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Differential impact of paired patient-derived BPH and normal adjacent stromal cells on benign prostatic epithelial cell growth in 3D culture

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Abstract

Background: Benign prostatic hyperplasia (BPH) is an age-related disease characterized by nonmalignant abnormal growth of the prostate, which is also frequently associated with lower urinary tracts symptoms (LUTS). The prostate with BPH exhibits enhanced growth not only in the epithelium but also in the stroma, and stromal-epithelial interactions are thought to play an important role in BPH pathogenesis. However, our understanding of the mechanisms of stromal-epithelial interactions in the development and progression of BPH is very limited.

Methods: Matched pairs of glandular BPH and normal adjacent prostate specimens were obtained from BPH patients undergoing simple prostatectomy for symptomatic BPH. Tissues were divided further into fresh specimens for culture of primary prostatic stromal cells, and specimens were embedded in paraffin for immunohistochemical analyses. Proliferation assays, immunohistochemistry, and immunoblotting were used to characterize the primary prostate stromal cells and tissue sections. Co-culture of the primary stromal cells with benign human prostate epithelial cell lines BHPe1 or BPH-1 was performed in 3D Matrigel to determine the

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Author contributions

WC, KW, AMS, and RC performed the experiments and WC, LEP and ZW analyzed the data and wrote the manuscript. RD provided prostate tissues and pathology assessment. WC and ZW conceived and designed the study. All authors read and approved the final manuscript.

Disclosure of conflicts of interest

The authors have nothing to disclose.

impact of primary stromal cells derived from BPH on epithelial proliferation. The effect of stromal conditioned medium on BHPe1 and BPH-1 cell growth were tested in 3D Matrigel as well.

Results: BPH stromal cells expressed less smooth muscle actin and calponin and increased vimentin, exhibiting a more fibroblast and myofibroblast phenotype compared to normal adjacent stromal cells both in culture and in corresponding paraffin sections. Epithelial spheroids formed in 3D co-cultures with primary BPH stromal cells were larger than those formed in co-culture with primary normal stromal cells. Furthermore, conditioned medium from BPH stromal cells stimulated epithelial cell growth while conditioned medium from normal primary stromal cells did not in 3D culture.

Conclusions: These findings suggest that the stromal cells in BPH tissues are different from normal adjacent stromal cells and could promote epithelial cell proliferation, potentially contributing to the development and progression of BPH.

Keywords

BPH; Stromal-epithelial interaction; 3D culture; BPH-1; BHPe1

Introduction

Benign prostatic hyperplasia (BPH) is characterized by nonmalignant pathological growth of the prostate which tends to constrict the urethra and can cause debilitating urinary symptoms. BPH is one of the most common diseases in men, particularly in older men^{1,2}. BPH prevalence increases with age, ranging from 50% incidence in men between the ages of 60–69 to almost 90% incidence in men between the ages of 70–89³. Although BPH is not typically life threatening, it is associated with symptoms such as lower urinary tract symptoms (LUTS) and bladder outlet obstruction (BOO), which can be debilitating and significantly impact quality of life⁴. As male life expectancy increases, the number of men affected by BPH and the economic burden of treating BPH are likely to increase. In 2006, the cost of BPH treatment was estimated to be more than \$4 billion per year in the USA^{4,5}. Treatment for BPH-associated LUTS includes lifestyle changes, medication and/or surgery^{6,7}. Lifestyle changes including caffeine and alcohol avoidance, fluid management and bladder retraining have been shown to improve LUTS in some patients^{7,8}. FDA approved medications currently used to treat BPH-associated LUTS include 5 alpha-reductase inhibitors (5ARIs), finasteride and dutasteride, and alpha adrenoreceptor blockers, such as terazosin and doxazosin^{9–13}. Unfortunately, not all patients benefit from lifestyle changes and/or medication and elect surgery for symptomatic relief, which is costly and as with any surgery carries risks. Thus, there is an urgent need for new approaches for prevention and more effective treatment of LUTS associated with BPH, which will require a greater understanding of the basic mechanisms underlying BPH development and progression.

