transcribed from the cDNA using the GeneChip® IVT Labeling kit (Affymetrix, Inc.). Labeled cRNA (15 µg) was then fragmented and hybridized to the Affymetrix Rat

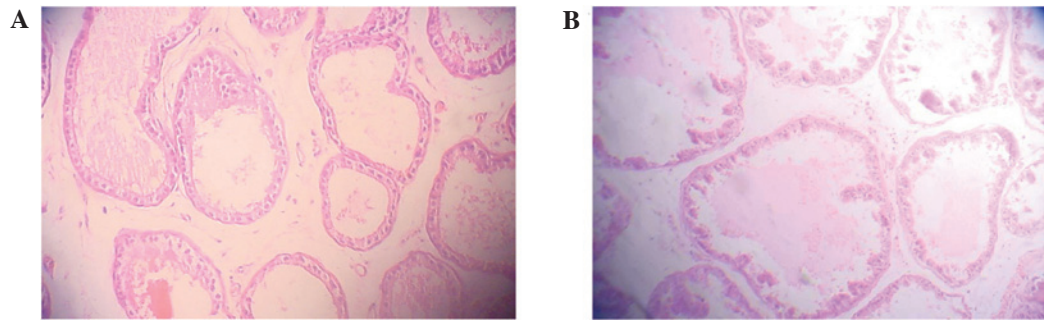


Figure 1. Prostate epithelia of the (A) control and (B) TP groups, stained with hematoxylin and eosin. Compared with the control group, the area of the prostate glandular cavity was significantly different in rats exposed to TP (magnification, x10). TP, testosterone propionate.

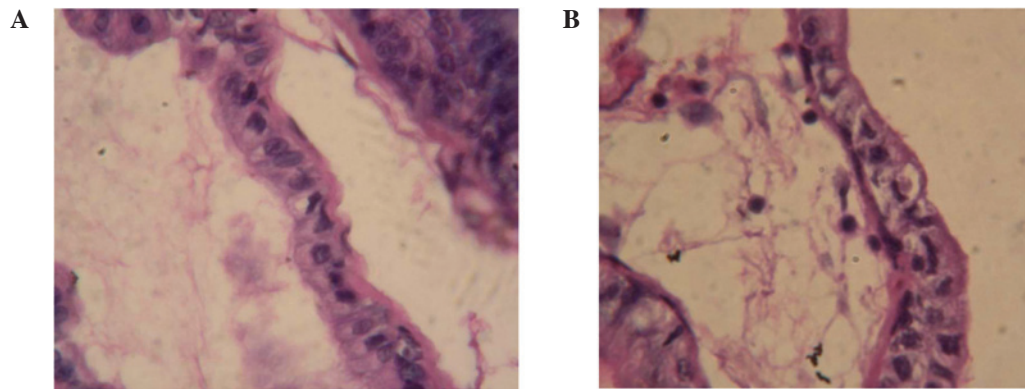


Figure 2. Mitotic orientation of prostate epithelia in the (A) control and (B) TP groups. Mitotic orientation of the prostate epithelium in the control group was perpendicular to the basement membrane, while that of the TP group was parallel to the basement membrane (magnification, x400). TP, testosterone propionate.

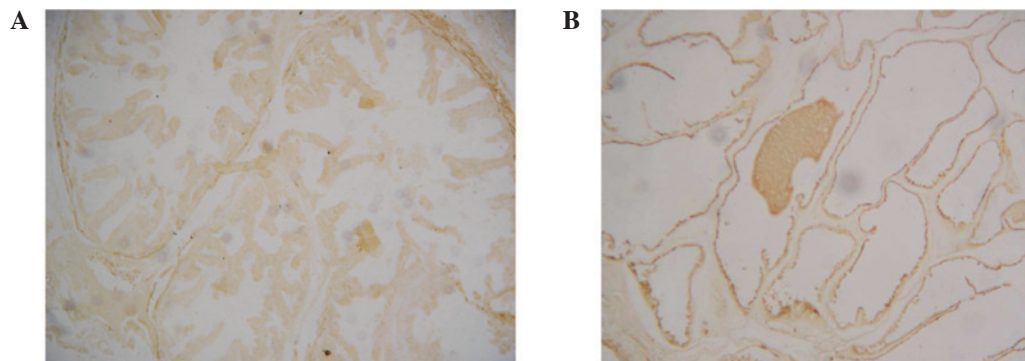


Figure 3. Analysis of AR expression in the (A) control and (B) TP groups. Brown-colored cells were positively stained for AR. Few AR-positive cells were seen in the control group, whereas increased AR staining was observed in the prostate glandular epithelia of the TP-treated rats (magnification, x100). AR, androgen receptor; TP, testosterone propionate.

Genome 230 2.0 Array for 16 h at 45°C in an Affymetrix GeneChip® Hybridization Oven 640 (Affymetrix, Inc.). The hybridized array was washed and stained in the GeneChip® Fluidics Station 450 (Affymetrix, Inc.), and fluorescent signals were detected using the Affymetrix GeneChip® Scanner 3000 (Affymetrix, Inc.). These data were initially documented with Affymetrix GeneChip® operating software GCOS 1.2 (Affymetrix, Inc.), which generated an expression report file.

