Asian Journal of Andrology (2010) 12: 735–743

www.nature.com/aja

Original Article

Antagonism of estrogen-mediated cell proliferation by raloxifene in prevention of ageing-related prostatic hyperplasia

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Abstract

Estrogen has important roles in the initiation and development of benign prostatic hyperplasia (BPH). Regulators of the estrogen receptor (ER) are tissue- and cell-specific. We evaluated the effect of estrogen antagonist, raloxifene (Ral), on the prevention and treatment of BPH by investigating its effect on the proliferation of two different prostate cell lines: a stromal cell line, WPMY-1, and a benign prostatic hyperplasia epithelial cell line, BPH-1. We additionally evaluated its effect on prostatic hyperplasia induced by estrogen and androgen in a rat model. The effect of Ral on the prevention of prostatic hyperplasia was analyzed by haematoxylin and eosin staining and quantitative immunohistochemistry (IHC) for proliferating cell nuclear antigen and α-smooth muscle actin. *In vitro* and *in vivo*, tamoxifen (Tam), another anti-estrogen drug, and finasteride (Fin), a drug for the clinical treatment of BPH, served as efficacy controls. The *in vitro* data showed that neither Ral nor Tam alone affected the proliferation of WPMY-1 and BPH-1, but both antagonized the effect of oestradiol in promoting the proliferation of the two cells. Results from the IHC staining of the rat prostates indicated that, similar to Tam and Fin, Ral inhibited the proliferation of stromal cells *in vivo*. Interestingly, in contrast to Tam, both Ral and Fin inhibited the proliferation of epithelial cells. Furthemore, Ral treatment much strongly decreased the number of prostatic acini and the surrounding layers of smooth muscle cells than Fin ($P < 0.05$). Our data showed for the first time that Ral may have a role in the response of the rat prostate to selective ER modulators.

Asian Journal of Andrology (2010) 12: 735–743. doi: 10.1038/aja.2010.24; published online 17 May 2010.

Keywords: estrogen, prostatic hyperplasia, raloxifene, rats

1 Introduction

The prostate is a target organ of sex hormones. Estrogen and androgen together regulate the growth and development of the prostate. In older men, the circulating and intra-prostatic ratio of estrogen to androgen (E:T) increases, and is accompanied by an increased expression of estrogen receptor (ER) in the prostate [1–3]. There is evidence that enhanced estrogenic effect correlates with prostatic stromal hyperplasia [4]. A distinct prostatic stromal hyperplasia was observed in castrated rats that were treated with 1:100 E/T, a ratio that is similar to the circulating E/T ratio in older men [5]. Zhang *et al*. [6] reported that oestradiol (E2) pro-

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Received: 2 February 2010 Revised: 15 March 2010
Accepted: 29 March 2010 Published online: 17 May 2010

motes the phenotype of prostate smooth muscle cells (SMCs) in cultured prostate stromal cells (PrSCs). Using microarray analysis, Bektic *et al*. [7] compared the mRNA expression patterns in cultured PrSCs treated with or without E2, and found that estrogen regulates the expression of genes involved in cell proliferation and differentiation. It has been reported that E2 activates a non-genomic ERK pathway through the ERα, leading to the proliferation of PrSCs [8]. Our recent study showed that the effect of E2 on enhancing the SMC phenotype in cultured PrSCs is mediated by both non-genomic and genomic actions [9]. Besides its direct effect on PrSCs, E2 also regulates the proliferation and differentiation of PrSCs through prostatic epithelial cells in a paracrine manner [10]. Taken together, these findings support the possibility that anti-estrogen drugs may be effective in treating prostate stromal hyperplasia.

Selective ER modulators (SERMs), a class of compounds that act on ERs, have attracted intensive interest as alternatives of hormone replacement therapy. One distinct difference between these substances, the pure receptor agonists and antagonists, is that their action is tissue-specific, thereby allowing for the possibility to selectively inhibit or stimulate estrogen-like action in various tissues [11]. For example, tamoxifen (Tam) is an ER antagonist in the breast, but a partial stimulator in the uterus. Raloxifene (Ral) has estrogenic effects on the bone, and is used in the prevention of osteoporosis in post-menopausal women. Ral also has anti-estrogenic effects in some tissues, and it has been shown that Ral is as effective as Tam in reducing the incidence of breast cancer in certain high-risk group of females [12]. Based on the evidence from five recent large trials, Lee *et al*. [13] suggested that Ral might be the most attractive SERM agent, because it is effective in the preventing fractures in osteoporosis and decreasing the incidence of invasive breast cancer.

