Stromal Insulin-Like Growth Factor Binding Protein 3 (IGFBP3) Is Elevated in the Diseased Human Prostate and Promotes ex Vivo Fibroblast-to-Myofibroblast Differentiation

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Dysregulation of the IGF axis is implicated in the development of benign prostatic hyperplasia (BPH) and prostate cancer (PCa), 2 of the most common diseases affecting elderly males. PCa is the second leading cause of male-related cancer death in Western societies. Although distinct pathologies, BPH and PCa are both characterized by extensive stromal remodeling, in particular fibroblast-to-myofibroblast differentiation, thought to be induced by elevated local production of TGFβ1. We previously showed that TGFβ1-mediated fibroblast-to-myofibroblast differentiation of primary human prostatic stromal cells resulted in the dysregulation of several components of the IGF axis, including the induction of IGF binding protein 3 (IGFBP3). Using isoform-specific lentiviral-mediated knockdown, we demonstrate herein that IGFBP3 is essential for TGFβ1-mediated differentiation. Although recombinant human IGFBP3 alone was not sufficient to induce differentiation, IGFBP3 synergistically potentiated TGFβ1-mediated stromal remodeling predominantly via an IGF-independent mechanism. Consistent with these in vitro findings, IGFBP3 immunohistochemistry revealed elevated levels of IGFBP3 in the hyperplastic fibromuscular stroma of BPH specimens and in the tumor-adjacent stroma of high-grade PCa. Collectively these data indicate that the dysregulation of the stromal IGF axis, in particular elevated IGFBP3, plays a crucial role in fibroblast-to-myofibroblast differentiation in the diseased prostatic stroma and indicate the therapeutic potential of inhibiting stromal remodeling and the resulting dysregulation of the stromal IGF axis as a novel strategy for the treatment of advanced PCa and BPH. (Endocrinology 154: 2586–2599, 2013)

Signaling via the multicomponent IGF axis regulates the physiological and pathophysiological growth of many tissues and organs, including the prostate (1). The mitogenic ligands IGF-I and IGF-II predominantly mediate their effects by binding to transmembrane tyrosine kinase receptors, IGF-I receptor (IGF1R) and IGF-II receptor, and activating downstream signaling cascades, eg, AKT, resulting in enhanced cell proliferation and survival (2). There are 7 IGF-binding proteins (IGFBPs; IGFBP1–7, the latter also known as IGFBP related protein-1) of which IGFBP3 is the principal carrier of IGF ligands in serum (3). Although IGFBP3 may potentiate IGF signaling, in many instances IGFBP3 action is inhibitory due to high-affinity IGF sequestration and/or via poorly understood IGF-independent mechanisms (4–7).

Abbreviations: bFGF, basic fibroblast growth factor; BPH, benign prostatic hyperplasia; CM, conditioned media; CNN1, calponin; ECM, extracellular matrix; IGFBP, IGF binding protein; IGF1R, IGF-I receptor; MOI, multiplicity of infection; NOX4, nicotinamide adenine dinucleotide phosphate oxidase 4; pAKT, phosphorylated AKT; PCa, prostate cancer; PrSC, primary human prostatic stromal cell; qPCR, quantitative real-time PCR; rhIGFBP3, recombinant human IGFBP 3; scr, scrambled; SF-RPMI, serum-free RPMI 1640; shRNA, short hairpin RNA; SMA, smooth muscle cell actin; Smad, phosphorylated mothers against decapentaplegic.
Epidemiological studies have implicated the IGF axis in the development of benign prostatic hyperplasia (BPH) and numerous solid tumors, including prostate cancer (PCa) (8–10). For example, circulating and prostatic tissue levels of IGF ligands are elevated in PCa, whereas elevated tissue levels of IGF1R in PCa-adjacent stroma positively correlated with Gleason grade (11–16). Although there does not appear to be a significant association between serum IGFBP3 levels and PCa incidence (12, 13), the tissue levels of epithelial IGFBP3 are reportedly decreased in PCa, whereas the stromal tissue levels of IGFBP3 are elevated in mouse models of PCa (17–19). Targeting the IGF axis is thus considered a promising therapeutic anticancer strategy (20–22).

BPH and PCa are 2 of the most common diseases affecting aging males (8, 23, 24). PCa is the second leading cause of male cancer-related death in Western societies (8, 23, 24). Although distinct pathologies, BPH and PCa are both associated with changes in the stromal microenvironment that actively promote disease development (25–28). In particular, the BPH- and PCa-adjacent stroma (the latter also termed reactive stroma) are characterized by increased extracellular matrix (ECM) deposition, capillary density, and differentiation of fibroblasts into myofibroblasts, whose mitogenic secretome promotes proliferation, angiogenesis, and tumorigenesis (28–33). Initial systemic treatment for BPH and local-confined PCa targets androgen signaling/metabolism resulting in the apoptosis of androgen-dependent cells and reduced prostate volume (34, 35). Approximately 90% of patients respond to current first-line androgen deprivation therapy; however, many patients experience disease progression and succumb to castration-resistant PCa within 3 years (36, 37). Importantly, neither approach specifically addresses the stromal component of disease. Thus, understanding the mechanisms underlying stromal remodeling, in particular fibroblast-to-myofibroblast differentiation, may facilitate the development of preventative or more effective treatment strategies.

