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TITLE: IN VIVO ACTIVITY OF INSULIN-LIKE
GROWTH FACTOR BINDING PROTEIN-3 IN
PREVENTION OF PROSTATE CANCER
PROGRESSION

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14. ABSTRACT Insulin-like growth factor (IGF) and IGF-binding protein-3 have been implicated in the growth and progression of prostate cancer. The effects of IGFBP-3 have been shown to be mediated by both an inhibition of the actions of IGF on the type 1 receptor (IGF-1R) and through IGF-independent mechanisms. Although these effects have been demonstrated in prostate cancer cells in culture, there has been no in vivo demonstration in a relevant model of prostate cancer. Using the established mouse model of early prostate cancer (LPB-Tag or LADY) and two transgenic lines that overexpress IGFBP-3 or mutant IGFBP-3, we have tested the hypothesis that IGFBP-3 can inhibit or delay the progression of prostate cancer. We have established that tumor growth is reduced and progression delayed by both IGF-dependent and IGF-independent mechanisms. We have assessed the relative contributions of cell proliferation and apoptosis to tumor growth and demonstrated an early IGF-dependent effect on proliferation, and a later IGF-independent on cell survival. The pathways involved include PI-3 Kinase, pAKT and GSK3, and the ras/raf pathway for transcriptional activation. Our findings support further investigation of the potential of IGFBP-3 as a therapeutic agent in combination with inhibitors of IGF-1R.					
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Introduction

A growing body of evidence supports a critical role for signaling through the insulin-like growth factor type I receptor (IGF-1R) in tumorigenesis, including prostate cancer. The original observation that circulating IGF-1 and IGFBP-3 levels might be markers for risk of prostate cancer (1,2) have been supplanted by studies of the role of the IGF pathway on tumor growth and progression. Recent attention has implicated IGF-1 and IGFBP-3 in the local tissue as the physiologically relevant molecules and considerable effort is underway to develop reagents that can inhibit signaling through the IGF-1R (more than a dozen clinical trials are underway). Expression of the IGF-1R receptor is upregulated in primary prostate cancer and overexpression of this receptor is associated with progression to androgen-independence in LAPC-9 and LNCaP xenografts (3). IGF-I is a potent stimulator of proliferation of many cell types including prostate cells (4). IGFs are normally present in the blood and other biological fluid bound to the IGFBPs. IGFBP-3 is the most abundant in plasma and the most widely expressed. IGFBP-3 is able to inhibit the proliferation and induce apoptosis of prostatic cells *in vitro*. These effects of IGFBP-3 are mediated both by inhibition of IGF action at the IGF-1R receptor and by IGF-independent effects mediated by the interaction of IGFBP-3 with specific cellular receptors. The latter has been conclusively demonstrated using mutant IGFBP-3 that does not bind IGFs but still induces apoptosis in PC-3 cells (5). IGF-I and IGFBP-3 have the opposing effects when added individually to cultured cells. When added in combination, cell proliferation or growth inhibition can be observed depending upon the molar concentrations and experimental conditions. Two reports using transgenic mouse models have confirmed the importance of the IGF pathway *in vivo* in prostate cancer. In the C3-Tag model, loss of the growth hormone receptor and thus downregulation of IGF-1 levels, led to reduced tumor formation (6). In the TRAMP model, loss of the receptor for the growth hormone releasing hormone, (GHRH-R or *lit* mutation) also led to reduced growth hormone and IGF-1, and reduced tumor formation (7). However, *in vivo* confirmation of the ability of endogenous IGFBP-3 to influence the growth and progression of prostate cancer has been elusive, and until this study was undertaken, there was no appropriate experimental model to address the question.