Sex hormones have important roles in both prostatic hyperplasia and prostate cancer. Recent progress on the use of SERMs in treating prostatic disease is highly encouraging. Tam can induce apoptosis in human PrSCs [14]. In addition, toremifene, Ral and Tam inhibit the proliferation of prostatic epithelial cells and PrSCs, and inhibit ER activity [15]. Ral significantly inhibits tumour metastases in rats and induces apoptosis in androgen-dependent and androgen-independent human prostatic cancer cell lines [16–18]. However, in a study of 17 benign prostatic hyperplasia (BPH) patients treated with Tam, Hanus and Matouskova [19] suggested that Tam monotherapy is not effective enough, and the authors recommended the administration of anti-estrogens in combination with other drugs. Such clinical studies suggest a need to screen other SERMs that may be more effective in preventing BPH. Using our previously published rat model, in which E/T treatment induced prostatic stromal hyperplasia after castration, we examined whether Ral, an experimental SERM being tested for other diseases, is also effective in the prevention and treatment of prostate stromal hyperplasia.

2 Materials and methods

2.1 Cell culture

WPMY-1 and BPH-1 cells were maintained in phenol red-free Dulbecco's modified Eagle's medium (DMEM) (Invitrogen, Carlsbad, CA, USA) supplemented with 100 mg mL^{-1} penicillin or streptomycin (Hyclone, Logan, UT, USA) and 5% fetal bovine serum (FBS; Invitrogen) at 37ºC in 5% CO_2.

2.2 Cell proliferation assays

Cell proliferation was analysed by the MTT assay. WPMY-1 and BPH-1 cells were seeded in 24-well and 96-well plates in quadruplicate at a density of 20 000 and 4 000 cells per well respectively in quadruplicate at a density of 20 000 cells per well, in a phenol red-free DMEM medium supplemented with 100 mg mL^{-1} penicillin or streptomycin and 2.5% charcoal-dextran-treated FBS (CDS; Invitrogen) for 2 days in a steroid-free environment. 17-β-Oestradiol (E2), finasteride (Fin), Tam and Ral (Sigma-Aldrich, St. Louis, MO, USA) were added at concentrations of 0.1 or 1 μmol L^{-1} with a final CDS concentration of 2.5%. ICI182,780 (ICI; Sigma-Aldrich), which is a kind of anti-estrogen, was added at concentrations 1 μmol L^{-1} as a positive control. After 48 h, MTT assay and cell counting were performed. To keep the hormone levels constant, the medium was refreshed every 24 h.

2.3 Animals and hormonal manipulations

A total of 96 adult male Wistar rats (250–300 g body weight) were obtained from the Weitong-Lihua Experimental Animal Center (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 Animal Care and Use Committee of Nankai Univer-

sity. Orchiectomy was carried out under ether anaesthesia through the scrotal route. Except for eight sham-operated controls, 88 rats were castrated and maintained for 3 weeks. Then, they were randomly assigned to 11 experimental groups with eight animals per group. Oestradiol benzoate/testosterone propionate (E/T; Jinyao Amino Acid Manufacturer, Tianjin, China), in a ratio of 1:100 (10 μg/1 000 μg), was given to the castrated rats by daily intra-peritoneal injections in 0.1 mL of corn oil, which was used as a vehicle, for 17 days. The daily doses of E/T, Fin, Tam and Ral in different treatment groups are shown in Table 1. Rats were killed under ether anaesthesia by cervical dislocation 48 h after the last injection. The prostate was dissected and the ventral prostate was fixed in phosphate-buffered formalin and embedded in paraffin for histological and immunohistochemical studies.

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 haematoxylin and eosin (H&E) for histological examination, and other sections were processed for immunohistochemistry (IHC) using the avidin–biotin–peroxidase complex method: endogenous peroxidase activity was blocked with 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 following primary antibodies were used: mouse monoclonal anti-proliferating cell nuclear antigen (PCNA) (1/100; Santa Cruz, CA, USA) and anti-α-smooth muscle actin (SMA) (1/400; Sigma-Aldrich). A biotinylated secondary antibody was added for 30 min at 37ºC followed by peroxidase-labelled streptavidin. The chromogen 3′, 3-diaminobenzidine was added and the slides were counterstained with hematoxylin. For a negative control, the primary antibody was replaced by non-specific immunoglobulin.

