Differential expression of genes in co-cultured versus separately cultured fibroblasts and epithelial cells from human benign hyperplastic prostate tissues

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Abstract. The prostate is composed mainly of epithelial and stromal cells, whose dynamic interaction is vital to a broad array of cellular processes, including proliferation, differentiation, growth, and apoptosis. To understand inter-cellular communication in the development and progression of prostatic diseases, we examined gene expression in tissues from five patients diagnosed with benign prostatic hyperplasia (BPH). Fibroblasts and epithelial cells derived from these tissues were grown in a primary co-culture system that retains many characteristics of the intact human prostate. The mRNA levels of expressed genes as assessed by differential-display reverse transcription-PCR revealed that 110 genes were differentially expressed in co-cultured fibroblasts and epithelial cells, compared with expression in separately cultured cells. Eighty-four of these were confirmed by reverse Northern blotting, and 68 were successfully sequenced. Of the sequenced genes, 43 were differentially expressed in epithelial cells (37 upregulated, 6 downregulated), and 25 were differentially expressed in fibroblasts (6 upregulated, 19 downregulated) in co-cultures versus separate cultures. Semi-quantitative RT-PCR analysis of 12 genes with known functions showed that five of these were differentially expressed in co-cultured cells. Human kallikrein gene 7 (*KLK7*) was markedly upregulated in co-cultured compared with separately cultured epithelial cells ($P<0.001$), whereas S100 calcium binding protein A11, tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein, cyclin I, and latexin were significantly downregulated in co-cultured fibroblasts ($P<0.05$). Quantitative real-time RT-PCR and Western blot analysis confirmed *KLK7* up-regulation at both the mRNA and protein levels, respectively. Thus, epithelial-stromal cell interaction and communication are likely to be important in BPH. Epithelial cells and fibroblasts may interplay coordinately or collaboratively to influence cellular growth and death through dynamically differential gene expression in response to physiological and patho-physiological changes.

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Abbreviations: KLK, kallikrein; DDRT-PCR, differential display reverse transcription polymerase chain reaction; PSA, prostate specific antigen; hK7, human tissue kallikrein 7 protein; HSCCE, human stratum corneum chymotrypsin enzyme; *KLK7*, human kallikrein gene 7

Key words: benign prostatic hyperplasia, kallikrein, differential expression, epithelia, fibroblasts, epithelial/stromal interaction

Introduction

Benign prostatic hyperplasia (BPH) is an almost inevitable feature of the aging male (1) and is considered to be responsible for lower urinary tract symptoms in the majority of men >50 years of age. Histopathologically, BPH is characterized by an increased number of epithelial and stromal cells in the periurethral area of the prostate. The increased cell numbers may result from epithelial and stromal cell proliferation or from impaired apoptosis (1). Androgens, estrogens, growth factors (2), and neurotransmitters may participate, either individually or in combination, in the etiology and pathogenesis of the hyperplastic process, but their molecular mechanisms in BPH remain unknown. Many other putative factors, including epithelial-stromal cell interaction, may also be involved in the regulation of prostate growth. Abundant experimental evidence has demonstrated that prostatic epithelial and stromal cells maintain sophisticated paracrine-type communications, although the precise details are not yet entirely understood.

In a multicellular organism, cells function as an integral unit, and thus homeostasis cannot be regulated without inter-cellular communication. The exchange of information and

signals helps to balance the vital opposing processes of proliferation and programmed cell death. A defect in this network leads to either the excessive growth of hyperplasia and neoplasia or the excessive cell death of hypoplasia and atrophy. In rats, normal epithelial cells are capable of inducing the differentiation of prostatic smooth muscle in urogenital sinus mesenchyma (3). Stromal cells from the prostate have been shown to inhibit clonal growth of the prostatic carcinoma cell lines PC-3 (hormone-independent) and LNCaP (hormone-sensitive) in co-culture (4). A recent study has shown that most histological carcinomas and atypical adenomatous hyperplasia lesions are found in enlarged prostates with intense hypertrophy (5), suggesting that BPH and prostate cancer may share some common mechanisms. In that regard, the interaction between epithelial and stromal cells has recently become an area of intense interest. Bayne *et al* have described a co-culture model that reflects more closely the *in vivo* environment of human BPH and is thus far more suitable than current *in vitro* systems for investigating the molecular and cellular interaction underlying BPH (6). We hypothesize that the interaction is mediated by specific growth factors, and no studies to date have searched for the signal molecules involved in fibroblast-epithelial interaction in BPH.

The present study was designed to identify regulatory factors involved in epithelial-stromal interaction and communication in BPH. We used primary fibroblasts and epithelial cells derived from benign hyperplastic prostate tissues and a co-culture model system that maintains many characteristics of the intact human prostate. The gene expression profiles of co-cultured and separately cultured epithelial cells and fibroblasts were determined by differential display reverse transcription-PCR (DDRT-PCR), revealing 110 genes that were differentially expressed in co-cultured versus separately cultured cells. These differentially expressed genes may be responsible for the increased proliferation and decreased apoptosis of prostate cells in BPH.

Patients and methods

Primary cell culture of prostate epithelial cells and fibroblasts from BPH tissues. Prostate tissues were obtained from five BPH patients undergoing suprapubic prostatectomy, and the prostate tissues in the transition zone with hyperplasia were selected. The use of human specimens from patients in the current study was approved by the Ethics Committee of the First Hospital of Guangxi Medical University, and all experiments were performed in accordance with the ethical standards of the 1995 Declaration of Helsinki, as revised in Tokyo in 2004. Written informed consent was obtained from all patients. Histological diagnosis of tissues was confirmed by an independent pathologist.

