

## Cellular localization of fibroblast growth factor 2 (FGF-2) in benign prostatic hyperplasia

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**Summary.** Fibroblast growth factor 2 (FGF-2, basic fibroblast growth factor) has been reported to be elevated in tissues from benign prostatic hyperplasia (BPH), the most frequent neoplastic disease in aging men. This suggests that FGF-2 may play a significant role in the development of BPH. In this study the cellular distribution pattern of FGF-2 in tissues from BPH has been investigated by immunohistochemical and molecular biological methods. Radioimmunoassay revealed high concentrations of FGF-2, ranging between 450 and 950 ng per g tissue. Immunoblots confirmed the presence of a 18 kDa FGF-2 in tissue extracts. By immunohistochemistry done with a polyclonal antibody to recombinant FGF-2 on paraffin sections, FGF-2 was localized in fibroblasts, endothelial cells and smooth muscle cells of tissue samples of BPH. Nuclei of these cells were labelled distinctly. Moreover the cytoplasm of smooth muscle cells was labelled moderately. No immunostaining was seen in prostatic epithelium. Non-radioactive in situ hybridization with digoxigenin-labelled oligonucleotides revealed the presence of mRNA for FGF-2 in smooth muscle cells of the prostatic stroma. These results provide evidence that FGF-2 may be produced locally in the human prostate as a stroma-specific mitogen and may play a causal role in the development of BPH.

**Key words:** BPH, FGF-2, Immunocytochemistry, In situ hybridisation

### Introduction

Benign prostatic hyperplasia (BPH) is regarded as the most commonly occurring neoplastic disease in the aging human male (Isaacs and Coffey, 1989). Etiology and pathogenesis of this disease are still poorly understood. Although there are a number of hypotheses

on the etiology of BPH (Isaacs and Coffey, 1989; Aumiiller, 1992) none of them has been fully proven. The role of androgens in the pathogenesis of BPH has been intensely studied (Griffiths et al., 1991; Sinowatz et al., 1995). Dihydrotestosterone (DHT) is the active intracellular androgen formed from testosterone by 5 $\alpha$ -reductase. The concentration of DHT appears to be more increased in BPH tissue than in normal prostatic tissue (Griffiths et al., 1991), and there is no doubt that the DHT-receptor complex modulates gene expression. Current studies suggest that DHT is essential but not sufficient for proliferation in BPH, and that other regulatory factors, including peptid growth factors (Byrne et al., 1996; Culig et al., 1996; Cussenot et al., 1997; De Bellis et al., 1998) are a prerequisite.

The presence and possible function of various growth factors and their receptors in the normal and diseased human prostate have been reported in recent studies. Epidermal growth factor (EGF) (Sciarra et al., 1995; Di Silverio et al., 1998) epidermal growth factor-related peptides, fibroblast growth factors (FGFs) (Mydlo et al., 1988; Sherwood et al., 1992; Begun et al., 1995; Story, 1995; Schmitt et al., 1996) as well as their mRNAs (Mori et al., 1990) have been demonstrated in intact and diseased human prostate, but their role in prostatic growth is not fully understood. Stimulation of both prostatic stromal and epithelial cells by FGF-2 (Mydlo et al., 1988; Luo et al., 1996) has been described and it was postulated that an imbalance in growth factor concentration may contribute to BPH. To date, little information is available on the cellular localization of growth factors in BPH. In this study we examined the cellular distribution pattern of FGF-2 and its mRNA using immunocytochemical and molecular biological methods.

### Material and methods

#### *Tissue samples*

Tissue samples of BPH (18 cases) were obtained at open prostatectomy. Small pieces of tissue (1 cm<sup>3</sup>) were

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fixed in Bouin's fluid for 12 hours and embedded in paraffin. 5- $\mu$ m sections were cut on a Leitz microtome and used for immunohistochemistry. A selection of slides was also routinely stained with hematoxylin-eosin. Tissue for electrophoresis, immunoblot and radioimmunoassays was snap-frozen in liquid nitrogen and stored at -70 °C until use.

