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Title Page

Intermediate-conductance Ca^{2+}-activated K^+ channel, $K_{Ca}3.1$, as a novel therapeutic target of benign prostatic hyperplasia

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Running title: K_{Ca}3.1 as a novel therapeutic target of BPH

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Abbreviations: BPH: benign prostate hyperplasia; **REST**: a repressor element 1-silencing transcription factor; **UGS**: urogenital sinus; **K_{Ca} channel**: Ca^{2+}-activated K^+ channel,**IK_{Ca} channel**: intermediate-conductance K_{Ca} channel; **AP-1**: nuclear transcription factor activator protein-1; **PCNA**: proliferating cell nuclear antigen; **TRAM-34**: 1-[(2-Chlorophenyl)diphenylmethyl]-1H-pyrazole; **TRP**: Transient receptor potential; **CRAC channel**: Ca^{2+}-release-activated Ca^{2+} channel; **STIM**: stromal interaction molecule.

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Abstract

Recently, a new experimental stromal hyperplasia animal model corresponding to clinical benign prostatic hyperplasia (BPH) was established. The main objective of this study was to elucidate the roles of the intermediate-conductance Ca^{2+}-activated K^+ channel ($K_{Ca}3.1$) in the implanted urogenital sinus (UGS) of stromal hyperplasia BPH model rats. Using DNA microarray, real-time PCR, Western blot, and/or immunohistochemical analyses, we identified the expression of $K_{Ca}3.1$ and its transcriptional regulators in implanted UGS of BPH model rats and prostate needle-biopsy samples and surgical prostate specimens of BPH patients. We also examined the *in vivo* effects of a $K_{Ca}3.1$ blocker, TRAM-34, on the proliferation index of implanted UGS by measurement of UGS weights and PCNA immunostaining. $K_{Ca}3.1$ genes and proteins were highly expressed in implanted UGS rather than in the normal host prostate. In implanted UGS, the gene expressions of two transcriptional regulators of $K_{Ca}3.1$, a repressor element 1-silencing transcription factor (REST) and c-Jun, were significantly down- and up-regulated, and the regulations were negatively or positively correlated with $K_{Ca}3.1$ expression, respectively. Positive signals of $K_{Ca}3.1$ proteins were detected exclusively in stromal cells, whereas they were scarcely immunolocalized to basal cells of the epithelium in implanted UGS. *In vivo* treatment with TRAM-34 significantly suppressed the increase in implanted UGS weights compared with the decrease in stromal cell components. Moreover, significant levels of $K_{Ca}3.1$ expression were observed in human BPH samples. $K_{Ca}3.1$ blockers may be a novel treatment option for patients suffering from BPH.

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Introduction

Benign prostatic hyperplasia (BPH) is noncancerous enlargement of the prostate gland with aging. BPH is demonstrated in 50% of men by the age of 60 years, and is evident in up to 90% of men by 85 years (Berry et al., 1984). BPH causes increased resistance to urine flow through the urethra and sometimes kidney damage, bladder stones, and urinary tract infections, and thereby affects the quality of life (QOL) in one third of men older than 50 years (Djavan, 2003). α_1-adrenoceptor antagonists are clinically used to decrease the periurethral tone of the prostatic smooth muscle (Anderson & Gratzke, 2007), and can regulate prostatic growth by inducing apoptosis of epithelial, stromal and smooth muscle cells, but their long-term use can induce therapeutic tolerance (Kojima et al., 2009a & 2009b). Anti-androgen therapy or surgery is used to resolve mechanical outlet obstructions (Geller, 1989).

BPH is a proliferative process of both the stromal and epithelial elements of the prostate arising in the periurethral and transition zones of the gland, and is hormonally dependent on testosterone and dihydrotestosterone production (Berry et al., 1984).The major component of clinical BPH specimens is stromal extracellular matrix, which is organized by smooth muscle, fibrous tissue element, and collagen (Shapiro et al., 1992); however, this feature is histologically different from the testosterone-induced rodent BPH model (Ishigooka et al., 1996),and this animal BPH model is not adequate to elucidate the pathological mechanisms of stromal hyperplasia. Recently, Mori *et al.* (2009) established a new experimental rodent model characterized by stromal hyperplasia. Urogenital sinuses implanted into the ventral prostate, 'implanted UGS', contain a much higher ratio of stroma than the age-matched ventral prostate and the testosterone-induced BPH model, and are similar to those in men with BPH.

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Ca^{2+}-activated K^+ (K_{Ca}) channel can directly communicate Ca^{2+} signal pathways to changes in membrane potential required for various cellular processes. In excitable cells, K_{Ca} channel activation contributes to the post-hyperpolarization that follows an action potential, and generally suppresses membrane excitability, whereas in non-excitable cells, hyperpolarization by K_{Ca} channel activation increases the driving force for Ca^{2+} entry, strongly associating with sustained Ca^{2+} influx (Stocker, 2004). Intermediate-conductance Ca^{2+}-activated K^+ (IK_{Ca}) channel is present in undifferentiated vascular and urinary bladder smooth muscles, and participates in the control of cellular functions such as cell proliferation and maintenance of resting tone (Köhler et al., 2003; Ohya et al., 2000). IK_{Ca} channel has been molecularly identified as $K_{Ca}3.1$ (Ishii et al., 1997), and is a possible molecular target for pharmacological intervention in restenosis, urinary incontinence, prostate cancer, and autoimmune diseases (Köhler et al., 2003; Wulff et al., 2007; Ohya et al., 2009). Additionally, at least two transcriptional regulators of $K_{Ca}3.1$ have been identified in vascular smooth muscle cells and T-lymphocytes: repressor element-1 silencing transcription factor (REST) and nuclear transcription factor activator protein-1, AP-1 (Fos/Jun) (Cheong et al., 2005; Ghanshani et al., 2000).

The main objective of this study was to elucidate the possibility that IK_{Ca} channel can be a potential therapeutic target in human BPH using a stromal hyperplasia BPH model. Expressions of $K_{Ca}3.1$ genes and proteins in implanted UGS of stromal hyperplasia BPH model rats were compared with the normal host prostate by DNA microarray, real-time PCR, Western blot and immunohistochemical analyses. We also determined whether implanted UGS weights were suppressed by treatment with TRAM-34, a specific IK_{Ca} channel inhibitor. Similar examinations were performed using human

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prostate needle-biopsy samples and surgical prostate specimens of BPH patients.

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Methods

RNA extraction, RT-PCR, and real-time PCR

Experimental model rats for BPH with stromal hyperplasia were prepared as previously reported (Mori et al., 2009).Implanted urogenital sinuses (UGS) and normal host prostate in stromal hyperplasia BPH model rats were dissected 3 weeks after implantation. All experiments were carried out in accordance with the guiding principles for the care and use of laboratory animals (the Science and International Affairs Bureau of the Japanese Ministry of Education, Science, Sports and Culture) and also with the approval of the ethics committee of Nagoya City University and Taiho Pharmaceutical Corp. Total RNA from human normal prostate was purchased from BD Biosciences (San Jose, CA, USA) and BioChain (Hayward, CA, USA) (21-50 years old, three distinct Lot No. samples). Total RNA extraction and reverse-transcription were performed as previously reported (Ohya et al., 2009).We enrolled 7 patients with BPH aged 54–86 (mean age: 70.4 ± 4.5 years). Prostate needle-biopsy samples and surgical prostate specimens of BPH patients were obtained with informed consent from all patients before the study, explaining the purpose and methods. The study was approved by the ethics committee of Nagoya City University. BPH was diagnosed on the basis of the International Prostatic Symptom Score (IPSS), the Quality of Life (QOL) index, digital rectal examination, ultrasonography, uroflowmetry and prostate needle-biopsy. The resulting cDNA product was amplified with gene-specific primers, designated using Primer Express^{TM} software (Ver 1.5; Applied Biosystems, Foster City, CA, USA).