Primary cultures of isolated fibroblasts and epithelial cells were established as described by Habib *et al* (7). Briefly, prostate tissues were washed with phosphate-buffered saline (PBS) to remove all traces of blood and were diced into ~1-mm^3 pieces using forceps and scissors. Diced tissues were incubated in a collagenase solution (33 IU/ml; Worthington Biochemical Corp., Lakewood, NJ, USA) for 16 h at 37˚C with shaking. After digestion with collagenase, epithelial cells were separated by sedimentation for 30 min. Epithelial cells and fibroblasts were washed separately with PBS and centrifuged twice to remove collagenase. Epithelial cells were resuspended in epithelial growth medium consisting of WAJC 404 (Invitrogen Life Technologies, Carlsbad, CA, USA) with 6.7 g/l HEPES, 1.2 g/l $NaHCO_3$, 10 U/l epidermal growth factor, and 392 μg/l dexamethasone, supplemented with 0.5 mg/l insulin, 0.5% fetal calf serum (FCS), 100,000 IU/ml penicillin, and 100,000 μg/ml streptomycin. Fibroblasts were resuspended in RPMI-1640 (Life Technologies, Paisley, Scotland) supplemented with 200 mM L-glutamine, 100,000 IU/l penicillin, 100,000 μg/ml streptomycin, and 10% FCS. Cells were separately incubated at 37˚C in a humidified atmosphere of 95% air - 5% CO_2. After 4 days incubation in flasks, epithelial cells demonstrated good spread, and confluence was usually reached by day 5.

The purity of the fibroblasts and epithelial cells was verified by phase-contrast microscopy and immunocytochemical staining with mouse monoclonal anti-human desmin (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA) and mouse monoclonal anti-human PSA antibodies (Santa Cruz Biotechnology), as described by Habib *et al* (7).

Co-culture and separate culture of prostate epithelial cells and fibroblasts. Confluent cultures of epithelial cells were harvested by trypsinization, and seeded onto six-well plates at a density of 20,000 cells/well in 2 ml of a 50:50 (v/v) mix of epithelial cell growth medium, supplemented with 0.5% FCS, 100,000 IU/ml penicillin, 100,000 μg/ml streptomycin, and 0.5 μg/l insulin, and fibroblast growth medium RPMI-1640, supplemented with 200 mM L-glutamine, 100,000 IU/l penicillin, 100,000 μg/ml streptomycin, and 10% FCS. Epithelial cells were then incubated at 37˚C in 95% air - 5% CO_2 for 24 h, to allow attachment. Fibroblasts harvested after initial culturing were seeded at a density of 4,000 cells/well in 2 ml of the same 50:50 (v/v) mix of supplemented growth media, onto micro-porous membrane inserts (Millipore Corp., Billerica, MA, USA) placed into six-well plates. After cells attached for 2 h, the inserts containing fibroblasts were removed using sterile forceps and placed into the wells containing epithelial cells, allowing for interpopulation communication by means of diffusible elements while preventing direct contact between epithelial cells and fibroblasts. For separate cultures, epithelial cells or fibroblasts were seeded onto six-well plates in the same medium, to serve as controls. Co-cultured and separately cultured cells were maintained for up to 12 days; medium was changed every 3 days.

Total RNA isolation. After 12 days of co-culture, medium was removed, and cells were lysed in Trizol reagent (Invitrogen Life Technologies) and total cellular RNA was extracted according to the manufacturer's protocol. Cellular RNA was treated with RNase-free DNase I (Takara Biotechnology Co., Ltd., Dalian, China) as follows. A 50-μl reaction mix containing ~20 μg of total cellular RNA, 5 μl of 10x reaction buffer, 20 units RNase-free DNase I and 5 units of RNasin was incubated at 37˚C for 30 min and extracted with 50 μl of phenol/chloroform (1:1, v/v) (Sigma-Aldrich, St. Louis, MO, USA). The aqueous phase was transferred to a fresh tube and extracted with chloroform and isopropanol, in turn. The RNA was precipitated with 3 M sodium acetate and 75% ethanol, and then dissolved in DEPC-treated water.

Differential display of mRNA transcripts. Differential display experiments were performed using a Hieroglyph mRNA profile kit with a FluoroDD TMR-fluorescent anchored primer adapter kit, a Genomyx LR programmable DNA sequencer, and a Genomyx SC fluorescence scanner (all from Beckman Instruments, Inc., Fullerton, CA, USA), following the manufacturer's instructions as modified below.

Total RNA was reverse transcribed with four of the twelve T7(dT_{12})AP anchored primers (ACGACTCACTATA GGGCT_{12}GC; ACGACTCACTATAGGGCT_{12}GG; ACGA CTCACTATAGGGCT_{12}CC; and ACGACTCACTATAGGG CT_{12}CG), at 25˚C for 5 min, 42˚C for 5 min, 50˚C for 60 min, and 70˚C for 15 min. PCR amplifications with one TMR-anchored primer and one arbitrary primer (ACAATTTCACA CAGGACGACTCCAAG; ACAATTTCACACAGGAGCTA GCATGG; or ACAATTTCACACAGGAGACCATTGCA) were carried out using a RapidCycler PCR machine (PerkinElmer Cetus DNA Thermal Cycler) according to the following program, 95˚C for 5 min, followed by four cycles at 94˚C for 30 sec, 50˚C for 30 sec, and 72˚C for 90 sec and 25 cycles at 94˚C for 30 sec, 60˚C for 30 sec, and 72˚C for 90 sec, with a final extension at 72˚C for 7 min.