Table 1. Grouping method and drug manipulation.

Group	Daily dose
Control	0.1 mL corn oil
Model	0.1 mL E/T
Fin (low)	0.075 mg + 0.1 mL E/T
Fin (middle)	0.150 mg + 0.1 mL E/T
Fin (high)	0.300 mg + 0.1 mL E/T
Tam (low)	0.075 mg + 0.1 mL E/T
Tam (middle)	0.150 mg + 0.1 mL E/T
Tam (high)	0.300 mg + 0.1 mL E/T
Ral (low)	0.075 mg + 0.1 mL E/T
Ral (middle)	0.150 mg + 0.1 mL E/T
Ral (high)	0.300 mg + 0.1 mL E/T
Ral + Fin	0.150 mg + 0.150 mg + 0.1 mL E/T

Abbreviations: E/T, oestradiol benzoate/testosterone propionate; Fin, finasteride; Ral, raloxifen; Tam, tamoxifen.

2.5 Assessment and quantification of immunohistochemical staining

Light microscopy was carried out with an Olympus microscope (CX-41; Olympus, Tokyo, Japan). The thickness of the SMC layer surrounding the prostatic acini was measured by an ocular micrometer (AX0067, Olympus) in units of 2.5 μm at × 400 magnification. PCNA-positive cells and acini were counted using an ocular micrometer 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 Statistical analysis

Data are expressed as mean ± SD. SPSS software (SPSS Inc., Chicago, IL, USA) was used for statistical analysis. Comparison of group data was done by one-way analysis of variance (ANOVA) with a *post hoc* test. Differences were considered significant at $P < 0.05$.

3 Results

3.1 Ral antagonized estrogen-stimulated proliferation in PrSCs

E2 promoted the proliferation of the PrSC line WPMY-1 by 31% ($P < 0.05$), whereas Ral, Tam and ICI had no the effect. However, Ral, Tam and Fin antagonized the proliferation of WPMY-1 cells when used together with E2 by 17%, 16% and 25%, respectively ($P < 0.05$, Figure 1A). Cell counting provided the same results ($P < 0.05$, Figure 1B).

3.2 Ral antagonized estrogen-stimulated proliferation in benign prostatic hyperplasia epithelial cells

E2 promoted the proliferation of the BPH-1 by 23% ($P < 0.05$), whereas Tam, Ral and ICI had no the effect.

However, when used in combination with E2, Ral antagonized the estrogenic effect in promoting BPH-1 cell proliferation by 21% ($P < 0.05$). Similarly, Tam reduced estrogen-stimulated proliferation by 19% ($P < 0.05$). Fin alone inhibited BPH-1 cell proliferation by 26%, whereas it reduced estrogen-stimulated BPH-1 cell proliferation by 39% ($P < 0.05$, Figure 2A). Cell counting provided the same results ($P < 0.05$, Figure 2B).

3.3 Effect of Ral on preventing prostatic stromal hyperplasia in E/T-induced BPH rats

3.3.1 Quantitative analysis of rat prostate histology

H&E staining results of the rat prostate indicated that the lumens of prostate acini were normal in the sham-operated control group and that the epithelial cells in the prostatic acini appeared cuboidal or columnar and there was no obvious stromal hyperplasia (Figure 3A). In E/T induced hyperplasia model group, the thickness of SMC layers surrounding the acini was increased remarkably accompanying with slight glandular hyperplasia ($P < 0.05$, Figure 3B, Figure 4A). Compared with the model group, the number of acini in the middle