Quantitative, real-time PCR performed with the use of SYBR Green chemistry on an ABI 7700 sequence detector system (Applied Biosystems) as previously reported (Ohya

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et al., 2009). The following PCR primers for rat clones were used for real-time PCR: K_{Ca}3.1 (GenBank accession number: NM_023021, 837-937), 101 bp; RE1-silencing transcription factor (REST) (NM_031788, 2367-2489), 123 bp; c-Fos (NM_022197, 897-1025), 129 bp; FosB (NM_001013146, 1075-1209), 135 bp; Fra-1 (NM_012953, 848-968), 121 bp; Fra-2 (NM_012954, 99-227), 129 bp; c-Jun (NM_021835, 1195-1317), 123 bp; JunB (NM_021836, 357-492), 136 bp; JunD (NM_138875, 1036-1160), 125 bp; β-actin (NM_031144, 419-519), 101 bp. The following PCR primers for human clones were used: K_{Ca}3.1 (NM_002250, 172-293), amplicon = 122 bp; REST (NM_005612, 1415-1545), 131 bp; c-Fos (NM_005252, 533-658), 126 bp; FosB (NM_006732, 1075-1209), 135 bp; Fra-1 (X16707, 680-781), 102 bp; Fra-2 (X16706, 102-226), 125 bp; c-Jun (NM_002228, 1851-1999), 149 bp; JunB (NM_002229, 271-391), 121 bp; JunD (NM_005354, 1027-1134), 108 bp; β-actin (NM_001101, 411-511), amplicon = 101 bp. Regression analyses of the mean values of three multiplex RT-PCRs for $\log_{10}$ diluted cDNA were used to generate standard curves. Unknown quantities relative to the standard curve for a particular set of primers were calculated, yielding transcriptional quantitation of gene products relative to the endogenous standard, β-actin. For PCR cloning of full-length K_{Ca}3.1, the following PCR primers were used; K_{Ca}3.1 (GenBank accession number: NM_023021, 77-1459), 1383 bp. To confirm the nucleotide sequences, amplified PCR products and plasmid constructs were sequenced with an ABI PRIZM 3100 genetic analyzer (Applied Biosystems).

Genome-wide DNA microarray analysis

To determine the quantity and purity of the RNA samples, purified DNase-free RNA

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from normal host prostate and implanted UGS was analyzed on a ND-1000 spectrophotometer (Nanodrop Technologies, Wilmington, DE) and a 2100-Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA). We then used 12 Affymetrix (Santa Clara, CA, USA) Rat Genome 230 2.0 Arrays. These microarrays contain over 31,000 genes. Labeling, hybridization (for 16 hrs), washing, and scanning of the microarray were performed by Bio Matrix Research (Chiba, Japan), following the manufacturer's specifications (i.e. One-Cycle Target Labeling kit). The arrays were scanned on the GCS 3000 Affymetrix high resolution scanner and analyzed using GeneChip Operating Software v1.4 (GCOS; Affymetrix) and GeneSpring v 7.3.1 (Agilent Technologies). Gene expression data were normalized by preparing 'Per-Chip_Only', and were confirmed by Pearson correlation. Comparative analysis among expression profiles of two samples was carried out using Gene Spring software. Only gene expression levels with statistical significance ($p<0.05$) were recorded as being "present" above background levels, and genes with expression levels below this statistical threshold were considered as "absent", as previously reported (Kojima et al., 2009a).

Western blot analysis

Plasma membrane protein fraction was prepared from rat tissues as previously reported (Ohya et al., 2005),and nuclear protein fraction was prepared using a ProteoJET^{TM} Cytoplasmic and Nuclear Protein Extraction kit (Fermentas UAB, Vilnius, Lithuania). Equal amounts of proteins were subjected to SDS-PAGE (10 %). Blots were incubated with anti-K_{Ca}3.1 (Alomone Labs, Jerusalem, Israel), anti-REST (Millipore, Temecula, CA, USA), and anti-c-Jun antibodies (Cell Signaling Technology, Danvers, MA, USA), and then incubated with anti-rabbit horseradish peroxidase-conjugated IgG (Millipore).

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An enhanced chemiluminescence detection system (GE Healthcare Japan, Tokyo, Japan) was used for detection of the bound antibody. The resulting images were analyzed by a LAS-1000 device (Fujifilm, Tokyo, Japan).

Immunohistochemical analysis

The implanted UGS from BPH model rats (4 weeks after implantation) and surgical prostate specimens from BPH patients were fixed in 10 % neutral-buffered-formalin, embedded in paraffin, and thin-sectioned at 2 μm (Mori et al., 2009). Tissue sections were deparaffinized in xylene followed by rehydration in graded alcoholic solutions and PBS(-). Endogenous peroxidase activity was blocked with 0.3 % hydrogen peroxide in methanol for 30 min at 4 °C followed by washing with PBS(-). Three different anti-K_{Ca}3.1 antibodies [Alomone Labs (APC-064), Abnova, Taipei, Taiwan (PAB7986), Abcam, Cambridge, UK (ab83740)] were used for immunostaining. All sections were developed by the avidin-biotin-complex method. The sections were also stained with hematoxylin and eosin staining and Masson's trichrome. Microscopic images of each section were captured on a computer and analyzed with an image analysis system (Win ROOF; Tech-Jam, Osaka).

Measurement of cell proliferation by PCNA immunohistochemistry

To examine the proliferation of stromal and epithelial compartments of implanted UGS after treatment with TRAM-34(Sigma-Aldrich, Saint Louis, MO, USA), the expression of proliferating cell nuclear antigen (PCNA) was analyzed as previously reported (Mori et al., 2009). After sectioning and staining, PCNA positive and negative cells were counted in ten separate microscopic fields for each graft. The percentage of

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PCNA-positive cells to total epithelium cells and stromal cells was calculated as the PCNA labeling index.

In vivo proliferation assay

To examine the effect of a selective IK_{Ca} channel blocker, TRAM-34 (150, 300, 600 , 1200 μg/kg) on prostatic growth in BPH model rats, the *in vivo* proliferation assay was performed as previously reported (Kojima et al., 2009a).The compound was subcutaneously administrated through 7 to 21 days after UGS implantation (150, 300, 600, 1200 μg/kg, s.c.). Vehicle was given in the same way. On day 21, the implanted UGS were collected and weighed, and then fixed in formalin and embedded in paraffin.

Statistical analysis

Statistical significance between two groups or among multiple groups was evaluated using Welch's *t*-test or Tukey's test after the F-test or ANOVA. Data are presented as the means ± S.E.M.

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Results

Gene and protein expression of $K_{Ca}3.1$ in implanted UGS and normal host prostate

We first examined the expression of $K_{Ca}3.1$ transcripts in normal prostate and implanted UGS of stromal hyperplasia BPH model rats using DNA microarray and real-time PCR analyses. As shown in Figure 1A, the expression of $K_{Ca}3.1$ transcripts in implanted UGS was much higher than in normal host prostate (23-fold, probe ID 1368930_at).Similarly, real-time PCR analysis showed that the expression of $K_{Ca}3.1$ transcripts in implanted UGS was approximately 6-fold higher than in normal host prostate (Fig. 1B). Expressions of $K_{Ca}3.1$ relative to β-actin were 0.0032 ± 0.0004 (n=8) and 0.022 ± 0.003 (n=8) in normal prostate and implanted UGS, respectively. No significant differences in Ct values at threshold 0.2between two groups were found: 17.6 ± 0.2 (n=8) and 17.6 ± 0.1 (n=8) in normal host prostate and implanted UGS. We next cloned full-length $K_{Ca}3.1$ from the host prostate and implanted UGS. By repetitive PCR cloning using at least three independent cDNA products, no spliced variants were identified in both tissues. The expression levels of $K_{Ca}3.1$ proteins in implanted UGS were verified by Western blot analysis. To confirm that equal amounts of protein (20 μg) were loaded in each lane, the blots were stained with 0.2 % Ponceau S aftertransfer. As shown in Figure 1C (upper panel), the anti-$K_{Ca}3.1$ antibody recognized a single band at approximately 50 kDa, similar to the predicted molecular weight of $K_{Ca}3.1$ protein. Densitometric analysis revealed that $K_{Ca}3.1$ expression levels were significantly higher in implanted UGS than in normal host prostate. When expressing the calculated values in normal host prostate group as 1.0, the expressions of $K_{Ca}3.1$ protein were 7.9 ± 1.6 in the implanted UGS group (n=4) (Fig. 1C. lower panel). When anti-$K_{Ca}3.1$ antibody was pre-incubated

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with the excess antigen peptide, against which the antibody was generated, a band of approximately 50 kDa specifically disappeared but non-specific, weak bands did not (not shown). Furthermore, immunohistochemical examination showed that the expression of $K_{Ca}3.1$ proteins was rarely detected in normal rat ventral prostate (not shown), whereas larger amounts of $K_{Ca}3.1$-positive signals were detected in the stromal compartments of implanted UGS (Fig. 1D).