Stromal-epithelial interactions play key roles in prostate development and differentiation throughout embryonic life, puberty and adulthood (Reviewed in^{14,15}). For example, stromal cells are able to modulate the differentiation pattern of epithelium^{16–19}. Prostatic stromal cells secrete many growth factors such as fibroblast growth factors and insulin-like growth

factors I and II to induce proliferation and differentiation of epithelial cells and the stroma itself^{20–23}. Stromal cells may also impact the development and progression of prostate cancer (Reviewed in^{15,24}). More recently, a role for stromal-epithelial interaction in BPH development and progression has been explored^{25–27}.

Epithelial cell lines in monolayer cultures have been commonly used to study prostate cancer and BPH. However, prostate epithelial cell cultures do not express luminal epithelial markers or androgen receptors²⁸. The importance of the stroma as a target for prostate carcinogenesis has been highlighted by using 3D cultures, which provide a more physiologically relevant *in vitro* system for assessing the stromal/epithelial cell interactions^{29–31}. Recently, prostatic stroma was reported to increase the viability and maintained the branching phenotype of human prostate organoids³². Although stromal-epithelial interactions play an important role in BPH pathogenesis, it is not clear if and how BPH stroma is different from the paired normal prostatic stroma in their influence on prostatic epithelial cells.

Here, we used primary cultures from paired stromal cells of BPH and normal adjacent prostate (NAP) tissues from patients with clinical glandular BPH. We used a 3D heterotypic human prostate model to reveal a differential effect of BPH versus NAP primary stromal cells on the growth of prostatic epithelial cells lines.

Materials and Methods

Prostate tissue collection and processing

Residual prostate tissue specimens used in this study included paraffin embedded tissue blocks and fresh specimens obtained from patients undergoing simple prostatectomy for symptomatic glandular BPH at the University of Pittsburgh Medical Center, under an approved Institutional Review Board protocol through the Pitt Biospecimen Core at the University of Pittsburgh. The cohort of tissue blocks included specimens from 10 patients who were treatment naïve and who also underwent prostatectomy because of BPH. The cohort of 6 fresh paired specimens of BPH and normal adjacent prostate (NAP) were derived from patients who were treated for symptomatic BPH by transurethral resection of the prostate or prostatectomy. No incidental foci of carcinoma were present in this cohort. Fresh prostatic tissue specimens were dissected under sterile conditions and histologically classified by a board-certified genitourinary pathologist (R. Dhir) immediately after surgery. Areas of BPH and NAP were confirmed by examining frozen sections of either NAP or BPH tissues procured adjacent to the sample submitted for clinical histologic assessment. All tissues were confirmed free of cancer. Normal adjacent tissues were without any histopathological changes and taken from the transition zone and/or central zone of the prostate. Thereafter, the tissue samples were transported to the cell culture laboratory in 50/50 Dulbecco's modified Eagles medium (DMEM)/F12 (10–090-CV, Corning Inc., Corning, NY, USA), supplemented with 1 µg/ml insulin-transferrin-selenium-X (51500056, Invitrogen, Waltham, MA, USA), 0.4% bovine pituitary extract (13028014, Gibco, Waltham, Massachusetts, USA), and 3 ng/ml epidermal growth factor (S0155, Gibco). A total of 6 paired BPH and NAP samples were used. Each sample was divided into two parts. One part

was formalin-fixed and then embedded in paraffin for subsequent immunohistochemical analyses, and the second part was further processed for primary cell culture.

Ki-67 staining

Slides were deparaffinized in xylene and rehydrated in graded concentrations of ethanol in water, ending with a final rinse in water. Antigen retrieval was performed using a Diva Decloaker (DV2004, Biocare Medical, Pacheco, CA, USA) retrieval solution pH 6.2 and a Decloaking chamber at 120°C. The slides were stained using an Autostainer Plus (Dako, Carpinteria, CA) with TBST rinse buffer (Dako). The Ki-67 antibody was applied using a 1:100 dilution. The secondary consisted of Envision Dual Link + (Dako) HRP polymer. The substrate used was 3,3', Diaminobenzidine + (Dako). Lastly, the slides were counterstained with Hematoxylin (Dako).

