CLINICAL STUDY

Fibroblast growth factor receptor 1 (FGFR1) is over-expressed in benign prostatic hyperplasia whereas FGFR2-IIIc and FGFR3 are not

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Abstract

Objective: Benign prostatic hyperplasia (BPH) is one of the major public health problems among men: 50% of men over 55 are concerned with this disease. Prostate growth is under the control of androgens which act by means of several growth factors such as fibroblast growth factors (FGFs), epidermal growth factor and transforming growth factor β . Basic FGF (bFGF) has been shown to stimulate prostatic stromal growth. In BPH, bFGF concentration is two- to threefold higher than in normal prostate. In this work, the bFGF receptors (FGFR1, FGFR2-IIIc and FGFR3) genes expression was evaluated to study the correlation between the expression of bFGF receptors and induction of BPH.

Methods: The expression of FGFRs was analyzed by RT-PCR, FGFR1 was localized by immunohistochemistry and protein expression was evaluated by Western blot.

Results: A two- to eightfold over-expression of FGFR1 was observed in BPH compared with normal prostates. FGFR1 was localized in the stroma both in BPH and in normal prostates and 1.5- to 2.5-fold over-expression of the protein was observed. The expression of FGFR2-IIIc and FGFR3, more secondary receptors, was not significantly different between BPH and normal prostates.

Conclusions: bFGF receptors and particularly FGFR1 seem to be involved in the induction and evolution of BPH and probably potentiate bFGF over-expression effects in BPH.

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Introduction

Benign prostatic hyperplasia (BPH) is one of the most common diseases among aging men (1), and is responsible for the main surgical intervention on men over 55 years old (2). The initial lesion of BPH seems to be a stromal nodule in the transition zone of the prostate that induces proliferation and organization of epithelial cells into new acini (3). Age is the principal risk factor since the prevalence of BPH is approximately 8% at age 25–30, 50% at age 51–60 and 90% at age 85 and over (3). Relatively little is known about growth regulation of the prostate and the mechanisms involved in BPH. The mechanisms underlying the development of BPH seem to be similar to those responsible for normal growth (3). Prostatic hyperplasia appears when the concentration of androgens decreases in man; but growth factors seem to act in a dysregulated manner, as if they have escaped from androgen control (4).

Prostate regulation is under the control of androgens (5- α -dihydrotestosterone mainly) and, locally, prostate

growth depends on positive control by growth factors (5, 6) such as fibroblast growth factors (FGFs) (7) (basic fibroblast growth factor (bFGF or FGF2), keratinocyte growth factor (KGF or FGF7)...), epidermal growth factor (EGF), and transforming growth factor β (TGF β) which acts as an inhibitor for both stromal and epithelial growth (8).

In BPH, the local concentration of bFGF is two- to threefold higher than in normal prostate (9, 10). This fact suggests that bFGF is probably involved in BPH.

In the prostate, three of the four FGFs receptors are expressed: FGFR1 (which is the principal receptor for bFGF), FGFR2 in two isoforms (FGFR2-IIb, which is the receptor for KGF, and FGFR2-IIIc, a receptor for bFGF) and FGFR3 (which is a receptor for acidic FGF (aFGF) and FGFs in general).

FGFR1 concentration seems to increase in BPH (11) but conflicting studies exist about FGFR1 localization (epithelium (12), both stroma and epithelium (13) or only stroma (14–16)). However, FGFR2-IIIc and FGFR3 expressions were not quantified (Table 1).

Table 1 bFGF receptors in BPH in the literature.

Reference	FGFR1		FGFR2-IIIc		FGFR3	
	Tissue localization	Expression in BPH compared with normal prostates	Tissue localization	Expression in BPH compared with normal prostates	Tissue localization	Expression in BPH compared with normal prostates
Story (4)	Stroma	No difference	Stroma	No difference	ND	ND
Hamaguchi <i>et al.</i> (11)	Stroma and epithelium (weak)	5-fold over-expression in BPH	ND	ND	ND	ND
Ittmann & Mansukhani (14)	Stroma	ND	Stroma	ND	Stroma and epithelium	ND
Saez <i>et al.</i> (16)	Stroma and epithelium (weak)	ND	Stroma and epithelium	ND	ND	ND
This study	Stroma	2- to 8-fold over-expression in BPH	ND	No over-expression	ND	No over-expression

ND, not determined.