Effects of TRAM-34 on the *in vivo* proliferation of implanted UGS

We next evaluated the *in vivo* effects of a potent $K_{Ca}3.1$ blocker, TRAM-34, on the growth of implanted UGS in stromal hyperplasia BPH model rats. A potent $K_{Ca}3.1$ blocker, TRAM-34, was subcutaneously administrated through day 7 to 21 after UGS implantation: 300, 600, 1200 mg/kg, s.c., and the weights of implanted UGS on day 22 were compared between TRAM-34-treated and vehicle (DMSO)-treated groups. Daily administration of TRAM-34 for 2 weeks prevented the increase of implanted UGS weight in a dose-dependent manner [17.7 % inhibition at a dose of 300 μg/kg (n=26), $p<0.05$ vs. vehicle (n=36), 35.7 % inhibition at a dose of 600 μg/kg (n=22), $p<0.01$, and 59.8 % inhibition at a dose of 1200 μg/kg (n=10), $p<0.01$] (Fig. 2).

To assess the *in vivo* effect of TRAM-34 on the proliferation index in the stromal hyperplasia BPH model, implanted UGS samples on day 22 were analyzed by PCNA immunostaining (Mori et al., 2009). PCNA-positive cells were quantified by counting brown-stained cells within the total number of cells in 10 randomly selected fields at x400 magnification (Fig. 3). In the TRAM-34-treated group (600 and 1200 μg/kg, s.c.), qualitative macroscopic examination of PCNA-stained sections showed a substantial decrease in PCNA-positive cells in the stromal components compared with epithelial

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cells whereas, in the vehicle control, no significant changes in the ratio of stromal to epithelial components were detected (Fig. 3A-C). Without affecting the quantification of PCNA staining in epithelial components (Fig. 3D, closed circles), that in stromal components showed a significant decrease in PCNA-positive cells at 600 (39.6 ± 1.7 %, n=8, $p<0.05$) and 1200 (31.7 ± 3.0 %, n=8, $p<0.01$) μg/kg, s.c. TRAM-34-treated groups compared with the vehicle control (47.2 ± 2.2 %, n=7) (Fig. 3D, open circles). These results suggest that pharmacological blockade with $K_{Ca}3.1$ may be effective for human stromal hyperplasia BPH.

Expressions of $K_{Ca}3.1$ transcriptional regulators in implanted UGS and normal host prostate

In vascular smooth muscle cells, the gene encoding $K_{Ca}3.1$ is suppressed by a functional repressor element 1-silencing transcription factor (REST) (Cheong et al., 2005).The expression of the $K_{Ca}3.1$ channel is also regulated by nuclear transcription factor activator protein-1 (AP-1) in T-lymphocytes (Ghanshani et al., 2000). AP-1 is composed of dimeric complexes of the Fos (c-Fos, FosB, Fra-1, and Fra-2) and Jun (c-Jun, JunB, and JunD) members (Wisdom, 1999).We therefore determined the expression levels of REST and Fos/Jun members. By real-time PCR analysis, a significantly lower expression of REST transcripts was found in implanted UGS than in the normal prostate (Fig. 4Aa). Expressions of REST relative to β-actin were 0.021 ± 0.003 and 0.010 ± 0.003 in the normal prostate and implanted UGS, respectively. In addition, significantly higher expressions of c-Jun were found in implanted UGS than in the normal prostate (Fig. 4Ae). Expressions of c-Jun were 0.009 ± 0.001 (n=8) and 0.018 ± 0.002 (n=8) in normal prostate and implanted UGS, respectively. No significant

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differences in the expressions of the other AP-1 components were detected between two groups (Fig. 4Ab, Ac, Ad, Af, Ag). Expressions of Fra-1 transcripts were under 0.003 in both groups (not shown).

The expression levels of REST and c-Jun proteins in the nuclear protein fractions of normal prostate and implanted UGS were verified by Western blot analysis. As shown in Figure 4Ba and 4Bb, anti-REST and anti-c-Jun antibodies recognized a single band at approximately 130 kDa and 40 kDa, similar to the predicted molecular weight of REST and c-Jun proteins, respectively. Densitometric analysis revealed that REST and c-Jun expression levels were significantly lower and higher in implanted UGS than in normal host prostate, respectively. When expressing the calculated values in normal host prostate group as 1.0, the expressions of REST and c-Jun proteins were 0.35 ± 0.05 and 3.43 ± 0.38 in the implanted UGS group, respectively (n=4) (Fig. 4Bc). When anti-REST and c-Jun antibodies were pre-incubated with the excess antigen peptides, against which the antibodies were generated, respectively, bands of approximately130 kDa (REST) and 40 kDa (c-Jun) specifically disappeared but non-specific, weak signals did not (not shown).

Expressions of K_{Ca}3.1 and its transcriptional regulators in prostate from human BPH patients

We next determined the expressions of K_{Ca}3.1, REST, and Fos/Jun member transcripts in prostate needle-biopsies from BPH patients by real-time PCR analysis. The mean serum PSA was 12.3 ± 4.6 ng/ml in BPH patients (n=7). Normal prostates showed a low expression of K_{Ca}3.1, whereas seven BPH samples showed a high expression of K_{Ca}3.1 (Fig. 5A). Expressions of REST transcripts were inversely correlated to those of

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$K_{Ca}3.1$ transcripts in both groups (Fig. 5A). Expressions of $K_{Ca}3.1$/REST transcripts were 0.005 ± 0.001/0.043 ± 0.004 (n=3) and 0.026 ± 0.004/0.020 ± 0.003 (n=7) in normal prostate and BPH samples, respectively (p<0.01 for each) (Fig. 5A). We also determined the expression levels of Fos/Jun members (Fig. 5B). Significantly higher expressions of Fra-2 and c-Jun were found in BPH samples than in normal prostate (Fig. 5B). Expressions of Fra-2/c-Jun transcripts were 0.018 ± 0.003/0.016 ± 0.002 (n=3) and 0.040 ± 0.007/0.037 ± 0.008 (n=7) in normal prostate and BPH samples (p<0.05 for each). There were no significant differences in the expressions of c-Fos and JunD between two groups (p>0.05) (Fig. 5B). Expressions of FosB, Fra-1, and JunB transcripts were under 0.007 in both groups (not shown).

We further performed immunohistochemical staining of $K_{Ca}3.1$ proteins using surgical prostate specimens (n=12) from BPH patients. We enrolled 10 patients with BPH aged 61-81 (mean age: 73.0 ± 1.8 years), and the prostate volume was 166.3 ± 63.0 ml (n=10). In the normal human prostate, the volume was 15.0 ± 3.6 ml (n=3). We used three different anti-$K_{Ca}3.1$ antibodies from Alomone Labs, Abnova, and Abcam. Strong background signals were observed with anti-$K_{Ca}3.1$ antibodies (1:100) from Alomone Labs (not shown), whereas there were relatively weak background signals with the other antibodies: Abnova (1:25) and Abcam (1:100). We considered the double-positive samples for both $K_{Ca}3.1$ antibodies obtained from Abnova and Abcam as '$K_{Ca}3.1$-positive'. In the normal human prostate, no $K_{Ca}3.1$-positive signals were detected (n=3, not shown). As shown in Figure 5C, in most surgical prostate specimens (8/10) from BPH patients, $K_{Ca}3.1$-positive signals were detected in stromal but not in epithelial compartments.