Body

We proposed to test directly, *in vivo*, the hypothesis that IGFBP-3 inhibits prostate cancer growth and progression using complementary transgenic mouse lines, an established model for prostate cancer (8) and two established transgenic lines overexpressing IGFBP-3 (9) or mutant IGFBP-3 (10). Our specific aims were:

- 1) To determine the potential of IGFBP-3 to limit the growth and/or progression of prostate cancer *in vivo*.
- 2) To determine whether IGFBP-3 exerts its effects through IGF-dependent or IGF-independent mechanisms.
- 3) To determine the receptor/ signal transduction pathways activated in response to elevated IGFBP-3 and mutant IGFBP-3.

The approved Statement of Work was:

STATEMENT OF WORK

- Task 1.** To determine the potential of elevated IGFBP-3 to limit the growth and/or progression of prostate cancer *in vivo*. (Months 1-24)
- a. Establish breeding to obtain double transgenic males LPB-Tag/PGKBP-3 (Months 1- 18)
 - b. Perform PCR-based analysis of tail biopsy DNA from male offspring to confirm genotypes as
LPB-Tag / wt LPB-Tag / PGKBP-3 wt /PGKBP3 wt / wt
 - c. Confirm overexpression of IGFBP-3 mRNA in males by RNase Protection Assay (RPA) and large T antigen protein by Western blot analyses (Months 6 – 18)
 - d. Determine incidence, latency and growth rate of IGFBP-3 by collecting prostate tissue / tumors at 7, 11, 15 and 19 weeks of age (Months 3-18). Extend to 26 weeks of age if tumor growth is markedly reduced by elevated IGFBP-3.
 - e. Perform immunoblot analysis of prostate tissue / tumor protein extracts to determine expression of dorsolateral prostate proteins (DLPs), a marker of differentiated function of the prostatic epithelium, androgen receptor and PCNA as markers of early tumor progression (Months 6-24)
 - f. Perform Radioimmunoassays (RIAs) to determine plasma levels of IGF-1 and IGFBP-3, and circulating testosterone (Months 12-24)
 - g. Perform histopathology and immunohistochemical analyses on prostate tissue / tumors to determine incidence of PIN-like lesions and progression to invasive carcinoma (Months 18 – 24)
 - h. Perform data analysis to establish statistical significance of differences in tumor weight (growth) and tumor gene expression (progression of PIN to carcinoma) between genotypes (Months 12-24)
- Task 2.** To determine the anti-tumor effects of IGFBP-3 through IGF-dependent versus IGF-independent mechanisms. (Months 12 – 30)
- a. Establish breeding to obtain double transgenic males that overexpress mutant PGKBP-3 which does not bind IGFs (Months 12 – 24)
 - b. Perform PCR-based genotyping of DNA from tail biopsies of male mice to determine genotypes as
PGKmutBP-3 / wt PGKmutBP-3 / LPB-Tag wt / LPB-Tag
wt/wt

- c. Confirm overexpression of mutant IGFBP-3 by RNase protection assay (12 – 24)
- d. Determine incidence, latency and growth rate of prostate tissue / tumors in response to mutant IGFBP-3 by collecting tissue samples at 7, 11, 15 and 19 weeks of age. If tumor growth is markedly inhibited by mutant IGFBP-3, extend to 26 weeks. (Months 12 – 30).
- e. Compare DLP, AR, PCNA, and Tag expression by immunoblot analyses as in Task 1. Complete histological evaluation as in Task 1.
- f. Complete data analysis of the relative abilities of IGFBP-3 and mutant IGFBP-3 to inhibit prostate tumor growth and progression of PIN-like lesions to invasive cancer.