Electrophoresis in a 5.6% denaturing polyacrylamide gel (HR-1000 5.6% denaturing high-resolution differential display gel; Genomyx Co., Beckman Instruments) was performed using a Genomyx LR programmable DNA sequencer at 50˚C, 3000 V, and 100 W for 4.5 h. After scanning the gel on a Genomyx SC scanner, duplicate cDNA bands were excised, and gel pieces were immersed in 30 μl of Tris/EDTA (10 mM Tris-HCl, 1 mM EDTA), incubated at 37˚C for 3 h, and stored at 4˚C for future use.

To re-amplify gel bands, a 20-μl PCR reaction mix containing 2 μl each of gel supernatant, 2 μM M13 reverse (-48) 24-mer primer (5'-AGCGGATAACAATTTCACACA GGA-3'), and 2 μM T7 promoter 22-mer primer (5'-GTAAT ACGACTCACTATAGGGC-3') (Genomyx Co., Beckman Instruments) with dNTPs, Amplitaq DNA polymerase, and buffer (PerkinElmer, Branchburg, NJ, USA) was run on a RapidCycler PCR machine at 95˚C for 2 min, followed by 30 cycles of 94˚C for 30 sec, 60˚C for 30 sec, and 72˚C for 90 sec, with a final extension at 72˚C for 7 min. This PCR product was used as a template for direct DNA sequencing on an ABI Prism Genetic Analyzer (Applied Biosystems/ PerkinElmer, Carlsbad, CA, USA).

Reverse Northern blot analysis. The PCR products, in duplicate, were re-amplified, blotted, and fixed onto a positively charged nylon membrane (Roche, Pleasanton, CA, USA) by using a spot-blot apparatus according to the manufacturer's instructions. RNA from co-cultured and separately cultured epithelial cells and fibroblasts was reverse transcribed. Radiolabeled first-strand cDNA was generated by reverse transcription in the presence of [α-^{32}P]dCTP, dNTP, and random primer. Probes were purified by Sephadex G-50 chromatography. Membrane containing the PCR products was prehybridized in hybridization solution (6x SSC, 2x Denhardt's solution, and 0.1% SDS) containing salmon sperm DNA for 3 h at 68˚C, followed by hybridization in the same solution containing ^{32}P-labeled cDNA probes at 68˚C overnight. The membrane was washed twice in 2x SSC with 0.5% SDS at 68˚C for 20 min and then washed in 0.2x SSC with 0.5% SDS at 68˚C for 20 min. Autoradiography was performed at -70˚C.

Intensities of the hybridization signals on autoradiograms of the reverse Northern blots were determined by densitometry. The absorbance over the scan-length was evaluated and corrected for baseline absorbance by the GeneSnap 5.0 software package (Synoptics Ltd., Cambridge, England).

Gene cloning and sequencing. PCR products were ligated into pGEM-T Easy vector (Promega Co.), and the insert was confirmed by EcoRI (Life Technologies) digestion, according to the manufacturer's instructions. Sequence analysis was performed with an ABI Prism Genetic Analyzer 3100 (Applied Biosystems/PerkinElmer). cDNA sequences were analyzed using the BLAST program to search for homologous matches in the GenBank database.

After the cDNAs were identified by searching the GenBank database, we designed new primers to amplify the open reading frames of genes of interest. The PCR products were subcloned into pGEM-T Easy vector and confirmed by EcoRI digestion. Sequence analysis was performed with an ABI Prism Genetic Analyzer 3100.

RNA preparation and semi-quantitative RT-PCR analysis. Tissues were obtained from five different BPH patients, and fibroblasts and epithelial cells were separated and cultured as described above. Cells were lysed with Trizol reagent (Invitrogen Life Technologies), mixed extensively, and stored at -80˚C until RNA extraction. RNA was extracted according to a protocol provided by Invitrogen Life Technologies. The concentration of RNA was determined based on the A_{260}/A_{280} ratio (PerkinElmer UV/Vis spectrometer Lambda 20, Waltham, MA, USA) and was equalized among samples by adding the appropriate amount of DEPC-treated water. RNA was reverse transcribed using a RevertAid™ first-strand cDNA synthesis kit (Fermentas, Glen Burnie, MD, USA) according to the manufacturer's instructions.

Semi-quantitative determination of 12 EST transcript concentrations was performed by RT-PCR, with *G3PD* as an internal control. Each PCR reaction was performed in a 25-μl volume containing 0.5 μg of the diluted RT product, 2 μl of 2.5 mM dNTPs, 2.5 μl of 10x ExTaq buffer, 0.2 μl of ExTaq (Takara Biotechnology), 1 μl each of the forward and reverse intron-spanning primers (20 μM), and 1 μl each of the *G3PD* forward and reverse intron-spanning primers (20 μM). The optimal annealing temperature and cycle number required to optimize amplification efficiency for each individual gene are shown in Table I. The optimized PCR program was 95˚C for 10 min, followed by 28-35 cycles of 95˚C for 60 sec, 54-62˚C for 60 sec, and 72˚C for 90 sec, with a final extension at 72˚C for 10 min. PCR reactions were run on an MJ-PTC-220 thermocycler (MJ Research, Inc., Waltham, MA, USA). PCR products were separated in 1.5% agarose gels containing ethidium bromide and visualized by UV transillumination (Gel Doc 2000; Bio-Rad Laboratories, Hercules, CA, USA). All semi-quantitative RT-PCR analyses were repeated.

Quantitative real-time RT-PCR analysis. Based on the mRNA sequences of *KLK7* and the internal standard (*G3PD*), gene-specific primers were designed, *KLK7* forward, 5'-CCTTAG

Table I. Primers used in the semi-quantitative RT-PCR analysis of some differentially expressed genes in co-cultured epithelial cells and fibroblasts.