### Immunohistochemistry

Polyclonal antibodies to recombinant human FGF-2 (Boehringer Mannheim, Germany) were raised in rabbits. The antibody did not crossreact with FGF-1. Localization of immunoreactive FGF-2 was done using the avidin-biotin technique (Hsu et al., 1981) according to the following protocol:

Sections were deparaffinized, rehydrated and exposed for 15 min to 0.5% hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>) in phosphate-buffered saline (PBS) to block endogenous peroxidase. Non-specific binding was minimized by incubating the slides with 10% normal goat serum for 1 hour at 20 °C. Incubation with rabbit anti-FGF-2 antibodies (1:1000 diluted in PBS) was performed overnight at 4 °C followed by an incubation with biotinylated goat anti-rabbit IgG (Sigma, Munich, Germany) diluted 1:150 in PBS for 2 hours at 20 °C. Visualization of the bound antibodies was carried out with ABC kit reagents (DAKO, Glostrup, Denmark) and diaminobenzidine/H<sub>2</sub>O<sub>2</sub> as chromogenic substrates. All incubations were performed in a humidified chamber. Sections were left unstained or counterstained in Mayer's haematoxylin.

Controls were performed by a) omission of the primary antibodies; b) by replacing the antibodies to FGF-2 by normal mouse serum (Sigma, Munich, Germany) in different concentrations 1:5; 1:10; 1:100. c) pre-absorption of FGF-2 antibodies was done by preincubation with 15 ng/ml recombinant human FGF-2 in a siliconized polystyrene tube for 24 h at 4 °C before incubation of the slides.

### Tissue extraction

Tissue samples from human BPH were homogenized in a 10-fold volume of ice-cold PBS supplemented with 0.1M mercapto-ethanol. After centrifugation of the suspension the supernatant was measured for protein and used for Western blot analysis.

### Electrophoresis and immunoblot

SDS-polyacrylamide gel electrophoresis was done according to Laemmli (1970). Proteins were stained with Coomassie blue. Rabbit anti-FGF-1 and anti-FGF-2 antibodies were diluted 1:15.000 for immunoblot analysis. Chemiluminescence was performed using goat anti-rabbit IgG-peroxidase-conjugate according to standard procedures (ECL, Amersham, UK). Protein concentrations were determined by the BCA-method.

### Radioimmunoassay

FGF-2 concentrations in tissue samples of BPH were determined by radioimmunoassay using a rabbit antiserum to recombinant human FGF-2 (kindly provided by Dr. D. Gospodarowicz, San Francisco, USA). This antiserum did not crossreact with FGF-1. Recombinant bovine FGF-2 (Boehringer Mannheim, Germany) was used as standard and also for iodination by the chloramin-T-method (Vaisman et al., 1990). Separation of bound and free iodine was done by heparin-Sepharose column chromatography (sensitivity of the assay was 1 ng/ml).

### Synthesis of oligodeoxynucleotides

Antisense oligodeoxynucleotides were synthesized using standard cyanoethyl-phosphoramidite chemistry on an ABI instrument (Applied Biosystems, Weiterstadt, Germany). Base sequences according to Guthridge et al., 1992 were: 5'-GAC ACA ACC CCT CTC TCT TCT G'CT TG'-3' and 5'-CTA GTA ATC TTC CAT CTT CTT TCA TAG'-3. Chemical 5'-biotinylation was performed with biotin phosphoramidite (Cambridge Research Scientific Ltd., UK), attaching the biotin reporter group to the 5'hydroxyl group of the oligonucleotide via C6 spacer. Reverse phase HPLC on a CI18 column (Beckman Instruments, Munich, Germany) was done in order to purify DNA with dimethoxytrityl groups (DITT) attached to biotin. After incubation in 80% acetic acid in order to remove DMT groups, the oligonucleotide was purified by chromatography on Sephadex material (NAP column, Pharmacia, Freiburg). The yield of the purified product was determined by UV absorption (254 nm, Beckman DU65 spectrophotometer). Digoxigenin-labeling was done using the DIG Oligonucleotide Tailing Kit (Boehringer, Mannheim, Germany) according to instructions of the supplier, however, with two minor modifications: 1) the final volume was 25 ml, and 2) tailing was done for 2.5 hours.