Reverse transcription-polymerase chain reaction (RT-PCR) analysis of gene expression. Total RNA prepared for microarray analysis was also used for RT-PCR analysis. Total RNA (2 µg) from each sample was subjected to reverse transcription using

a Superscript First-Strand cDNA Synthesis kit (Henan Huamei Bioengineering Co., Ltd., Henan, China) according to the manufacturer's instructions. PCR was subsequently conducted by mixing 1 µl cDNA, 2.5 µl 10X PCR buffer, 1.5 µl 25 mM MgCl₂, 2.5 µl 2.5 mM dNTP, 1 µl 10 µM specific gene primer pairs, 1 µl 10 µM β-actin or GAPDH primer pairs, 25 µl H₂O and 1 U Taq polymerase (Ambion; Thermo Fisher Scientific, Inc.). The primers used in the PCR reaction are presented in Table I. PCR products were then amplified for 30 cycles, with each cycle consisting of the following steps: Denaturation for 10 sec at 95°C, annealing for 20 sec at 72°C and polymerization for 5 min at 72°C. The PCR products were resolved using an Affymetrix Hybridization Oven Model 640 (model 800138;

Table II. Wnt- and AR-associated genes that were up- or downregulated following testosterone propionate treatment in rats.

Probe ID	Gene	Character and function	Regulation
AT 1367590	<i>Ran</i>	AR-associated protein	Up
AT 1371022	<i>Tgm4</i>	Pca-associated gene	Up
AT 1393927	<i>Wnt2</i>	Wnt signaling transduced element	Up
AT 1370328	<i>Dkk3</i>	Negative regulation element of Wnt	Down
AT 1397221	<i>Fas</i>	Apoptosis	Down
AT 1370163	<i>Odc1</i>	Cell cycle control	Up
AT 1370235	<i>Dbi</i>	GABA receptor regulation factor	Up
AT 1371150t	<i>Ccnd1</i>	Cell cycle control	Up
AT 1368785	<i>Pitx2</i>	Transcription regulation factor	Down
AT 1369788	<i>Jun</i>	Transcription regulation factor	Down

AR, androgen receptor; Pca, prostatic carcinoma; GABA, γ -aminobutyric acid.

Table III. Reverse transcription-polymerase chain reaction analysis of the five selected genes and β -actin.

Gene	Control	TP
β -actin	1.95×10^{-3}	2.66×10^{-3}
<i>Dkk3</i>	2.93×10^{-4}	1.18×10^{-5}
<i>Ran</i>	1.07×10^{-4}	2.37×10^{-4}
<i>Fas</i>	9.47×10^{-4}	8.85×10^{-6}
<i>Tgm4</i>	1.09×10^{-4}	2.46×10^{-3}
<i>Wnt2</i>	1.24×10^{-3}	1.44×10^{-4}

TP, testosterone propionate.

Affymetrix, Inc.) according to a DNA standard curve. Data were analyzed using Light Cyclers 480 software version 1.5 (Roche Diagnostics, Basel, Switzerland).

Statistical analysis. Data were expressed as the mean \pm standard deviation. One-way analysis of variance (ANOVA) and P-values were used to evaluate significant differences between the groups. Image-Pro Plus 6.0 (Media Cybernetics, Inc., Rockville, MD, USA) was used to analyze image data. Data analysis to determine differential gene expression profiles was performed by importing MAS 5.0 intensity data (Affymetrix, Inc.) into the Partek Genomics Suite Version 6.4 (Partek Incorporated, St. Louis, MO) for ANOVA based on time in culture. $P < 0.05$ was considered to indicate a statistically significant difference. Post-hoc Fisher's exact test adjusted to correct for false discovery rates ($P < 0.05$) was used to compare data from multiple groups. Only differentially expressed genes ($-2.0 > \text{fold-change} > 2.0$; $P < 0.05$) were included in each IPA core analysis.

Results

Mitotic orientation of prostate epithelia in rats. Prostate epithelia from TP-treated rats exhibited typical prostate proliferation morphology, in which the organ quotient, volume

and area of the prostate glandular cavity were increased compared with the control group. H&E staining revealed that the prostate epithelia were highly stylolitic, with intrusions in the glandular cavity, and the area of the prostate glandular cavity was large (Fig. 1). The mitotic orientation of prostate epithelial cells in TP-treated rats was parallel to the basement membrane, while the orientation in the control rats was perpendicular to the basement membrane (Fig. 2). These data demonstrate that the mitotic orientation of prostate epithelial cells changed following long-term exposure to TP.

Analysis of AR expression. Notably, immunohistochemical staining revealed an increased number of AR-positive cells in the prostate epithelia of the TP-treated rats when compared with the control rats (Fig. 3), indicating that TP treatment upregulated AR expression.

