

Proprotein convertases modulate budding and branching morphogenesis of rat ventral prostate

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ABSTRACT The onset of prostate morphogenesis is involved in the interaction between mesenchyme and epithelium. Proprotein convertases (PCs) activate a variety of growth and differentiation factors including mesenchymal and epithelial factors, such as insulin-like growth factor (IGF) and transforming growth factor- β (TGF- β), which induce ductal budding and branching. In this study, we provide evidence that PCs play a critical role in prostatic budding from the urogenital sinus (UGS) and ductal branching morphogenesis of the neonatal rat ventral prostate. PCs were expressed only in the epithelial cells of neonatal rat prostate. PC activity in the ventral prostate was modulated by endogenous androgen. PC inhibition suppressed prostatic budding and branching. Taken together, our data indicates that androgen-induced PCs initiate the development of the prostate.

KEY WORDS: *proprotein convertase, prostate, development, furin, PCI*

Proprotein convertases (PCs) have been known as processing proteases that convert a wide variety of precursor proteins to their mature active form. To date, eight PCs have been identified including furin, PC1/3, PC2, PC4, PACE4, PC5/6 (and its isoform PC5B), PC7/8 and SKI-1. All PCs except for SKI-1 cleave precursor proteins at basic residues within the consensus motif (K/R)-(X)_n-(K/R)_↓, when n=0, 2, 4 or 6 and X is usually not Cys. All PCs except for SKI-1 are responsible for processing of matrix metalloproteinases (MMPs), growth factors, membrane receptors and adhesion molecules (Khatib *et al.*, 2004). Therefore, these processing enzymes are suggested to have a fundamental role in embryogenesis, development and disease via the activation of their substrates (Thomas 2002).

The prostate is a male sex accessory gland and develops from the embryonic urogenital sinus (UGS). Prostatic buds emerge from urogenital sinus epithelium (UGE) and extend into urogenital sinus mesenchyme (UGM) where they undergo ductal branching morphogenesis under androgenic stimulation of interactions between mesenchymal and epithelial cells (Sugimura *et al.*, 1986). In the developing prostate, both growth and differentiation factors are derived from mesenchyme via androgenic stimulation which induce prostatic epithelial growth (Hayward *et al.*, 2000). As candidates of these factors, insulin-like growth factor (IGF-1), hepatocyte growth factor (HGF), transforming growth factor- β (TGF- β), bone morphogenetic protein-4 (BMP-4) and prostate differentiation factor (PDF) were reported previously (Hayward *et al.*, 2000, Lamm *et al.*, 2001, Paralkar *et al.*, 1998). Many growth and differentiation factors are first

synthesized as larger biologically inactive precursors, which are proteolytically activated by PCs. Previously we reported that the inhibition of PC activity resulted in the loss of the luminal cell phenotype and induction of the basal cell phenotype in LNCaP (Uchida *et al.*, 2003). Our results suggest that PCs activity may be involved in the regulation of prostate epithelial cell differentiation. The role of PCs in the development of the prostate remains largely unknown. Therefore, we hypothesized that PCs regulate budding and branching morphogenesis of the prostate epithelium via activation of growth and differentiation factors.

In this study, we investigated the distribution of PCs and the effect of the PCs inhibitor, decanoyl-Arg-Val-Lys-Arg-chloromethylketone (CMK) on prostatic bud formation from the UGS and the development of rat ventral prostate.

Results and Discussion

PC expression in the UGS and neonatal rat prostate

All PCs except for PC4, which is confined to the testis and ovary, are expressed widely in a variety of tissues and cells (Hatsuzawa *et al.*, 1990). However, the expression, localization

Abbreviations used in this paper: CMK, decanoyl-Arg-Val-Lys-Arg-chloromethylketone; IGF, insulin-like growth factor; PC, proprotein convertase; TGF, transforming growth factor; UGS urogenital sinus; VP, ventral prostate.