Elevated production of the cytokine TGFβ1 is observed in BPH and preneoplastic prostatic intraepithelial neoplasia lesions with tissue and circulating levels positively correlating with disease risk and more rapid PCa progression (38, 39). Moreover, we and others demonstrated that TGFβ1 induces fibroblast-to-myofibroblast differentiation and stromal remodeling both in vitro and in vivo (40–42). Thus, TGFβ1 is widely considered a key inducer of pathogenic stromal reorganization; however, the downstream effectors and hence potential therapeutic targets remain largely unknown. Using Affymetrix expression profiling, we previously showed that TGFβ1-mediated fibroblast-to-myofibroblast differentiation of primary human prostatic stromal cells (PrSCs) is associated with the dysregulation of several components of the IGF axis, in particular the induction of IGFBP3 (40, 42). Similar findings were also reported by others (44, 45). Although the epithelial IGF axis has been intensely studied, particularly with respect to PCa development (1, 46, 47), relatively little is known about the function and regulation of IGF signaling components in the benign hyperplastic and malignant prostatic stroma. This study aimed to determine the functional significance of elevated stromal IGFBP3 during fibroblast-to-myofibroblast differentiation, a key process involved in the pathogenesis of BPH and PCa.

Materials and Methods

Reagents

Reagents were from Sigma Aldrich (St Louis, Missouri) unless otherwise specified. Recombinant human TGFβ1 and IGFBP3 (rhIGFBP3) were from R&D Systems (Minneapolis, Minnesota). Antibodies were obtained as follows: α-tubulin (Santa Cruz Biotechnology, Inc, Santa Cruz, California); IGFBP3 and phospho-phosphorylated mothers against decapentaplegic (Smad)-2 (R&D Systems); β-actin, calponin, α-smooth muscle cell actin (α-SMA) and fluorescein isothiocyanate-α-SMA (Sigma); glyceraldehyde-3-phosphate dehydrogenase (GAPDH; Abcam, Cambridge, Massachusetts); phospho-specific antibodies against AKT, IGF1R, ERK1/2, and c-Jun N-terminal kinase and total AKT and total IGF1R (Cell Signaling Technology, Inc, Danvers, Massachusetts); IGF1R neutralizing antibody, aIR3 (Millipore, Bedford, Massachusetts); horseradish peroxidase-conjugated secondary antibodies (Jackson ImmunoResearch Europe, Newmarket, United Kingdom); and AlexaFluor secondary antibodies (Invitrogen, Vienna, Austria). For immunohistochemistry and immunofluorescence, anti-IGFBP3 antibody (H-98) was from Santa Cruz Biotechnology. Additional competition immunofluorescent staining experiments in which the antibody was preincubated with a 20-fold molar excess of rhIGFBP3 (200 ng/mL) were performed to confirm the specificity of the H-98 IGFBP3 antibody (Supplemental Figure 1, published on The Endocrine Society’s Journals Online web site at http://endo.endojournals.org).

Primary cell culture

Human PrSCs were established from regularly supplied prostate biopsy tissue as described (42), which provided a regular source of benign material from different donors for PrSC isolation. PrSCs from a total of 34 patients were used in this study. All patients gave written informed consent. Each experiment was repeated at least 3 times with primary cells of passages 2–4 from different donors. PrSCs were maintained for routine culture at
37°C in a humidified atmosphere of 5% CO₂ in stromal cell growth medium (Lonza, Basel, Switzerland), which contains basic fibroblast growth factor (bFGF). For differentiation, PrSCs were incubated for 12 hours in serum-free RPMI 1640 supplemented with L-glutamine and antibiotics (PAA Laboratories, Pasching, Austria), hereafter referred to as SF-RPMI. Cells were subsequently stimulated with rhIGFBP3, IGF-I, or LongR3-IGF1 at the indicated concentration in SF-RPMI alone or in the presence of either 1 ng/mL bFGF (as control to maintain the fibroblast phenotype) or 1 ng/mL TGFβ1 for the indicated duration. For IGF1R neutralization experiments, and where the cells were treated with a combination of rhIGFBP3 and IGF-I or LongR3-IGF1, media were prepared with the indicated concentration of reagents and incubated at room temperature with end-over-end rotation for 2 hours prior to the addition to cells.

RNA isolation, cDNA synthesis, and quantitative real-time PCR (qPCR)

RNA isolation, cDNA synthesis, and qPCR were performed as described (40). Primer sequences are given in Supplemental Table 1. cDNA concentrations were normalized against hydroxymethylbilane synthase (HMBS), a moderate copy number housekeeping gene not regulated under the experimental conditions used. Fold change in gene expression was determined from the change in cycle threshold values using the mathematical model ratio 2^{-ΔΔCT} as described (48).