This study evaluated the expression of the three receptors by reverse-transcription and polymerase chain reaction (RT-PCR) to detect a possible increase in receptor mRNA in agreement with bFGF. FGFR1 localization was studied by immunohistochemistry to find whether its distribution was different from normal tissue. Western blot analysis was performed for FGFR1 to observe if the protein expression had the same profile as the gene expression.

This work confirmed the increase in FGFR1, but localized it in the prostatic stroma only, and showed no significant difference in FGFR2-IIIc and FGFR3 genes expression between hyperplastic and normal tissues.

Materials and methods

Tissue samples

Tissue samples of BPH were collected from 25 men aged 48 to 79 undergoing transurethral resection of the prostate. For each patient, some clinical and biological information was provided by the urological department, indicating the age of the patient, weight, specific treatment, a possible associated disease, date of the first symptoms, prostate weight, prostate specific antigen (PSA) blood concentration, sampling weight and prostate zone origin. The samples were analyzed by the Pathological Anatomy Department to define the microscopic structure of the hyperplasia (stromal or glandular) and to detect a possible cancer which was an exclusion criterion in our study. Only 2 hyperplastic prostates out of 25 were glandular, the 23 others were mixed (stroma plus glandular), which is the most common type. Normal tissues were obtained from 8 patients aged 21 to 46 years without prostatic disease and undergoing vesical surgery. Eight normal or pathological prostate fragments of about 0.1 to 1 g each were randomly sampled during the surgical intervention. Sampling was limited as most of the tissue had to be checked in the Pathological Anatomy

Department. For immunohistochemistry, three fragments were fixed in 4% paraformaldehyde, dehydrated in alcohol and then paraffin embedded. For RT-PCR and Western blot analysis, five fragments were immediately frozen and kept at -80°C .

RNA extraction and reverse-transcription

RNA was extracted from small amounts of frozen tissues with Trizol (Gibco BRL, Life Technologies, Gaithersburg, MD, USA). The extracted RNA was treated with 2 units of DNase I (Gibco BRL) to eliminate contaminant DNA, and was purified by sequential extraction with phenol-chloroform. The RNA pellet was diluted in Tris-EDTA 10:1, quantified by absorptiometry at 260 nm (GeneQuant II spectrophotometer, Pharmacia Biotech, Uppsala, Sweden) and stored at -20°C . One microgram total RNA was reverse-transcribed into cDNA at 42°C for 60 min in PCR buffer (16.6 mmol/l ammonium sulfate, 2 mmol/l MgCl_2 , 6.7 $\mu\text{mol/l}$ EDTA, 67 mmol/l Tris-HCl, pH 8.8, 0.17 mg/ml BSA) with 10 units MMLV Reverse-Transcriptase (Gibco BRL), 300 $\mu\text{mol/l}$ of each dNTP (Roche Diagnostics, Meylan, France) and 1 mmol/l of downstream primers (Table 2) for bFGF receptors FGFR1, FGFR2-IIIc and FGFR3 with glyceraldehyde phosphate dehydrogenase (GAPDH) used as an internal standard due to its relatively constant expression in prostate cells.

For each reverse transcription reaction, a negative control (without Reverse-Transcriptase) was included to verify the absence of DNA contamination.

Polymerase chain reaction

Primers for RT-PCR of FGFR1, FGFR2-IIIc, FGFR3 and GAPDH were designed using human FGF receptors and GAPDH mRNAs (Table 2). The specificity of the primers was verified (Blast, GenBank) and the primers' complementarity was tested with DNASTar software (Primer

Table 2 Primers used in reverse transcription and polymerase chain reaction.

Gene	Primer sequence	Nucleotide number	Product size (bp)
FGFR1	fgfr1-1 (upstream primer) 5'-GGAGGATCGAGCTCACTCGTGG-3'	13–34	421
	fgfr1-2 (downstream primer) 5'-CGGAGAAGTAGGTGGTGTAC-3'	433–413	
FGFR2-IIIc	fgfr2-1 (upstream primer) 5'-(GTTAACACCACGGACAAAGAGATTGAG-3'	1128–1154	831
	fgfr2-2 (downstream primer) 5'-AACACGGTTAATGTCATAGG-3'	1958–1939	
FGFR3	fgfr3-1 (upstream primer) 5'-CGTGGGGCGAGCGGCAGAAAGTC-3'	132–153	383
	fgfr3-2 (downstream primer) 5'-TCCGCTCGGGCCGTGTCCAGTAAG-3'	514–491	
GAPDH	Gap1 (upstream primer) 5'-GCGGGGCTCTCCAGAACATCAT-3'	659–680	301
	Gap2 (downstream primer) 5'-CCAGCCCCAGCGTCAAAGGTG-3'	959–939	

GAPDH, glyceraldehyde-phosphate dehydrogenase, control gene; bp, base pairs.