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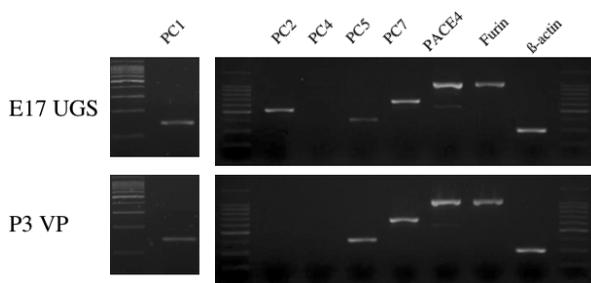


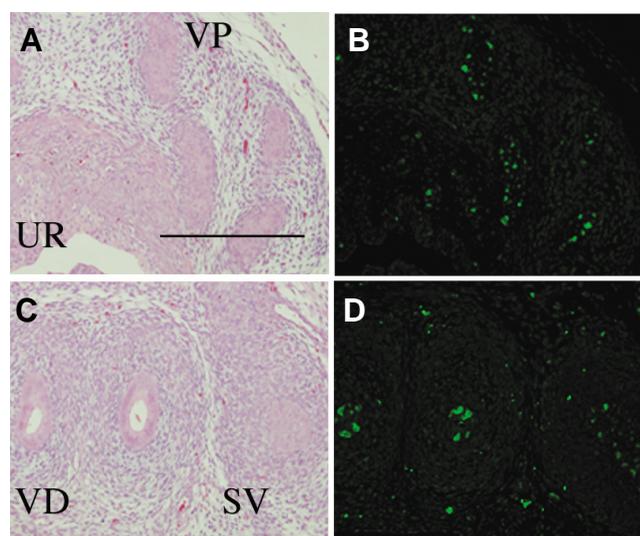
Fig. 1. Proprotein convertase (PC) mRNA expression in E17 urogenital sinus (UGS) and P3 ventral prostate. Amplification products for PC1 and other PCs were separated by 2 % and 4 % agarose gel electrophoresis, respectively. PC2 mRNA was detected in the E17 UGS, not in the P3 ventral prostate

Fig. 2. PC1 distribution in the developing prostate. Light micrographs of HE staining of P3 rat prostate (A,C) and immunostaining for PC1 (B,D). PC1-positive cells were detected in the epithelial cells of the VP (B), SV and VD (D). PC1 staining was not observed in the epithelial cells of the urethra and mesenchymal cells of the prostate. Abbreviations: SV, seminal vesicle; UR urethra; VD, vas deferens; VP, ventral prostate. Bar, 200 μ m.

and function of PCs in the prostate are still unknown. We first examined the gene expression and protein distribution of PCs in the developing prostate. Gene expressions of all PCs except for PC4 were detected in E17 UGS by RT-PCR. In contrast, PC2 and PC4 gene expression was not detected in P3 VP, whereas mRNAs of PC1, PC5, PC7, furin and PACE4 were observed (Fig. 1). Next we confirmed PCs expression and distribution by immunofluorescence staining. We examined the distribution of furin and PC1 in the developing prostate by immunofluorescence staining. At P3, cytoplasmic staining of PC1 was observed in the epithelial cells of the prostate, seminal vesicle and vas deferens. PC1 immunoreactivity was not observed in the mesenchymal cells of the prostate (Fig. 2). PC1 was also detected in endothelial cells and red blood cells. The distribution of furin-positive cells was similar to that of PC1-positive cells (data not shown). Furin and PC1 expressions were not observed in urethral mesenchyme and epithelium. At E17, furin and PC1 were detected in the epithelial cells of the vas deferens, but not epithelial cells of the UGS (data not shown). The discrepancy in PCs expression at UGS between RT-PCR and immunofluorescence staining could be due to the lower expression of PCs or the contamination of the vas deferens.

The modulation of PC activity in the neonatal ventral prostate

Previous studies demonstrated that androgenic effects on epithelial growth, budding and ductal branching morphogenesis of the prostate were mediated by mesenchymal androgen receptors (Cunha *et al.*, 1980). Therefore, we hypothesized that the activity of epithelial PCs could be regulated by testosterone via epithelial-mesenchymal interaction. To investigate regulation of PCs activity by testosterone, E17 UGS and P1 VPs were cultured with or without testosterone. Comparable cultures were treated with CMK, the PC inhibitor. Results demonstrated that PCs activity was significantly increased by the addition of testosterone and PCs activity induced by testosterone was significantly inhibited by CMK at 50 μ M in P1 VP (Fig. 3B), but not in E17 UGS (Fig. 3A).