Task 3. To determine the receptor/ signal transduction pathways activated in mice with elevated IGFBP-3 (and elevated mutant IGFBP-3) (Months 12 – 36)

- a. Perform PCNA index, BrdU labeling and TUNEL assays to determine the relative contributions of proliferation, cell survival and cell death to prostate tumor progression in LPB-Tag mice with and without IGFBP-3 and with and without IGF-1 (Months 6 – 24)
- b. Perform analysis of PIN-like lesions for expression of known biomarkers for progression by immunohistochemistry of tissue samples and/or immunoblotting to determine the effect of IGFBP-3 and IGF-1 on tumor progression (Months 12 – 30)
- c. Examine IGF receptor levels in response to IGFBP-3 expression and IGF-1 levels and assess receptor autophosphorylation using phosphor-specific antibodies (Months 18 – 24)
- d. Determine the impact of IGFBP-3 and IGF-1 on downstream signal transduction pathways by immunoblot and phosphorylation status of kinase cascades including MAPK, pAKT, PI3K, and putative alternate pathways involving p21 and p53. (Months 18-36)
- e. Based on experience with the double transgenic mice, present to the scientific community on its potential utility as an experimental model for testing compounds or drugs that influence the IGF axis for prostate cancer treatment or prevention.

Work completed:

In relation to the original Statement of Work (above), the research to date includes:

Task 1	a - h	completed.
Task 2	a - f	completed.
Task 3	a - e	completed.

The major experimental observations (reported previously) are: We examined the progression from mouse prostatic intraepithelial neoplasia (mPIN) to carcinoma (11) in F1 crosses of PGKBP-3 transgenic mice which ubiquitously over express IGFBP-3 (9) with the LPB-Tag ('LADY') transgenic model for prostate cancer, line T5 (8). In addition we have tested the contribution of IGF-independent actions of IGFBP-3 by crossing LPB-Tag mice with PGKmutBP-3 mice (10), that is, transgenic mice which express mutant IGFBP-3 that does not bind IGFs. We determined incidence, latency and rate of prostate tumor growth in these mice and control littermates, and examined the expression of biomarkers for differentiation and progression. The enhanced expression of IGFBP-3 delayed significantly the onset of tumor formation and growth in this model. Mutant IGFBP-3 was not able to delay tumor formation suggesting an effect of IGF-1 in early tumorigenesis. The unexpected finding was that mutant IGFBP-3 did inhibit later tumor growth, suggesting an IGF-independent mechanism of action of IGFBP-3 after tumor formation. Tumor tissues have been examined for histopathology, cellular proliferation and apoptosis (12) [publication appended].

As reported in our Third Annual Report, we went on to examine the activation of the IGF-1R cell surface receptor and intracellular signal transduction pathways involved in the growth inhibition and cell survival / apoptosis pathways. As the level of IGF-1R was significantly higher in the prostates of LPB-Tag mice and all the double transgenic mice than in non-transgenic littermates ($P < 0.05$), we examined the two major downstream pathways associated with IGF-1R activation:

- i) PI 3-kinase pathway that leads to the activation of AKT and GSK3, and is inhibited by PTEN
- ii) Ras/Raf, MEK cascade that leads to the activation of ERK1/2 and transcriptional activation

The data indicated that phospho-Akt levels were reduced in the presence of high IGFBP-3. In the presence of high IGFmutBP-3, phospho-Akt levels were not reduced, however apoptosis was enhanced, reaching statistically significant levels by 17 weeks. Nur77 has been implicated as a possible effector of the IGF-independent actions of IGFBP-3 due to its potential for rapid intracellular translocation in response to increased IGFBP-3 (14). In the PGKmutBP-3xT5 animals, total Nur77 levels were higher at 15 weeks of age consistent with increasing cell death in the later stage tumors and an IGF-independent effect of IGFBP-3.

In normal cells destruction of p27 following its phosphorylation in the late G1 phase of the cell cycle permits G1/S transition. Phospho-p27 is abundant in the LPB-Tag prostates with the highest levels seen when tumor growth is most rapid. In the mice

expressing PGKBP-3, phospho-p27 is lower, and cell proliferation is reduced; in mice expressing PGKmutBP-3, phospho-p27 levels were highly elevated and proliferation is not inhibited. This is consistent with IGF-1R activation leading to proliferation as previously observed in prostate cancer cell lines.

