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The role of transforming growth factor-β and its receptors in human prostate smooth muscle cell fibronectin production

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Abstract

In the present study, the role of transforming growth factor-β (TGFβ) on the production of the extracellular matrix component, fibronectin, in the prostate has been studied. The mRNA levels of fibronectin, TGFβ and the two TGFβ receptors, ALK5 (activin like kinase) and type II, were measured using reverse-transcription polymerase chain reaction (RT-PCR). TGFβ increased fibronectin mRNA and protein (7-fold) in a concentration-dependent fashion. An interesting relationship between the two TGFβ receptors was found in that TGFβ caused an upregulation of its type I receptor mRNA (5–6-fold) and a downregulation of the type II receptor mRNA (5-fold). Time-course experiments revealed that the change in expression of the TGFβ receptors reached maximum at 24 h with an early increase at 4–5 h, whereas the fibronectin gene expression was not significantly stimulated until about 24 h. These data provide evidence that TGFβ stimulates extracellular matrix production in prostate cells. © 2001 Published by Elsevier Science B.V.

Keywords: TGF-$_\beta$ (transforming growth factor-β); Fibronectin; Smooth muscle, human prostate; Prostatic hyperplasia, benign; Activin-like kinase 5; Extracellular matrix

1. Introduction

Benign prostatic hyperplasia is a nonmalignant enlargement of the prostate gland. Despite its name, benign prostatic hyperplasia appears to involve cellular hypertrophy and increases in extracellular matrix production in addition to proliferation of epithelial cells. TGFβ is an important cytokine in the regulation of extracellular matrix production (Hocevar and Howe, 2000; Massague, 1990). Within the prostate there is evidence that stromal cells express TGFβ (Nemeth et al., 1997) and that TGFβ can inhibit proliferation of prostatic epithelial cells (Sutkowski et al., 1999). There is a lack of data, however, with regards to the effect of TGFβ on prostate smooth muscle cell matrix production. In the present study, we have therefore evaluated the effect of TGFβ on fibronectin expression. In addition, since TGFβ has also been reported to alter its own expression and that of its receptors, especially in vascular smooth muscle tissue (Siegert et al., 1999), we also investigated these effects on prostate smooth muscle cells.

2. Materials and methods

2.1. Cell maintenance and treatment

Human prostate smooth muscle cells (HPSM, Clonetics, Walkersville, MD) were grown in smooth muscle basal media supplemented with growth factors and 5% fetal bovine serum included in smooth muscle growth medium −2 bullet kit (Clonetics). Cells were maintained in a humidified incubator at 37 °C in 5% CO_2 and grown in monolayer in T-75 flasks until confluent and passaged 1:10 into T-150 flasks. Once confluent, these were passaged 1:20 into new T-150 flasks. Cells from passages between 4 and 9 were used for study. Cells used for experiments in plates were diluted into new growth media and seeded into 24-well plates at 6×10^4 cells/well and 6-well plates at 1×10^5 cells/well. Subculturing was performed by trypsinization (0.025% trypsin, 1 mM EDTA)

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for 4–5 min at 37 °C. Cells were counted by hemocytometry under a microscope.

Cell media was replaced with serum and growth factor-free media for 24 h prior to study. In experiments involving RNA extraction, flasks were treated with TGFβ (R&D Systems, Minneapolis, MN) at confluency using new serum-free media. In experiments involving plates, cells were left to attach overnight, and were treated with TGFβ following 24 h of serum-free media. Cells used for protein expression experiments were incubated with TGFβ for 24, 48 and 72 h. In all experiments, inhibitors were serial diluted in new serum-free media and added to the cells 4 h prior to TGFβ treatment.

2.2. RNA extraction

RNA was extracted by a modified acid phenol–chlorophorm extraction method (Chomczynski and Sacchi, 1987). Briefly, cells were lysed by addition of a buffer containing 4 M guanidium isothiocyanate, 25 mM sodium citrate, 0.5% sarcosyl and 0.1 M β-mercaptoethanol. The cell homogenate was added to 300 μl 2 M sodium acetate (pH 4), 3 ml phenol and 600 μl Sevag (49:1 chloroform:isoamyl alcohol). The aqueous phase was removed, added to 100% ethanol and RNA was left to precipitate at −20 °C overnight. The RNA pellet was washed in 75% ethanol and then in 100% ethanol. The resultant pellet was air dried and dissolved in 20 μl RNase free water. Yield and purity were determined by UV spectrophotometry at 260/280 nm. 10 μg total RNA was checked by electrophoresis in a 1% agarose gel.