Select, Lasergen, London, UK). mRNA expression of FGF receptors was semi-quantified using GAPDH as an internal control. GAPDH mRNA was co-transcribed and co-amplified with bFGF receptors mRNA. The signal intensity of PCR products for FGF receptors was compared with that of GAPDH for each sample.

The PCR mixtures contained PCR buffer with 100 µmol/l of each of the four dNTPs (Roche Diagnostics, Meylan, France), 0.75 units of *Taq* DNA Polymerase (Eurobio, Les Ulis, France), 10% DMSO, 0.8 mmol/l of each primer (Eurogentec, Seraing, Belgium) and 7 µl reverse transcription product in a total volume of 50 µl. Samples were amplified for 35 cycles for FGFR1 and FGFR3 and for 40 cycles for FGFR2-IIIc, comprising denaturation at 95 °C for 60 s, annealing for 60 s at 55 °C for FGFR1, 56 °C for FGFR2-IIIc and 59 °C for FGFR3, and elongation at 72 °C for 90 s for FGFR1 and FGFR2-IIIc and for 60 s for FGFR3. The amplification products were separated with electrophoresis, visualized by ethidium bromide and analyzed by densitometry (BioImage densitometry software, Millipore Corporation, Bradford, MA, USA). The mRNA intensity of the FGF receptors was determined by calculating the receptors/GAPDH optical density (OD) ratio.

Statistics

Statistical evaluation was performed using a non-parametric Mann–Whitney U-test. Statistical significance was inferred at $P < 0.01$.

Analysis and sequencing of RT-PCR products

The amplified products for FGFR1, FGFR2-IIIc, FGFR3 and GAPDH were analyzed by electrophoresis on 1.5% agarose gel with a DNA ladder (Roche Diagnostics) as a molecular weight standard. Bands were cut off and DNA was purified using Qiaquick gel extraction kit (Qiagen, Hilden, Germany). Purified products were then analyzed by electrophoresis on a 2% agarose gel with a quantitative DNA ladder (MBI Fermentas, Hanover, MD, USA) for quantification. DNA was amplified by PCR for 25 cycles each comprising denaturation for 10 s at

96 °C, annealing for 5 s at 50 °C and elongation for 4 min at 60 °C, with 1.6 pmol of upstream primer in a total volume of 20 µl, using the ABI Prism DNA sequencing kit (Applied Biosystems, Perkin Elmer, Warrington, Cheshire, UK). The amplified products were purified by precipitating with 95% and 70% alcohol followed by centrifugations. DNA was diluted in sequencing buffer (Applied Biosystems) and purified PCR products were sequenced using an ABI Prism sequencer (Applied Biosystems) using dye-primer cycle sequencing.

Each gene sequence (Blast, internet) was compared with the sequenced amplified products using Sequence Navigator software (Applied Biosystems).

Immunohistochemistry (17, 18)

Tissue sections (4 µm) were collected on silane-coated slides. Sections were deparaffinized, rehydrated in decreasing concentrations of alcohol and washed in water. Endogenous peroxidases were inhibited by hydrogen peroxide. Biostain Biomed kit (Biomed, Foster City, CA, USA) was used for protein blocking and for biotin-labeled secondary antibody and streptavidin-biotin-peroxidase complex reactions. Sections were incubated with protein blocker containing goat serum (Sigma, St Louis, MO, USA). Polyclonal rabbit antibody, specific to human FGFR1, (Flg(C15); TEBU Santa Cruz, Heidelberg, Germany) (19) with no cross-reactivity for other FGF receptors (FGFR2, FGFR3, FGFR4) was used at a concentration of 0.1 ng/µl. Sections were incubated with biotinylated secondary goat anti-rabbit antibody, followed by streptavidin-biotin-peroxidase contact. FGFR1 was stained brown by diaminobenzidine (DAB) chromogen (Microm, Francheville, France), peroxidase substrate. Stained sections were counterstained with hematoxylin (Biomed) to demonstrate nuclei.

