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Original Article

Proliferation and phenotypic changes of stromal cells in response to varying estrogen/androgen levels in castrated rats

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Abstract

It is known that human benign prostatic hyperplasia might arise from an estrogen/androgen (E/T) imbalance. We studied the response of castrated rat prostate to different ratios of circulating E/T. The castrated male Wistar rats were randomly injected with E/T at different ratios for 4 weeks. The prostates of E/T (1:100) group showed a distinct prostatic hyperplasia response by prostatic index, hematoxylin and eosin staining, and quantitative immunohistochemical analysis of α-smooth muscle actin (SMA). In this group, cells positive for Vimentin, non-muscle myosin heavy chain (NMMHC) and proliferating cell nuclear antigen (PCNA) increased in the stroma and epithelium. Furthermore, the mRNA levels of smooth muscle myosin heavy chain (SMMHC) and NMMHC increased. So E/T at a ratio of 1:100 can induce a stromal hyperplastic response in the prostate of castrated rats. The main change observed was an increase of smooth muscle cells, whereas some epithelial changes were also seen in the rat prostates.

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Keywords: androgens, castration, estrogen, prostatic hyperplasia, rat, stromal cells

1 Introduction

Benign prostatic hyperplasia (BPH) is the most common urogenital disorder that affects older men, and stromal hyperplasia is one of the prominent pathological changes in BPH [1]. The circulating and intraprostatic estrogen/androgen (E/T) ratio increases in older men [1, 2] and is accompanied by an increased expression of the estrogen receptor in the stroma [3]. The enhanced estrogenic effect, mediated through the stroma, is correlated with the development of prostatic stromal hyperplasia [4].

One major characteristic of prostatic stromal hyperplasia is an increase in the proportion of smooth muscle cells (SMCs) [5]. There are two phenotypes of SMCs, 'synthetic' and 'contractile' [6]. The former indicates immature SMCs with a proliferating ability [7], whereas the latter characterizes fully differentiated contractile

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cells [8]. In most of the fetal and early postnatal periods, SMCs are of the synthetic phenotype. During development, a shift from the synthetic to the contractile phenotype occurs [9]. Prostatic stromal cells also show phenotypic plasticity in response to changes in culture conditions *in vitro* [10]. *In vivo*, apparent changes occur in proliferation and phenotype in BPH with regard to the expression of estrogen receptor-α [11].

Estrogen plays a physiological role during prostate development with regard to the programming of stromal cells and early morphogenesis [12]. The proliferation of stromal cells can be stimulated by an increasing ratio of E/T [13]. Estradiol acts in concert with sex hormone binding to globulin to produce an eightfold increase in cAMP, which can induce the proliferation of prostatic stromal cells [14]. Estrogen can also activate the extracellular signal-regulated kinase pathway through the estrogen receptor-α pathway, which leads to the proliferation of prostatic stromal cells [15]. However, the prostate is more prone to developing inflammation and dysplasia when treated with a high level of estrogen during development [12]. 17 β-estradiol might exert a triggering effect on stroma-predominant BPH [16]. Estradiol enhances the protein expression of SMC-specific markers in human primary cultured prostatic stromal cells [17]. Estrogen can also regulate the expression of genes that are involved in cell proliferation and differentiation, as detected by DNA microarray analysis [18]. Thus, a relative increase in the estrogen level may have a significant impact on stromal cells.

The concomitant administration of estrogen with androgen promotes prostatic growth in an animal model [19]. This synergistic hormonal effect has been studied on 5-α-reductase activity [19], which has androgen-responsive gene expression [10, 21], and its effect on the development of glandular hyperplasia [22]. However, a detailed study on hormone-induced prostatic stromal hyperplasia is still lacking.

In this study, we examined which ratio of E/T could induce distinct prostatic stromal changes in castrated rats with particular regard to proliferative and phenotypic responses.