Primary stromal cell culture

BPH and NAP tissue specimens were minced and dissociated by incubation in 2.4 U/ml Dispase II (04942078001, Roche Applied Science, Penzberg, Germany) at 37 °C for 1 hr to form a suspension. A volume of 1 ml cell suspension was diluted with 4 ml of 50/50 Dulbecco's modified Eagles medium (DMEM)/F12 included 1 µg/ml insulin-transferrin-selenium-X, 0.4% bovine pituitary extract, and 3 ng/ml epidermal growth factor in 6 cm dishes. Plated cells were incubated at 37 °C, 5% CO₂ for 5 days without disturbing. On day five, 2 ml of fresh medium was added to each dish to compensate for evaporation. Stromal cell proliferation was typically evident by day seven. When stromal cells reached 95% confluence, cells were serially passaged using trypsin:EDTA (0.25%:0.53 mM) solution and neutralized with medium. Primary cells were used in experiments from passages 1–15.

Proliferation Assay

A total of 3000 primary stromal cells per well were seeded in 6-well plates and grown in 50/50 Dulbecco's modified Eagles medium (DMEM)/F12 supplemented with 1 µg/ml insulin-transferrin-selenium-X, 0.4% bovine pituitary extract, and 3 ng/ml epidermal growth factor. After attachment and growth overnight, the cells were trypsinized and cell density was calculated using a hemocytometer at 48 and 96 hours in triplicate for each time point.

Western blotting

Primary cultures of BPH and NAP cells were collected for Western blot assay. RIPA lysate solution was used to prepare cell lysates, and the protein concentration was measured using a BCA Protein Assay Kit (23225, Thermo Fisher Scientific, Waltham, MA, USA). After Western transfer, the membranes were blocked and then incubated with primary antibodies for SMA (1:1000), calponin (1:1000), vimentin (1:1000) and GAPDH (1:3000) (Table 1). Subsequently, the membranes were incubated with corresponding secondary antibodies and were assessed with chemiluminescence. Quantification of band intensity was determined using ImageJ³³. The band intensities were normalized to GAPDH.

Immunohistochemistry

Immunohistochemical staining of patient tissues was performed on 5 μm sections of paraffin blocks. Sections were dewaxed in xylene and ethanol, then rehydrated through a graded series of ethanol. Heat induced epitope retrieval was performed using 10 mmol/L of citrate buffer (pH 6), followed by rinsing in TBS buffer. Sections were then incubated in 3% H_2O_2 for 30 min followed by blocking with 3% BSA for 30 min at room temperature. Primary antibodies (Table 2) were diluted in 3% BSA and incubated overnight at 4°C. ImmunoCruz Rabbit ABC staining (sc-2018, Santa Cruz Biotechnology, Dallas, Texas, USA) and ImmunoCruz Mouse ABC staining (sc-2017, Santa Cruz) were used. Slides were then counterstained in hematoxylin and cover-slipped. Immunostained sections were imaged with a Leica DM LB microscope (Leica Microsystems Inc, Bannockburn, IL, USA) equipped with an Imaging Source NII 770 camera (The Imaging Source Europe GmbH, Bremen, Germany) and NIS-Elements Documentation v 4.6 software (Nikon Instruments, Inc., Mellville, NY, USA).

Immunostaining was quantitated using the color deconvolution plugin to separate the positive staining (brown, 3,3'-diaminobenzidine (DAB)) and then ImageJ³⁴. Five fields from each section were analyzed and an average score for each tissue type was calculated for each patient. Immunostained slides were also evaluated using the H-Score method by assessing the percentage of stromal cells exhibiting each level of staining intensity, i.e., no staining, or none, faint, moderate or intense³⁵. A minimum of 5 fields from each section was analyzed and an average score for each tissue type was calculated for each patient. All scores were reviewed and confirmed by a board-certified genitourinary pathologist (R.D.).