In all experiments, various negative controls were included to detect any non-specific coloration (primary and secondary antibody controls and streptavidin-biotin-peroxidase complex control). The primary antibody specificity was verified by preincubating it with a corresponding antigenic peptide (TEBU Santa Cruz)

prior to immunohistochemistry; no immunostaining was observed in these sections.

Western blot

Frozen tissue fragments were cut into small pieces and crushed at 4 °C in RIPA buffer (1×PBS with 1% Igepal (Sigma-Aldrich, St. Louis, MO, USA), 0.5% sodium deoxycholate, 0.1% SDS and a protease inhibitor cocktail: 0.6 mmol/l phenylmethylsulfonyl sulfate (PMSF), 2 µg/ml aprotinin and 2 µg/ml leupeptin); 18 mmol/l PMSF were then added and the tissue homogenate was incubated on ice for 30 min. It was then centrifuged twice at 15 000 *g* for 20 min at 4 °C. Total protein was quantified and the cell lysate was conserved at −70 °C.

Proteins at a concentration of 2 mg/ml were denatured in 125 µl Tris-HCl (0.5 mol/l, pH 6.8), 200 µl 10% SDS, 50 µl β-mercapto-ethanol, 100 µl 88% glycerol and boiled for 5 min at 100 °C. One hundred micrograms total proteins were loaded on to 8% acrylamide gel (with 4% acrylamide stacking gel) and electrophoresis was carried out for 1 h at 130 V. Proteins were transferred from the gel to a PVDF membrane (Amersham, Freiburg, Germany) for 1 h at 15 V and 500 mA. The membrane was then soaked in 1×PBS containing 1% milk for 1 h to block non-specific binding. It was then incubated with the

primary antibody Flg(C15) (TEBU Santa Cruz) at 1/1000 dilution in PBS, 0.05% Tween 20 and 1% milk for 3 h at 37 °C and washed 3 times in PBS-Tween 20. The membrane was then incubated with horseradish peroxidase conjugated anti-rabbit antibody for 90 min and washed twice in PBS-Tween 20 and twice in water. ECL+ chemiluminescence substrate (Amersham) was added to the membrane for 5 min. The membrane was drained and exposed for 15 min to a film. The bands were analyzed densitometrically after development of the film.

Negative controls without primary and secondary antibodies were also included to detect any non-specific bands. A negative control with an Flg(C15) corresponding antigenic peptide was added to verify the primary antibody specificity.

Results

RT-PCR

For each patient, 2 samples of prostatic tissue were obtained, each sample being tested in triplicate. Between 2 fragments of 2 different regions of the prostate of the same patient, the standard deviation for each mRNA expression was slight (5 to 15% of the mean value), showing some homogeneity in the prostatic tissue for each patient.

Figure 1 Amplification products of FGFR1 (A), FGFR2-IIIc (B), FGFR3 (C) and GAPDH mRNAs and their expression evaluated versus an internal standard (GAPDH) in 8 normal prostates and 25 BPH. Total mRNA was reverse transcribed and amplified by PCR. RT-PCR products were separated by electrophoresis, visualized with ethidium bromide (top panels) and analyzed by densitometry (bottom panels; BioImage software). Lanes 1 and 2 are amplification products of FGFRs and GAPDH alone. Lanes 3 to 9 are co-amplification products of each FGFR with GAPDH as internal standard. The insets represent the mean values for normal prostates and for BPH ($\pm$ standard deviation). (A) FGFR1 gene expression is 2 to 8 times more important in BPH than in normal prostates. The difference between values in normal prostates and BPH is significant ($P < 0.01$) (identical results for 3 patients). (B and C) FGFR2-IIIc and FGFR3 genes expression shows no difference between normal prostates and BPH.

By RT-PCR, the size of the PCR products was as predicted. DNA sequencing of the amplified products for FGFR1, FGFR2-IIIc, FGFR3 and GAPDH confirmed that they corresponded to the expected segments of targeted cDNA.