2 Materials and methods

2.1 Animals and hormonal manipulations

A total of 40 adult male Wistar rats (250–300 g body weight) were obtained from Weitong-Lihua Experimental Animal (Beijing, China). The rats were maintained in a controlled environment with free access to food and water. Animal care and experiments were conducted in accordance with the guidelines of the Chinese Council on Animal Care and approved by the NanKai University Animal Care and Use Committee.

Orchiectomy was carried out under ether anesthesia through the scrotal route. Except for the five sham-operated control group rats, the other 35 rats were castrated and maintained for 3 weeks, after which they were randomly assigned to seven experimental groups with five animals in each. Different ratios of estradiol benzoate/testosterone propionate (Jinyao Amino Acid Manufacturer, Tianjin, China) were given to the castrated rats by daily intraperitoneal injections in 0.1 mL corn oil, as a vehicle, for 4 weeks. The daily dose ratios of E/T in the different groups were as follows: control group (0 μg/0 μg); castrated group (0 μg/0 μg); castrated + T group (0 μg/500 μg); castrated + E group (10 μg/0 μg); castrated + 1:50 E/T group (10 μg/500 μg); castrated + 1:100 E/T group (10 μg/1000 μg); castrated + 1:200 E/T group (10 μg/2000 μg) and castrated + 1:300 E/T group (10 μg/3000 μg).

Rats were killed under ether anesthesia 48 h after the last injection. Femoral arterial blood was collected, and the prostate was dissected and weighed for the prostatic index (PI). One lobe of the ventral prostate was fixed in phosphate-buffered formalin and embedded in paraffin for histological and immunohistochemical studies. Another lobe was frozen and stored at –80ºC for RNA extraction.

2.2 Determination of serum estradiol and testosterone

Rat blood samples were centrifuged 2 500 × *g* for 10 min. The translucent serum was collected and stored at –80ºC. Serum estradiol and testosterone concentrations were determined with a radioimmunoassay (Larwin Bio-technological, Shenzhen, China) [20], and the hormone ratio was calculated. The assay range was 20–2000 pg mL^{-1} for estradiol and 0.05–15 ng mL^{-1} for testosterone. The sensitivity of the estradiol assay was 0.05 pg mL^{-1}, and the intra- and inter-assay coefficients of variation were < 5.6% and 7.9%, respectively. Cross-reactivity with estriol, estrone and testosterone was < 0.1%, 0.5% and 0.01%, respectively. The sensitivity of the testosterone assay was 0.2 ng per 100 mL, the intra-assay coefficient of variation was < 4.5%, and the inter-assay coefficient of variation was < 6.4%. Cross-reactivity with dihydrotestosterone, dehydroepiandrosterone and estradiol was < 1%, 0.1% and 0.01%,

respectively.

2.3 Calculation of PI

The following formula was used for PI calculation: PI = gross weight of prostate/weight of whole animal × 100%.

2.4 Histological and immunohistochemical studies

Five-μm sections were deparaffinized in xylene and rehydrated in a graded series of alcohol. One section was stained with hematoxylin and eosin for histology, and other sections were processed for immunohistochemistry using the avidin–biotin–peroxidase complex method: endogenous peroxidase activity was blocked by 0.3% hydrogen peroxide for 10 min, followed by incubation with 10% serum for 30 min at room temperature. Sections were incubated with primary antibodies at room temperature for 2 h. The primary antibodies used were mouse monoclonal anti-SMA (α-smooth muscle actin) (1/400; Sigma-Aldrich, St. Louis, MO, USA), anti-Vimentin (1/200; Santa Cruz, CA, USA), anti-PCNA (proliferating cell nuclear antigen) (1/100; Santa Cruz) and anti-NMMHC (non-muscle myosin heavy chain) (1/3000; SMemb, Yamasa, Tokyo, Japan), which is the embryonic form of myosin heavy chain (MHC) that is predominantly expressed in dedifferentiated SMC [23]. A biotinylated secondary antibody was added for 30 min at 37ºC, followed by peroxidase-labelled streptavidin. The chromogen 3',3-diaminobenzidine was added and counterstained with hematoxylin. For a negative control, the primary antibody was replaced by non-specific immunoglobulin.