3D Matrigel co-culture

In 3D Matrigel co-cultures, primary stromal cells and epithelial cells (BPH-1 or BHPe1) were mixed in a 2:1 ratio. Mixed cells (2500 cells) were suspended in 40 μl Matrigel and seeded into a 24-well plate, and gels were allowed to solidify for 15 min at 37 °C before adding 1 ml medium into each well. Cultures were maintained for 6 days, and the medium refreshed every 2 days.

Epithelial spheroids (about 30 spheres/well) were imaged using an inverted phase-contrast microscope (Nikon TE2000-U) equipped with a Hamamatsu digital camera (Hamamatsu Inc) and NIS-Elements Documentation v 4.6 software (Nikon Instruments, Inc., Mellville, NY) with a 20x objective at the 6th day of culture. Cells were fixed using 2% paraformaldehyde (PFA) at room temperature for 30 mins. The diameter of the spheres was measured using ImageJ (Wayne Rasband, National Institutes of Health, USA)³³. All experiments were performed in triplicate.

3D Matrigel stromal conditioned medium culture

For stromal conditioned medium experiments, primary stromal cells derived from BPH or NAP were seeded into 6 cm dishes and grown in complete medium for 2 days to reach 100% confluence prior to collection. Conditioned medium (CM) was collected from 100% confluent plates by aspirating from the culture dish into a 15 ml falcon tube. The stromal CM was centrifuged at 180 x g for 5 min to remove cellular debris and the supernatant was

collected and stored at 4 °C. BPH-1 or BHPRE1 (2500 cells) were mixed with 40 µL of Matrigel then added carefully at the center of each well of a 24-well plate, and gels were allowed to solidify for 15 min at 37 °C. After Matrigel solidification, 500 µl stromal CM plus 500 µl fresh complete medium were added to each well and cultured without disturbing for 2 days at 37 °C. The media in each well was replaced with a fresh mixture of 500 µl CM and 500 µl completed medium every 2 days.

Epithelial spheroids (about 20 spheres/well) were imaged using an inverted phase-contrast microscope (Nikon TE2000-U) equipped with Hamamatsu digital camera (Hamamatsu Inc) and NIS-Elements Documentation v 4.6 software (Nikon Instruments, Inc., Melville, NY) with a 20x or 40x objective. On day 6, cells were fixed using 2% paraformaldehyde (PFA) at room temperature for 30 mins. The diameter of the spheres was measured using ImageJ (Wayne Rasband, National Institutes of Health, USA).

Statistics and Calculations

Statistical analyses were performed with GraphPad Prism 7 (GraphPad Software, La Jolla, California, USA). Data were expressed as mean $\pm$ standard deviation. Differences between groups were analyzed by Multiple t-tests-one per row. Statistical significance was defined as $P < 0.05$.

Results

Proliferation in BPH and NAP patient specimens

Increased epithelial proliferation has been reported in BPH tissues³⁶. The proliferation marker Ki-67 was used to detect dividing cells in paraffin embedded prostate tissue sections from a cohort of 10 patients with glandular BPH. Areas of BPH and NAP were identified by a board-certified genitourinary pathologist (RD), and Ki-67 positive epithelial and stromal cells were quantified. The average number of Ki-67-positive epithelial and stromal cells (five 20X-fields/patient) was significantly increased in areas of glandular BPH compared to NAP (Fig.1). These results suggest that the proliferation of epithelial and stromal cells was higher in BPH tissue compared to NAP tissue.

Characterization of primary human prostatic stromal cells derived from BPH patients

In order to explore potential differences in BPH and NAP stroma, six pairs of primary BPH and NAP stromal cells were derived from patients with symptomatic glandular BPH (Fig. 2A). The morphology of the monolayer cultures of BPH stromal cells and NAP cells were different. Generally, NAP stromal cells displayed a more spindle-shaped morphology, while BPH stromal cells appeared to be slightly larger and more irregular in shape (Fig. 2B). The cell growth was routinely determined at various passages for all of the six patient-derived paired stromal cells. Proliferation of stromal cells derived from BPH was higher than stromal cells derived from NAP in four patient pairs, however two patient pairs exhibited no significant difference in growth rate between BPH and NAP derived stromal cells and a much lower growth rate than the other four patient pairs (Fig.2C). These proliferation rates for BPH and NAP for each patient remained consistent over the passages examined.