Our results suggested that PC were expressed in the epithelium of the prostate during the period of budding and branching morphogenesis and that the activity of epithelial PCs was regulated by testosterone via epithelial-mesenchymal interaction. Our results also showed that testosterone treatment had minimal effect on P1 VP and did not affect PCs activity in UGS. These could be due to the lower activity of specific PC modulated by

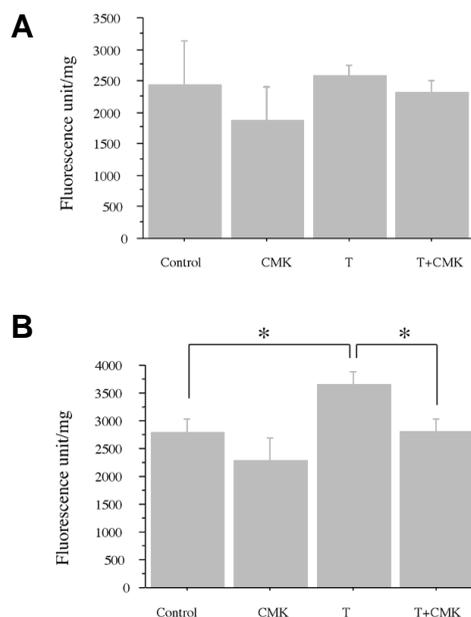


Fig. 3. Proprotein convertase (PC) activity as measured by boc-RVRR-AMC processing. (A) The effect of 10 nM testosterone and 50 μ M CMK on PC activity in the urogenital sinus. No significant differences were observed. (B) The effect of 10 nM testosterone and 50 μ M CMK on PC activity of the rat P1 ventral prostate. PC activity was significantly increased by the addition of 10 nM testosterone. Testosterone-induced PC activity was significantly decreased by 50 μ M CMK. * p < 0.05

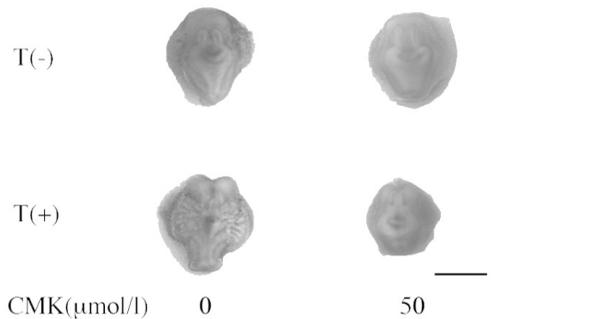


Fig. 4. Effect of the proprotein convertase inhibitor, CMK, on prostatic bud formation. Representative images of rat urogenital sinus (UGS) cultured for 4 days in serum free medium without testosterone and CMK, with 10 nM testosterone alone, with both 50 μ M CMK + 10 nM testosterone and 50 μ M CMK alone. Prostatic budding was observed by androgen stimulation. Androgen-stimulated budding was inhibited by treatment with 50 μ M CMK. Bar, 1 mm.

testosterone or the selectivity of boc-RVRR-AMC for specific PC, because wide variety of PCs was expressed in VP and UGS.

Effect of the proprotein convertase inhibitor, CMK, on prostatic bud formation and branching morphogenesis

We next focused the effect of PCs on the prostatic budding and branching morphogenesis using *in vitro* organ culture system. Rat E17 UGS were cultured for 4 days and P1 VPs for 6 days. In the presence of testosterone at 10 nM, prostatic buds emerged from the UGE and elongated into the UGM. Testosterone-induced prostatic budding was inhibited by the addition of 50 μ M CMK (Fig. 4). Neonatal VPs grew and underwent ductal branching morphogenesis extensively with 10 nM testosterone. The addition of CMK inhibited the growth of neonatal prostate and significantly induced the reduction of the number of ductal tips with or without 10 nM testosterone in a dose- and a time-dependent manner (Fig 5A, B).

Histological and immunohistochemical analysis of cultured VPs was examined. Microscopically, the higher frequency of canalization was observed in the prostate treated with testosterone alone, not in the prostate with CMK (Fig. 6A). The diameter of ducts was significantly increased by the addition of CMK (Fig. 6B). In the prostate treated with testosterone alone, nuclear