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Correlation between $K_{Ca}3.1$ and transcription factor (REST and c-Jun) expressions in implanted UGS and prostate needle-biopsies from BPH patients

The wet weight of implanted UGS was positively correlated with the expression of $K_{Ca}3.1$ transcripts relative to β-actin (arbitrary unit) in implanted UGS (n=8, R (correlation coefficient for linear fitting) =0.91, p=0.002) (Fig. 6A). We evaluated the correlation between transcript expressions of $K_{Ca}3.1$ and REST/c-Jun in implanted UGS. Expressions of REST (Fig. 4Aa), and c-Jun (Fig. 4Ae) were plotted against that of $K_{Ca}3.1$ (arbitrary unit) (Fig. 1B),as $K_{Ca}3.1$ vs. REST (Fig. 6B) and $K_{Ca}3.1$ vs. c-Jun (Fig. 6C), respectively. The $K_{Ca}3.1$ expression showed a negative correlation with REST expression (n=8, R=-0.78, p=0.021) (Fig. 6B), and a positive correlation with c-Jun expression (n=8, R=0.84, p=0.010) (Fig. 6C), respectively. Similarly, expressions of REST (Fig. 5A), and c-Jun (Fig. 5B) in prostate needle-biopsies from BPH patients were plotted against $K_{Ca}3.1$ (arbitrary unit) (Fig. 5A), as $K_{Ca}3.1$ vs. REST (Fig. 6D) and $K_{Ca}3.1$ vs. c-Jun (Fig. 6E), respectively. The $K_{Ca}3.1$ expression showed a negative correlation weith that of REST (n=7, R=-0.81, p=0.026) (Fig. 6D), and a positive correlation with that of c-Jun (n=7, R=0.95, p<0.001) (Fig. 6E). These findings strongly support our assumption that the down-regulation of REST and up-regulation of c-Jun contribute to facilitate $K_{Ca}3.1$ expression in implanted UGS and prostate from BPH patients.

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Discussion

The identification of novel targets for BPH therapy is of great interest. The main findings of the present study indicated: 1) high level expression of $K_{Ca}3.1$ in the stromal components of implanted UGS in a new experimental stromal hyperplasia BPH model recently established by Mori et al. (2009) and human BPH samples and 2) the inhibitory effect of a $K_{Ca}3.1$ blocker, TRAM-34, on the *in vivo* proliferation of implanted UGS in stromal components.

The intermediate-conductance Ca^{2+}-activated K^+ ($IK_{Ca}/K_{Ca}3.1$) channel is a regulator of cell proliferation in undifferentiated smooth muscles, tumors and lymphocytes, and provides new therapeutic target for restenosis, cancer and autoimmune disease (Köhler et al., 2003; Wulff et al., 2007; Ohya et al., 2009). Up-regulation of activator protein-1 (AP-1) and down-regulation of repressor element-1 silencing transcription factor (REST) are associated with up-regulation of $K_{Ca}3.1$ (Cheong et al., 2005; Ghanshani et al., 2000; Tharp et al., 2008; Park et al., 2011).

AP-1 regulates cellular processes such as differentiation, proliferation and apoptosis in response to a variety of stimuli. Ricote *et al.* (2003) have shown that no immunoreaction to AP-1 is detected in the normal human prostate; however, immunoreaction to AP-1 is positive in BPH. In the present study, we showed a significant increase in the gene and protein expressions of c-Jun but not other AP-1 components (Fos, FosB, Fra-1, Fra-2, JunB, JunD) in implanted UGS compared with the normal rat prostate (Fig. 4). Similarly, up-regulation of $K_{Ca}3.1$ and c-Jun were observed in human BPH samples (Fig. 5B). Li *et al.* (2007) have shown that c-Jun are abundantly expressed in the prostatic stroma but not in the epithelium of human BPH samples, and c-Jun is expressed at high levels in BPH nodules enriched with stroma. In

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the present study, positive correlations between $K_{Ca}3.1$ and c-Jun were significant in both implanted UGS and prostate needle-biopsies from BPH patients (Figs. 4Ae, 5B, 6C, 6E). These strongly suggest that the increased expression of $K_{Ca}3.1$ mediating the up-regulation of c-Jun may contribute to the promotion of proliferation in prostatic cells in the stromal region. In the present study, Fra-2 was also significantly up-regulated in human BPH samples (Fig. 5B). A recent report has shown that Fra-2 contributes to prostate cancer cell growth (Kajanne et al., 2009), suggesting that the Fra-2 signal pathway may be responsible for the cell proliferation of prostatic cells in epithelial region components but not in stromal regions.

Down-regulation of REST can be correlated to the up-regulation of $K_{Ca}3.1$ in implanted UGS of stromal hyperplasia BPH model rats (Fig. 4Aa, 4Ba, 4Bc). Negative correlations between $K_{Ca}3.1$ and REST were significant in both implanted UGS and prostate needle-biopsies from BPH patients (Figs. 4Aa, 5A, 6B, 6D).The expression of REST declines in undifferentiated vascular smooth muscle cells, showing an inverse relationship with functional $K_{Ca}3.1$ (Cheong et al., 2005). Neylon et al. (1999) have shown that smooth muscle cells exhibiting contractile function express $BK_{Ca}/K_{Ca}1.1$predominantly, whereas proliferative smooth muscle cells express $IK_{Ca}/K_{Ca}3.1$predominantly. In addition to the up-regulation of $K_{Ca}3.1$ and down-regulation of REST, DNA microarray assay indicated the significant down-regulation of $K_{Ca}1.1$ in the implanted UGS in the stromal hyperplasia BPH model (supplemental Fig. S1). Stromal hyperplasia BPH is characterized by stromal reorganization due to the trans-differentiation of fibroblasts to myofibroblasts and smooth muscle cells (Bartsch et al., 1979). This suggests that the transcriptional regulation of $K_{Ca}3.1$ may be involved in stromal reorganization in stromal hyperplasia

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BPH. Taken together, similar to the recent reports showing correlation between $K_{Ca}3.1$, REST, and c-Jun expressions (Tharp et al., 2008; Park et al., 2011), concomitant up-regulation of AP-1 and down-regulation of REST can be suggested as a possible mechanism underlying the up-regulation of $K_{Ca}3.1$ in implanted UGS of stromal hyperplasia BPH model rats, and may be responsible for the progressive growth of stromal compartments of BPH.

A selective $K_{Ca}3.1$ blocker, TRAM-34, reduces atherosclerosis development by the inhibition of both vascular smooth muscle cell proliferation and T-lymphocyte and macrophage activity (Wulff et al., 2007). The present study clarified the inhibitory effects of TRAM-34 on the *in vivo* proliferation of implanted UGS of the stromal hyperplasia BPH model in a dose-dependent manner (Fig. 2). Moreover, in the TRAM-34-treated group, qualitative macroscopic examination of PCNA-stained sections showed a correlative decrease in PCNA-positive cells in stromal compartments including smooth muscle cells without any changes in those in epithelial compartments (Fig. 3). Recent studies have shown that K_{Ca} channel such as $K_{Ca}3.1$ and $K_{Ca}2.2$ participates in the modulation of cell proliferation in several types of cells, and their pharmacological blockade and down-regulation inhibit cell proliferation by G_0/G_1 arrest (Wang et al., 2007; Grgic et al., 2009; Morimoto et al., 2007).This suggests that the inhibitory effects of TRAM-34 on *in vivo* cell proliferation in the implanted UGS of stromal BPH model rats may be responsible for the termination of cell cycle progression from G_0/G_1 by TRAM-34 treatment.

Although α_1-adrenoceptor antagonists are considered an appropriate treatment for all BPH patients, irrespective of prostate size, combination therapy with α_1-adrenoceptor antagonists and 5α-reductase inhibitors is a recently accepted treatment for patients with

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clinically enlarged prostates (McConnell et al., 2003). 5α-reductase inhibitors are well tolerated, but the most common adverse effects are sexual dysfunction, including a reduced libido, erectile dysfunction and, less frequently, ejaculation disorders (Gravas et al., 2010); therefore, other options are required to reduce prostate volume and the risk of the clinical progression of BPH. Daily administration of TRAM-34 at relatively high concentration did not induce any changes in blood chemistry, hematology or at necropsy of the major organs of rodents (Si et al., 2006). Pharmacological blockade of $K_{Ca}3.1$ may be relatively safe and well tolerated as a therapeutic target of BPH. In addition, pharmacological blockade of $K_{Ca}3.1$ prevents up-regulation of $K_{Ca}3.1$ and down-regulation of REST in coronary arteries of post-angioplasty restenosis model (Tharp et al., 2008). This suggests that pharmacological blockade of $K_{Ca}3.1$ prevents phenotypic modulation of prostatic stromal cells in stromal hyperplasia BPH. Moreover, Tharp et al. (2006 & 2008) have shown that in proliferative, dedifferentiated vascular smooth muscle cells, up-regulation of $K_{Ca}3.1$ is followed by $K_{Ca}3.1$ promoter histone acetylation. These suggest that TRAM-34 also prevents $K_{Ca}3.1$ promoter activation at the epigenetic level. Further study will provide more detailed mechanisms underlying TRAM-34-induced anti-proliferative effects in prostatic stromal cells from BPH patients.