A putative substrate of GSK3 in the prostate is beta-catenin. Elevated pAKT can lead to phosphorylation and inactivation of GSK3, leading to an increase in beta-catenin. We found a highly significant increase in the steady state level of beta-catenin in mice expressing PGKmutBP-3 prostates relative to the normal allele. This is consistent with the enhanced signaling through the IGF-1R causing inactivation of GSK3.

The relative levels of phosphorylated ERK were used as a downstream marker of Raf/MEK signaling. Activation of ERK1 was significantly reduced in mice overexpressing the normal IGFBP-3 allele, but significantly increased in mice expressing the mutant IGFBP-3. This is consistent with IGF-dependent effects on transcription working through the Ras/MEK pathway.

The striking inhibition of prostate tumor growth and the delay of tumor progression from PIN to cancer were seen early in the model (before 15 weeks of age). IGF-independent effects of IGFBP-3 were not evident until later (after 17 weeks of age). In an editorial review of work from our lab and others, Pinchas Cohen has commented on the apparent separation of the IGF-dependent and IGF-independent effects of IGFBP-3 in cancer. The case is building for IGF acting to promote tumor growth early in cancer while in more advanced cancer IGFBP-3 acts as a tumor suppressor influencing cell survival in an IGF-independent manner (13). Our data are consistent with that interpretation.

Extension

In October 2007, we requested a 'no cost' extension to the award period in order to complete work with Dr. Suresh Mishra who replaced the late Dr. Liam Murphy on the project. This included further elucidation of the signal transduction pathways involved in the IGF-dependent effects early in tumor progression, and the IGF-independent effects not evident until late in tumor progression.

We previously reported on the expression of total Nur77 protein in the tumor model (Third Annual Report). We have now extended that analysis to assessing the phosphorylation status of Nur77 using pNur77-specific antibody (Ser-78) from Santa Cruz (Figure 1). Nur77 has been implicated as a possible effector of IGF-independent actions of IGFBP-3 due to its potential for rapid intracellular translocation in response to increased IGFBP-3 (14). Nur77 forms homodimers or heterodimers with the nuclear receptor RXR in its role as a transcription factor. Nur77 translocation to the mitochondria is a pro-apoptotic event. When Nur77 is phosphorylated, both transactivation and mediation of apoptosis are inhibited (19). Although the level of pNur77 varies from tumor to tumor (Figure 1), at 15 weeks pNur77 is significantly higher in mice with mutant IGF-BP3 ($p < 0.001$) (Figure 2). Although we cannot follow the

translocation of Nur77 in these fixed tissue samples, the high level of pNur77 suggests is consistent with the reduced apoptotic index seen in the mutant mice at this time.

In addition to Nur77, RXR is also an intracellular binding partner with another member of the steroid receptor superfamily that is implicated in control of cell proliferation and apoptosis - PPAR γ (20). This receptor is involved in the differentiation of adipocytes but has also been implicated in a number of human cancers including prostate (21). PPAR γ has two isoforms - γ 2 is high in adipose tissue, while γ 1 is more common in non-adipose tissues. As PPAR γ 2 is high in PIN lesions as well CaP, it suggests a role in progression of PIN to prostate cancer and a potential site for therapeutic intervention in early disease (21). PPAR γ 2 expression is elevated in the LPB-Tag model as well (Figure 3). At 13 weeks, overexpression of the normal allele of IGF-BP3 in the double-transgenic mice was associated with a significantly higher level ($p < 0.05$) of PPAR γ 1 expression, typical of normal, prostate tissue; at the same time, PPAR γ 2 expression was reduced (Figure 4). This is coincident with the higher rate of apoptosis seen in these mice relative to the parental line (LPB-Tag), and the delay in tumor progression. The expression of PPAR γ 1 is an IGF-dependent effect, as the mice overexpressing the mutant form of IGF-BP3 continue to express predominantly the PPAR γ 2 isoform.