2.3. Quantitative PCR (TaqMan)

Ten-microgram total RNA samples were first treated with RQ1 DNase (Promega, Madison, WI) to digest any traces of DNA. Two-microgram total RNA from this reaction was reverse transcribed using SuperScript reverse transcriptase enzyme with random hexamers (GibcoBRL, Rockville, MD). This was incubated at 37 °C for 60 min and the reaction was terminated at 70 °C for 10 min. The cDNA samples were diluted 20-fold and used in the subsequent PCR reactions. Standard curves were determined by making a serial dilution of control sample cDNA from 5- to 160-fold dilution. PCR was then carried out on all samples. The 25-μl PCR reaction mixture contained 2 μl diluted cDNA, 1 × TaqMan universal PCR master mix (Perkin Elmer Applied Biosystems, Foster City, CA), 200 nM of gene-specific forward and reverse primers and 200 nM of gene-specific fluorescent oligonucleotide probes. Primers specific for all target gene sequences were deduced from the full-length sequences obtained from GenBank (rpL32-NM_000994, human FN-X02761), checked for homology and synthesized by Perkin Elmer Applied Biosystems (rpL32 forward GAAAC-TGGCG-GAAAC-CCA, reverse GGATC-TGGCC-CTTGA-ATCTT-C, probe 6fam-AGGCA-TCGAC-AACAG-GGTGC-GG-tamra, FN forward GAAAG-TACAC-CTGTT-GTCAT-TCAAC-A, reverse ACCTT-CACGT-CTGTC-TGTCA-CTTCC-A, probe CCACT-GGCAC-CCCAC-CGCTC-A). (Human ALK5 forward ACATCTATGCAATGGGCTTAG-TATTCT, reverse ATAAGGCAGTTGGTAATCTTCAT-GAA, probe 6fam-CGACCCAGAGGCCCGTCTCACA-TAMRA). (Human TGFβ1 forward CGAGCCTGAGGC-CGACTAC, reverse AGATTTCGTTGTGGGTTTCCA, probe 6fam-CCAAGGAGGTCACCCGCGTGC-TAMRA).

The PCR reaction was carried out in the ABI Prism 7700 Sequence Detector (Perkin Elmer Applied Biosystems) with 40 cycles of 95 °C for 20 s and 60 °C for 30 s. The quantity of gene-specific cDNA in the unknown samples was calculated by comparing the cycle time of the unknown (at which the fluorescent signal is three times above the threshold level) to the cycle times measured for the standards. The quantity of the 20-fold dilution was arbitrarily set at one. All the data presented are standardized to the signal that measures mRNA for ribosomal protein L32.