Microarrays

PrSCs from 3 independent donors incubated overnight in RPMI 1640 supplemented with 1% charcoal-treated bovine calf serum (Hyclone Laboratories, Logan, Utah) were stimulated with either 1 ng/mL bFGF as mock control or 1 ng/mL TGFβ1 for 48 hours. Two micrograms of total RNA from each donor were pooled, and hybridization to Affymetrix Human Genome U133 Plus 2.0 GeneChips was performed at the Microarray Facility (Tübingen, Germany). A technical replicate array was performed. Raw expression data were normalized using the GRCMA algorithm at CARMAweb (49, 50). The complete microarray data set is available at ArrayExpress (E-MEXP-2167) from the European Bioinformatics Institute at www.ebi.ac.uk/arrayexpress.

Cell proliferation

PrSCs were seeded in quadruplicate in 96-well plates at 4000 cells/well in SF-RPMI. The following day, cells were treated with 1 ng/mL bFGF or TGFβ1 as indicated for the duration stated. Proliferation was determined after differentiation with 10 μM 5-bromo-2′-deoxyuridine for 14 hours using a colorimetric ELISA according to the manufacturer’s instructions (Roche Applied Science, Indianapolis, Indiana).

Lentiviral-mediated knockdown of IGFBP3

IGFBP3 and scrambled short hairpin RNA (shRNA) sequences and generation of lentiviral particles have been described previously (40, 51). For viral transduction, 1 × 10⁵ PrSCs were seeded in 6-cm dishes in stromal cell growth medium. The following day, media were replaced, supplemented with 8 μg/mL polyanene and virus-containing supernatant at a multiplicity of infection (MOI) of 4.0. After 24 hours, puromycin was added directly to existing media to a final concentration of 1 μg/mL and incubated for a further 72 hours. For qPCR and Western blotting, cells were subsequently incubated overnight in SF-RPMI before stimulation with 1 ng/mL bFGF or TGFβ1 as indicated for the duration stated. For proliferation analyses, cells transduced with lentiviruses as above in 6-cm dishes were trypsinized 96 hours after transduction before seeding in 96-well plates and treating for proliferation analysis as described above.

Western blotting and IGFBP3 ELISA

Isolation of total cell lysates and Western blotting were performed as described (40) and normalized for total protein content via bicinchoninic acid protein assay according to the manufacturer’s instructions (Thermo Fisher Scientific, Vienna, Austria). Conditioned media (CM) were harvested from cells treated for the indicated duration with 1 ng/mL bFGF or TGFβ1 in SF-RPMI. After centrifugation for 5 minutes at 700 rpm, CM were normalized against the total protein content of the corresponding cell lysate and stored at −80°C. Secreted IGFBP3 was analyzed by Western blotting or ELISA according to the manufacturer’s instructions (Mediagnost, Reutlingen, Germany).

Immunofluorescence and immunohistochemistry

Immunofluorescence was performed as previously described (52) with the exception that 5 hours prior to fixation in 4% paraformaldehyde, cells were incubated with 10 μg/mL Brefeldin A to reduce IGFBP3 secretion enabling immunofluorescent detection of intracellular IGFBP3. For competition experiments, H-98 IGFBP3 antibody was preincubated with 200 ng/mL rhIGFBP3 for 2 hours at room temperature with end-over-end rotation. Confocal microscopy was performed with a micro lens-enhanced Nipkow disk-based confocal system UltraVIEW RS (PerkinElmer, Wellesley Massachusetts) mounted on an Olympus IX-70 inverse microscope (Olympus, Nagano, Japan). Images were captured with a cooled digital charge-coupled device camera (Hamamatsu Photonics K.K., Hamamatsu City, Japan) in combination with the UltraVIEW RS from PerkinElmer. Prostate tissue sections from paraffin blocks of formalin-fixed, whole-biopsy specimens were obtained from the archives of the Institute of Pathology at the University Hospital Basel (Basel, Switzerland). All patients gave written informed consent. Samples were processed for immunohistochemistry as described (40) with the exception that antigen retrieval was performed in Target Antigen Retrieval solution, pH 9 (Dako, Vienna, Austria) for 20 minutes at 95°C and incubated with antirabbit IGFBP3 antibody (H-98; Santa Cruz Biotechnology) or control rabbit IgG (negative control) was performed at 37°C for 2 hours before transferring to 4°C for a further 10 hours. Stained sections were analyzed and imaged by a uropathologist (G.S.) on an Olympus BX 50 microscope (Olympus, Tokyo, Japan) with a Pixelink PL-A642 microscope camera using PicEd software (Pixelink, Ottawa, Canada).