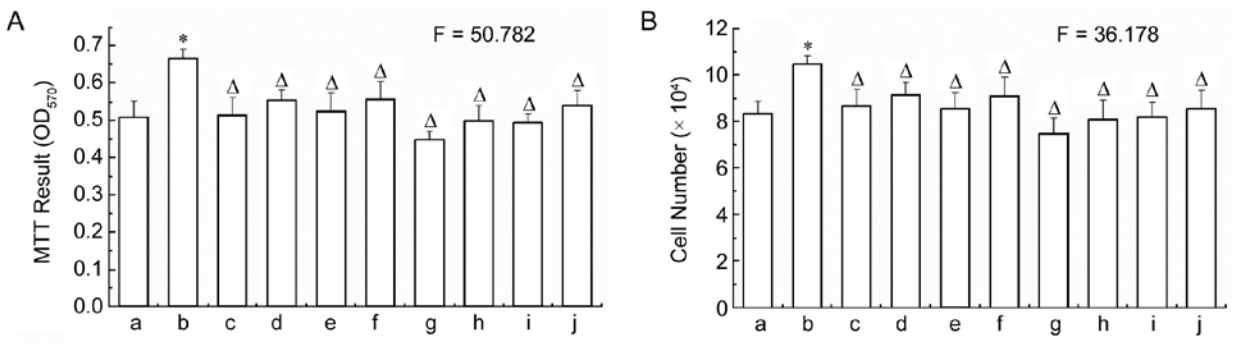

Figure 1. Raloxifene (Ral) antagonized estrogen-stimulated WPMY-1 cell proliferation. Prostate stromal WPMY-1 cells were seeded into a 24-well plate with 2×10^4 cells per well. After serum starvation for 24 h, cells were treated with 0.1 μmol L^{-1} 17-β-oestradiol (E2), tamoxifen (Tam), Ral, finasteride (Fin), and ICI alone or together with E2. After 48 h of incubation, proliferation was measured by MTT assay (A) and cell counting (B). a, control; b, 0.1 μmol L^{-1} E2; c, 1 μmol L^{-1} Ral; d, 1 μmol L^{-1} Ral + 0.1 μmol L^{-1} E2; e, 1 μmol L^{-1} Tam; f, 1 μmol L^{-1} Tam + 0.1 μmol L^{-1} E2; g, 1 μmol L^{-1} Fin; h, 1 μmol L^{-1} Fin + 0.1 μmol L^{-1} E2; i, 1 μmol L^{-1} ICI; j, 1 μmol L^{-1} ICI + 0.1 μmol L^{-1} E2. $^{*}P < 0.05$, compared with control, $^{\Delta}P < 0.05$, compared with 0.1 μmol L^{-1} E2.

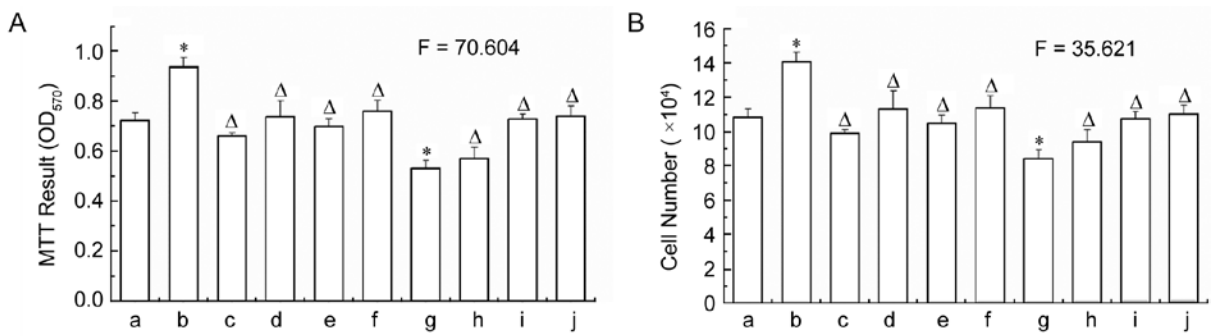

Figure 2. Ral antagonized estrogen-stimulated benign prostatic hyperplasia-1 (BPH-1) cell proliferation. Prostatic epithelial BPH-1 cells were seeded into a 96-well plate with 4×10^3 cells per well. After serum starvation for 24 h, the cells were treated with Tam, Ral, Fin or ICI alone or together with oestradiol (E2). After 48 h incubation, proliferation was determined by MTT assay (A) and cell counting (B). a, control; b, 0.1 μmol L^{-1} E2; c, 1 μmol L^{-1} Ral; d, 1 μmol L^{-1} Ral + 0.1 μmol L^{-1} E2; e, 1 μmol L^{-1} Tam; f, 1 μmol L^{-1} Tam + 0.1 μmol L^{-1} E2; g, 1 μmol L^{-1} Fin; h, 1 μmol L^{-1} Fin + 0.1 μmol L^{-1} E2; i, 1 μmol L^{-1} ICI; j, 1 μmol L^{-1} ICI + 0.1 μmol L^{-1} E2. $^{*}P < 0.05$, compared with control; $^{\Delta}P < 0.05$, compared with 0.1 μmol L^{-1} E2.