Gene name	Primer sequences (5'-3')	PCR product size (bp)	Annealing temperature (˚C)	PCR cycles (n)
NGRN	F: CACCCTGAAACGACAGAAAC R: TCA CGA GGC ATC TCC ATAAG	779	56	28
S100A10	F: CACCAAAATGCCATCTCAAATG R: TATCAGGGAGGAGCGAACTG	321	60	28
MAP1LC3B	F: ATGCCGTCGGAGAAGACCT R: CTGGTTTTACACTGACAATTTC	394	54	35
NAE1	F: GATCCCAAGCAGTATTGAAG R: AAGGAAGCCAGTGAGACAAG	556	54	35
SOD1	F: CCTAGCGAGTTATGGCGAC R: GCCTCAGACTACATCCAAG	502	60	30
KLK7	F: ATGGCAAGATCCCTTCTCC R: TTAGCGATGCTTTTTCATGGT	762	53	35
COL1A2	F: GTGGATACGCGGACTTTGTT R: AGGTTCACCCTTCACACCAG	610	61	28
S100A11	F: CTCGCTCAGCTCCAACATG R: GTGGGTTTGAAGGCCAGG	364	60	28
YWHAB	F: TGGGCTTAGGAAGGAAGAGG R: TGAACAGATCACAAAGCACGA	847	61	28
CCNI	F: AGGATATCATGAAGTTTCCAG R: AGTCTACCTTAGTTTACACTC	1,183	55	28
LXN	F: GCTCATCCGGAATGGAAATC R: GCTAGATGCCAGTGAAATTGG	748	61	28
RPL6	F: GTACTCAGCCGCTAAATCCA R: CAAACACAGATCGCAGGTAG	610	60	30
G3PD	F: ACCACAGTCCATGCCATCAC R: TCCACCACCCTGTTGCTGTA	452		

CCTTGGAAACTG-3'; reverse, 5'-CATGGGTGGGAGC CTCTT-3'; and *G3PD* forward, 5'-GGTGAAGGTCGGAG TCAAC-3', reverse, 5'-CCATGGGTGGAATCATATTG-3'. These primers spanned more than two exons, to avoid contamination by genomic DNA. Real-time monitoring of the PCR reactions was performed using the LightCycler™ system (Bio-Rad) and SYBR Green I dye, which binds preferentially to double-stranded DNA. The fluorescence intensity, which was proportional to the concentration of the PCR product, was measured at the end of each cycle and immediately displayed on a computer screen, permitting real-time monitoring of the PCR reaction. The threshold cycle is defined as the fractional cycle number at which the fluorescence passes a fixed threshold above baseline. For each sample, the amounts of the target and *G3PD* were determined using the respective calibration curves, and the target amount was divided by the control amount to obtain a normalized target value.

To construct the calibration curves, recombinant plasmids were prepared by respectively cloning the appropriate parts of the *KLK7* and *G3PD* coding regions into pGEM-T Vector (Promega Corp., Madison, WI, USA). The recombinant plasmids were serially diluted and included in each run. LightCycler software automatically calculated the standard curves as the starting dilution of each standard sample versus the threshold cycle, and the sample concentrations were then calculated accordingly. Hence, all calculated concentrations are relative to the concentration of the internal standard. For each run, the master mix, prepared on ice, contained 1 μl of template cDNA, 0.25 μl of SYBR Green I mix, 0.25 μl of primers, 0.2 μl of ExTaq, 2 μl of 2.5 mM dNTP, 3 μl of 25 mM $MgCl_2$, and 2.5 μl of 10x buffer. The final volume was adjusted to 25 μl with H_2O. The cycling conditions were as follows, initial denaturation at 95˚C for 2 min, followed by 35 cycles of 95˚C for 5 sec, 54˚C for 10 sec, and 72˚C for 20 sec. The temperature transition rate was 20˚C/sec. The fluorescent product was measured at the annealing step. A melting curve was obtained by gradually increasing the temperature from 65 to 95˚C at a rate of 0.5˚C/10 sec, in the signal acquisition mode set.

Western blot analysis. As human tissue kallikrein 7 protein (hK7) is a secretory protein, we used both the culture medium and cell lysates for Western blot analysis. Medium containing 10% FCS was used as a negative control. Samples of cell lysates and culture media were mixed with gel loading buffer containing 10% ß-mercaptoethanol, denatured at 100˚C for 5 min, and separated on 12% denaturing SDS-polyacrylamide gels. Separated proteins were transferred (Trans-Blot; Bio-Rad) to nitrocellulose Hybond™ membranes (Amersham Biosciences, Buckinghamshire, England). Membranes were blocked with 5% nonfat milk in Tris-buffered saline (TBS;