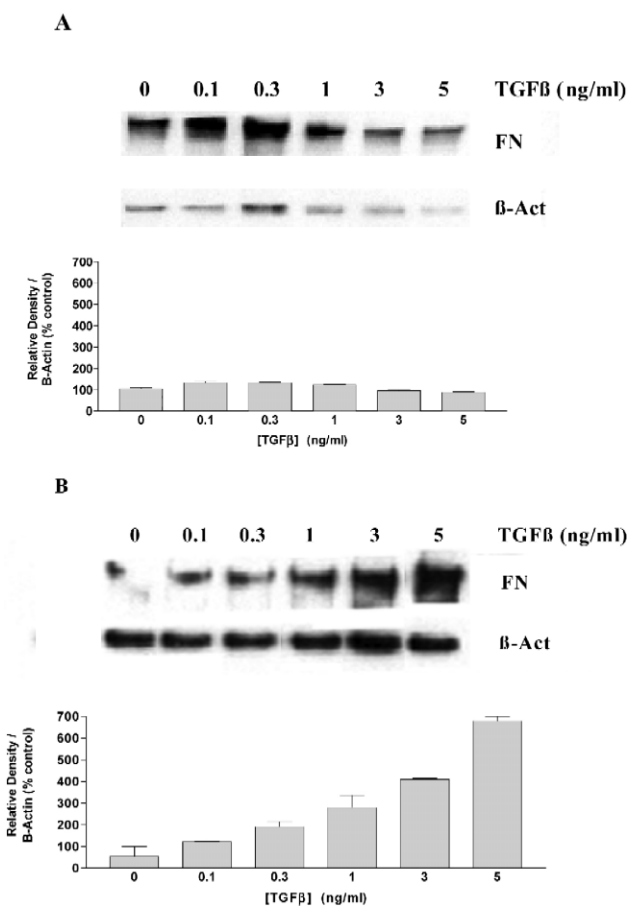

Fig. 1. Protein expression of fibronectin in cell lysate of normal HPSM cells following treatment with TGFβ. Western blots used antibodies for human fibronectin and human β-actin and the graphs quantify the intensity of the fibronectin normalized to the β-Actin. Panel A shows the effect on fibronectin synthesis after 24 h and panel B shows the effect after 72 h.

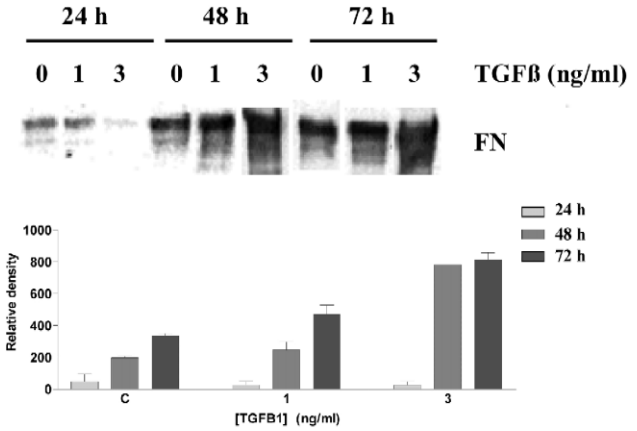

Fig. 2. Protein expression of fibronectin in the conditioned media of normal HPSM cells following treatment with TGFβ. Western blots used antibodies for human fibronectin and the graph quantifies the intensity of the bands (data not normalized).

2.4. Western blotting

Cell media was removed and 500 μl was concentrated to 10 μl using Microcon centrifugal filter devices (Millipore, Bedford, MA) following the manufacturer's guidelines. Cells were washed three times with cold PBS and lysed in 200 μl 2 × loading buffer (100 mM Tris–Cl (pH 6.8), 4% SDS, 0.2% Bromophenol blue, 20% glycerol and 50 μl/ml β-mercaptoethanol). Twenty microliters of each sample plus a protein ladder (Amersham Life Sciences, Piscataway, NJ) were electrophoresized on a NuPAGE 4–15% gradient gel at 200 V for 45 min in gel running buffer (Novex, San Diego, CA). The resultant gel was transferred to a nitrocellulose membrane in semi-dry transfer apparatus (Bio-Rad transblot SD) at 15 V for 15 min. Protein transfer was confirmed using Ponceau stain (Sigma, St. Louis, MO).

The membrane was blocked using blocking solution (Zymed, San Francisco, CA) for 1 h at room temperature. Following this, a 1:500 dilution of primary antibody in the same blocking solution was added for 1 h at room temperature. After washing with 0.1% PBS-Tween, the membrane was then incubated with a 1:5000 dilution of secondary antibodies in blocking solution for 1 h at room temperature. Following this period, the membrane was washed in the same way and treated with ECL chemiluminescent reagent (Amersham Life Sciences). The membrane was exposed to photographic film (Kodak) and analyzed using a scanner and the program "imagequant" (Molecular Dynamics, Sunny Vale, CA).

Primary antibodies used in the detection of specific proteins were rabbit monoclonal antibody to human fibronectin and mouse monoclonal antibody to β-Actin. Secondary antibodies used were goat-derived anti-rabbit