2.5 Assessment and quantification of immunohistochemical staining

Light microscopy was carried out with the Olympus microscope, CX-41 (Olympus, Tokyo, Japan). The thickness of the SMC (SMA-positive) layer surrounding the prostatic acini was measured by ocular micrometer (AX0067, Olympus, Tokyo, Japan) in units of 2.5 μm at × 400 magnification. Vimentin, NMMHC or PCNA-positive cells were counted by ocular micrometer (AX0071, Olympus, Tokyo, Japan) in a unit area of 250 μm × 250 μm at × 400 magnification. The identity of each specimen was blinded to the evaluator. The slide area was divided into 4 × 4 squares, and 10 randomly selected fields were examined from each section with four sections analysed per animal. The mean thickness and positive cell numbers for the animals of each group were then obtained.

2.6 Real-time quantitative PCR analysis

Total RNA was isolated from frozen prostatic tissue using Trizol reagent (Invitrogen, Carlsbad, CA, USA). One microgram of RNA was reverse-transcribed with 200 U of MMLV-RT (Invitrogen). Real-time quantitative PCR with EVA Green (Invitrogen) was carried out with the following PCR primers: smooth muscle myosin heavy chain (SMMHC) forward: CTTAGCCAAGGCCACTTATGAG, reverse: ATGCCCTCTCGTTGGTACTCTT; NMMHC forward: GGATTGGCAGGTCTCTCTATCAG, reverse: ATTGGGATCCTGGATATTGCT; and GAPDH (glyceraldehyde 3-phosphate dehydrogenase) forward: TGCTGAGTATGTCGTGGAGT, reverse: GGATGCAGGGATGATGTT. The PCR mixture on ice contained 1× PCR buffer, 2.5 mmol L^{-1} $MgCl_2$, 200 m mol L^{-1} dNTP, 400 nmol L^{-1} primers, 1 × EVA Green, 0.625 U Taq DNA polymerase and 1 μL cDNA. The PCR conditions included an initial incubation at 95ºC for 5 min, followed by 35 cycles of 95ºC for 30 s, 55ºC for 30 s and 72ºC for 30 s. The reaction products were normalized to that of GAPDH.

2.7 Statistical analysis

Data were expressed as means ± standard deviation (s.d.). SPSS software was used. Comparison of group data was analysed by one-way ANOVA (analysis of variance) with a *post hoc* test. Differences were considered statistically significant at $P < 0.05$.

3 Results

3.1 Determination of serum estradiol and testosterone

Except for the testosterone level in the 1:300 E/T group and the estradiol level in the control and castrated groups, which were out of the assay range, the measured serum hormone concentrations showed that the E/T ratios were similar to the injected ratios (Table 1).

3.2 Prostatic index

Castration or estrogen treatment caused a significant reduction in the PI compared with controls ($P < 0.05$). E/T or androgen injection resulted in a significant increase of the PI ($P < 0.05$) compared with controls, with the most significant effect seen for E/T ratios of 1:100 and 1:200 ($P < 0.05$) in contrast to E/T of 1:50 (Table 1).

3.3 Histological analysis

The ventral prostate of a normal rat in puberty is composed of tubuloacinar structures formed by a layer

of tall columnar epithelial cells that are surrounded by stromal cells (Figure 1A). Atrophy in some prostatic acini showing flat epithelial cells was seen in the castration and estrogen groups (Figures 1B, D). The lumens of these prostate glands were markedly reduced in size, whereas the stromal compartment appeared to be ex-

Table 1. Ratio of serum testosterone/estradiol (E/T), prostatic index and thickness of the SMC layers surrounding the acini (means ± s.d.).