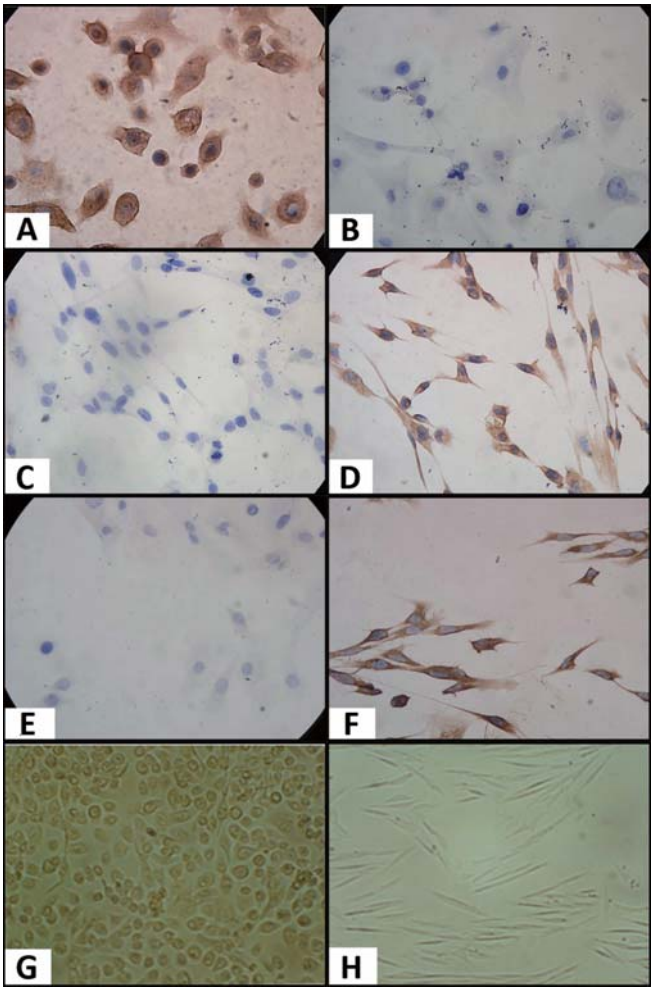

Figure 1. Characterization of cultured epithelial cells and fibroblasts from human BPH tissues, as determined by phase-contrast microscopy and immunocytochemical staining. Primary epithelial cells and fibroblasts were cultured as described in Patients and methods. Cell morphology was observed under a light microscope after staining with an antibody and under a phase-contrast microscope without staining. Representative photomicrographs of human epithelial cells and fibroblasts in culture are shown. A, Prostate epithelial cells stained with anti-PSA antibody (original magnification, x400). B, Prostate epithelial cells stained with anti-desmin antibody, x400. C, Prostate fibroblasts stained with anti-PSA antibody, x400. D, Prostate fibroblasts stained with anti-desmin antibody, x400. E, Prostate epithelial cells stained with anti-prolyl 4-hydroxylase antibody, x400. F, Prostate fibroblasts stained with anti-prolyl 4-hydroxylase antibody, x400. G, Phase contrast micrograph of prostate epithelial cells, x100. H, Phase contrast micrograph of prostate fibroblasts, x100.

50 mM Tris-HCl, pH 7.5, 150 mM NaCl) for 1 h at room temperature and then incubated with anti-hK7 antibody (1:150 dilution; R&D Systems, Minneapolis, MN, USA) in TBS-Tween 20 containing 5% nonfat milk for 2 h at room temperature, followed by incubation with horseradish peroxidase-conjugated rabbit anti-goat antibody (1:4,000 dilution; KPL, Gaithersburg, MD, USA) for 1 h at room temperature. Signals were visualized on X-ray film by enhanced chemiluminescence (SuperSignal West Pico System; Pierce Biotechnology, Rockford, IL, USA). ß-Actin (LabVision Corp., Fremont, CA, USA) was used as an internal control.

Statistical analysis. Bands from semi-quantitative RT-PCR were quantitated with Quantity One version 4.1.1 software

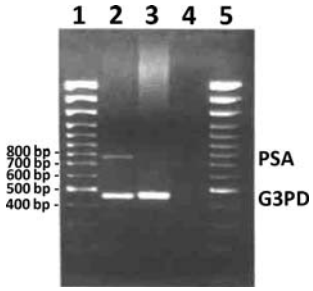

Figure 2. Detection of the biomarker PSA in cultured prostate epithelial cells and fibroblasts, using semi-quantitative RT-PCR analysis. Total RNA was isolated from prostate epithelial cells and fibroblasts and was converted to cDNA. The PSA mRNA expression level was determined by semi-quantitative RT-PCR, as described in Patients and methods. PCR products were separated in 1.5% agarose gels containing ethidium bromide and visualized by UV transillumination. Shown is a photograph representing the analysis of PSA mRNA levels in one of three independent experiments. Lanes 1 and 5, markers; 2, prostate epithelial cells; 3, prostate fibroblasts; 4, negative control.

(Bio-Rad). Comparisons between co-cultured and separately cultured cells were made using a paired-sample t-test (SPSS 11.5). Results were considered significant at $P<0.05$. Real-time RT-PCR data were analyzed with a Wilcoxon signed ranks test.

Results

Characterization of fibroblasts and epithelial cells of prostate tissues from BPH patients. Cultures of prostatic fibroblasts and epithelial cells were verified by immunocytochemical staining with antibodies against PSA and desmin. Epithelial cells were uniformly immunostained for PSA (Fig. 1A), but not for desmin (Fig. 1B). In contrast, the fibroblasts were positively labeled for desmin (Fig. 1D), but not for PSA (Fig. 1C). Prolyl 4-hydroxylase, another fibroblast marker, was also used to characterize prostate fibroblasts. Fibroblasts were immunostained by anti-prolyl 4-hydroxylase antibody (Fig. 1F), but epithelial cells were not (Fig. 1E). In addition to immunostaining, phase-contrast microscopy was used to examine the cells. An analysis of the photomicrographs demonstrated that epithelial cells and fibroblasts had no contaminants (Fig. 1G and H). Lastly, we characterized the cells by semi-quantitative RT-PCR analysis of the PSA biomarker, showing clearly that PSA was expressed in epithelial cells (Fig. 2, lane 2), but not in fibroblasts (Fig. 2, lane 3).