Statistical analysis

Numerical data are presented as mean ± SEM from at least 3 independent experiments using PrSCs isolated from independent donors. Statistical differences were calculated after 1-way ANOVA and subsequent pairwise comparisons analyzed via Dunnett’s post hoc test (SPSS software version 20; SPSS Inc, Chicago, Illinois). Statistically significant differences are denoted: *, P < .05; **, P < .01; ***, P < .001; ns, not significant where P > .05.
Results

Dysregulation of the stromal IGF axis during prostatic fibroblast-to-myofibroblast differentiation

To investigate molecular changes during BPH/PCa-associated fibroblast-to-myofibroblast differentiation, we previously established an in vitro model system in which PrSCs treated with TGFβ1 undergo differentiation into myofibroblasts as defined by their continued expression of the mesenchymal marker vimentin and induction of early smooth muscle differentiation markers, such as α-SMA and calponin (CNN1) (41, 42). Recently we reported Af-fymetrix expression profiles of TGFβ1-induced differentiated and nondifferentiated PrSCs, which revealed an essential role of nicotinamide adenine dinucleotide phosphate oxidase 4 (NOX4) in the downstream induction of SMA during TGFβ1-mediated PrSC differentiation (40). Additional pathway analyses revealed significant dysregulation of several components of the IGF axis during differentiation, a finding validated in this study by qPCR (Table 1). Under basal conditions, PrSCs express low levels of IGF1 and IGF2 and moderate levels of IGF1R, whereas differentiated PrSCs express significantly higher levels of IGF1 and IGF1R (Table 1). Of the 7 IGFBPs, PrSCs under basal conditions express low levels of IGFBP5 and -7 (Supplemental Figure 2) but no detectable IGFBP1. These IGFBP isoforms were thus not investigated further. PrSCs, however, express much higher levels of IGFBP2, -3, -4, and -6 (Supplemental Figure 2). IGFBP3 was strongly up-regulated during differentiation (9.5 ± 2.8-fold by qPCR), whereas IGFBP6 was slightly down-regulated (−3.2 ± 1.2-fold by qPCR). No differential regulation of IGFBP2 or -4 was observed during differentiation (Table 1).

Table 1. Dysregulation of the IGF Axis During Fibroblast-to-Myofibroblast Differentiation

<table>
<thead>
<tr>
<th>Gene Symbol</th>
<th>Gene Identification</th>
<th>Affymetrixa</th>
<th>qPCRB</th>
</tr>
</thead>
<tbody>
<tr>
<td>IGF1</td>
<td>3479</td>
<td>12.1 ± 3.2</td>
<td>24.8 ± 6.9</td>
</tr>
<tr>
<td>IGF1R</td>
<td>3480</td>
<td>2.1 ± 0.3</td>
<td>3.1 ± 0.7</td>
</tr>
<tr>
<td>IGFBP2</td>
<td>3485</td>
<td>1.0 ± 0.2</td>
<td>−1.3 ± 0.8</td>
</tr>
<tr>
<td>IGFBP3</td>
<td>3486</td>
<td>6.4 ± 2.1</td>
<td>9.5 ± 2.8</td>
</tr>
<tr>
<td>IGFBP4</td>
<td>3487</td>
<td>−1.1 ± 0.8</td>
<td>1.5 ± 1.1</td>
</tr>
<tr>
<td>IGFBP6</td>
<td>3489</td>
<td>−2.3 ± 0.1</td>
<td>−3.2 ± 1.2</td>
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PrSCs from 3 independent donors were treated with 1 ng/mL TGFβ1 or bFGF for 48 hours.

IGFBP3 is abundantly secreted during prostatic stromal differentiation

Of the 4 abundantly expressed IGFBP isoforms in PrSCs under basal conditions, IGFBP3 exhibited the highest degree of differential expression in PrSCs treated with TGFβ1 relative to bFGF control. Subsequent experiments therefore focused on determining the functional significance of elevated IGFBP3 during stromal remodeling. The temporal dynamics of IGFBP3 up-regulation were examined in time-course assays of TGFβ1 stimulation. In contrast to the rapid (<2 hours) up-regulation of the reactive oxygen species-producing enzyme NOX4, which we previously showed to be an essential mediator of TGFβ1-induced PrSC differentiation (40), induction of IGFBP3 occurred with delayed dynamics (12–24 hours; Figure 1A). Temporal induction of IGFBP3 closely correlated with that of the early smooth muscle cell differentiation markers SMA and CNN1. Notably, however, maximal induction of NOX4, CNN1, and SMA mRNAs was reached after 12 hours, whereas induction of IGFBP3 continued to increase, even after 48 hours of TGFβ1 stimulation (Figure 1A). Similar findings were also observed at the protein level (Figure 1, B–D).