An over-expression of FGFR1 mRNA was observed in BPH compared with normal tissues (Fig. 1A). Densitometric analysis showed a two- to eightfold increase of FGFR1 expression in BPH. No correlation was observed between mRNA expression levels and the clinical and biological data of different patients. For example, 6 of the patients were treated with α -blockers, 5 with Finasteride, 4 with plant extracts and 10 were not treated at the time of intervention, but mRNA expression did not seem to be influenced by the existence of a treatment. The size of the hyperplastic prostates was between 30 and 80 g and no correlation could be observed with mRNA expression. All the correlation studies (comparing mRNA expression with PSA levels, sampling weight, associated diseases, hyperplasia type...) were nil. Yet, the most important expressions were generally observed when the symptoms had occurred over a long period (5 to 15 years) and weak results were observed when the symptoms were recent (a few months). The mean FGFR1 mRNA expression was significantly higher in BPH than in normal prostates ($P < 0.01$).

For FGFR2-IIIb and FGFR3, no over-expression was observed as densitometric analysis showed no significant difference between BPH and normal population prostates (Fig. 1B and 1C).

Immunohistochemistry

For each patient, 3 fragments of prostate were obtained and at least 5 sections out of each fragment were tested. Flg(C15) is a polyclonal antibody raised against a peptide corresponding to the amino acids 802 to 822 mapping at the carboxy terminus of human FGFR1 receptor; it specifically recognises FGFR1 and is not reactive with other FGFRs. All controls were negative. The preincubation of Flg(C15) with the corresponding antigenic peptide before immunohistochemistry showed no immunoreaction.

As in normal prostates, a brown staining was observed only in the stromal cells in BPH. The epithelial

Figure 2 Immunohistochemical localization of FGFR1. bFGF receptor was localized with a very specific primary antibody. A biotinylated secondary antibody fixed a streptavidin-biotin-peroxidase complex. The immunoreactivity towards FGFR1 was visualized with DAB chromogen. Nuclei were counterstained with hematoxylin. Original magnification, $\times 100$. (A) Negative control for FGFR1. (B) Localization of FGFR1 in patient 8: it is localized in the prostatic stroma; no immunoreactivity was observed in the epithelium. Immunoreactivity is moderately positive. (C) Localization of FGFR1 in normal prostate. It is localized in stroma with a weak immunoreactivity.

Figure 3 Total proteins were extracted, loaded on to polyacrylamide gel and transferred on PVDF membrane and incubated with a FGFR1 specific antibody. FGFR1 bands were visualized by chemiluminescence and analyzed by densitometry. Two bands of 120 and 80 kDa were obtained and a 1.5 to 2.5 times over-expression of the protein FGFR1 was observed in BPH compared with normal tissues. Values presented are means $\pm$ standard deviation.

tissue was not immunoreactive for FGFR1 (Fig. 2). The immunoreactivity was intense in almost all the BPH tissues showing important concentrations of this receptor. No correlation was observed between the intensity of staining and the clinical and biological data from patients. Yet we noticed that the immunoreactivity was all the more intense when the symptoms were of long standing.

Western blot

For each patient, the samples were studied in duplicate with similar results for each patient. All controls were negative and the preincubation of the Flg(C15) corresponding antigenic peptide prior to immunoblotting with the primary antibody showed no band.

Western blot analysis using antibody against FGFR1 showed 2 bands with molecular masses of about 120 and 80 kDa which correspond to the expected sizes of the membrane receptor and the secreted form of FGFR1 respectively. Densitometric analysis showed a 1.5- to 2.5-fold over-expression of the protein FGFR1 (Fig. 3).

Discussion

The identification of growth factors implicated in abnormal cell proliferation is important in understanding the mechanisms of development of BPH. Elucidating these mechanisms may have clinical and therapeutic import. Indeed, inhibiting the growth factors or the synthesis or activities of their receptors involved in this hyperplasia could be of great interest in the prevention and treatment of prostatic pathologies. This study was undertaken to evaluate the importance of bFGF receptors in the development of BPH.

This work was performed on 25 samples from patients with benign prostatic hyperplasia and on 8 normal samples from young unrelated patients undergoing vesical surgery and taken under exactly the same conditions as the BPH tissues. About 6 to 10% of the total removed tissue was studied.