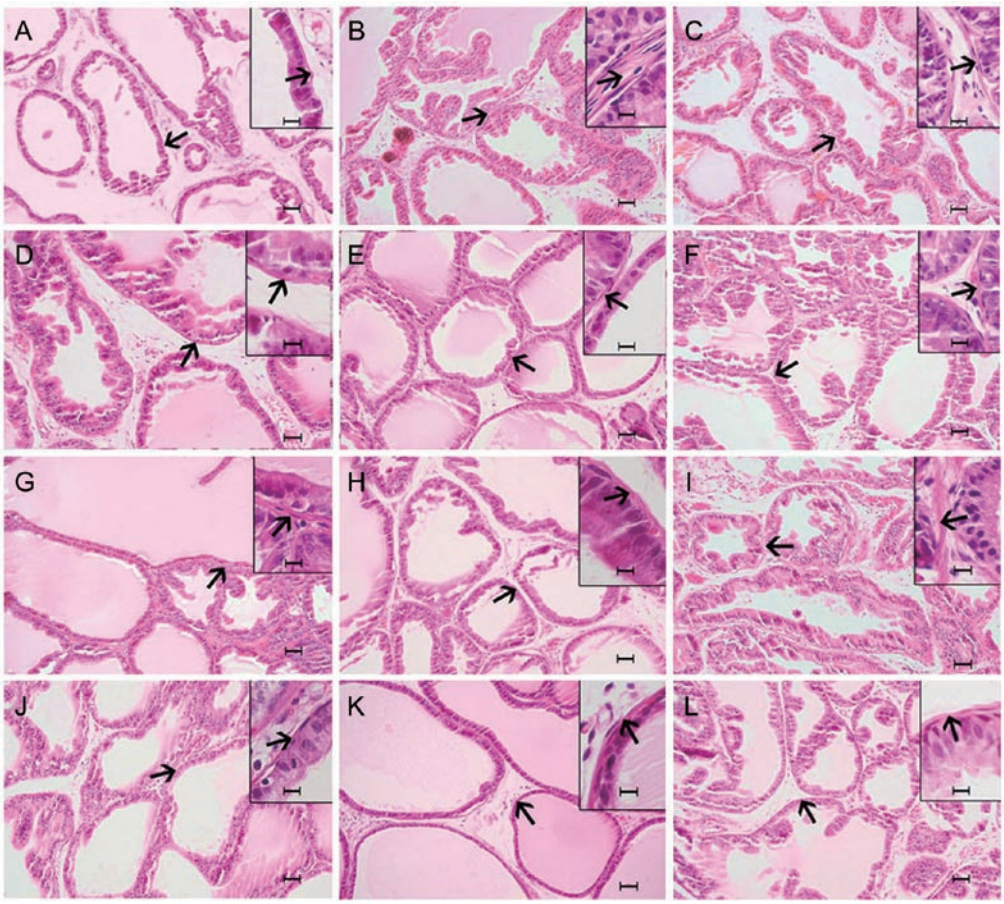

Figure 3. Histology of rat prostates. (A): Sham-operated control, (B): model, (C)–(E): Fin (low, middle and high doses), (F)–(H): Tam (low, middle, high doses), (I)–(K): Ral (low, middle and high doses), (L): Ral + Fin. The arrows point to the stromal cells. Magnifications: originals × 200; insets × 400. Scale bars are 50 μm for the originals and 25 μm for the insets.