Like all members of the IGFBP family, IGFBP3 is a secreted glycoprotein (6). To investigate whether elevated IGFBP3 during TGFβ1-mediated differentiation is secreted by PrSCs, IGFBP3 levels were determined in PrSC CM by Western blotting and ELISA (Figure 1, C and D). Under basal conditions (bFGF control), IGFBP3 was detected at low concentrations in PrSC CM after 24 hours (6.0 ± 1.8 ng/mL), which progressively increased over the time course examined (7.7 ± 1.1-fold at 72 hours; P < .001; Figure 1, C and D). TGFβ1 significantly increased secreted IGFBP3 levels. After 24 hours of TGFβ1 treatment, secreted IGFBP3 concentrations were comparable with those in the CM from bFGF-treated cells after 48 hours (15.3 ± 2.6 relative to 18.1 ± 4.5 ng/mL, respectively; Figure 1, C and D). Moreover, after 72 hours of TGFβ1 treatment, secreted IGFBP3 levels were 111.3 ± 18.3 ng/mL compared with 30.0 ± 7.7 ng/mL after 72 hours of bFGF treatment (P = .018; Figure 1, C and D). Collectively these data indicate that IGFBP3 is secreted at elevated levels during TGFβ1-mediated differentiation of PrSCs.

IGFBP3 is essential for TGFβ1-mediated fibroblast-to-myofibroblast differentiation

To determine whether IGFBP3 is required for TGFβ1-induced fibroblast-to-myofibroblast differentiation, lentiviral-mediated, isoform-specific knockdown was used (Figure 2). Under basal (bFGF) conditions, IGFBP3 shRNA significantly reduced IGFBP3 expression at both
the mRNA (−7.5 ± 3.2-fold; \( P < .001 \)) and protein level compared with cells treated with scrambled (scr) shRNA (Figure 2, A and B). Similar findings were observed for a second shRNA sequence targeting a distinct region of IGFBP3 (not shown). Moreover, in TGFβ1-treated cells, the 4.7-fold (±1.4-fold) induction of IGFBP3 mRNA in scr shRNA-treated cells was strongly inhibited in cells treated with IGFBP3 shRNA (−4.6 ± 0.5-fold; \( P = .003 \); Figure 2A). Similar findings were observed at the protein level (Figure 2B). Furthermore, immunofluorescent staining revealed that TGFβ1-mediated induction of IGFBP3 was uniformly attenuated in cells treated with IGFBP3 shRNA, indicating high homogeneity of lentiviral infection (Figure 2C).

We next investigated the effect of IGFBP3 knockdown on TGFβ1-mediated induction of differentiation markers, including NOX4, SMA, and CNN1 (Figure 2, A–C). After 72 hours TGFβ1 treatment, IGFBP3 shRNA significantly attenuated induction of NOX4, SMA and CNN1 mRNAs relative to scr shRNA, a finding also observed at the protein level for SMA and CNN1 at 24 and 72 hours (Figure 2, A–C). Furthermore, immunofluorescent staining revealed that IGFBP3 knockdown reduced the appearance of SMA-immunoreactive filaments induced by TGFβ1, indicating that fibroblast-to-myofibroblast differentiation was also attenuated at the morphological level (Figure 2C). Collectively these data indicate that induction of IGFBP3 is essential for TGFβ1-mediated fibroblast-to-myofibroblast differentiation.

IGFBP3 synergistically potentiates TGFβ1-mediated differentiation

The functional significance of increasing IGFBP3 levels during differentiation was further investigated in PrSCs incubated under serum-free conditions with 300 ng/mL rhIGFBP3 (Figure 3), a concentration selected to mimic that secreted by PrSCs after 72 hours of TGFβ1 treatment.
(111.3 ± 18.3 ng/mL; see Figure 1D), taking into consideration that recombinant purified protein may exhibit reduced activity relative to native IGFBP3 and is similar to concentrations used by others (53, 54). Relative to bFGF-treated PrSCs, rhIGFBP3 alone did not significantly alter expression of the differentiation markers investigated either at the mRNA or protein level (Figure 3, A–C, and data not shown). In addition, rhIGFBP3 alone exerted no change on the phosphorylation status of Smad2, ERK, or c-Jun N-terminal kinase (Supplemental Figure 4), which in addition to AKT represents the key downstream signaling transducers of TGFβ1-mediated differentiation in PrSCs (40, 55). Consistent with IGF sequestration, however, rhIGFBP3 reduced phosphorylated AKT (pAKT) levels, demonstrating that rhIGFBP3 used in this study is biologically active (Supplemental Figure 4). These data indicate that elevated IGFBP3 alone is not sufficient to induce fibroblast-to-myofibroblast differentiation.

Given that IGFBP3 is first significantly induced when phenotypic transformation becomes morphologically ap-
IGFBP3 promotes differentiation in an IGF-independent manner

To investigate whether IGFBP3 promotes differentiation as a consequence of attenuated IGF signaling, we next examined AKT phosphorylation, which serves as a marker of IGF signaling (56). There was no significant difference in TGFβ1-induced AKT phosphorylation up to 8 hours after TGFβ treatment of scr and IGFBP3 shRNA-treated cells (Figure 4, A and B). Thereafter pAKT levels subsequently decreased in scr control cells (−1.5 ± 0.3-fold from 8 to 24 hours), which temporally correlates with IGFBP3 induction (Figure 4, A and B, and Figure 1B). Upon IGFBP3 knockdown, this decrease was initially attenuated (1.0 ± 0.1 from 8 to 24 hours). Thereafter, however (24–72 hours), pAKT levels declined at a rate comparable with scr control (Figure 4, A and B), indicating that during differentiation pAKT levels are predominantly determined by factors other than IGFBP3, which exerts only a minor and temporally defined effect (at 8–24 hours) on AKT phosphorylation.