### In situ hybridization

All solutions were prepared using 0.02% diethylpyrocarbonate (DEPC) treated water (Fluka, Buchs, Switzerland) and glassware sterilized at 200 °C. Slides were preincubated in 200 ml of prehybridization mix (5x SSC; 5x Denhardt's solution; 0.05M PIPES; 0.5 mg/ml salmon sperm DNA; 50% formamide) for at least 1 h at 37 °C. This mix was replaced by an equal volume of hybridization mix, containing about 500 ng/ml of digoxigenin-labelled oligonucleotides. After 16 h of incubation at 37 °C in a humidified chamber, the hybridization mix was removed and slides were washed in 5x SSC at room temperature, 1x SSC at 37 °C, and 0.1x SSC at room temperature. After a final wash in PBS at room temperature, hybridization signals were visualized using anti-digoxigenin-alkaline phosphates Fab-fragments (Boehringer, Mannheim, Germany) and

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5-bromo-4 chloro 3 indolyl phosphate (Boehringer, Mannheim, Germany) according to the instructions of the manufacturer.

Controls were performed by a) omitting the digoxigenin-labelled probe, b) using the sense probe, c) pretreatment of the sections with RNase

### Results

In paraffin sections immunoreactive FGF-2 was found in fibroblasts, smooth muscle cells and endothelial cells of the prostatic stroma (Fig. 1). Distinct staining was seen in most nuclei of these cells and a somewhat less intense immunoreaction occurred in the cytoplasm of smooth muscle cells (Fig. 2). No immunostaining was seen in the prostatic epithelium (Fig. 2). The staining reaction in stromal cells was clearly specific, because controls using non immune IgG were negative and also preabsorption of the polyclonal FGF-2 antiserum with recombinant FGF-2 preparation abolished the staining (Fig. 4).

When using non-radioactive in situ hybridization with digoxigenin-labelled oligonucleotides, a distinct staining was seen in the smooth muscle cells (Fig. 3), demonstrating transcripts of FGF-2 mRNA in this cell population.

The presence of FGF-2 in prostatic tissue from patients with BPH was confirmed by Western blotting as shown in Fig. 5. Immunoblot analysis showed the presence of an immunoreactive band at 18 kDa that comigrated with purified FGF-2. FGF-1, which shares a

high homology (55%) with FGF-2, and did not show any crossreaction with the antiserum. Using radioimmunoassay, FGF-2 concentrations between 450 and 950 ng were found in extracts from BPH-tissue.

### Discussion

Androgens play an important role in the normal and abnormal growth of the prostate. It is known that BPH never develops in men who were castrated prior to puberty (Mori et al., 1990). However there are many clinical and experimental observations on the development of BPH that cannot solely be explained by the action of androgens. Jacobs and associates (1979) were the first to report the presence of a growth promoting factor in extracts of human BPH. It was then demonstrated by amino terminal sequencing and immunological studies that prostate growth factor was structurally related to FGF-2 (Story et al., 1987). Several investigators have recently isolated FGFs from extracts of rat and human prostatic tissue (Fiorelli et al., 1991; Sherwood et al., 1992; Begun et al., 1995) and identified FGF-2 as the primary FGF in the human prostate. However, the site(s) of FGF-2 synthesis and the identity of FGF-2-responsive cells in the human prostate have not been fully ascertained.

Immunoblots proved the presence of FGF-2 of 18 kDa size in the extracts of tissue from BPH. By radioimmuno-assay high concentrations, ranging between 450 and 950 ng, of FGF-2 per 1 g of BPH-tissue were found. This result confirms the findings of