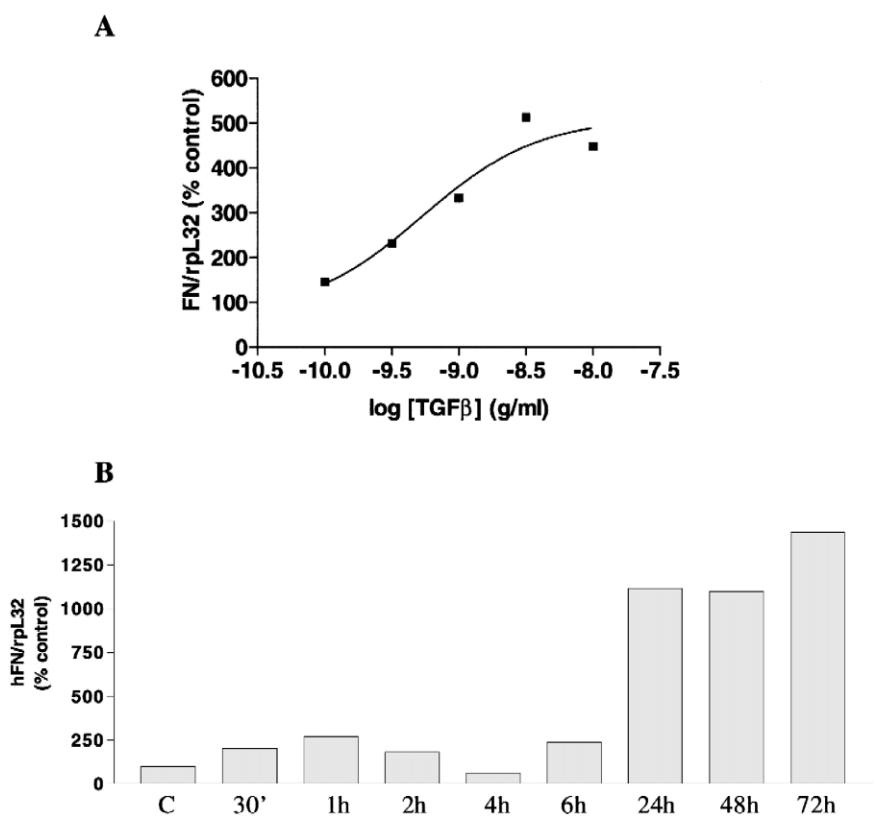

Fig. 3. Effect of TGFβ on mRNA expression of fibronectin as shown by RT-PCR (TaqMan). Panel A shows the dose-dependent increase of fibronectin with TGFβ (24 h). Panel B shows the time-dependency of fibronectin stimulation by TGFβ (5 ng/ml).

IgG for fibronectin and goat-derived anti-mouse IgG for β-Actin.

2.5. Data analysis

All data are reported as mean and standard error of the mean. All studies were performed in duplicate or triplicate with the exception of the time-course schedule shown in Figs. 3 and 4, where multiple observations were made over time.

3. Results

The presence of fibronectin was observed using Western blot analysis in both the cell lysate (cytoplasmic and cell-surface bound) and the conditioned media (released) of normal HPSM cells (Fig. 1). Treatment with TGFβ resulted in a dose-dependent increase of cytoplasmic and cell-surface bound fibronectin after 72 h (Fig. 1B) demonstrating a 6- to 7-fold increase with 3 ng/ml TGFβ and an EC_{50} of approximately 2 ng/ml. However, no response to TGFβ was observed at 24 (Fig. 1A) or 48 h (data not shown).

TGFβ (3 ng/ml) increased fibronectin protein in the media at 48 h (6-fold) and 72 h (8-fold) in a dose-dependent fashion (Fig. 2). There was no change after 24 h. At higher concentrations of TGFβ, a smear of lower molecular weight products was seen below the main band. This is typical when high levels of expression are seen since there is a commensurate increase in partially translated and