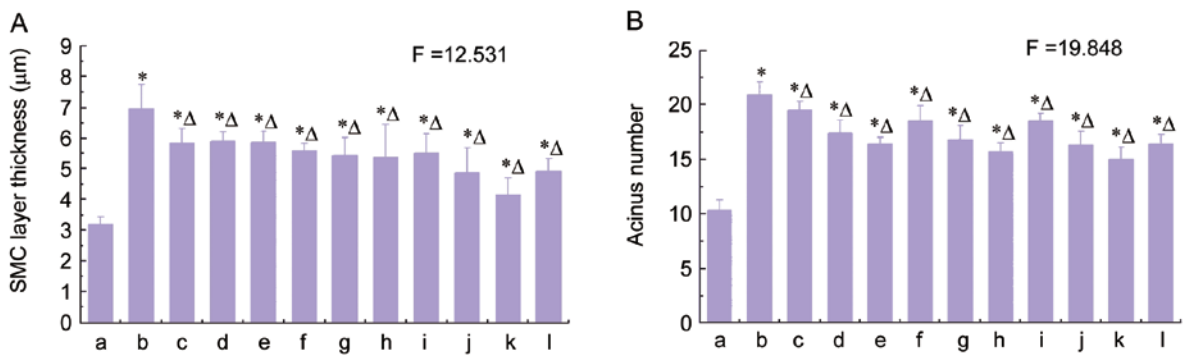

Figure 4. Quantitative analysis of histology of rat prostates. a, sham-operated control; b, model; c–e, Fin (low, middle and high doses); f–h, Tam (low, middle and high doses); i–k, Ral (low, middle and high doses); i, Ral + Fin. Five haematoxylin and eosin (H&E) stained pathologic slides were chosen from each group. In each slide, 8–10 fields were picked randomly, the thickness of the smooth muscle cell (SMC) layers surrounding the acini (A) was measured and the number of acini (B) were counted with original (× 100) and enlarged (× 400) magnifications, respectively. $^{*}P < 0.05$, compared with control, $^{\Delta}P < 0.05$, compared with model.

and high doses of the Fin group decreased (Figure 4B). The epithelial cells appeared low columnar or flat, and the thickness of the SMC layer surrounding the acini decreased slightly (Figures 3C–E and Figure 4A). In the Tam (Figures 3F, 3G and 3H) and Ral (Figure 3I–K) groups, the number of acini also decreased (Figure 4B), and the epithelial cells changed from a columnar to a low columnar or flat shape. In addition, the SMC layer surrounding the acini became thinner (Figure 4A). A combination of Fin and Ral (Figure 3L) did not show further improvement compared with that of Ral alone.

Quantitative analysis of the thickness of the SMC layers surrounding the acini and the number of acini was performed for all groups. Compared with the model group, the thickness of the SMC layers surrounding the acini decreased in all treatment groups, and the effect was most obvious in the Ral group, with a reduction of 30% and 40% at the middle and high doses, respectively ($P < 0.05$). The effect of Ral + Fin was not different from that of Ral alone (Figure 4A).

Compared with the model group, the number of prostate acini decreased in the Ral group (low, middle and high doses) by 11%, 22% and 28% ($P < 0.05$), in the Tam group (low, middle and high dose) by 11%, 20% and 25% ($P < 0.05$), in the Fin group (low, middle and high dose) by 7%, 17% and 22% ($P < 0.05$) and in the Ral + Fin group by 22% ($P < 0.05$). Furthermore, the effect was more dramatic in the Ral and Tam groups than in the Fin group ($P < 0.05$). The Ral + Fin was not different from that of Ral alone (Figure 4B).

3.3.2 IHC and quantitative analysis of PCNA or SMA in rat prostate

Compared with sham-operated controls, PCNA-positive cells increased in prostatic acini by 400% ($P < 0.05$) and stroma by 198% ($P < 0.05$) in the model group (Figures 5A, 5B and 6). Compared with the model group, positive cells in the acini and stroma in the Fin-treated group were reduced by 69% and 40% ($P < 0.05$, Figures 5B, 5C and 6). In addition, compared with the model group, the Tam-treated group had similar amounts of positive cells in the acini, but a slightly decreased by 29% ($P < 0.05$) number of positive cells in the stroma (Figures 5B, 5D and 6). Com-

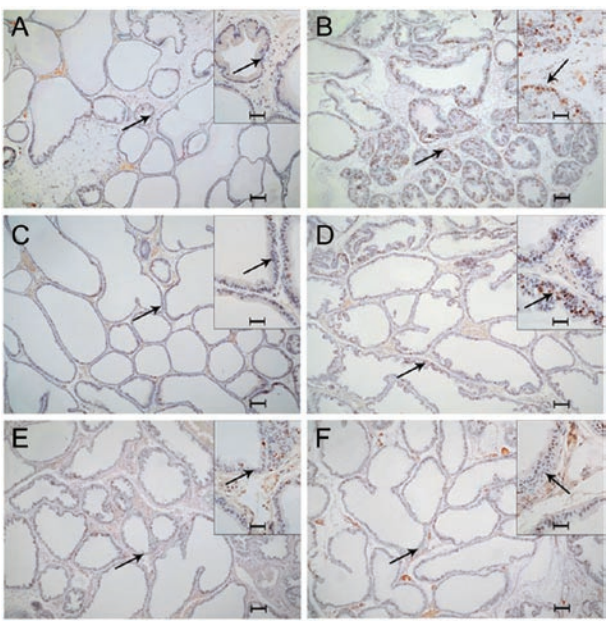

Figure 5. Proliferating cell nuclear antigen (PCNA) expression in rat prostates. (A): Sham-operated control, (B): model, (C): Fin, (D): Tam, (E): Ral, (F): Ral + Fin. The arrows point to the positive cells. Magnifications: originals × 100; insets × 400. Scale bars are 100 μm for originals and 25 μm for insets.