Group E/T ratio	Estradiol concentration (pg mL^{-1})	Testosterone concentration (ng mL^{-1})	Ratio of testosterone/ estradiol	Prostatic index	SMC layer thickness (μm)
Control	0	3.95 ± 3.20	0	0.20 ± 0.04	4.60 ± 1.89
0:0	0	0.10 ± 0.04	0	0.04 ± 0.01*	5.49 ± 2.10
0:500	22.67 ± 3.79	10.71 ± 7.44	468.84 ± 53.10	0.25 ± 0.07*	5.22 ± 1.60
10:0	83.00 ± 43.31	0.56 ± 0.45	8.74 ± 6.90	0.05 ± 0.04*	6.58 ± 3.32*
1:50	112.00 ± 61.12	6.08 ± 3.12	54.62 ± 9.03	0.27 ± 0.08*	5.61 ± 2.15*
1:100	92.75 ± 44.72	8.79 ± 4.99	91.36 ± 21.26	0.36 ± 0.05$^{*\Delta}$	6.99 ± 2.64$^{*\Delta}$
1:200	90.25 ± 43.71	13.01 ± 2.30	163.64 ± 33.62	0.32 ± 0.02$^{*\Delta}$	5.25 ± 1.80
1:300	103.25 ± 24.68	> 15.00	152.09 ± 38.52	0.29 ± 0.01*	3.95 ± 1.58
F value	—	—	—	5.906**	7.605**

Abbreviation: SMC, smooth muscle cells.
$^{*}P < 0.05$, $^{**}P < 0.01$, compared with the control group.
$^{\Delta}P < 0.05$ compared with the 1:50 E/T group.