Prostate stroma contains a mixture of smooth muscle cells, fibroblasts and myofibroblasts, as denoted by their expression of smooth muscle actin (SMA), calponin and vimentin^{30,37,38}. Normal adult prostate stroma is composed predominantly of smooth muscle cells with few fibroblasts and myofibroblasts, while BPH stroma has an increased composition of fibroblasts and myofibroblasts and fewer smooth muscle cells (Fig. 3A)^{39,40}. Western blot analysis of the six paired patient stromal cells showed that primary stromal cells derived from NAP tissues displayed a higher level of SMA and calponin compared to the cells derived from BPH (Fig. 3B), suggesting that stromal cultures derived from NAP are predominantly composed of smooth muscle cells. Primary stromal cells derived from BPH expressed increased vimentin levels compared to NAP, suggesting an increased prevalence of fibroblasts and myofibroblasts. These results were consistent across all six patients and were maintained over the passages examined (1–15).

Immunohistochemical staining of corresponding paraffin embedded tissue sections from BPH and normal adjacent areas was in an agreement with the western blot results for all ten patient pairs. Overall, SMA staining intensity was similar between NAP and BPH (Fig. 4A, B), while calponin expression was significantly decreased in BPH compared to NAP (Fig. 4C, D). Vimentin expression was evident as strong intensity staining of the plasma membrane in BPH stromal cells, while expression was low in NAP tissues (Fig. 4E, F). Distribution of staining intensity was also determined for the percentage of stromal cells displaying no staining (none), faint, moderate or intense staining. There was no difference between NAP and BPH in the distribution of stromal cells displaying SMA staining (Supplemental Fig. S1A). There was a significant decrease in the percentage of BPH stromal cells exhibiting intense and moderate staining and a significant increase (22.9 % in NAP, vs 48.7 %, $p < 0.001$) in the percentage of BPH stromal cells exhibiting no calponin staining (Supplemental Fig. S1B). BPH stromal cells displayed a statistically significant increase in moderate-intense vimentin staining and a decrease in the percentage of stromal cells with no staining (Supplemental Fig. S1C). These changes further suggest that the BPH stromal composition contains more fibroblasts and myofibroblasts and decreased smooth muscle cells.

BHPrE1 and BPH-1 spheroid formation in 3D co-cultures

In prostate, stromal cells surround and support the epithelium and provide important signaling for the maintenance and differentiation of the glandular epithelium (Reviewed in^{23,41}). To determine whether stromal cells derived from BPH are more potent than normal stromal cells in stimulating proliferation of epithelial cells, paired stromal cells derived from BPH and NAP from three different patients were co-cultured with the BHPrE1 prostatic epithelial cell line in Matrigel for 6 days. Stromal pairs included two patients with different proliferation rates in BPH compared to NAP derived stromal cells, and one patient pair with similar proliferation rates (see Fig. 2C). BHPrE1 or BPH-1 prostatic epithelial cells were able to form acinus-like spheroids within 6 days in 3D culture (Fig. 5A). Overall, the spheroid volume of BHPrE1 and BPH-1 was not influenced by co-culture with NAP stromal cells (Fig. 5B). In the presence of primary BPH stromal cells, the volume of spheroids formed by both BHPrE1 and BPH-1 was significantly larger compared to cells cultured in Matrigel alone or co-cultured with NAP stromal cells on Day 6 (Fig. 5C, D, E). These

results were reproducible in three pairs of stromal cells derived from three different patients with BPH, suggesting that BPH stromal cells could stimulate epithelial growth. In contrast, NAP stromal cells did not stimulate epithelial cell growth in 3D culture in parallel experiments.