We have previously shown that IGF-BP3 is able to bind to Autocrine Motility Factor (AMF/PGI) in breast cancer cells (22). AMF/PGI is an anti-apoptotic cytokine that acts in an autocrine manner on the AMF receptor to stimulate proliferation and cell migration (23). When IGF-BP3 binds to AMF/PGI, it blocks its autocrine function. In addition, AMF/PGI is able to inhibit IGF-BP3 induced apoptosis (22). Using Western blot analysis of tumor tissue at various time points (Figure 6), we examined the expression of AMF/PGI in our model. Early in tumor progression, the parental LPB-Tag and mice overexpressing mutant IGF-BP3 demonstrated high levels of AMF/PGI, coincident with the period of low apoptosis. The mice overexpressing the normal IGF-BP3 allele demonstrated lower levels of AMF/PGI, coincident with the period of higher apoptotic rate. At later time points (after 15 weeks), AMF/PGI levels increase in this strain as well, coincident with the drop in apoptosis and tumor progression. In our analysis the level of AMF/PGI is reduced by the overexpression of normal IGF-BP3 but not mutant IGF-BP3. This suggests an action of the IGF signaling pathway on the regulation of AMF/PGI expression *per se*, although we cannot discount the possibility that IGF-BP3 and mutant IGF-BP3 may interact differentially with AM/PGI and alter the protein's stability.

One of the downstream effectors of the IGF signaling pathway in our model is phosphoAkt. Akt is also implicated in the formation of autophosphorylating complex that includes Heat Shock Protein 27(Hsp-27). In human prostate cancer cell lines, PC-3 and LNCaP, elevated Hsp-27 is associated with down-regulation of apoptosis (24). Hsp-27 expression has also been demonstrated to promote androgen-independent progression of prostate cancer (25). Evidence is mounting that Hsp-27 regulates cancer cell metastasis and survival through level of expression and differential phosphorylation. Phospho-HSP27 modulates cell motility; blocking phosphorylation blocks cell migration. High

levels of Hsp-27 inhibit apoptosis. We examined the level of phospho-Hsp-27 in our model relative to age-matched non-transgenic mice. We observed peak levels of phospho-Hsp-27 at 13 weeks in all the transgenic lines (Figure 7). The pattern of pHsp-27 is consistent with an IGF-dependent effect on phosphorylation, presumably through modulation of pAkt complex. Of the many downstream effectors of IGF signaling that we have examined, pHsp-27 is the only one to show such a marked peak of activity, as opposed to a continual increase/decrease with tumor progression. How this *transient* activation by pHsp-27 is implicated in tumor progression remains to be determined, but it does precede the point of most active cellular proliferation, and the histological progression of mouse PIN to carcinoma. In this regard, a recent study has identified Hsp-27 expression at diagnosis as a strong predictor of poor clinical outcome, independent of ETS-gene rearrangements (26).

Key Research Accomplishments

- Demonstrated the potential of IGFBP-3 to significantly limit the growth and delay the progression of prostate cancer *in vivo*.
- Demonstrated that IGFBP-3 exerts its effects through both IGF-dependent and IGF-independent mechanisms.
- Determined that receptor/ signal transduction pathways activated in response to elevated IGFBP-3 and mutant IGFBP-3 include both of the major intracellular signaling pathways linked to IGF-1R: the PI-3Kinase, pAKT and p27 regulation of cell proliferation, and the ras/MAPK leading to transcriptional activation.
- Determined that expression and phosphorylation status of receptors of the steroid receptor superfamily (Nur77 and PPAR γ) are altered in the response to IGF-BP3 expression consistent with expected effects on cell survival.
- Determined that IGF-BP3 expression influences expression of putative binding partners (AMF/PGI) and downstream effectors (pHsp-27) in prostate cancer progression *in vivo*.