As a more direct approach to determine whether IGFBP3 potentiates PrSC differentiation in an IGF-dependent manner, we asked whether IGF-I or an IGF-I analog with low affinity for IGFBP3 (LongR3-IGF1) could modulate TGFβ1-induced differentiation or ablate IGFBP3 potentiation of TGFβ1-induced differentiation. Although LongR3-IGF1 increased PrSC proliferation and thus was biologically active (Supplemental Figure 5), it had no effect on TGFβ1-mediated induction of differentiation markers NOX4, IGFBP3, or SMA (Figure 4, C–E). Similar findings were observed for wild-type recombinant IGF-I (not shown). Moreover, the synergistic induction of differentiation markers SMA and CNN1 by IGFBP3 and TGFβ1 still occurred in the presence of IGF-I or LongR3-IGF1 (Figure 4, D and E, and data not shown). Furthermore, the IGF1R neutralizing antibody α-IR3, which effectively attenuated IGF-I-mediated phosphorylation of IGF1R and its downstream target AKT (Figure 4F), had no effect on TGFβ1-mediated induction of differentiation markers (Figure 4G). It may be noted that neither IGFBP3 mRNA nor protein levels are altered by IGF-I or LongR3-IGF1 treatment in PrSCs (Figure 4D and not shown). Moreover, IGF1R is not detectably phosphorylated during TGFβ1-mediated fibroblast-to-myofibroblast differentiation (Figure 4D). Collectively these data indicate that IGFBP3 potentiates differentiation in an IGF-independent manner.

Stromal IGFBP3 is elevated in BPH and PCA

The above-mentioned in vitro data indicated that IGFBP3 may play a role in stromal remodeling associated with BPH and PCA, in particular in myofibroblast differentiation. Thus, we next examined the localization and

Figure 3. IGFBP3 potentiates TGFβ1-mediated differentiation. A, qPCR of the indicated genes in PrSCs treated for 72 hours with the indicated concentration of rhIGFBP3 in SF-RPMI treated with 1 ng/mL bFGF or TGFβ1 as stated. PrSCs treated with TGFβ1 alone served as a positive control. Values represent mean fold change in gene expression (±SEM) using PrSCs isolated from 3 independent donors. B, Western blotting using the indicated antibodies of cell lysates from PrSCs treated for 72 hours as in panel A. Images are representative of 4 independent experiments using PrSCs from different donors. C, Densitometric quantification of assays are as shown in panel B. Values denote mean fold change in densitometric intensity (±SEM) from 4 independent experiments using PrSCs from different donors. A and C, Statistical significance is indicated (where P < .05; *, P < .01; **, P < .001). GAPDH, glyceraldehyde-3-phosphate dehydrogenase; ns, not significant.
abundance of IGFBP3 in BPH and PCa prostatic biopsy specimens via immunohistochemistry (Figures 5 and 6). In BPH specimens, strong IGFBP3 staining was observed in the epithelial cells of benign glands with weak/moderate staining of only a few interspersed cells in the periglandular stroma (Figure 5A). However, consistent with the above-mentioned in vitro data and increased numbers of myofibroblasts in BPH (57), the abundance of IGFBP3 positively stained cells was markedly increased in the hyperplastic fibromuscular stroma, which displayed a heterogeneous and nonuniform distribution of stromal IGFBP3 (Figure 5B). Specificity of antibody staining was verified in parallel-stained sections with control rabbit IgG and in competition experiments with rhIGFBP3 (Supplemental Figures 1 and 6). A similar staining pattern was observed with a second anti-IGFBP3 antibody (data not shown).

Although there did not appear to be a significant difference in staining intensity of tumor cells in PCa specimens compared with the adjacent benign epithelium (Figure 6A), moderate/strong IGFBP3 immunoreactivity was observed in the tumor-adjacent reactive stroma (Figure 6, A and B). This is in contrast to much lower levels of stromal IGFBP3 staining in benign areas of the same tissue section (Figure 6A). Thus, consistent with the above-mentioned in vitro observations, these findings indicate that IGFBP3 is elevated in the prostatic stroma of BPH and PCa patients.