BHPrE1 and BPH-1 spheroid formation in 3D cultures with stromal conditioned medium

Prostate stromal cells have been shown to induce changes in epithelial cells through secreted factors and do not require cell-cell contact^{22,23,42}. To compare the ability of stromal secreted factors from NAP and BPH-derived stromal cells to impact prostate epithelial cell growth *in vitro*, we compared growth of BHPrE-1 and BPH1 spheroids in 3D Matrigel when paired NAP and BPH stromal cell conditioned medium from three patients. The spheroid volume of BHPrE1 and BPH-1 was not influenced by NAP stromal cell conditioned medium except for a slight increase in spheroid volume for patient 2 (Fig. 6). BPH conditioned medium induced an increase in epithelial sphere volume compared to spheres cultured with NAP conditioned medium or complete medium alone (Fig. 6). These observations suggest that paracrine factors secreted from BPH stromal cells were capable of enhancing spheroid volume in 3D culture.

Discussion

Dysregulated stromal-epithelial interactions are thought to play an important role in BPH development and progression^{17,18}. In this study we explored potential differences between BPH and NAP stroma in their ability to stimulate epithelial cell growth using paired primary BPH stromal and NAP stromal cells derived from BPH patients. To the best of our knowledge, this represents the first study using paired primary BPH stromal and NAP stromal cells derived from patients with glandular BPH. The primary stromal cells derived from either BPH or NAP prostate express markers for smooth muscle cells, fibroblasts and myofibroblasts, which is consistent with a previous report that prostate stromal cells are a mixture of fibroblasts, myofibroblasts, and smooth muscle cells³⁸.

Our studies showed that primary BPH stromal cells, but not normal stromal cells, could stimulate prostatic epithelial cell growth in 3D culture and that stromal conditioned medium from BPH-derived stromal cells could promote epithelial spheroid growth. BPH stromal stimulation of prostatic epithelial growth in 3D culture provides further supportive evidence that the stroma plays a critical role in uncontrolled prostatic growth in BPH pathogenesis. John McNeal first proposed the concept that the neo-formation of prostatic ductal-acinar tissue in BPH pathogenesis was caused by the re-awakening of embryonic inductive activity in adult prostatic stroma^{43,44}. This idea was supported by subsequent studies showing the existence of reactive stroma in BPH¹⁸ and that prostatic stroma plays an important role in regulating prostatic epithelial cell proliferation^{30,45}. Stimulation of epithelial spheroid growth appeared to be independent of BPH stromal cell growth rate, as even slow-growing BPH-derived stromal cells (see Fig. 2C, Patient 3) induced an increase in epithelial spheroid growth compared to NAP-derived stromal cells. These results suggest that secreted factors from both proliferative and slow-growing BPH stromal cells are capable of stimulating epithelial growth in 3D culture. The growth rate of stromal cells may be impacted by many

factors, such as age, BMI and lifestyle habits. The patients included in this study ranged from age 50 to 80, however our tissue acquisition protocol did not include specific patient data. Future expanded studies will be required to determine whether patient demographics may have an impact on prostate stromal cell growth. It is likely that BPH stroma cells can produce chemokines, cytokines, and/or growth factors capable of stimulating prostatic epithelial cell growth. Chemokines and cytokines, including IL-1 α ⁴⁶, IL-8⁴⁷, IL-15 and IL-17⁴⁸, have been shown to be associated with BPH. It will be interesting to determine if these cytokines are preferentially produced by primary BPH stromal cells compared to the primary NAP stromal cells.

The Macoska lab has published a series of papers showing that expression and secretion of cytokine CXCL12 by older prostate stromal fibroblasts derived from BPH patients was increased as compared with younger healthy stromal fibroblasts and that CXCL12 can enhance human prostate epithelial proliferation^{20,42,49}. These observations suggest that aged prostatic stroma is likely to produce and secrete more cytokines and/or chemokines than younger prostatic stroma. Since BPH is prevalent in aged men^{50,51}, these findings are also consistent with an increased production of cytokines and chemokines in BPH stroma than in normal prostatic stroma. Here, we provide evidence that media from BPH stromal cells could stimulate epithelial growth in 3D culture, suggesting that BPH stromal cell secretions may play a role in the increased epithelial proliferation observed in BPH.