Results of gene chip microarray analysis. A rat genome microarray technique was used to analyze the differences in gene expression between the control and TP-treated rats. Genes that were altered in the TP group were assumed to be associated with changes in prostate cell mitotic orientation and proliferation. A number of the differentially expressed genes were associated with the AR and Wnt signal transduction pathways (Table II). Upregulation was observed in genes that promote cellular proliferation, including *Ran*, *Tgm4*, *Odc1* and *Wnt2*, while downregulation was observed in genes that inhibit cell proliferation, such as *Dkk3* and *Fas*.

RT-PCR analysis of selected genes. To confirm the observed changes in gene expression, RT-PCR analysis was performed for a number of the genes, including *Dkk3*, *Ran*, *Fas*, *Tgm4* and *Wnt2* (Table III). The results of RT-PCR analysis were consistent with the microarray data.

Discussion

To the best of our knowledge, the present study showed for the first time that the mitotic orientation of rat prostate epithelial cells changes following exposure to TP for an extended period.

In addition, a microarray analysis of over 28,000 rat genes, followed by verification using RT-PCR, indicated that the Wnt and AR signal transduction pathways were involved in the observed changes in the mitotic orientation of prostate epithelial cells in response to TP treatment.

The present study revealed that the mitotic orientation of rat prostate epithelial cells is altered following cell exposure to TP, providing a new method for the study of TP-dependent prostate cell proliferation. Additionally, the immunohistochemistry and H&E results of previous studies demonstrated that the prostate may be a target organ for TP (8,9). Therefore, changes in cell proliferation may result from the reorientation of the mitotic complex following exposure of the prostate to TP. In a previous study, in control cells, mitotic orientation was parallel to the basement membrane; therefore, changes in proliferation may occur via the AR signal transduction pathway (10).

In the present study, TP-treated rats exhibited upregulation of *Wnt2* and other positive regulatory genes, while negative regulatory genes, such as *Dkk3*, were downregulated compared with the control group. The Wnt pathway has been widely studied (11,12), and is crucially involved in numerous proliferative diseases, such as Pca. In the classical Wnt signaling pathway, *Wnt2* stimulates cell proliferation and regulates the aging process of fibroblasts and epithelial cells. In addition, *Wnt2* signal reduction has been observed in senescent cells (13). Increased expression of *Wnt2* is able to stimulate prostate epithelial cell proliferation, activate the Wnt signaling pathway and cause changes in prostate epithelial cell polarity (13), which may result in chromosomal changes in prostate epithelial cells.

Dkk3 is a member of the Dkk family, which inhibits the Wnt signaling pathway by interacting with the LRP5/6 receptor and promotes lysosome endocytosis (14,15). Although it has not been as well studied as Wnt, the *Dkk3* gene has been shown to be downregulated in prostate tumor cells (16), suggesting that increased *Dkk3* expression may inhibit tumor cells development (17). In the present study, TP-treated rats exhibited downregulated *Dkk3* expression compared with the control group. As it is known that TP induces BPH, it may be concluded that downregulation of the *Dkk3* gene reduces the inhibition of cell proliferation and cell differentiation, in addition to causing BPH (18).

Tgm4 is a gene involved in the AR signaling pathway and is expressed in normal and abnormal prostate tissues. In numerous studies, *Tgm4* has been shown to be a candidate marker for prostate cells (19,20). In the TP group, *Tgm4* upregulation promoted prostate cell proliferation. Additionally, TP treatment resulted in the downregulation of *Fas*, resulting in reduced apoptosis (21), and therefore enhanced prostate cell proliferation.

Ran is a member of RAS family and has been shown to be involved in the androgen signaling pathway in addition to the regulation of DNA transcription (22). Although numerous studies have investigated the association between Ran and prostate cancer (23), more recent studies have investigated Ran in the context of benign proliferation of prostate cells (24,25). In the present study, the upregulation of the *Ran* gene was confirmed using RT-PCR, and the results suggested that *Ran* upregulation may contribute to prostate cell proliferation in BPH.

AR serves a crucial function in the prostate and is coregulated by hormone/receptor combinations (26). Previous studies have demonstrated that numerous coregulatory events are associated with the Ras family. Furthermore, recent studies have reported that Ras response element binding protein 1 (RREB-1) is a ligand for AR and coregulates AR. Therefore, this Ras-associated protein has the potential to inhibit AR function. The Ras/MAPK kinase pathway is able to counteract RREB-1, inhibiting the androgen signaling pathway (27). As a member of the Ras family, Ran is involved in this inhibitory process and functions to promote prostate cell proliferation. In addition, Ran is involved in mitotic spindle organization and biogenesis, which may affect mitotic orientation of epithelial cells. In Ras-mediated transformation, Krüppel-like factor 5 regulates the cyclin B1/Cdc2 proteins, in addition to activating the cell cycle, and thereby promotes cell mitosis (27,28). Thus, collectively, the present data suggest that TP treatment altered the mitotic orientation of prostate epithelial cells via a pathway involving Wnt and AR signaling. Further studies are required to determine the precise mechanisms through which Krüppel-like factor 5 is involved in this process.

In conclusion, mitotic reorientation of rat prostate epithelial cells was altered following exposure to subchronic TP, which may have promoted prostate cell proliferation via the Wnt and AR signaling pathways.

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