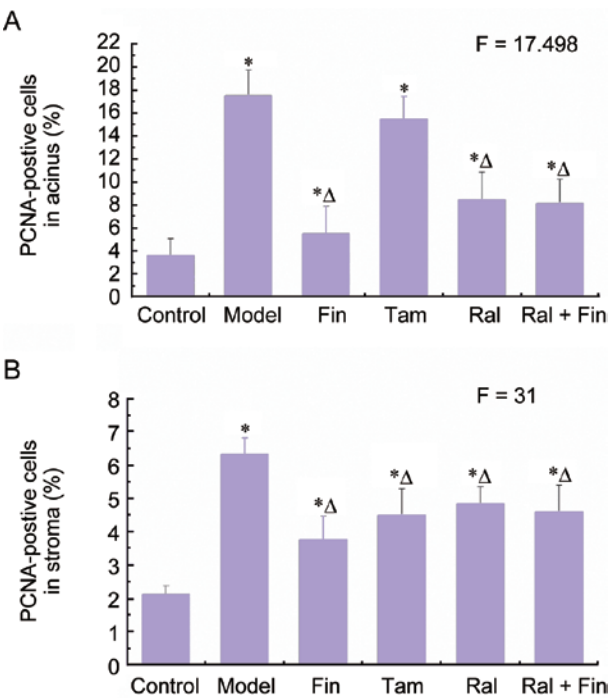

Figure 6. Quantitative analysis of PCNA expression in rat prostates. Five slides were chosen from each sample group for immunohistochemistry (IHC) staining. In each slide, 8–10 fields were picked randomly, and PCNA-positive stained cells were counted in the acini (A) and stroma (B) under the microscope with original magnification × 400 for the inserts. $^{*}P < 0.05$, compared with control; $^{\Delta}P < 0.05$, compared with model.

pared with the model group, positive cells in the acini in the Ral group and the Ral + Fin group were decreased by 52% and 54% ($P < 0.05$), whereas the decreases were 23% and 27% in the stroma, respectively ($P < 0.05$, Figures 5B, 5E, 5F and 6).

The thickness of the SMA-positive SMC layer surrounding the acini increased by 198% in the model group (Figures 7A, 7B and 8). This increase was reversed most significantly by Ral (46%, $P < 0.05$) and Ral + Fin (42%, $P < 0.05$) (Figures 7B, 7E, 7F and 8), and to a less degree by Fin (20%, $P < 0.05$) and Tam (35%, $P < 0.05$) (Figures 7B, 7C, 7D and 8). These results indicate that Ral, Tam and Fin are all capable of decreasing the thickness of the SMC layer, but that Ral was the most potent. There was no synergistic effect of the Ral and Fin combination.

4 Discussion

Estrogen regulates the proliferation and differentiation of stromal and epithelial cells through the autocrine or paracrine pathways [20]. Estrogen effects are tissue- and cell-specific, which is in part due to the differential expression of ERα and ERβ in different cell types. In the prostate, ERα is expressed mainly in stromal cells, and mediates estrogenic effect in promoting cell proliferation, whereas ERβ is expressed mainly in epithelial cells and mediates estrogenic effect on inhibiting proliferation [21–23]. Under pathological conditions, an increase in ERα expression in epithelial cells can mediate estrogenic effects to promote proliferation. Tam, one of the SERMs we used in this study, binds more selectively to ERα with a binding affinity higher than that of Ral but lower than that of estrogen. Tam mainly antagonizes the ERα-mediated estrogenic effect on promoting stromal cell proliferation. However, it has no influence on the estrogenic effect to promote epithelial cell proliferation mediated by ERβ. Ral selectively binds to ERβ with a higher affinity than Tam and estrogen. Ral can inhibit the proliferation of epithelial cells through ERβ, and it can also antagonize the ERα-mediated estrogenic effect in promoting stromal cell proliferation. Fin inhibits the production of the active form of androgen in prostate cells. Androgen promotes the proliferation of epithelial cells through the androgen receptor and also promotes stromal cell proliferation by regulating bFGF expression through the androgen receptor [24]. Therefore, Fin may also inhibit the proliferation of epithelial and stromal cells. Wu *et al*. [25] reported an increase in cell proliferation and a loss of cell differentiation in the prostate of mice lacking epithelial androgen receptors; therefore, the long-term effect of Fin treatment for BPH is uncertain.