TABLE 1

PRIMERS USED FOR RT-PCR AMPLIFICATION

Gene	Primers	fragment size (bp)	Reference
PC1/3	S ^a : 5'-GCTACTAATCTCACTCAAAGC-3' AS: 5'-CCTTCTCTTAATATGCCAAC-3'	143	Lee 1999
PC2	S: 5'-GCTAGACTTGAATGTGGC-3' AS: 5'-GTTGCACTCATCGTAGCT-3'	525	Rovere 1996
PC4	S: 5'-AGCCGCAACACATACATC-3' AS: 5'-GCCATCGCAGCATACAGTCA-3'	680	
PC5	S: 5'-CTGCTGGTTAAAGGTGAGCCA-3' AS: 5'-TCACCAGCAGCTCTTTCTCC	403	Akamatsu 2000
PC7	S: 5'-GCCAGGAAGAGACATCAAT-3' AS: 5'-CATCTTGTCTCCTCCACTGA-3'	640	
PACE4	S: 5'-CCCTCTGGAACCAAGTCTCAACTT-3' AS: 5'-TGAAGCCAGCTTTACATCTGCTGC-3'	1041	Akamatsu 2000
Furin	S: 5'-AATGGTGTCTGTGGTGTAGG-3' AS: 5'-GTCAGCGTCCATAGTTGTT-3'	1050	

^aS, sense; AS, antisense

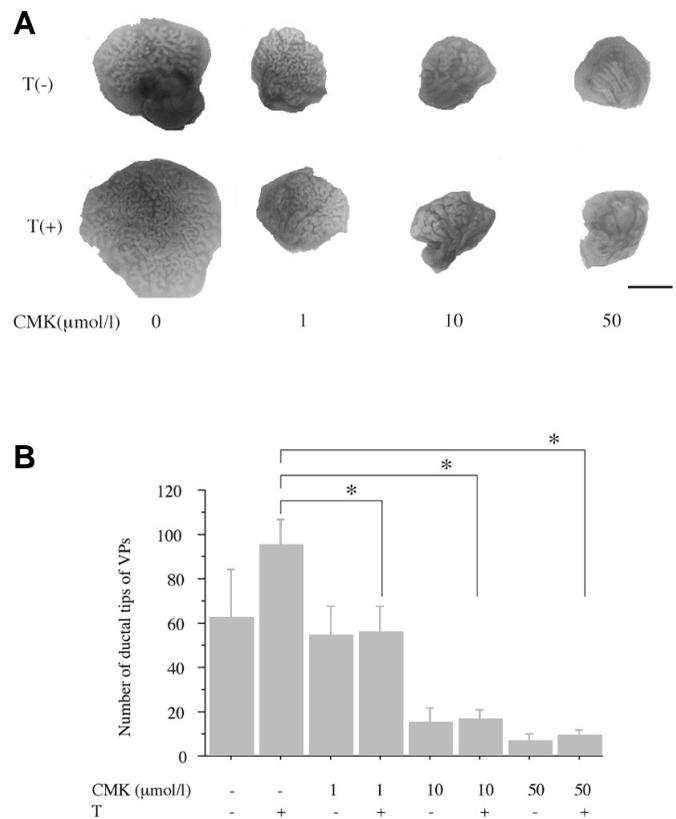


Fig. 5. Effect of the proprotein convertase inhibitor, CMK, on branching morphogenesis. (A) Representative images of rat P1 ventral prostates cultured for 6 days in serum free medium with/without 10 nM testosterone, with various concentrations of CMK. Prostatic ductal growth and branching were observed by androgen stimulation. Androgen-stimulated growth was inhibited by treatment with CMK in a dose-dependent manner. Bar, 2 mm. (B) Quantification of branching with CMK treatment. Branching was significantly increased with testosterone and significantly inhibited by the addition of CMK in a dose-dependent manner. * $p < 0.05$

staining of PCNA was more frequently observed in the ductal tip than in the base of the prostate by immunohistochemical staining. In contrast, PCNA expression was frequently observed in the tip and base of the prostate treated with testosterone + CMK (Fig. 6C).

Prostatic development is characterized by the bud formation, canalization and branching mediated via the epithelial-mesenchymal interaction under the balance of stimulatory and inhibitory factors. Among PCs substrates, IGF-1 and HGF are considered to be responsible for prostatic development as the stimulatory factors and BMP-4 is known as the inhibitory factor. TGF- β has the stimulatory effects on the periphery and inhibitory effects on the center of the prostate (Tomlinson *et al.*, 2004). In addition, previous study demonstrated that MMPs and integrins, which were considered as PCs substrates, also had the stimulatory effect on the branching of mammary gland and ureteric bud (Simian *et al.*, 2001, Pohl *et al.*, 2000, Zent *et al.*, 2001). PCs inhibition might cause an imbalance in the activation of stimulatory and inhibitory substrates. In fact, our macroscopic observa-