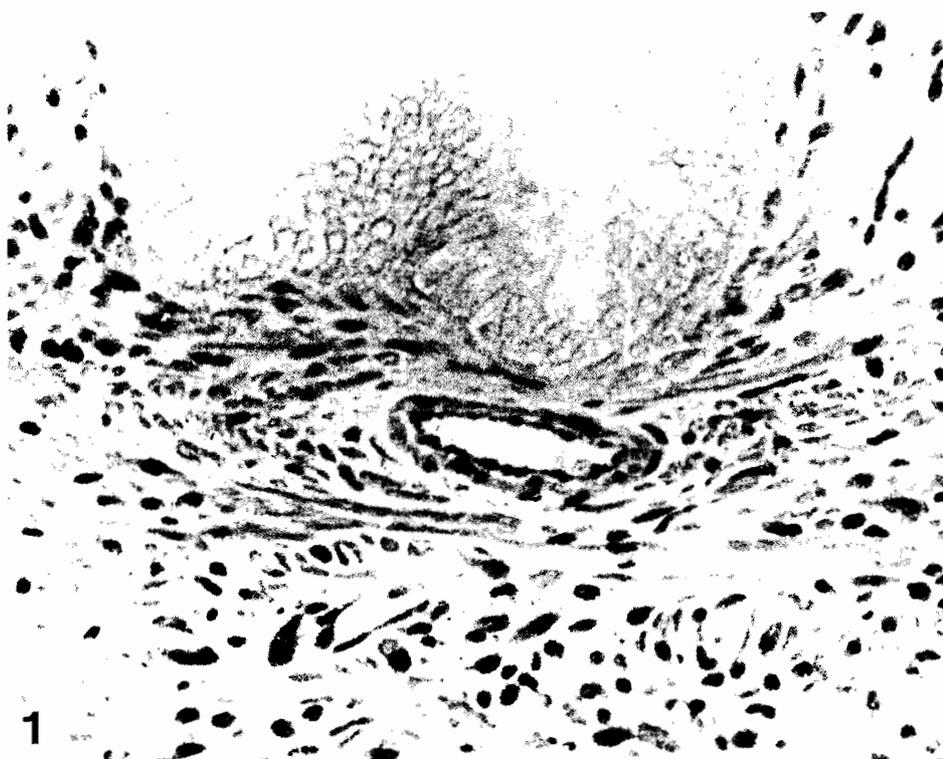


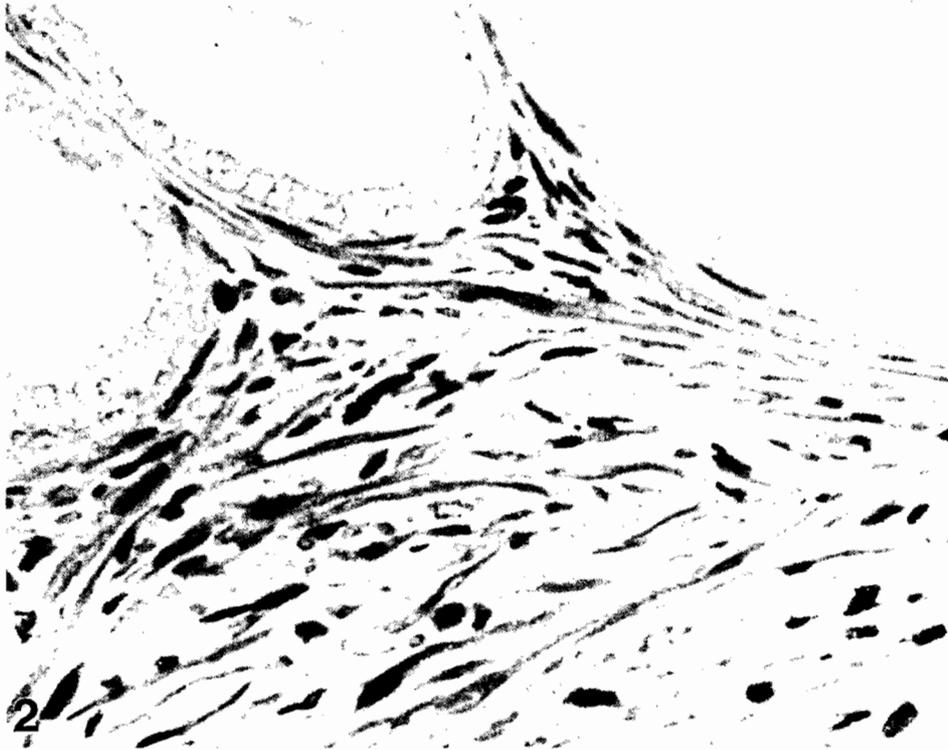
Fig. 1. Benign prostatic hyperplasia, paraffin section, labelled with polyclonal antibody to recombinant FGF-2. Distinct immunoreactivity can be seen in the nuclei of fibroblasts, smooth muscle cells and endothelial cells. Cytoplasm of smooth muscle cells is labelled moderately. x 350