Identification of differentially expressed genes by DDRT-PCR and reverse Northern blot analysis. To identify genes with altered expression in BPH, we examined mRNA levels in co-cultured and separately cultured prostate fibroblasts and epithelial cells, using DDRT-PCR analysis (Fig. 3). The mRNA transcripts of 110 genes were differentially expressed between co-cultured and separately cultured fibroblasts and epithelial cells. On reverse Northern blots, 84 bands showed differential expression in co-cultured versus separately cultured fibroblasts and epithelial cells, and 68 of these were successfully sequenced. Of these 68 cDNAs, 43 had altered expression in co-cultured epithelial cells (37 upregulated and 6 downregulated genes), and 25 showed altered expression in co-cultured

Table II. Identification of some genes differentially expressed in co-cultured versus separately cultured epithelial cells and fibroblasts from human BPH tissues.

Band no.	cDNA (gene) name	GenBank accession no.
Prostate epithelial cells		
A3	*Homo sapiens* neugrin (*NGRN*)	AF242770
A51	S100 calcium binding protein A10 (*S100A10*)	BC105786.1
B56	Microtubule-associated protein 1 light chain 3-ß (*MAP1LC3B*)	BC045759.1
B57	NEDD8 activating enzyme E1 subunit 1 (*NAE1*)	NM_003905.3
B58	Superoxide dismutase 1 (*SOD1*)	NM_000454.4
B61	Kallikrein 7 long variant protein (*KLK7*)	NM_139277
Prostate fibroblasts		
B27	Collagen, type I α 2, mRNA (*COL1A2*)	BC054498.1
B31	S100 calcium binding protein A11 (*S100A11*)	BC001410.2
B32	Tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein activation protein, ß polypeptide (*YWHAB*)	NM003404.3
B34	Cyclin I (*CCNI*)	BT020019.1
B37	Latexin mRNA (*LXN*)	BC008438.1
B50	Ribosomal protein L6 (*RPL6*)	BC031009.1

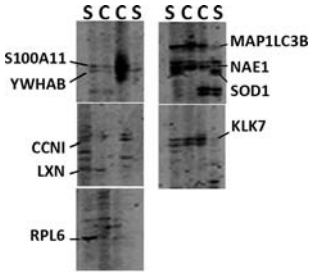

Figure 3. Identification of differentially expressed genes in co-cultured versus separately cultured prostate cells, determined by DDRT-PCR analysis. RNA was isolated from co-cultured and separately cultured epithelial cells and fibroblasts. Differential gene expression was assessed by DDRT-PCR analysis, as described in Patients and methods. A representative autoradiogram shows the DDRT-PCR products amplified by different primer sets after electrophoresis in a 5.6% denaturing polyacrylamide gel at 50˚C, 3000 V, and 100 W. Radiolabeled PCR products were visualized by autoradiography. Lanes in each panel, from left: S, separately cultured fibroblasts; C, co-cultured fibroblasts; C, co-cultured epithelia; S, separately cultured epithelia. Several differentially displayed bands are marked. S100A11, S100 calcium binding protein A11; YWHAB, tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein activation protein ß polypeptide; CCNI, cyclin I; LXN, latexin; RPL6, ribosomal protein L6; MAP1LC3B, microtubule-associated protein 1 light chain 3-ß; NAE1, NEDD8 activating enzyme E1 subunit 1; SOD1, superoxide dismutase 1; KLK7, kallikrein 7.

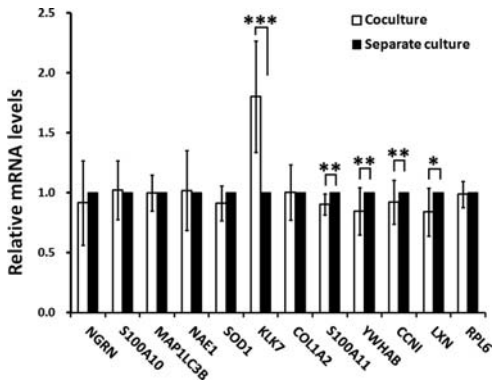

Figure 4. Differential gene expression in co-cultured versus separately cultured epithelial cells and fibroblasts from human BPH tissues, as assessed by semi-quantitative RT-PCR analysis. Total mRNA was isolated from co-cultured and separately cultured epithelial cells and fibroblasts and was converted to cDNA. The mRNA level of each gene was determined by semi-quantitative RT-PCR, as described in Patients and methods. Bands were detected automatically by Quantity One version 4.1.1. software. The housekeeping gene *G3PD* was used as an internal control for each sample, and the mRNA level of each gene was normalized to *G3PD* mRNA level. After normalization, the mRNA level of each gene in separately cultured epithelial cells and fibroblasts was set at 1 (100% control value). The mRNA level of each gene in co-cultured epithelial cells and fibroblasts are expressed relative to the level in separately cultured epithelial cells and fibroblasts, respectively. The results represent the mean ± SD of at least three independent experiments. *P<0.05; **P<0.01; ***P<0.001. NGRN, *Homo sapiens* neugrin; S100A10, S100 calcium binding protein A10; MAP1LC3B, microtubule-associated protein 1 light chain 3-ß; NAE1, NEDD8 activating enzyme E1 subunit 1; SOD1, superoxide dismutase 1; KLK7, kallikrein 7; COL1A2, collagen type I α 2; S100A11, S100 calcium binding protein A11; YWHAB, tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein activation protein ß polypeptide; CCNI, cyclin I; LXN, latexin; RPL6, ribosomal protein L6.

fibroblasts (6 upregulated and 19 downregulated genes). Representative cDNAs are shown in Fig. 3 and Table II.