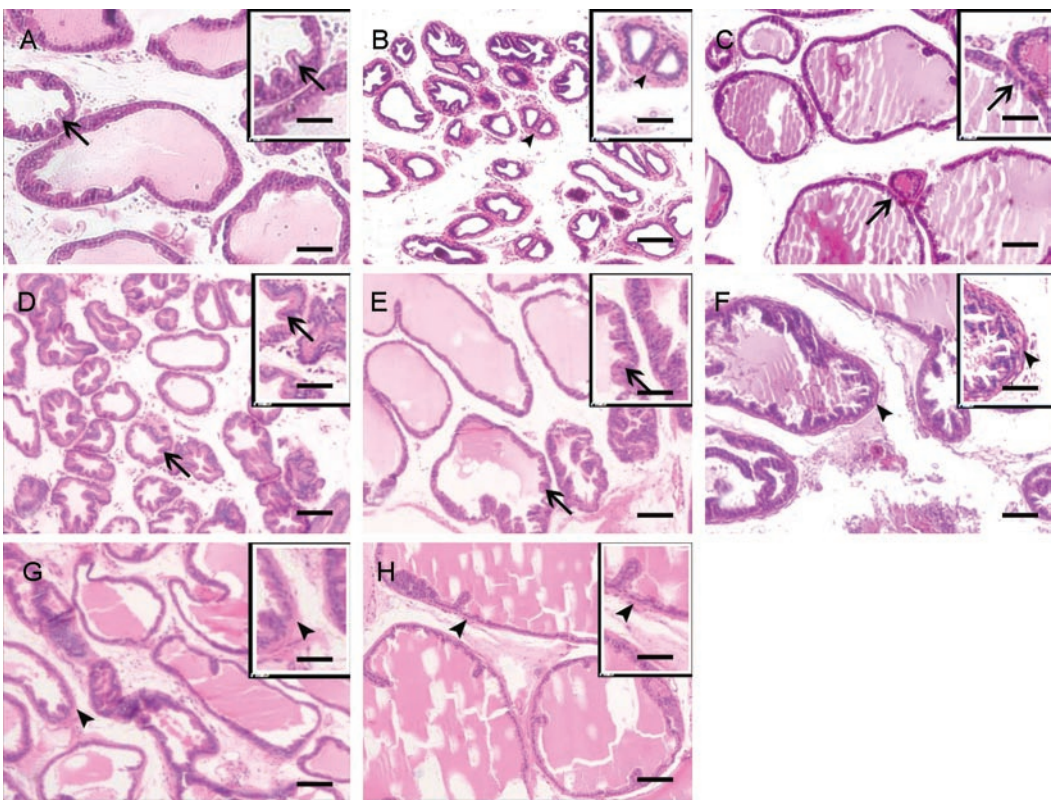

Figure 1. Histology of testosterone/estradiol (E/T)-treated rat prostates. (A) Sham-operated control, (B) castrated, (C) castrated + T, (D) castrated + E, (E) castrated + 1:50 E/T, (F) castrated + 1:100 E/T, (G) castrated + 1:200 E/T and (H) castrated + 1:300 E/T. The arrows point to the epithelial cells, and the arrowheads point to the stromal cells. Original magnification × 100; × 200 for the insets. Scale bars are 100 μm and 50 μm (for the insets).

panded. In the androgen group, glandular structures lined with cuboidal or columnar epithelial cells were restored (Figure 1C). Stromal hyperplasia was noted in the prostate after E/T treatment, with the glandular acini surrounded by multiple layers of stromal cells (Figures 1E, F, G, H).

3.4 Immunohistochemistry and quantitative analysis

In the prostates of control animals, the acinus was surrounded by a SMA-positive smooth muscle layer (Figure 2A). The surrounding SMC was dense in the androgen (Figure 2C) and various E/T groups (Figures 2E, F, G, H), but scattered in the castration and estrogen groups (Figures 2B, D).

Quantitative analysis of the SMC layer thickness surrounding the acinus was carried out. The 1:50 and 1:100 E/T groups showed thicker SMC layers ($P < 0.05$, Table 1) than controls. In addition, the layer of the SMC surrounding the acini increased most significantly in the 1:100 E/T group ($P < 0.05$, Figure 2F and Table 1) *vs*. the 1:50 E/T group.

There were a few Vimentin-positive fibroblasts in the stroma that were distributed among the acini in the control group prostates (Figure 3A). Compared with the control group, the number of Vimentin-positive cells increased 2.4-fold in the 1:100 E/T group, whereas the thickness of SMA layer increased 1.6-fold ($P < 0.05$, Table 2).

Few NMMHC-positive cells were found in the control group prostates, both in the stroma and in the epithelia (Figure 3C). In the 1:100 E/T group, the number of NMMHC-positive cells in the stroma and epithelia increased 2.7- and 2.4-fold, respectively ($P < 0.05$, Figure 3D and Table 2).