In vascular smooth muscle cells (VSMCs) with dedifferentiated, proliferating phenotype, hyperpolarization by K^+ channel activation increases the driving force for Ca^{2+} entry via voltage-independent Ca^{2+} channels such as store-operated Ca^{2+} channel (SOC), receptor-operated Ca^{2+} channel (ROC), and Ca^{2+}-release activating Ca^{2+} channel (CRAC), which are encoded by mammalian homologues of transient receptor potential (TRP) genes and Orai/STIM genes (Inoue et al., 2006; Albert et al., 2007; House et al.,

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2008; Guibert et al., 2008). However, molecular components directly involving in VSMC proliferation remain to be determined. Dedifferentiated VSMCs are characterized by loss of voltage-gated Ca^{2+} channel ($Ca_V1.2$), which is a major Ca^{2+} channel component in smooth muscle cells with differentiated, contractile phenotype. In implanted UGS of stromal hyperplasia BPH model rats, $Ca_V1.2$ was rarely expressed (not shown). In the present study, the molecular identification and functional characterization of voltage-independent Ca^{2+} channels remain to be determined either in implanted UGS or prostatic tissues from BPH patients.

Several researchers have reported that down-regulation of large-conductance Ca^{2+}-activated K^+ channel, $K_{Ca}1.1$ and voltage-gated K^+ channel, $K_V1.3$ is observed in lower urinary tracts in BPH and prostatitis patients (Chang et al., 2010; Liang et al., 2006). DNA microarray analysis showed that $K_{Ca}1.1$ transcript expression (probe ID 1372929_at) in implanted UGS was markedly lower than that in normal host prostate. No significant difference in $K_V1.3$ transcript expression (probe ID 1369802_at) was detected between them (supplemental Fig. S1), however, relative expression level of $K_V1.3$ was much lower than that of $K_{Ca}3.1$in implanted UGS and prostate from BPH patients (not shown). The possibilities of voltage-independent Ca^{2+} channels and other K^+ channel subtypes as therapeutic targets for BPH are also burning question.

In summary, our present study suggests that the activation of CRAC channels by up-regulation of the intermediate-conductance Ca^{2+}-activated K^+ channel, $K_{Ca}3.1$, may be implicated in the development of BPH, which represents a field of great interest for scientific and clinical research communities. We further showed that both c-Jun and REST are candidates for the transcriptional regulation of $K_{Ca}3.1$ in stromal

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compartments of BPH. *In vivo* treatment with TRAM-34 significantly suppressed cell proliferation in stromal compartments, supporting that $K_{Ca}3.1$ blockers may be a novel treatment option for patients suffering from stromal hyperplasia BPH.

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Authorship Contributions

Participated in research design: Ohya, Sasaki, Kohri, Imaizumi

Conducted experiments: Ohya, Niwa, Kojima, Sakuragi

Performed data analysis: Ohya, Niwa, Kojima, Sakuragi

Wrote or contributed to the writing of the manuscript: Ohya, Kojima, Sasaki, Kohri, Imaizumi

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References

Albert AP, Saleh SN, Peppiatt-Wildman CM and Large WA (2007) Multiple activation mechanisms of store-operated TRPC channels in smooth muscle cells. J Physiol **583:**25-36.

Andersson KE and Gratzke C (2007) Pharmacology of α_1-adrenoceptor antagonists in the lower urinary tract and central nervous system. Nat Clin Pract Urol **4:**368-378.

Bartsch G, Müller HR, Oberholzer M and Rohr HP (1979) Light microscopic stereological analysis of the normal human prostate and of benign prostatic hyperplasia. J Urol **122:**487-491.

Berry SJ, Coffey DS, Walsh PC and Ewing LL (1984) The development of human benign prostatic hyperplasia with age. J Urol **132:**474-479.

Chang S, Gomes CM, Hypolite JA, Marx J, Alanzi J, Zderic SA, Malkowicz B, Wein AJ and Chacko S (2010) Detrusor overactivity is associated with downregulation of large-conductance calcium- and voltage-activated potassium channel protein. Am J Physiol Renal Physiol **298:**F1416-F1423.

Cheong A, Bingham AJ, Li J, Kumar B, Sukumar P, Munsch C, Buckley NJ, Neylon CB, Porter KE, Beech DJ and Wood IC (2005) Downregulated REST transcription factor is a switch enabling critical potassium channel expression and cell proliferation. Mol Cell **20:**45-52.

Djavan B (2003) Lower urinary tract symptoms/benign prostatic hyperplasia: fast control of the patient's quality of life. Urology **62:**6-14.

Geller J (1989) Pathogenesis and medical treatment of benign prostatic hyperplasia. Prostate **15:**95-104.

Downloaded from jpet.aspetjournals.org at ASPET Journals on April 16, 2024

JPET Fast Forward. Published on May 20, 2011 as DOI: 10.1124/jpet.111.182782
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Ghanshani S, Wulff H, Miller MJ, Rohm H, Neben A, Gutman GA, Cahalan MD and Chandy KG (2000) Up-regulation of the *IKCa1* potassium channel during T-cell activation. Molecular mechanism and functional consequences. J Biol Chem **275:**37137-37149.

Gravas S and Oelke M (2010) Current status of 5α-reductase inhibitors in the management of lower urinary tract symptoms and BPH. World J Urol **28:**9-15.

Grgic I, Kiss E, Kaistha BP, Busch C, Kloss M, Sautter J, Müller A, Kaistha A, Schmidt C, Raman G, Wulff H, Strutz F, Gröne HJ, Köhler R and Hoyer J (2009) Renal fibrosis is attenuated by targeted disruption of $K_{Ca}3.1$ potassium channels. Proc Natl Acad Sci U S A **106:**14518-14523.

Guibert C, Ducret T and Savineau JP (2008) Voltage-independent calcium influx in smooth muscle. Prog Biophys Mol Biol **98:**10-23.

House SJ, Potier M, Bisaillon J, Singer HA and Trebak M (2008) The non-excitable smooth muscle: calcium signaling and phenotypic switching during vascular disease. Pflügers Arch **456:**769-785.

Inoue R, Jensen LJ, Shi J, Morita H, Nishida M, Honda A and Ito Y (2006) Transient receptor potential channels in cardiovascular function and disease. Circ Res **99:**119-131.

Ishigooka M, Hayami S, Hashimoto T, Suzuki Y, Katoh T and Nakada T (1996) Relative and total volume of histological components in benign prostatic hyperplasia: relationships between histological components and clinical findings. Prostate **29:**77-82.

Ishii TM, Silvia C, Hirschberg B, Bond CT, Adelman JP and Maylie J (1997) A human intermediate conductance calcium-activated potassium channel. Proc Natl Acad

Downloaded from jpet.aspetjournals.org at ASPET Journals on April 16, 2024

JPET Fast Forward. Published on May 20, 2011 as DOI: 10.1124/jpet.111.182782
This article has not been copyedited and formatted. The final version may differ from this version.

Sci U S A **94:**11651-11656.

Kajanne R, Miettinen P, Tenhunen M and Lappä S (2009) Transcription factor AP-1 promotes growth and radioresistance in prostate cancer cells. Int J Oncol **35:**1175-1182.

Kojima Y, Sasaki S, Hayashi Y, Tsujimoto G and Kohri K (2009a) Subtypes of α_1-adrenoceptors in BPH: future prospects for personalized medicine. Nat Clin Pract Urol **6:**44-53.