Validation of differentially expressed genes by semi-quantitative RT-PCR analysis. To confirm differential expression of specific genes, we used semi-quantitative RT-PCR to analyze the expression levels of 12 DDRT-PCR products that represented known genes with established functions (Fig. 4). The primers for RT-PCR amplification of these genes are shown in Table I. After normalization to the *G3PD* transcript level, the *KLK7* mRNA level was significantly higher in co-cultured epithelial cells compared with separately cultured epithelial cells (P<0.001), whereas there were no consistent differences in the mRNA levels of *Homo sapiens* neugrin (*NGRN*), S100 calcium binding protein A10 (*S100A10*), microtubule-associated protein 1 light chain 3-ß

Table III. Expression of KLK7 mRNA in co-cultured and separately cultured prostate epithelial cells, as assessed by quantitative real-time RT-PCR analysis.

Culture system	Mean[a]	SD[a]	Range[a]	P-value[b]
Co-cultured	1.50E-02	1.00E-02	2.38E-03-2.84E-02	0.028
Separately cultured	3.34E-03	3.59E-03	5.52E-04-8.93E-03	

[a]Values are expressed as the ratio of *KLK7* to *G3PD*; [b]Calculated by the Wilcoxon signed ranks test.

(*MAP1LC3B*), NEDD8 activating enzyme E1 subunit 1 (*NAE1*), and superoxide dismutase 1 (*SOD1*) between co-cultured and separately cultured epithelial cells (Fig. 4). In contrast, co-cultured fibroblasts exhibited reduced mRNA levels of four other genes, S100 calcium binding protein A11 (*S100A11*), tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein (*YWHAB*), cyclin I (*CCNI*), and latexin (*LXN*), compared with the levels in separately cultured fibroblasts (P<0.05) (Fig. 4). The mRNA levels of collagen type I α2 (*COL1A2*) and ribosomal protein L6 (*RPL6*) showed no consistent differences between co-cultured and separately cultured fibroblasts (Fig. 4).

Quantitative real-time RT-PCR analysis of KLK7 gene expression. Real-time RT-PCR was used to quantitate the mRNA expression of *KLK7*, based on a standard curve constructed by using serial dilutions of a plasmid containing a *KLK7* coding region. The *KLK7* mRNA level was normalized to that of *G3PD* in each sample, giving *KLK7*/*G3PD* ratios ranging from 2.38E-03 to 2.84E-02 (mean ±SD, 1.50±1.00E-02) in co-cultured epithelial cells, and ratios ranging from 5.52E-04 to 8.93E-03 (mean ±SD, 3.34±3.59E-03) in separately cultured epithelial cells (Table III). The *KLK7* mRNA expression level was significantly higher in co-cultured versus separately cultured epithelial cells (P<0.05) (Table III). The results with quantitative real-time RT-PCR agreed with those found by traditional semi-quantitative RT-PCR (Fig. 4).

Western blot analysis of hK7 protein levels. Western blots revealed that the *KLK7* gene product, hK7, was upregulated in the lysate and culture medium of co-cultured epithelial cells compared with hK7 expression in separately cultured epithelial cells (Fig. 5), consistent with *KLK7* mRNA expression. On Western blots, hK7 was detected as a 28-kDa band in epithelial cell lysates, but as a 60-kDa band in the culture medium (Fig. 5), suggesting that the secreted hK7 may bind another protein in the medium or may be modified, such as by glycosylation. Repetition of the Western blot analysis gave consistent results, and ß-actin levels verified relatively equal protein loads.

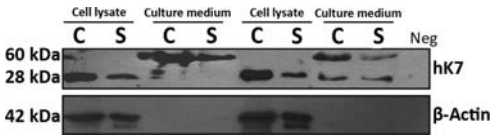

Figure 5. Western blot analysis of hK7 protein levels in culture medium and cell lysates of co-cultured and separately cultured epithelial cells. A total of 40 μg of protein isolated from co-cultured or separately cultured epithelial cells or from the culture medium was subjected to Tris-glycine gel electrophoresis and immunoblotted with an antibody against hK7. ß-Actin was used as a loading control. The Western blot data presented here are representative of those obtained from three separate experiments. Lane C, co-cultured epithelial cells; S, separately cultured epithelial cells; Neg, negative control.

Discussion

The mechanisms underlying the pathogenesis of BPH are not clear, despite intensive research efforts. Nevertheless, accumulating evidence supports a vital role for epithelial-stromal interaction in BPH development. Here, we demonstrated that 110 genes were differentially expressed in co-cultured, versus separately cultured, prostate epithelial cells and fibroblasts from BPH tissues. These results provide evidence for epithelial-stromal interaction in the BPH microenvironment. These interactions may be mediated through the secreted products of the differentially expressed genes and may affect BPH disease processes, including cell proliferation, differentiation, growth, and death.

Immortal cell lines may not be appropriate for studying some diseases, as they represent a highly selective cell population that might have undergone phenotypic changes and may therefore be distinctive from the cells of origin. In an attempt to overcome this limitation, we chose a co-culture system of epithelial cells and fibroblasts from benign hyperplastic prostate tissues, employing a defined medium. A morphological study (8) on cells from BPH tissues demonstrated that when grown in co-culture, the epithelial cells exhibited round nuclei, tonofibrils, and microvilli, and the fibroblasts had elongated nuclei, large amounts of Golgi apparatus, and cilia; these are characteristics of the differentiated state. However, there were no studies using a co-culture model to examine differential gene expression in cells derived from BPH tissues.