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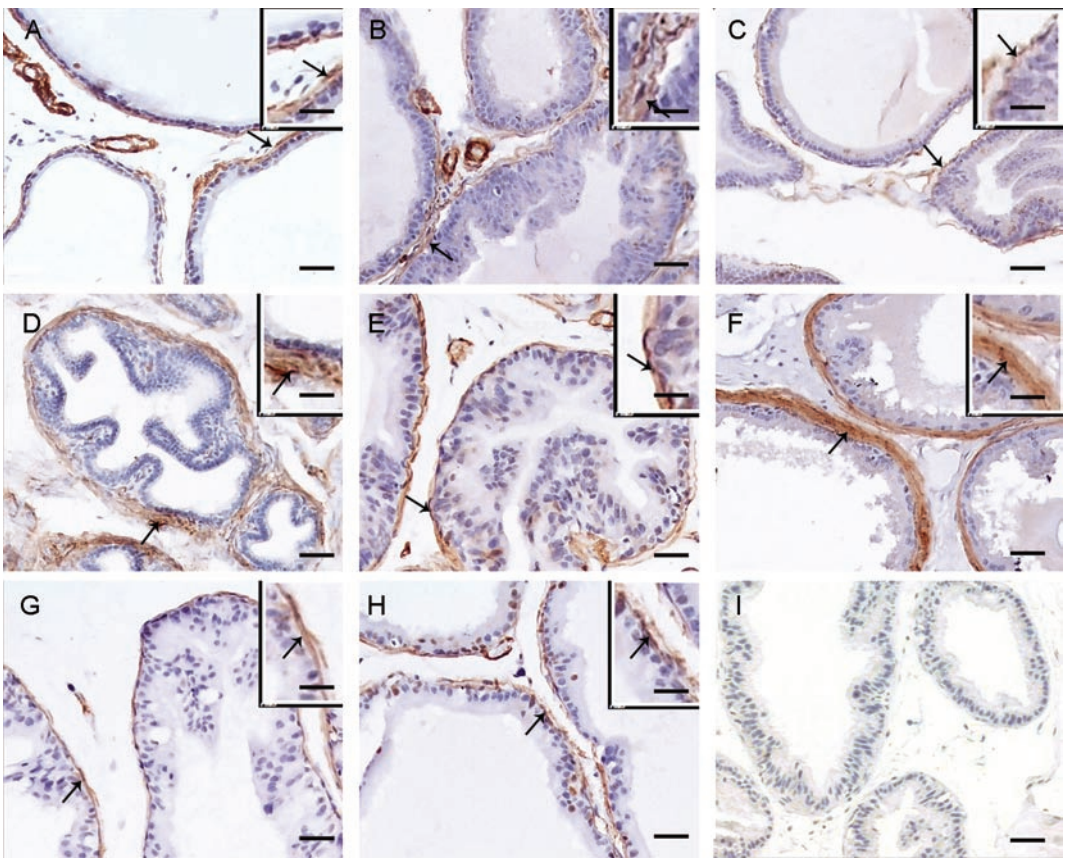

Figure 2. Smooth muscle actin (SMA) expression. (A) Sham-operated control, (B) castrated, (C) castrated + T, (D) castrated + E, (E) castrated + 1:50 E/T, (F) castrated + 1:100 E/T, (G) castrated + 1:200 E/T, (H) castrated + 1:300 E/T, and (I) negative immunohistochemistry control. The arrows point to the positive cells. Original magnification × 200; × 400 for the insets.Scale bars are 50 μm and 25 μm (for the insets).