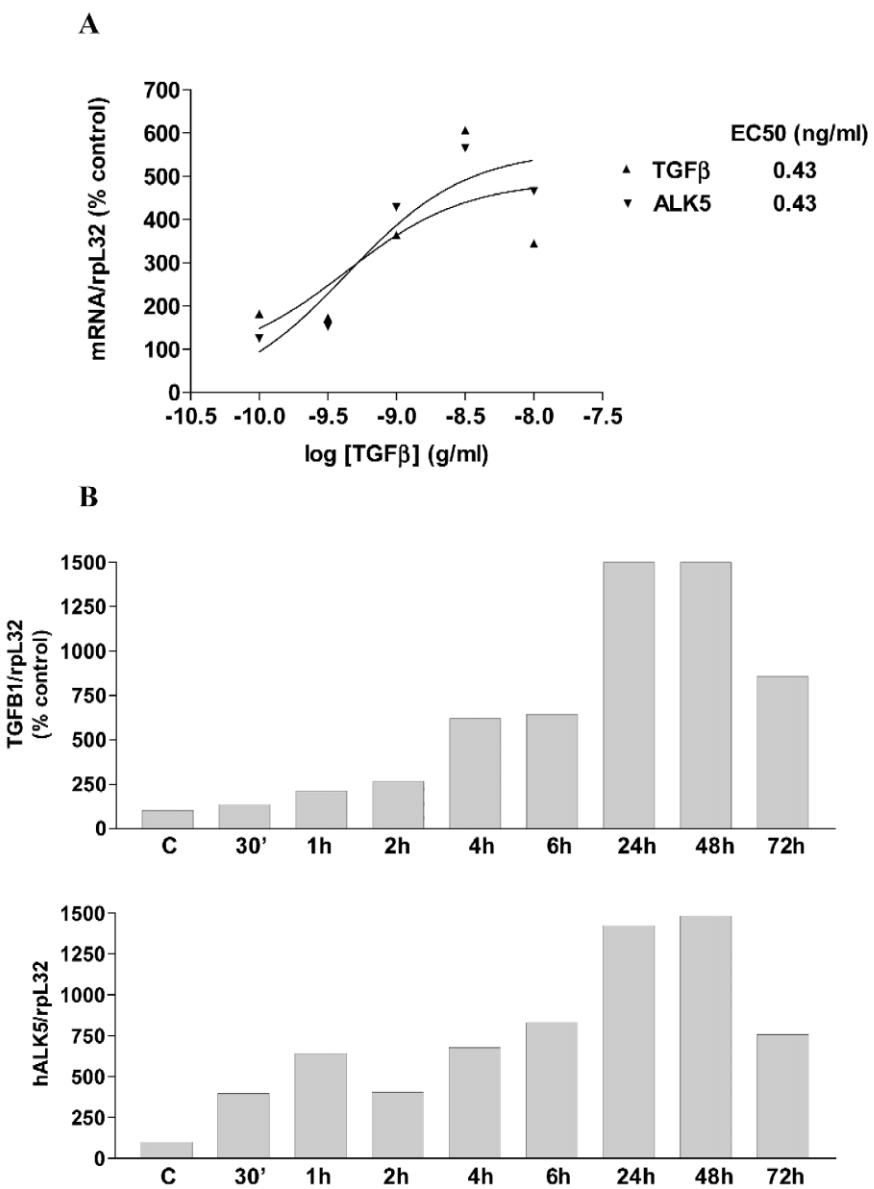

Fig. 4. Effect of TGFβ on mRNA expression of the ALK5 receptor and the TGFβ ligand as shown by RT-PCR (TaqMan). Panel A shows the concentration-dependent increase in expression of both genes after 24 h. Panel B shows the time-course of stimulation by TGFβ (5 ng/ml).

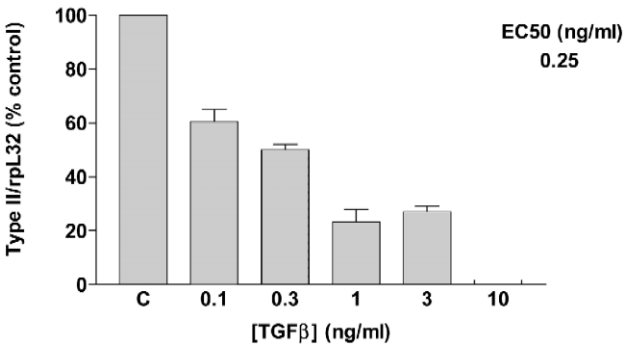

Fig. 5. Effect of TGFβ on mRNA expression of the TGFβ type II receptor as shown by RT-PCR (TaqMan). Panel A shows the dose-dependent decrease in expression of the receptor with TGFβ (24 h).

degraded protein recognized by the antibody. Basal levels of fibronectin in the media increased time-dependently with a 2-fold increase at 48 h and a 3–4-fold increase at 72 h. Fibronectin was not only present in the cell lysate and the media, but the stimulatory effects of TGFβ were seen in both locations, and with similar time courses.

RT-PCR experiments were carried out to determine if and when TGFβ causes fibronectin mRNA increases to explain the protein expression patterns observed in HPSM cells. TGFβ caused an increase in mRNA levels concentration-dependently up until about 5 ng/ml where stimulation was 5-fold (Fig. 3A). The EC_{50} for this response was 0.5 ng/ml. A time-course experiment demonstrated that TGFβ-mediated increases in fibronectin mRNA occurred at 24 h and were maintained for at least 72 h (Fig. 3B).