**Discussion**

This study aimed to investigate the functional role of IGFBP3 as part of the stromal IGF axis with respect to pathogenic stromal differentiation, a key hallmark of BPH and PCa. We
observed a dysregulation of numerous components of the IGF axis during TGFβ1-induced fibroblast-to-myofibroblast differentiation in PrSCs, in particular the up-regulation of IGFBP3, one of the most abundantly expressed IGFBP isoforms in PrSCs under basal conditions that was also detectable in the benign prostatic stroma in vivo. In contrast to rapid TGFβ1-mediated induction of NOX4, which we previously showed to be a driving mediator of TGFβ1-induced differentiation (40), the up-regulation of IGFBP3 occurred much later and continued to increase after the completion of morphological differentiation. Although IGFBPs share a similar overall 3-domain structure and possess highly conserved N- and C-terminal regions (58), IGFBP3-specific knockdown revealed that this member of the IGFBP family plays a unique and essential role during differentiation. However, although rhIGFBP3 alone did not induce differentiation, IGFBP3 synergistically potentiated TGFβ1-induced differentiation. This effect was not abrogated by either the addition of IGF-I or an IGF-I analog with low affinity for IGFBP3. Moreover, an IGF1R-neutralizing antibody failed to recapitulate the potentiation of TGFβ1-mediated differentiation by rhIGFBP3. In addition, IGF1R is not detectably phosphorylated during TGFβ1-mediated differentiation of PrSCs, suggesting that IGF1R signaling does not play a significant role during fibroblast-to-myofibroblast differentiation. These data strongly indicate that IGFBP3 is not merely sequestering mitogenic IGF to promote fibroblast-to-myofibroblast differentiation of PrSCs but rather is acting in an IGF-independent manner. This latter conclusion is consistent with reports that IGFBP3 inhibits the growth of fibroblasts containing a targeted disruption of IGF1R (59). Collectively findings herein indicate that dysregulation of stromal IGFBP3 plays an amplifying but permissive role during TGFβ1-mediated fibroblast-to-myofibroblast differentiation.

The precise mechanism by which IGFBP3 promotes PrSC differentiation in response to TGFβ1 is currently unclear. However, our observations are consistent with several in vitro and in vivo studies, which have implicated IGFBP3 as well as IGFBP5 as central mediators of the profibrotic phenotype in tissues of diverse histological origin. Moreover, elevated IGFBP3 levels were also reported in endometriosis, idiopathic pulmonary fibrosis, systemic sclerosis, Crohn disease, and hepatic fibrosis, diseases that exhibit many histopathological similarities with the BPH and PCa remodeled stroma (60, 61). Similar to our observations, IGFBP5 was shown to mediate its profibrotic effects on dermal and pulmonary fibroblasts independently of IGF-I (62–64). The mechanisms, however, by which IGFBP3/IGFBP5 mediate these effects remain unknown. Our finding that IGFBP3 alone was insufficient to induce differentiation but supported TGFβ1-induced differentiation in a manner that mimicked the action of TGFβ1 suggests that IGFBP3 mediates its effects by activating TGFβ1 signaling. Such synergistic effects of IGFBP3 on TGFβ1 signaling have been reported in a variety of cell types (53, 65–69). As demonstrated herein for PrSCs, however, these effects of IGFBP3 were dependent on the presence of TGFβ1 (68, 69). Analogously, IGFBP3 potentiated epidermal growth factor signaling with elevated phosphorylation of the cognate receptor in mammary epithelial cells (70). Collectively these data raise the intriguing possibility that the regulation of

Figure 5. Elevated IGFBP3 in the hyperplastic fibromuscular stroma of the BPH prostate. Immunohistochemical staining of IGFBP3 in a whole-tissue biopsy section from a BPH patient. Note the low number of IGFBP3-positive cells in the periglandular stroma (A, black arrows) compared with the abundant number of IGFBP3-immunoreactive cells in the hyperplastic fibromuscular stroma of the same tissue section (B). A and B, Boxed regions (left panels) are enlarged (right panels). Images are representative of whole-tissue section biopsy specimens from 6 independent donors.
growth factor signaling may be a generic mechanism by which IGFBP3 exerts some of its IGF-independent effects.

IGFBP3 and TGFβ cross talk may occur via direct interaction of IGFBP3 with TGFβ receptor type V/low-density lipoprotein receptor-related protein I or by IGFBP3 modulating interactions between TGFβ receptor type I and type II, the latter most likely in an indirect manner (53, 71–73). However, given that treatment of PrSCs with rhIGFBP3 alone did not alter the phosphorylation status of phosphorylated Smad2 or phosphorylated ERK1/2, which serve as markers of activated TGFβ receptor types I/II and TGFβ receptor type V, respectively (74–78), it is unlikely that IGFBP3-mediated potentiation of TGFβ1-induced differentiation is occurring via IGFBP3 interaction with TGFβ receptors.