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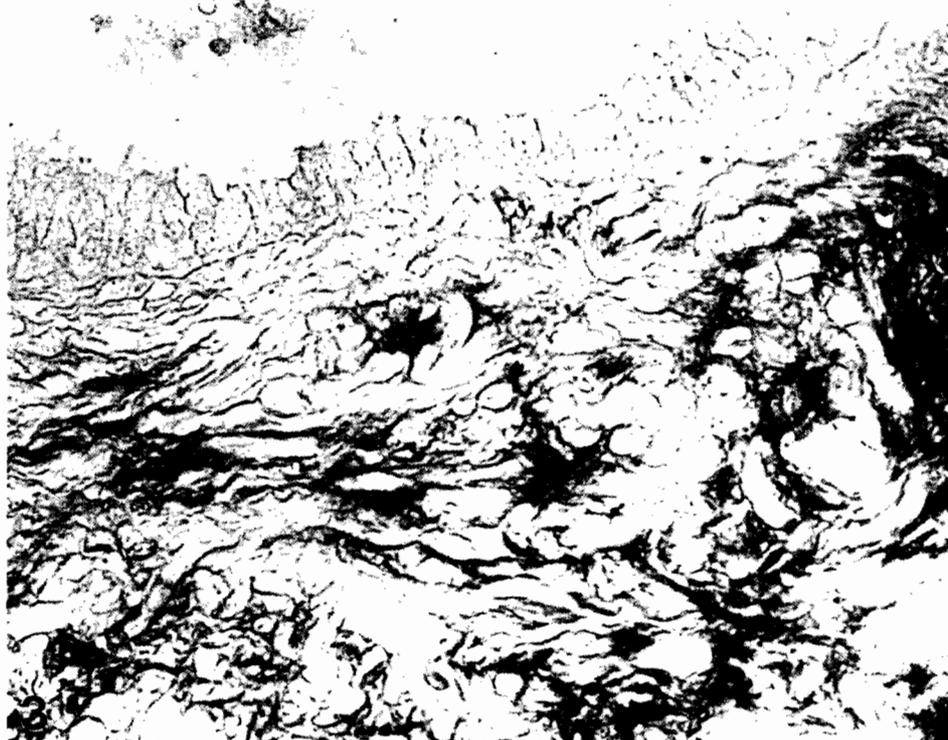
Mori et al. (1990) and Begun et al. (1995) on a significantly higher level of expression of FGF-2 in benign hyperplastic prostates as compared to normal prostates. Moreover, these findings indicate a causal role

of locally produced FGF-2 in the pathogenesis of BPH (Begun et al., 1995).

In several studies localization of FGF-2 in tissues and cells from various sources has been investigated



**Fig. 2.** Benign prostatic hyperplasia, paraffin section, labelled with polyclonal antibody to recombinant FGF-2. Nuclei of smooth muscle cells are labelled distinctly. Cytoplasm of these cells is labelled moderately. No immunostaining can be seen in the prostatic epithelium. x 480



**Fig. 3.** Benign prostatic hyperplasia, paraffin section, non-radioactive in situ hybridization, digoxigenin-labelled oligonucleotides of FGF-2 mRNA. Distinct staining in smooth muscle cells demonstrates transcripts of FGF-2 mRNA in these cells. x 560



**Fig. 4.** Benign prostatic hyperplasia, paraffin section, control, labelled with primary antibody (anti-FGF-2) which was preabsorbed with recombinant FGF-2. Immunostaining is completely abolished. x 560



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**Fig. 5.** Benign prostatic hyperplasia, immunoblot to FGF-2 (Western blot). The presence of a 18 kDa FGF-2 is demonstrated. Lanes 6A, 6B, 7A, 7B, 9A, 10A correspond to tissue samples of different patients.

(Tessler et al., 1990). FGF-2 was demonstrated in endothelial cells (Schweigerer et al. 1987), smooth muscle cells (Gospodarowicz et al., 1988), granulosa cells (Neufeld et al., 1987) and various types of tumor cells. With regard to the subcellular localization of FGF-2 in muscle cells, the results of different studies have been diverse. FGF-2 was found to be localized in the cytoplasm of smooth muscle cells (Joseph-Silverstein et al., 1989), and in the nuclei in another investigation (Tessler and Neufeld, 1990). In yet another study FGF-2 was localized in the extracellular matrix (Kardami and

Fendrich, 1987; Kalcheim and Neufeld, 1990).