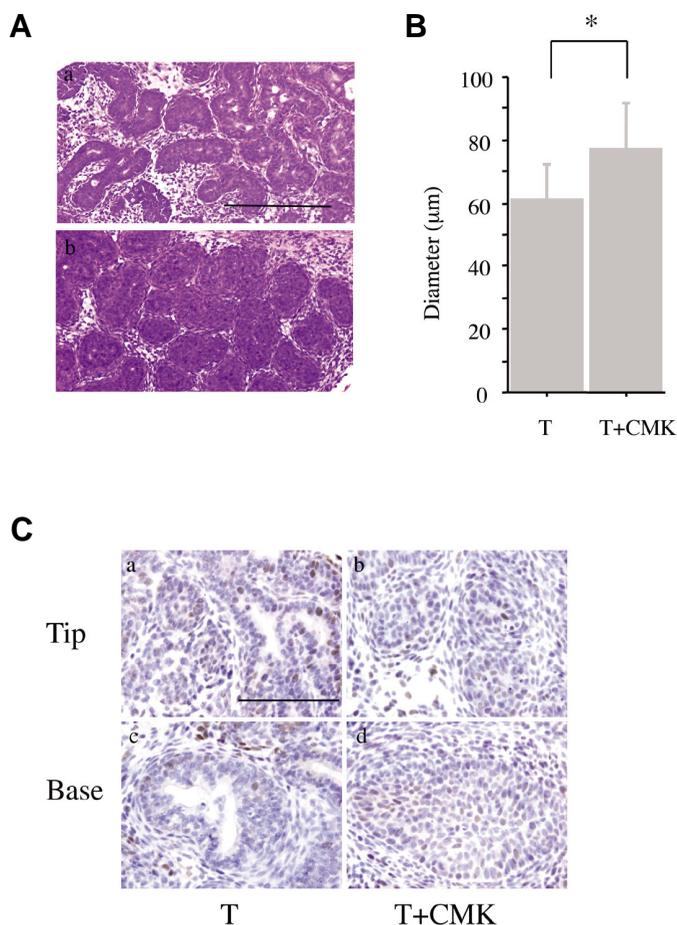


Fig. 6. Effect of the proprotein convertase inhibitor, CMK, on branching morphogenesis – microscopic and immunohistochemical analysis. (A) Representative microscopic images (HE staining) of rat P1 ventral prostates cultured for 6 days in serum free medium with 10 nM testosterone, with/without 10 μ M CMK. The higher frequency of canalization, the lower cell density of epithelium was observed in the prostate treated with testosterone without CMK (a), not in the prostate with CMK (b). Bar, 200 μ m. (B) The diameter of ducts with testosterone, with/without CMK. The diameter was significantly increased by the addition of CMK. * $p < 0.05$. (C) Immunostaining for PCNA of the prostate treated with testosterone alone (a, c) and with testosterone and CMK (b, d). PCNA expression was observed in the ductal tip (a, b) and base (c, d). Bar, 200 μ m.

tion showed that PCs inhibitor reduced prostatic budding and branching, suggesting that BMP-4 activation might be accelerated by the inhibition of PCs. On the other hand, microscopic observation showed that PCs inhibitor induced the enlargement of the prostatic epithelial cord and the reduction of canalization. These results could be associated with the inactivation of morphogens, such as TGF- β and PDF, by the inhibition of PCs. In addition, PCNA expression indicated that PCs inhibitor initiated the epithelial proliferation in the base, which might represent the imbalance of the prostatic growth and be resulted in the enlargement of the prostatic epithelial cord. Our results indicate PCs are involved in the usual process of the prostatic development, including the budding, cord formation, canalization and branching. Therefore, the difference of the phenotypic expression in the prostate treated with or without PCs inhibitor would be important.

The expression and localization of each PC, by which substrates are predominantly activated and responsible for the each steps of prostatic development, should be studied further.

In conclusion, our results suggest an important role of PCs in the prostatic morphogenesis. PCs modulated by androgen are expressed in neonatal VP and may be responsible for the processing of mesenchymal and epithelial factors, which initiate budding and branching of the prostate.

Materials and Methods

Materials

The animal use procedures were in accordance with the guide for the care and use of laboratory animals of Mie University Graduate School of Medicine. Sprague-Dawley (SD) rats were obtained from CLEA (Tokyo, Japan). Testosterone, insulin and transferrin were obtained from Sigma at MO. DMEM-Ham's F12 was purchased from Invitrogen (Carlsbad, CA), gentamycin from Wako (Osaka, Japan). N-t-butoxycarbonyl-Arg-Val-Arg-Arg-7-amino-4-methylcoumarine (Boc-RVRR-AMC) was obtained from Bachem (King of Prussia, PA) and decanoyl-Arg-Val-Lys-Arg-chloromethylketone (CMK) from Sigma (St. Louis, MO). Anti-furin and anti-PCNA antibodies were purchased from Santa Cruz (Santa Cruz, CA). Anti-PC1 antiserum was kindly gifted by Dr. Tanaka at Shizuoka University in Japan. FITC-labeled secondary antibody was purchased from MBL (Nagoya, Japan).