The patient group greatly differed in age which is unavoidable as BPH symptoms appear at various ages. According to the Pathological Anatomy Department reports, no prostatic intraepithelial neoplasia-change has been observed in the studied prostates. Still, we have imagined that this important difference in age could be responsible for a modified endocrine control (modified androgenic impregnation) which could have effects on the expression levels of FGFRs. However, we did not find any link between age and FGFRs expression.

To our knowledge, the first study on FGFR1 in BPH was performed by Hamaguchi *et al.* (11) on a Japanese population and their results were approximately the same as ours as a fivefold increase was observed in FGFR1 expression in BPH compared with normal tissue. FGFR2-IIIc and FGFR3 were not quantified. In our study, a two- to eightfold increase in FGFR1 expression is compatible with this work. However, no over-expression of FGFR2-IIIc and FGFR3 genes was observed.

Ittmann & Mansukhani (14) detected a moderate expression of FGFR1 in the stromal cells from BPH tissues. They concluded that stromal cells expressed FGFR1, FGFR2-IIIc and FGFR3 and thus were stimulated by bFGF and aFGF, while epithelial cells expressed FGFR2-IIIb and FGFR3 and were reactive to FGF7 (KGF) and aFGF.

By immunohistochemistry, we found that only the prostatic stroma was immunoreactive for FGFR1. This result is still much debated. Indeed, while it is accepted that both stromal and epithelial cells synthesize bFGF and that this growth factor has a paracrine and autocrine action (14, 20), opinions differ about FGFR1 localization. In some studies, FGFR1 was observed only in epithelium (12). Hamaguchi *et al.* (11) observed that FGFR1 was mainly localized in epithelium but also occasionally in stroma. Some authors propose that bFGF is responsible for cell proliferation and neo-vascularization both in stroma and epithelium through

its receptor FGFR1 (13). Our results seem to support most studies (14–16) which find FGFR1 only in the stroma and not in the epithelium. bFGF, synthesized by stroma and epithelial cells, would act on stroma cells through its receptor, FGFR1.

Our results do not refute a possible action of bFGF on epithelium. Indeed, FGFR1 is the principal receptor for bFGF, but this growth factor can also be fixed on FGFR3 at a lower rate. bFGF may also stimulate epithelium through its other receptors (20).

Moreover, these differences in FGFR1 localization could also be due to differences between the patients' origins, the protocol applied for localizing this receptor, the antibody origin etc.

We also observed that the immunoreactivity in immunohistochemistry was all the more intense as the duration of BPH symptoms was prolonged: more elevated results were generally observed when the BPH symptoms had lasted a long time.

Western blot analysis showed a more moderate difference in FGFR1 protein expression, as a twofold increase was observed in BPH when compared with normal prostates. These results support the work of Hamaguchi *et al.* (11) who found only a slight difference between BPH and normal tissues.

According to our results, the increase of FGFR1 in BPH tissue, associated with an increase in bFGF concentration, could potentiate cell growth and neo-vascularization implicated in the development of benign prostatic hypertrophy.

Concerning FGFR2-IIIb and FGFR3, as no significant difference in genes expression was observed between BPH and normal prostates, they may have a role in BPH through the over-expression of the ligand, but FGFR1, being over-expressed itself, probably takes a more important part in the development of the disease.

In normal prostate, bFGF is implicated in cell growth and neo-vascularization and is principally involved in physiological prostate growth at puberty and also in tissue reparation in adult human prostate (8). Lawson (20) presented two hypotheses to explain the increase of bFGF in BPH tissues. First, the dysregulation of endocrine and growth factors system and secondly, the many microscopic injuries during miction or infections, needing tissue reparation in the prostate.

In fact, prostate growth is under the direct influence of various growth factors such as FGFs, EGF, TGF β (6), and the influence of androgens. An increase in bFGF and its receptor FGFR1 probably results in a dysregulation of cell proliferation potentiating abnormal prostate stroma growth. In BPH, not only bFGF but also KGF (FGF7) is over-expressed (21). KGF is the major growth factor expressed in the prostate (22); it is synthesized by the stroma and stimulates epithelial growth. The over-expression of this growth factor together with bFGF and FGFR1 could lead to disordered cell proliferation in the epithelium and in the stroma respectively.

Our studies are now directed to other FGFs, such as KGF, and their receptors involved in normal prostate growth, which could be implicated in the development of BPH.

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