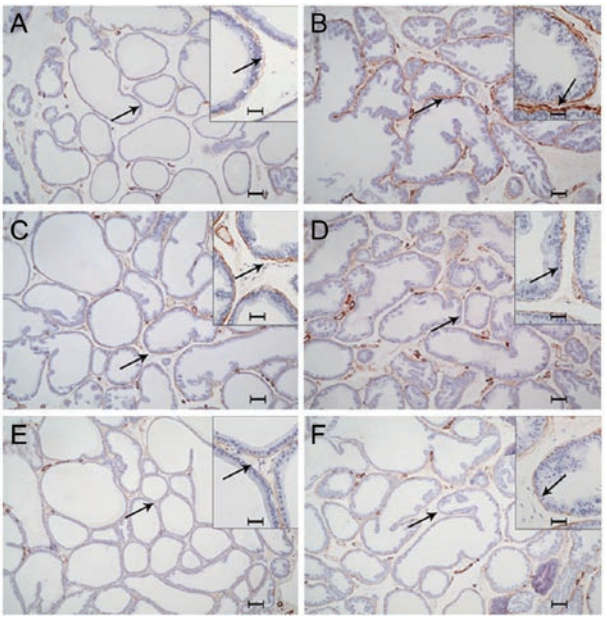

Figure 7. The α-smooth muscle actin (SMA) expression in rat prostates. (A): Sham-operated control. (B): Model. (C): Fin. (D): Tam. (E): Ral. (F): Ral + Fin. The arrows point to the positive cells. Magnifications: originals × 100; insets × 400. Scale bars: 100 μm for originals and 25 μm for insets.

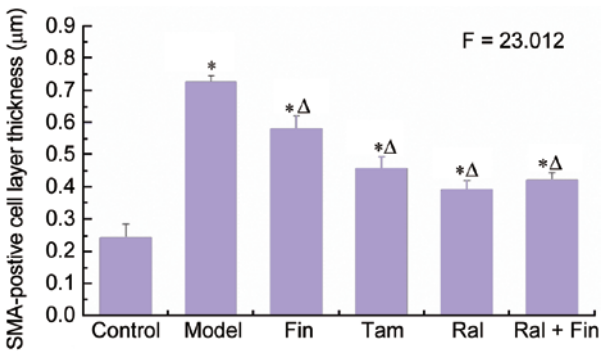

Figure 8. Quantitative analysis of SMA expression in rat prostates. Five slides were chosen from each sample group for IHC staining assay. In each slide, 8–10 fields were picked randomly. The thickness of the SMA-positive stained SMC layer was measured under the microscope with original magnification × 400 for the inserts. $^{*}P < 0.05$, compared with control; $^{\Delta}P < 0.05$, compared with model.

As BPH-1 and WPMY-1 express only ERα and

not ERβ, Ral and Tam cannot inhibit their proliferation on their own. However, they could antagonize the estrogenic effect on promoting the epithelial cell and the stromal cell proliferation mediated by ERα.

In our studies, PCNA was used as a proliferation marker in rat prostates. Our quantitative results showed that Ral, Tam and Fin could inhibit the proliferation of prostatic stromal cells. Ral and Fin inhibited the proliferation of prostatic epithelial cells, whereas Tam did not have this effect. The SMA IHC staining results showed that Ral was the most potent agent in the reversal of SMC thickness that was induced by E/T treatment. Our histology results also indicated that Ral was more effective than Fin in decreasing the SMC layers surrounding the acini and the number of acini. However, Ral and Fin have no synergistic effect on the prevention of BPH. Although Ral has been reported to inhibit cell proliferation and metastases in prostate cancer, there have not been any reports on its effect in BPH therapy. Herein, we have provided both *in vitro* and *in vivo* evidence showing for the first time that Ral may have a role in the response of the rat prostate to SERMs. Future studies are needed to further investigate whether Ral will serve as a new candidate drug for BPH therapy.

Acknowledgment

This research was funded by the following grants: the National Basic Research Programs, China (973 Programs, No. 2009CB918904, No. 2010CB945003), the National Natural Science Foundation of China (No. 30872592), Joint Research Fund for Overseas Chinese Scholars and Scholars in Hong Kong and Macao, China (No. 30928027), and the key research project of Tianjin Municipal Science and Technology Commission, China (No. 09ZCKFSF00800).

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