Kojima Y, Sasaki S, Oda N, Koshimizu TA, Hayashi Y, Kiniwa M, Tsujimoto G and Kohri K (2009b) Prostate growth inhibition by subtype-selective α_1-adrenoceptor antagonist naftopidil in benign prostatic hyperplasia. Prostate **69:**1521-1528.

Köhler R, Wulff H, Eichler I, Kneifel M, Neumann D, Knorr A, Grgic I, Kämpfe D, Si H, Wibawa J, Real R, Borner K, Brakemeier S, Orzechowski HD, Reusch HP, Paul M, Chandy KG and Hoyer J (2003) Blockade of the intermediate-conductance calcium-activated potassium channel as a new therapeutic strategy for restenosis. Circulation **108:**1119-1125.

Li W, Wu CL, Febbo PG and Olumi AF (2007) Stromally expressed c-Jun regulates proliferation of prostate epithelial cells. Am J Pathol **171:**1189-1198.

Liang CZ, Guo QK, Hao ZY, Yang S, Wang DB, Wu LX, Liu C, Wang KX and Zhang XJ (2006) K channel expression in prostate epithelium and its implications in men with chronic prostatitis. BJU Int **97:**190-192.

McConnell JD, Roehrborn CG, Bautista OM, Andriole GL Jr, Dixon CM, Kusek JW, Lepor H, McVary KT, Nyberg LM Jr, Clarke HS, Crawford ED, Diokno A, Foley JP, Foster HE, Jacobs SC, Kaplan SA, Kreder KJ, Lieber MM, Lucia MS, Miller GJ, Menon M, Milam DF, Ramsdell JW, Schenkman NS, Slawin KM and Smith

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JPET Fast Forward. Published on May 20, 2011 as DOI: 10.1124/jpet.111.182782
This article has not been copyedited and formatted. The final version may differ from this version.

JA (2003) The long-term effect of doxazosin, finasteride, and combination therapy on the clinical progression of benign prostatic hyperplasia. N Engl J Med **349:**2387-2398.

Mori F, Oda N, Sakuragi M, Sakakibara F, Kiniwa M and Miyoshi K (2009) New histopathological experimental model for benign prostatic hyperplasia: stromal hyperplasia in rats. J Urol **181:**890-898.

Morimoto T, Ohya S, Hayashi H, Onozaki K and Imaizumi Y (2007) Cell-cycle dependent regulation of Ca^{2+}-activated K^+ channel in Jurkat T-lymphocytes. J Pharmacol Sci **104**:94-98.

Neylon CB, Lang RJ, Fu Y, Bobik A and Reinhart PH (1999) Molecular cloning and characterization of the intermediate-conductance Ca^{2+}-activated K^+ channel in vascular smooth muscle: relationship between K_{Ca} channel diversity and smooth muscle cell function. Circ Res **85:**e33-e43.

Ohya S, Kimura K, Niwa S, Ohno A, Kojima Y,Sasaki S, Kohri K and Imaizumi Y (2009) Malignancy grade-dependent expression of K^+ channel subtypes in human prostate cancer. J Pharmacol Sci **109:**148-151.

Ohya S, Kimura S, Kitsukawa M, Muraki K, Watanabe M and Imaizumi Y (2000) SK4 encodes intermediate conductance Ca^{2+}-activated K^+ channels in mouse urinary bladder smooth muscle cells. Jpn J Pharmacol **84:**97-100.

Ohya S, Kuwata Y, Sakamoto K, Muraki K and Imaizumi Y (2005) Cardioprotective effects of estradiol include the activation of large-conductance Ca^{2+}-activated K^+ channels in cardiac mitochondria. Am J Physiol Heart Circ Physiol **289:**H1635-H1642.

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JPET Fast Forward. Published on May 20, 2011 as DOI: 10.1124/jpet.111.182782
This article has not been copyedited and formatted. The final version may differ from this version.

Park S, Kim JA, Joo KY, Choi S, Choi EN, Shin JA, Han KH and Suh SH (2011) Globotriaosylceramide leads to $K_{Ca}3.1$ channel dysfunction: a new insight into endothelial dysfunction in Fabry disease. Cardiovasc Res **89:**290-299.

Ricote M, Royuela M, García-Tuñón I, Bethencourt FR, Paniagua R and Fraile B (2003) Pro-apoptotic tumor necrosis factor-α transduction pathway in normal prostate, benign prostatic hyperplasia and prostatic carcinoma. J Urol **170:**787-790.

Shapiro E, Becich MJ, Hartanto V and Lepor H (1992) The relative proportion of stromal and epithelial hyperplasia is related to the development of symptomatic benign prostate hyperplasia. J Urol **147:**1293-1297.

Si H, Heyken WT, Wölfle SE, Tysiac M, Schubert R, Grgic I, Vilianovich L, Giebing G, Maier T, Gross V, Bader M, de Wit C, Hoyer J and Köhler R (2006) Impaired endothelium-derived hyperpolarizing factor-mediated dilations and increased blood pressure in mice deficient of the intermediate-conductance Ca^{2+}-activated K^+ channel. Circ Res **99:**537-544.

Stocker M (2004) Ca^{2+}-activated K^+ channels: molecular determinants and function of the SK family. Nat Rev Neurosci **5:**758-770.

Tharp DL, Wamhoff BR, Turk JR and Bowles DK (2006) Upregulation of intermediate-conductance Ca^{2+}-activated K^+ channel ($IK_{Ca}1$) mediates phenotypic modulation of coronary smooth muscles. Am J Physiol Heart Circ Physiol **291:**H2493-H2503.

Tharp DL, Wamhoff BR, Wulff H, Raman G, Cheong A and Bowles DK (2008) Local delivery of the $K_{Ca}3.1$ blocker, TRAM-34, prevents acute angioplasty-induced coronary smooth muscle phenotypic modulation and limits stenosis. Arterioscler

Downloaded from jpet.aspetjournals.org at ASPET Journals on April 16, 2024

JPET Fast Forward. Published on May 20, 2011 as DOI: 10.1124/jpet.111.182782
This article has not been copyedited and formatted. The final version may differ from this version.

Thromb Vasc Biol **28:**1084-1089.

Wang ZH, Shen B, Yao HL, Jia YC, Ren J, Feng YJ and Wang YZ (2007) Blockage of intermediate-conductance Ca^{2+}-activated K^{+} channels inhibits progression of human endometrial cancer. Oncogene **26:**5107-5114.

Wisdom R (1999) AP-1: one switch for many signals. Exp Cell Res **253:**180-185.

Wulff H, Kolski-Andreaco A, Sankaranarayanan A, Sabatier JM and Shakkottai V (2007) Modulators of small- and intermediate-conductance calcium-activated potassium channels and their therapeutic indications. Curr Med Chem **14:**1437-1457.

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JPET Fast Forward. Published on May 20, 2011 as DOI: 10.1124/jpet.111.182782
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Footnotes

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Legends for Figures

Figure 1. K_{Ca}3.1 transcript and protein expressions in normal prostate and implanted UGS of stromal hyperplasia BPH model rats. A: DNA microarray analysis of K_{Ca}3.1 gene expression. Expression levels express the values in normal host prostate as 1.0. B: Quantitative, real-time PCR analysis of K_{Ca}3.1 gene expression. Values are shown for steady state transcripts relative to β-actin in the same preparation. Results are expressed as the means ± SEM (normal prostate: n=8, implanted UGS: n=8). **: p<0.01 vs. normal prostate. C: K_{Ca}3.1 protein expressions in normal prostate and implanted UGS. Plasma membrane fractions were probed by immunoblotting with anti-K_{Ca}3.1 antibody (upper panel). Lower panel shows the summarized data of densitometric analyses (n=4). **: p<0.01 vs. normal prostate. D: Immunohistochemical staining of K_{Ca}3.1 expression in stromal compartments of implanted UGS. Brown-stained nuclei were considered 'positive'. Original magnifications, x 40.

Figure 2. *In vivo* inhibitory effects of TRAM-34 on the growth of implanted UGS in the stromal hyperplasia BPH model. TRAM-34 (300, 600, and 1200 μg/kg) was subcutaneously administrated through day 7 to 21 after UGS implantation, and the implanted UGS were weighed on day 22. Results are expressed as the means ± SEM: n=36 (vehicle control), n=26 (300 μg/kg, s.c.), n=22 (600 μg/kg, s.c.) and n=10 (1200 μg/kg, s.c.). *, **:p<0.05, <0.01 vs. vehicle control.