Tissue collection

Rat UGS and VPs were obtained from SD rats. The day of detection of a vaginal plaque and the day of birth was designed E0 and P0, respectively. UGS from E17 rats and VPs from P0 and P3 neonatal rats were dissected and used for immunofluorescence staining, PC activity assay and *in vitro* organ culture.

RT-PCR Analysis

Total RNA was prepared from E17 UGS and P3 VP using RNeasy (Qiagen, CA). cDNA was prepared using Superscript (Invitrogen, CA). The efficiency of each cDNA reaction was assayed by amplification of β -actin transcripts with primers for β -actin (R & D systems, MN). Primers used to amplify PCs gene were shown in Table 1. The viability of the primer for PC4 was conformed using neonatal rat testis. The first strand cDNA was amplified with amplitaq gold (Applied biosystems, NJ). The PCR condition was as follows: 1 cycle at 95°C at for 5 min; 35 cycles at 95°C for 15 s, 53-57°C for 30 s, 72°C for 1 min 30 s. Amplification products (5 μ l) were separated by 2-4% agarose gel electrophoresis.

Immunofluorescence staining

Dissected E17 UGS and P3 prostate were fixed with 10% formaldehyde for 3 h on ice, processed into paraffin and then sectioned at 3 μ m. Sections were deparaffinized with HistoClear. To enhance immunoreactivity for PCs, sections were oxidized before immunostaining with Gomori's oxidation mixture, as described previously (Kurabuchi and Tanaka, 1997). Rat pancreas was used as the positive control. Nonspecific binding was blocked with blocking buffer (DAKO, CA) for 1 hour at room temperature. Sections were sequentially incubated overnight at 4°C with following reagents: anti-furin antibody (1:50), anti-PC1 antiserum (1:500). The next day, sections were rinsed and incubated in a FITC-labeled secondary antibody and processed according to the manufacture's instructions.

Proprotein convertase activity assay

E17 UGS and P1 VP were cultured with and without 10 nM testosterone and 50 μ M CMK. After 24 h incubation, UGS and VP were washed with PBS and incubated with assay medium which consisted of the growth medium containing 0.25 % Triton X-100 to permeabilize cells and boc-RVRR-AMC (100 μ M) as a fluorogenic substrate. Fluorescence was

measured at 360 nm excitation and 460 nm emission wavelengths after 4 h of substrate addition. Tissue weights of UGS and VPs were measured for normalization before the addition of assay medium. The data were normalized to tissue weights

In vitro organ culture

UGS from E17 rats and VPs from P1 rats were grown in an organ culture system as previously described with modifications (Sugimura *et al.*, 1996). Briefly, dissected tissues were placed on Millicell membranes (Millipore, Bedford, MA) in 6-well plates (BD bioscience, Franklin Lakes, NJ) and cultured in DMEM-Ham's F12 without phenol red supplemented with insulin (10 µg/ml), transferrin (10 µg/ml) and gentamycin (50 µg/ml) at 5% CO₂. Testosterone (10 nM) and various concentrations of CMK were added and the medium was changed every day. Individual ductal tips were counted manually. Four to five VPs or UGSs were used for each group in one experiment. The experiment was repeated at least three times.

Histological and immunohistochemical analysis of cultured VPs

The prostates cultured with 10 nM testosterone with/without 10 µM CMK, were processed into paraffin and then sectioned at 3 µm. Sections were deparaffinized with Histoclear, used for Hematoxylin-Eosin (HE) staining and immunohistochemical staining. The diameter of the prostatic duct was measured under light microscope. Immunohistochemical staining was performed with Vectastain ABC kit (Vectastain Laboratories, CA). Briefly, sections were incubated overnight at 4°C with following reagents: anti-PCNA antibody (1:5000), The next day, sections were rinsed and incubated in a secondary antibody and processed.

Statistical analysis

Data are presented as the mean SD of four to five samples in each of two to six independent trials. Statistical analysis was performed using the Student *t* test. P value < 0.05 was considered significant.

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