IGFBP3 is known to bind integrins, ECM components, and ECM-bound factors (79–83). Thus, an alternative possibility is that IGFBP3 secreted by PrSCs in response to TGFβ1 potentiates differentiation by modulating ECM dynamics and/or the bioavailability of unknown ECM-sequestered factors. Analogously, IGFBP3 enhanced EGF-dependent signaling and downstream proliferation of breast epithelial cells in a fibronectin-dependent but IGF-independent manner (84). However, although IGFBP3 is known to bind latent TGFβ-binding protein 1, which tethers mature TGFβ to the ECM in an inactive state (79–83, 85, 86), we observed no effect of IGFBP3 on latent TGFβ1 activation and no difference in potentiation of differentiation in the presence or absence of fibronectin (not shown). Further investigations are thus required to discern the complex molecular mechanism underlying the synergistic effects of IGFBP3 during TGFβ1-mediated differentiation.

Figure 6. IGFBP3 is elevated in PCa-associated reactive stroma. A, Immunohistochemical staining of IGFBP3 in adjacent benign epithelial (BE) and tumor (T) areas of a single whole-tissue biopsy section from a patient with high-grade PCa. Bottom left and bottom right panels, enlargement of boxed areas (broken line and solid line, respectively). Note the low/weak staining of IGFBP3 in the periglandular stroma (black arrows) of benign glands (BE; bottom left panel), whereas IGFBP3-positive cells are more abundant in the reactive stroma (red arrows) of adjacent PCa (T; bottom right panel). T denotes foci of PCa. B, High-grade PCa area from the same tissue section as in panel A reveals intense nucleocytoplasmic staining of IGFBP3 in PCa-adjacent reactive stroma. Images are representative of whole-tissue section biopsy specimens from 8 independent donors.
Consistent with our in vitro observations, immunohistochemistry indicated that stromal IGFBP3 levels were significantly higher in BPH- and PCa-adjacent stroma compared with nondiseased regions of the prostate. To our knowledge, this is the first report demonstrating dysregulation of stromal IGFBP3 in pathological conditions of the prostate. Previous studies in the prostate have focused largely on epithelial localized IGFBP3, although 2 studies also reported weak or intense but heterogeneous staining of stromal IGFBP3 in vivo (17, 44). This most likely reflects the highly particular staining conditions required to robustly visualize the stromal IGFBP3 signal, including antigen retrieval under alkaline conditions (our observation). It may be noted that IGFBP3 has been reported in the stromal and epithelial compartments of other tissues, including the endometrium and colon (61, 87). Unfortunately, there is currently no molecular marker capable of distinguishing fibroblasts from myofibroblasts. Thus, it was not possible in the present study to precisely determine whether IGFBP3-positive cells in the BPH and PCa stroma represented myofibroblasts. However, given the induction of IGFBP3 in in vitro-differentiated myofibroblasts together with the well-documented elevated number of myofibroblasts in the prostatic stroma of BPH and PCa (41, 57, 88), the observed staining pattern is strongly suggestive of the myofibroblast phenotype and further supports the notion that stromal IGFBP3 plays a key role in pathogenic stromal remodeling associated with BPH and PCa.

IGFs promote cancer cell growth, and high circulating IGF levels are associated with an increased risk of several cancers, including PCa (12–16). Thus, a significant effort is being devoted to developing inhibitors of IGF action for the treatment of cancer (21, 89, 90). Many clinical trials to date have focused on targeting IGF1R; however, this remains a challenging field due to the complexity, widespread, and multifunctional role of the IGF/insulin axis (21, 22). As natural inhibitors of IGF but not insulin signaling, the anticancer potential of IGFBPs is consequently also being investigated, particularly with respect to reducing the toxic side effects that currently limit many IGF1R targeting approaches (21). However, data herein suggest that stromal IGFBP3 may exert protumorigenic effects by promoting stromal remodeling and differentiation of fibroblasts into myofibroblasts, whose mitogenic secretome exerts well-documented proproliferative and protumorigenic effects (28). Indeed, one such paracrine-acting mitogen may be stromal-derived IGF-I itself, whereby degradation of myofibroblast-derived IGFBP3 by epithelial proteinases such as prostate-specific antigen and matrix metalloproteinase-7 releases sequestered IGF-I and subsequently promotes epithelial cell and tumor growth (91–94). Along similar lines, the matrix metalloproteinase-7 cleavage of IGFBP5 releases bound IGF-II, which subsequently acted as a mitogen for colonic myofibroblasts (95). Given that reactivation of androgen receptor by nonandrogenic ligands such as IGF-I is a key process by which castration-resistant PCa can emerge (96), data herein indicate the therapeutic potential of inhibiting stromal remodeling and the resulting dysregulation of the stromal IGF axis in combination with current treatment modalities for advanced PCa.

In summary, data presented herein demonstrate that stromal IGFBP3 acts in an IGF-independent manner to play an amplifying and permissive role during TGFβ1-mediated fibroblast-to-myofibroblast differentiation, a hallmark of stromal remodeling associated with PCa and BPH. Because aberrant fibroblast-to-myofibroblast differentiation is implicated in the pathophysiology of impaired wound healing, the stromal response of many solid tumors and diverse fibrotic conditions, these data suggest that modulating stromal IGFBP3 may represent a therapeutic strategy not only in the treatment of prostatic disease but also for other pathologies associated with myofibroblast dysregulation.

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