Immunofluorescence studies done on frozen sections from benign human prostatic tumors revealed that the majority of FGF-2 is localized in the prostatic stroma (Sherwood et al., 1992). However, it was not clear whether staining of the cytoplasm or the nuclei occurred. In our investigation immunovisualization for FGF-2 was strong in the nuclei of the smooth muscle cells, fibroblasts and endothelial cells, and weaker in the cytoplasm. No immunostaining was found in normal prostatic epithelium. This is in contrast to the study of Deshmukh et al. (1997) who described distinct immunostaining for FGF-2 in the cytoplasm of all basal epithelial cells, but not in luminal epithelial cells of normal regions of the prostate gland. Basal expression of FGF-2 was reported to be diminished or absent in regions of mild epithelial dysplasia, particularly those strongly expressing FGF-1. Like in our study, FGF-2 occurred predominantly in smooth muscle-type stromal cells.

FGF-2 is a highly cationic molecule (pI 9.8) which might be expected to bind to DNA. Therefore an artifactual appearance of FGF-2 within the nuclei of prostatic tissue as a result of a fracture of the nuclear membrane during fixation must be considered. However, nuclear immunostaining was restricted to cells of the prostatic stroma (fibroblasts, smooth muscle cells, endothelial cells) and never occurred in nuclei of the prostatic epithelium. The staining reaction was clearly specific for FGF-2 because controls using nonimmune

IgG were negative and also absorption of the polyclonal FGF-2 antiserum to a recombinant FGF-2 preparation abolished the staining.

The presence of immunoreactive FGF-2 in prostatic stromal cells does not unequivocally prove the local production of FGF-2 by these cells. However, other studies and our own results clearly point in that direction. Mori et al. (1990) succeeded in identifying FGF-2 mRNA transcripts in prostatic tissues using Northern blots. Besides the 7.0 and 3.7 kb transcripts of FGF-2 mRNA reported in cultured cells two more transcripts of 2.1 and 1.2 kb were detected. Mydlo et al. (1988) reported expression of FGF-2 mRNA in hyperplastic and cancerous tissue of the human prostate. Using digoxigenin labelled oligonucleotides we were able to localize FGF-2 mRNA in the smooth muscle cells of BPH. In summary, these findings agree with data given by Story et al. (1987), who have shown that cultured prostatic stromal cells actively synthesize FGF-2. In previous studies it was hypothesized that FGF-2 produced by stromal cells of the prostate may act via an autocrine or paracrine mechanism and serve as stroma-specific mitogen in the human prostate. This idea is supported by the demonstration of high affinity receptors for FGF-2 on cultured stromal cell of the prostate. However, it still unclear how FGF-2 is secreted since it does not possess a classic signal peptide sequence, and no vesicles containing FGF-2 have been found in cells containing immunoreactive FGF-2 (Joseph-Silverstein et al., 1989).

Since secretion has conventionally been considered to be a prerequisite for growth factor activity, these observations have raised questions as to the mechanism of secretion of growth factor without a leader sequence. It was postulated that FGF-2 is secreted by atypical mechanisms or released only after cell death or damage. However, evidence is now emerging for another mode of action of growth factors, termed "intracrine" (Logan, 1990). Growth factors acting in this way need not be secreted, nor do they require receptors located on the cell surface to mediate their activity. Rather, they remain within the cell of origin and act directly as intracellular messengers to regulate cellular function. Our immunocytochemical study showed distinct nuclear and a weaker cytoplasmic staining for FGF-2 in smooth muscle nodules of BPH. It seems possible that in prostate stromal cells, as has been previously shown for other cell types which synthesize this growth factor, a cell cycle-dependent translocation of FGF-2 occurs between cytoplasm and nucleus (Hill and Logan, 1992). The significance of this for the biological actions of this growth factor is unknown. Further studies on a potential intracrine action of FGF-2 in BPH are required.

In conclusion, our results demonstrate an exclusive location of mRNA for FGF-2 and immunoreactive FGF-2 in the stroma of BPH-tissue and provide strong evidence for a causal role of FGF-2 as a stroma-specific mitogen in the human prostate.

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