The effects of dose and time of TGFβ on the expression of the two TGFβ receptors and the TGFβ ligand were investigated. Results show that expression of ALK5 and TGFβ increased dose-dependently, showing 5- and 6-fold increases with 3 ng/ml TGFβ after 24 h respectively (Fig. 4A). EC_{50} values for TGFβ were both 0.43 ng/ml. The expression of these genes increased in a time-dependent manner at 5 ng/ml TGFβ treatment, with no significant increase of TGFβ until 4 h (5 to 6-fold) and increases at around 1–2 h for ALK5 (5-fold) (Fig. 4B). Expression continued to increase until 48 h (15-fold) and then decreased to about 50% of maximum (7–8 fold) at 72 h. In contrast, the TGFβ type II receptor mRNA decreased dose-dependently after 24 h, showing a 5-fold decrease with 3 ng/ml TGFβ (Fig. 5).

4. Discussion

It was the aim of this study to investigate the role of TGFβ and its receptors in the production of fibronectin by HPSM tissue. We observed that TGFβ increased the expression of fibronectin. In addition, TGFβ caused an increase in expression of its own gene and that of its type I receptor, ALK5, but a decrease in the expression of its type II receptor.

To date most of the data concerning TGFβ and prostate cells have suggested that TGFβ is involved in cell proliferation and differentiation. The stromal cells express TGFβ (Nemeth et al., 1997), and its receptors are expressed in both the epithelial cells (Kim et al., 1998) and to a lesser degree in stromal cells (Kassen et al., 1996). TGFβ has been shown to inhibit proliferation within the epithelial cell layer (Sutkowski et al., 1999) and is proposed to cause differentiation of the stromal cells. Our data suggest that TGFβ may also play an important role in extracellular matrix production within the prostate.

Western blotting techniques demonstrated that TGFβ stimulated fibronectin levels in the cell lysate and the surrounding medium after 72 and 48 h, respectively. The delay in protein expression following TGFβ treatment can be partly explained by a relatively late rise in the expression levels of fibronectin mRNA (8–24 h), as seen using quantitative PCR (TaqMan).

An increase in expression of the ALK5 receptor by TGFβ would suggest that a positive feedback loop is in effect, which would in turn enhance extracellular matrix production by TGFβ. This theory is supported by an increase in expression of the TGFβ ligand, which would further mediate the positive feedback system. The function of decreases in the type II receptor is as yet unclear. One possible hypothesis is that a decrease in this subtype may cause the reversal of the positive feedback mechanism initiated by increases of ALK5. Consideration of the time-course of these events, however, reveals that changes in expression levels of these genes occur almost simultaneously, thus questioning this possibility. This finding provides further evidence that perhaps the current model of TGFβ signaling, involving specific interactions between ALK5 and the type II receptor, is not entirely accurate. If this was the mechanism operated by TGFβ, then a decrease in type II receptor expression would interrupt the positive feedback effects of an increase in ALK5 receptor expression. Kim et al. (1998) reported that in LNCaP cells, a human prostate cancer cell line, ALK5 was not expressed but the anti-proliferative effects of TGFβ are still observed. They concluded that ALK5 may not be required for TGFβ-mediated inhibition of proliferation and that there may be alternative mechanisms for TGFβ signal transduction regarding proliferation. It can be postulated then that perhaps either the two receptors can operate differently, mediating different effects, or that there are different, yet undiscovered receptor subtypes that are involved. The former is consistent with the differential regulation of ALK5 and the type II in HPSM cells as this would allow a positive feedback to occur, mediating the pro-fibrotic effects, whilst a decrease in the type II receptor would not affect this and might affect the anti-proliferative effects of TGFβ. Either hypothesis suggests that the pleiotropic effects of TGFβ are mediated via multiple

receptors that differentially affect subsets of TGFβ-mediated responses.

In summary, our data suggest that TGFβ plays a profibrotic role within the prostate through its action upon smooth muscle cells. It is known that benign prostatic hyperplasia is in part mediated by an increase in extracellular matrix levels, which suggests a role for TGFβ in the development of this disease.

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