Figure 3. Effects of the proliferation index on TRAM-34 in stromal and epithelial compartments of the stromal hyperplasia BPH model rats. Immunohistochemical

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staining for PCNA was performed as detailed in 'Materials and Methods'. A. vehicle control, B. 600 μg/kg, s.c., C. 1200 μg/kg, s.c. D. PCNA-positive epithelial (closed circles) and stromal cells (open circles) were quantified to determine the proliferation index in each group. Results are expressed as the means ± SEM: 47.2 ± 2.2 (vehicle control, n=7), 39.6 ± 1.7 (600 μg/kg, s.c., n=8) and 31.7 ± 3.0 % (1200 μg/kg, s.c., n=8). *, **: p<0.05, <0.01 vs. vehicle control.

Figure 4. Gene and protein expressions of REST and AP-1 components. A: Real-time PCR analysis of REST (a), c-Fos (b), FosB (c), Fra-2 (d), c-Jun (e), JunB (f), JunD (g)] in normal prostate and implanted UGS of stromal hyperplasia BPH model rats. Values are shown for steady state transcripts relative to β-actin in the same preparation. Results are expressed as the means ± SEM (native UGS: n=5, normal prostate: n=8, implanted UGS: n=8). **: p<0.01 vs. normal prostate. B: REST and c-Jun protein expressions in normal prostate and implanted UGS of stromal hyperplasia BPH model rats. Nuclear protein fractions were probed by immunoblotting with anti-REST (a) and anti-c-Jun (b) antibodies. 'c' shows the summarized data of densitometric analyses (n=4). *: p<0.05, 0.01 vs. normal prostate).

Figure 5. Expression of K_{Ca}3.1, REST, and AP-1 components in human BPH samples. A. Real-time PCR analysis of K_{Ca}3.1 and REST in human normal prostate (n=3, open columns) and BPH samples (n=7, closed columns). Values are shown for steady state transcripts relative to β-actin in the same preparation. Results are expressed as the means ± SEM. **: p<0.01 vs. normal prostate. B. Real-time PCR analysis of AP-1 component transcripts (c-Fos, Fra-2, c-Jun, and JunD) in human normal prostate

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(n=3, open columns) and BPH samples (n=7, closed columns). *: $p<0.05$ vs. normal prostate. C. Immunohistochemical analysis of $K_{Ca}3.1$ expression in human BPH samples. $K_{Ca}3.1$-positive, brown-stained signals, which are specific for anti-$K_{Ca}3.1$ antibody from Abnova, are detected in stromal but not in epithelial compartments. Original magnifications, x 40.

Figure 6. Correlation between $K_{Ca}3.1$ and transcription factor (REST and c-Jun) transcript expressions in implanted UGS and prostate from BPH patients. A: Correlation between wet tissue weight and $K_{Ca}3.1$ transcript expression in implanted UGS. Expression (arbitrary unit) of $K_{Ca}3.1$ transcripts was obtained from the data in Figure 1B (n=8). B, C: Correlation between $K_{Ca}3.1$, REST, and c-Jun transcript expressions, Expressions of $K_{Ca}3.1$, REST, and c-Jun transcript expressions in implanted UGS were obtained from the data in Figs. 1B, 4Aa, and 4Ae, and were plotted as $K_{Ca}3.1$ vs. REST and $K_{Ca}3.1$ vs. c-Jun (n=8). D, E: Expressions of $K_{Ca}3.1$, REST, and c-Jun transcript expressions in prostate needle-biopsies from BPH patients were obtained from the data in Figs. 5A and 5B, and were plotted as $K_{Ca}3.1$ vs. REST and $K_{Ca}3.1$ vs. c-Jun (n=7). Dotted line shows the best fitting line, and R value shows the correlation coefficient for linear fitting between the variable X-axis and the variable Y-axis.

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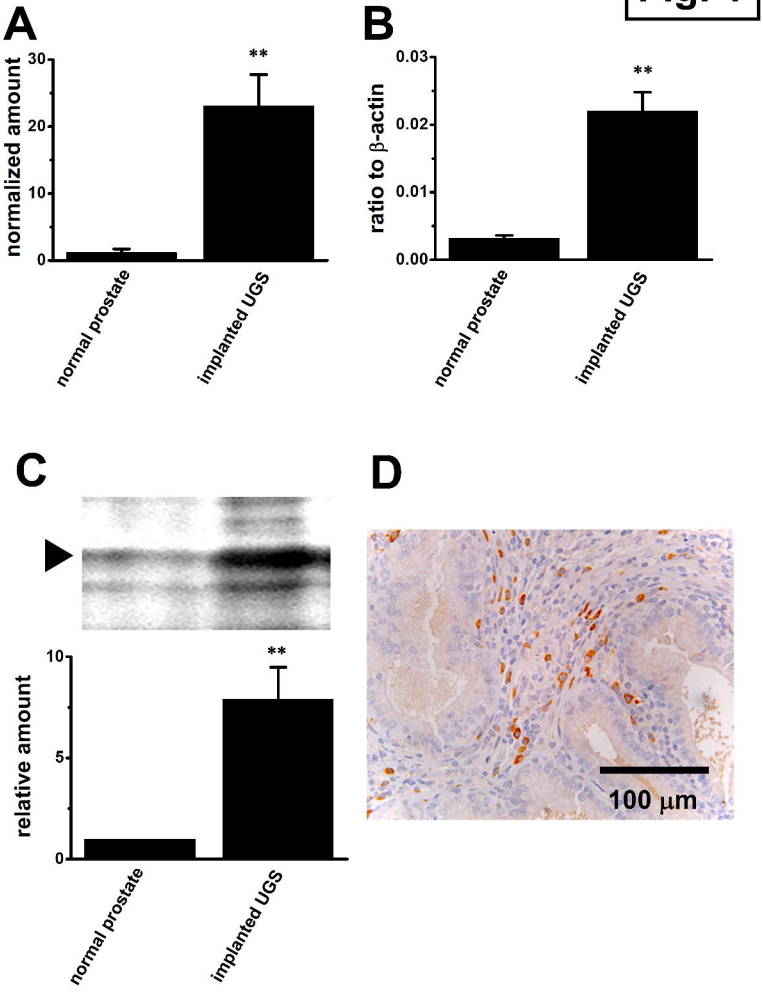
Fig. 1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D
100 μm