In summary, this study showed that primary BPH stromal cells and conditioned medium from BPH stromal cells were capable of stimulating prostatic epithelial cell growth, while NAP stromal cells exhibited little or no effect on epithelial growth. Our finding suggests that paired primary stromal cells from BPH and NAP could be used to identify and characterize epithelial cell growth stimulating factors, such as cytokines and chemokines, secreted by BPH stromal cells.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

Proliferation of epithelial cells and stromal cells in human BPH and NAP tissues. **A.** Immunostaining of Ki-67+ in human BPH and NAP tissue. Original magnification 20x, inset 40x. **B.** Quantification of the number of Ki-67+ epithelial and stromal cells in 10 BPH patient tissues (average of five 20x fields/patient; ** $p < 0.01$).

Figure 2. A.

A. Schematic of primary cultures and paraffin sections from patient-derived BPH and normal adjacent prostate (NAP) tissues obtained from simple prostatectomy. **B.** Morphology of primary stromal cells derived from prostate tissues isolated from human BPH through simple prostatectomy at passage 3. **C.** Growth of three patient-derived paired cell lines from BPH and NAP (age 60–69).

A **B** **Figure 3.**

Characterization of human prostatic stromal cells in primary culture derived from BPH patients. **A.** The typical markers for fibroblasts, myofibroblasts, and smooth muscle cells in prostatic stroma⁵². **B.** Western blot analysis of calponin, SMA and vimentin protein in human stromal cells derived from BPH and paired NAP tissues from 6 different patients.

Figure 4.

Immunostaining of stromal markers in BPH and NAP tissues. Immunostaining and H-score of the staining of SMA (**A, B**), Calponin (**C, D**), and Vimentin (**E, F**) were determined in human BPH and NAP specimens. Original magnification 10x, inset 40x. Immunostained slides were evaluated quantitatively by ImageJ using the color deconvolution plugin indicated in **B, D**, and **F**. * $P < 0.01$, ** < 0.001 .

Figure 5.

Human prostatic epithelial cells, BPH-1 and BHPPrE1, were cultured in 3D for 6 days separately with primary BPH stromal cells or NAP stromal cells derived from the patient with BPH. **A.** BHPPrE1 were cultured alone. **B.** BHPPrE1 were cultured with stromal cells derived from NAP tissue. **C.** BHPPrE1 were cultured with stromal cells derived from BPH tissue. Original magnification 20x. Quantification of the volume of spheres formed by BHPPrE1 and BPH-1 (**D.** and **E.**) * $P < 0.01$, ** < 0.001 , *** $P < 0.0001$.

Figure 6.

BPH epithelial cells (BHPe1 or BPH-1) were in 3D Matrigel cultured with conditioned medium (CM) from stromal cells (BPH and adjacent normal) for 6 days. **A.** BHPe1 cultured with completed medium. **B.** BHPe1 cultured with conditioned medium from stromal cells derived from normal adjacent tissue. **C.** The epithelial cells were cultured with conditioned medium from stromal cells derived from BPH tissue. Original magnification 40x. Quantification of the volume of spheres formed by BHPe1 and BPH-1 (**D.** and **E.**) * $P < 0.01$, ** $P < 0.001$, *** $P < 0.0001$.

Table 1.

Antibodies used for western blot

Antibody	Clone, Cat#	Source	Ratio
Calponin	ab-46794	Abcam	1:1000
SMA	sc-53142	Santa Cruz Biotechnology	1:1000
Vimentin	sc-73258	Santa Cruz Biotechnology	1:1000
GAPDH	sc-47724	Santa Cruz Biotechnology	1:3000

Table 2.

Antibodies used for IHC

Antibody	Clone, Cat#	Source	Ratio
Calponin	ab-46794	Abcam	1:500
SMA	sc-53142	Santa Cruz Biotechnology	1:200
Vimentin	sc-73258	Santa Cruz Biotechnology	1:200
Ki-67	M7240	Dako	1:100