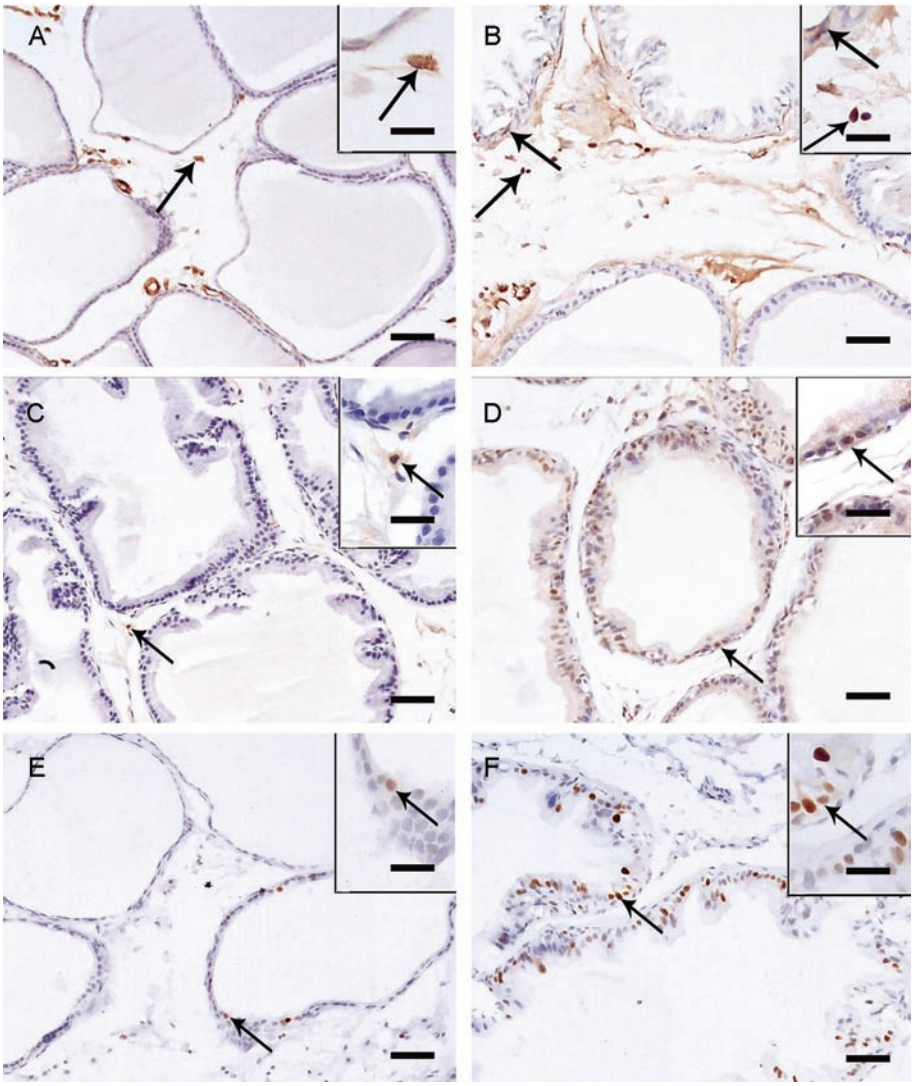

Figure 3. Immunohistochemistry data for (A) sham-operated Vimentin, (B) 1:100 E/T Vimentin, (C) sham-operated non-muscle myosin heavy chain (NMMHC) , (D) 1:100 E/T NMMHC, (E) sham-operated proliferating cell nuclear antigen (PCNA), and (F) 1:100 E/T PCNA. The arrows point to positive cells. Original magnification × 200; × 400 for the insets. Scale bars are 50 μm and 25 μm (for the insets).

Table 2. IHC-positive cells in the control and castrated + 1:100 E/T group (means ± s.d.).

	Control		Castrated+1:100 E/T	
	In stroma	In acinus	In stroma	In acinus
SMA[Δ]	4.10 ± 0.55	—	6.62 ± 0.52[*]	—
Vimentin[a]	7.78 ± 2.12	—	14.18 ± 2.74[*]	—
NMMHC[a]	1.90 ± 0.72	15.33 ± 3.60	5.07 ± 0.41[*]	37.13 ± 6.43[*]
PCNA[a]	1.81 ± 0.63	7.55 ± 1.55	3.42 ± 0.87[*]	14.95 ± 2.62[*]

Abbreviations: E/T, testosterone/estradiol; IHC, immunohistochemistry; NMMHC, non-muscle myosin heavy chain; PCNA, proliferating cell nuclear antigen; SMA, smooth muscle actin.
[Δ]Quantitative analysis of SMA-positive cells was determined by the thickness (μm) of the layer surrounding the acinus.
[a]Quantitative analysis was determined for the number of positive cells in the unit area at a magnification of × 400.
[*]$P < 0.05$, compared with the corresponding control group.

increased 2.7- and 2.4-fold, respectively ($P < 0.05$, Figure 3D and Table 2).

PCNA-positive cells were also rare in both in the stroma and in the epithelia of controls (Figure 3E). In the E/T 1:100 group, the number of PCNA-positive cells in the stroma and epithelia increased 1.9- and 2-fold, respectively ($P < 0.05$, Figure 3F and Table 2).

3.5 Determination of SMMHC and NMMHC expression by real time RT-PCR

Compared with controls, the prostates of the 1:100 E/T group showed a 2.4- and 1.9-fold increase in SMMHC and NMMHC expression, respectively ($P < 0.05$, Figure 4).