Fig. 2

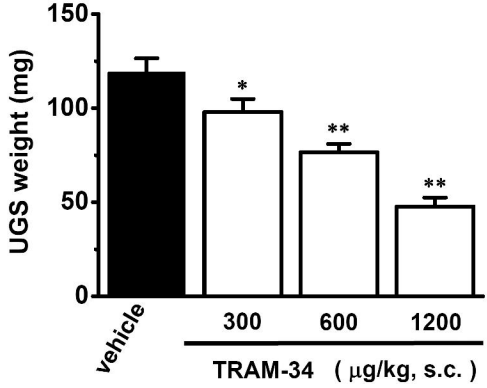


Fig. 3

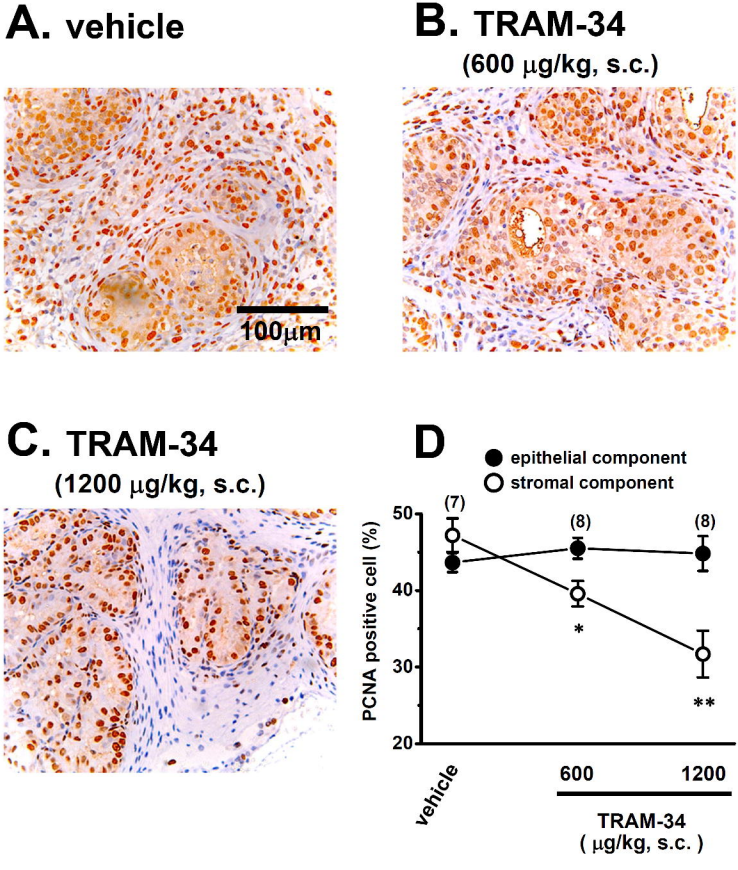

A. vehicle
B. TRAM-34
(600 μg/kg, s.c.)
100μm
C. TRAM-34
(1200 μg/kg, s.c.)
D
epithelial component
stromal component
PCNA positive cell (%)
50
40
30
20
(7)
(8)
(8)
*
**
vehicle
600
1200
TRAM-34
(μg/kg, s.c.)

Fig. 4

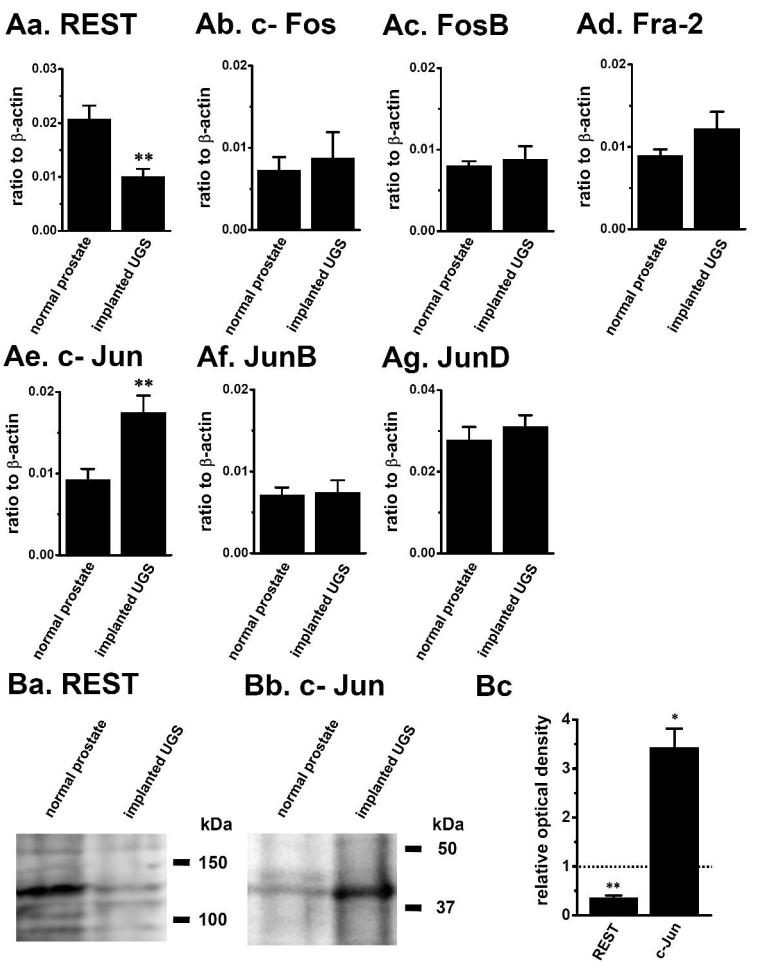
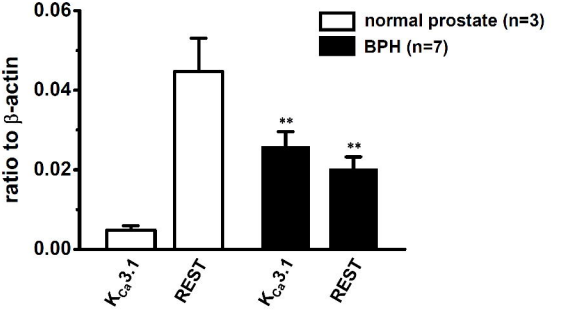
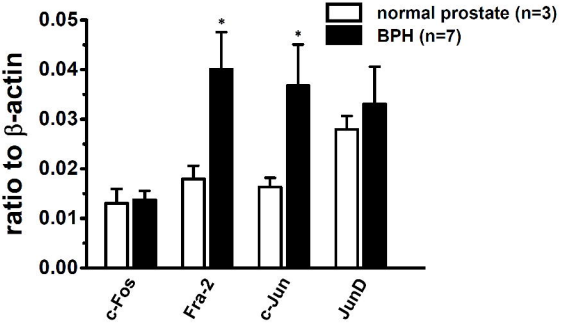

Aa. REST
Ab. c- Fos
Ac. FosB
Ad. Fra-2
ratio to β-actin
normal prostate
implanted UGS
**
Ae. c- Jun
Af. JunB
Ag. JunD
Ba. REST
Bb. c- Jun
Bc
kDa
150
100
50
37
relative optical density
REST
c-Jun
*

Fig. 5

A



B



C. anti-$K_{Ca}3.1$

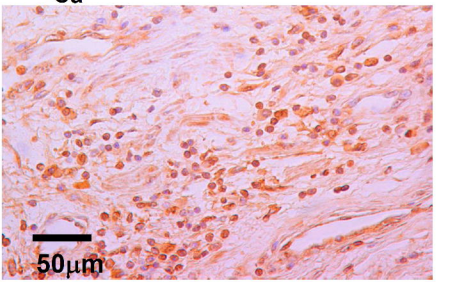
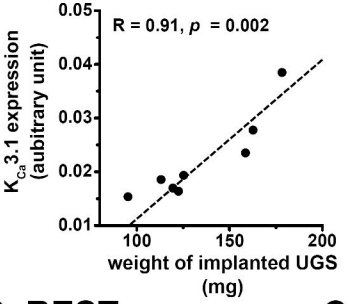
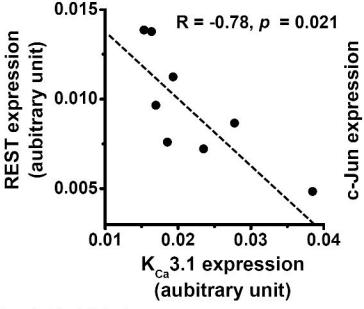


Fig. 6

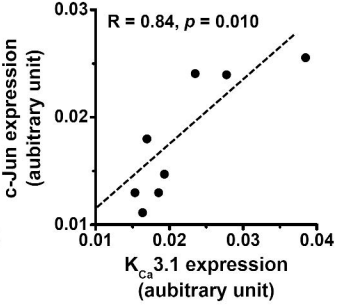
A. weight of implanted UGS



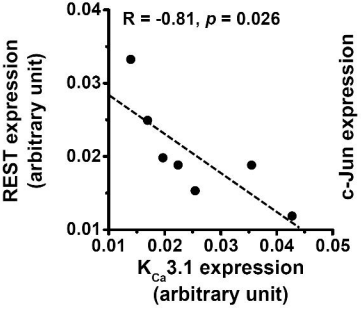
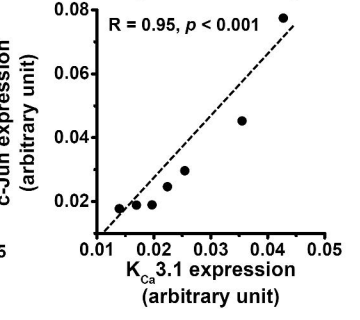
B. REST (UGS)



C. c-Jun (UGS)



D. REST (human BPH)



E. c-Jun (human BPH)