Initially, we used DDRT-PCR to identify differentially expressed genes. This method has several advantages for analyzing differential gene expression: it requires only small amounts of RNA and no specifically designed genetic tools. On the other hand, DDRT-PCR has a high false-positive rate and an uncertain degree of genome coverage. Therefore, to confirm differentially expressed mRNA, we performed a reverse Northern blot analysis, and further validated the data with RT-PCR, to minimize the occurrence of technically false positive results. The genes identified as being differentially expressed between co-cultured and separately cultured epithelial cells and fibroblasts include genes whose products are involved in energy metabolism, prosthetic groups, carriers, transporters, and the biosynthesis of cofactors and the cell envelope.

One gene differentially expressed in epithelial cells was *KLK7*. This gene belongs to the 15-member human tissue kallikrein (*KLK*) family of serine proteases, which includes prostate specific antigen (hK3). The *KLK7* product, hK7 (stratum corneum chymotryptic enzyme, SCCE) (9), was originally identified from a keratinocyte library, and the enzyme was purified from stratum corneum of human skin. According to previous studies, hK7 degrades the cohesive structures between individual corneocytes in the stratum corneum, thereby contributing to cellular desquamation or shedding of skin cells (10), and may have the potential to degrade the surrounding matrix during tumor progression (11). In addition, kallikreins may promote or inhibit cancer cell growth, angiogenesis, invasion, and metastasis through the activation of growth factors and other proteases, the release of angiogenic or anti-angiogenic factors, and the degradation of extracellular matrix components (12). Recent studies have revealed altered hK7 expression in hormone-independent (13) and hormone-dependent cancers, including ovarian (11,14) and breast cancer (15). However, the function of *KLK7* in prostate epithelial cells remains unknown. We propose that *KLK7* may be an important messenger for epithelial-stromal interaction and may play a role in the modulation of prostate cell growth. Further *in vitro* and *in vivo* studies are needed to elucidate the biological role of *KLK7* in BPH and other prostatic diseases.

PSA is a widely used serum biomarker for prostate cancer. Studies have confirmed that the expression of PSA and hK2 is higher in noncancerous than in cancerous prostatic tissues (16), although the levels of PSA and hK2 are higher in the serum of prostate cancer patients. One possible explanation for this phenomenon is that PSA may be released into the blood when the basal membranes of acini are damaged by cancer cells. PSA shares 41% amino acid identity with hK7 (17), and both are secreted by prostate epithelial cells. Previously, several reports have recommended hK7 as a candidate tumor marker with prognostic value in ovarian and breast cancers (11,14,15,18). Further investigation is necessary to evaluate hK7 as a new adjuvant tumor marker, in addition to PSA, for prostate cancer.

Another gene found to be differentially expressed is S100A11, which has been shown to induce apoptotic cell death (19). Furthermore, nuclear S100A11 may be involved in contact inhibition of cell growth (20). In our experiments, S100A11 was downregulated in co-cultured fibroblasts from BPH tissues, suggesting that it may participate in the modulation of cell proliferation during the pathophysiological processes of BPH.

Based on the presence of a cyclin box motif in its deduced amino acid sequence, cyclin I has been added to the cyclin family of proteins. Cyclin I is post-transcriptionally regulated by ubiquitination (21), and dysregulation of ubiquitin-proteasome-mediated degradation of cyclin I may be related to aberrant cell cycle regulation and/or resistance to apoptosis, which are characteristics of invasive tumor cells (22). A strong association was shown between cytoplasmic cyclin I staining and vascular endothelial growth factor (VEGF) as well as VEGF receptor in human breast cancer (22). This association may indicate a role of cyclin I in angiogenesis, a potentially separate function from cell cycle regulation (22). Immunohistochemical results have suggested that cyclin I is involved in pancreatic cancer carcinogenesis and progression (23). Cyclin I is upregulated by dihydrotestosterone (24), but the biological function of cyclin I in the prostate remains to be determined.

Tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein, ß polypeptide (*YWHAB*), another gene differentially expressed in co-cultured cells from BPH tissues, belongs to the 14-3-3 family of proteins, which bind to phosphoserine-containing proteins to mediate signal transduction. YWHAB has been proposed to have an important role in the negative regulation of mitogen-activated cell growth, proliferation, and differentiation via the p90 ribosomal S6 kinase 1 pathway (25). The *LXN* gene, which was down-regulated in co-cultured fibroblasts, encodes latexin, a protein inhibitor of the zinc-dependent metallocarboxypeptidases. Liang and colleagues (26) have shown that latexin expression was inversely correlated with the number of hematopoietic stem cells, indicating that latexin may be an important regulatory factor in cell proliferation. Nonetheless, the biological roles of latexin and YWHAB, as well as their implications in the pathogenesis of BPH and other prostatic diseases, remain elusive.

In summary, we showed by DDRT-PCR that 110 genes were differentially expressed in co-cultured versus separately cultured fibroblasts and epithelial cells of BPH tissues. To validate these results, we used semi-quantitative RT-PCR to analyze 12 genes with known functions and found that five genes were differentially expressed in the co-culture system. Among them, *KLK7* was upregulated in co-cultured epithelial cells, whereas four other genes were downregulated in co-cultured fibroblasts. We believe that these differentially expressed genes play important roles in the processes of epithelial-stromal cell interaction through the secretion of their gene products into the microenvironment at disease sites. Although previous reports have linked these five differentially expressed genes to cell cycle regulation, cell proliferation, and cell differentiation, the precise functions of these genes in biological and disease processes of the prostate remain unclear. Our studies are underway to determine the roles and mechanisms of the differentially expressed genes in the pathogenesis of BPH and other prostatic diseases and to elucidate the molecular basis for the signaling pathways involved in epithelial-stromal communications under both physiological and pathophysiological conditions.

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