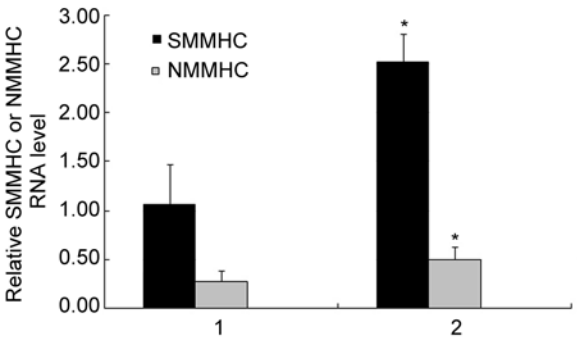

Figure 4. Smooth muscle myosin heavy chain (SMMHC) and non-muscle myosin heavy chain (NMMHC) gene expression in (1) sham-operated and (2) 1:100 E/T. $^{*}P < 0.05$, compared with the sham-operated group.

4 Discussion

There are two peaks in human prostatic growth [24, 25]. One occurs at puberty and the other occurs around age 50, when there is an increase in the ratio of E/T [2, 3]. The ratio of circulating E/T is about 1:150 in young men, whereas it ranges from 1:120 to 1:80 in older men [26]. In this study, we treated castrated rats with different ratios of E/T and showed that E/T at a ratio of 1:100 could induce distinct stromal hyperplasia. The rat prostate consists of ventral, dorsal and lateral lobes. As the ventral lobe is a common site for prostatic hypertrophy [27], the ventral prostates were collected and analysed. Interestingly, the ratio of 1:100 E/T is similar to that seen in older men. In addition, the main cell types in the human prostatic stroma are SMCs and fibroblasts. Rat stroma has SMA-positive SMCs that surround the epithelia and Vimentin-positive fibroblasts distributed among the acini. Although spontaneous prostatic hyperplasia develops infrequently in aged rats, we found that treatment with E/T could induce a significant thickening of the SMC layer. An increase in the thickness of this layer is characteristic of prostatic stromal hyperplasia. In this regard, rats can be used as an animal model for the study of human prostatic stromal hyperplasia.

The proportion of SMC increases in human prostatic stromal hyperplasia [5]. The phenotypes of the SMCs, which are the synthetic or contractile phenotype [28], are well illustrated by the expression of SMA and MHC [29]. There are two isoforms of MHC. One is SMMHC, which is associated with the contractile phenotype, and the other is NMMHC, which is associated with the synthetic phenotype. NMMHC is a non-muscle type of MHC that is predominantly expressed in embryonic smooth muscle and can be used as a molecular marker for dedifferentiated SMC [30]. SMCs can manifest phenotypic plasticity in response to physiological and pathological conditions, regardless of the tissue origin [6, 31]. The effect of estradiol on phenotypic modulation and proliferation was observed in rabbit aortic SMCs [32]. In this study, a prostatic stromal hyperplasia was induced in castrated rats treated with E/T, and the proliferation and phenotypic changes of stromal cells were investigated. Cells positive for SMA, NMMHC and PCNA in the stroma increased. The immunohistochemistry staining results showed a significant increase of the synthetic phenotype, which may derive from dedifferentiation of the contractile SMC induced by the E/T treatment.

The importance of the stromal interaction with the epithelium is well accepted [33, 34]. In this study, the NMMHC and PCNA-positive cells were found to increase in the rat epithelia. *In situ* hybridization also revealed NMMHC expression in the epithelial cells of human BPH [35]. The developing epithelium induces differentiation and morphological changes of the smooth muscle compartment [36]. In turn, the stromal cells can affect the structure and function of the epithelia [37]. McNeal [1] suggested that the stromal cells reversed to an embryonic state in BPH, which, in turn, could induce epithelial proliferation. Another viewpoint is that epithelial differentiation in the prostate takes place parallel to that of the stroma [38]. The triggering event of prostatic hyperplasia, whether it is in the stroma or epithelium, is still unknown.

In conclusion, we have showed that E/T at a ratio of 1:100 could induce distinct prostatic stromal changes in castrated rats. An increase of the synthetic SMC phe-

notype was a main finding, and epithelial dedifferentiation might also accompany the stromal changes.

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