Reportable Outcomes

- 1) Manuscript: Silha et al. *Endocrinology* 2006 [appended]
- 2) Abstracts presented at the prostate cancer research meetings as listed in the Bibliography [appended].
- 3) Editorial comment on the importance of the research: Dr. Pinchas Cohen in *Endocrinology* 2006 May; 147(5): 2109-11.
- 4) Patent Filed - New United States Patent Application Serial Number 12/036,091 entitled: Methods of Attenuating Prostate Tumor Growth by Insulin-like Growth Factor Binding Protein-3 (IGFBP-3).
We have engaged the Technology Transfer Office at the University of Manitoba to seek industrial private sector partners for the further development of this resource.
- 5) Novel animal models: The novel double transgenic mouse models that have been created overexpress the IGFBP-3 protein or the mutant IGFBP-3 protein in the context of a predisposition for prostate cancer development (LBP-Tag or 'LADY'

- model). These models are being utilized further to examine the impact of signaling through the Peroxisome Proliferator-Activated Receptor (PPAR) in early prostate cancer, and to examine the role of Autocrine Motility Factor in prostate cancer.
- 6) Trainee outcomes: Dr. Josef Silha - Post-Doctoral research fellow on the project has obtained a Post-graduate Medical Education residency position in Clinical Endocrinology. Dr. Suresh Mishra - initially a Post-Doctoral research fellow and then Co-Investigator on the project was appointed as Assistant Professor in Department of Internal Medicine, University of Manitoba.
- 7) Funding applications:
Dr. Suresh Mishra was a successful applicant to the Prostate Cancer Research Foundation of Canada in 2008 for a project entitled: Autocrine mobility factor: A potential target for IGFBP-3 in prostate cancer.
Dr. Janice Dodd received funding from the Manitoba Health Research Council in 2008 for a project entitled: The role of PPAR and IGF signaling pathway on progression of prostatic intraepithelial neoplasia (PIN) to prostate cancer.

Conclusions

The double transgenic mouse models established in our laboratories afforded the opportunity to determine whether the anti-proliferative and pro-apoptotic effects of IGFBP-3 seen in cells in culture can be capitalized on in a true physiological setting of prostate cancer. Enhanced expression of IGFBP-3 does delay prostate tumor formation and reduce growth in the LPB-Tag model. Enhanced expression of mutant IGFBP-3 does not delay the initial formation of prostate tumors in this model, implicating IGF in early tumor formation; however, later tumor growth is markedly inhibited by mutant IGFBP-3, suggesting an IGF-independent effect of IGFBP-3 on tumor progression.

Our data is consistent with current efforts to target IGF-1R in order to treat prostate cancer. The IGF axis has significant impact on prostate cancer cell growth in cell culture models and in mouse models, including ours. Clinical trials for agents that target IGF-1R, its partners and immediate downstream effectors are underway and show promise. Our data suggests that there is merit in considering the IGF axis as a site for chemoprevention as well as treatment, including the early progression of PIN to prostate cancer.

We conclude that IGFBP-3 also should remain a target for further exploration based on its ability to regulate cell survival via mechanism(s) independent of its ability to bind IGF. Our study identified the importance of local rather than systemic IGFBP-3 in modulating tumor progression. This has led others to examine how local IGFBP-3 production in the prostate can influence stromal-epithelial interactions, known to be critical for both normal prostate development and tumorigenesis (17). Recent work by several research groups have continued to document putative mechanisms for IGFBP-3 effects on prostate cancer cells, and have begun to map the sites in the IGFBP-3 molecule that are necessary for IGF-independent effects (16, 18). Such efforts have the potential to lead to both novel therapeutics (and chemopreventive strategies) that the agents currently undergoing clinical trials that focus on the IGF-IR.

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Appendices

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List of Personnel

Published manuscript: Silha et al *Endocrinology* 147: 2112 -2121 (2006)

Supporting data

Figures and Legends begin on page 25.

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Publication:

Silha, J. Sheppard, P., Mishra, S., Gui, Y., Schwartz, J., Dodd, J.G. and L.J. Murphy (2006)
IGFBP-3 INHIBITS PROSTATE TUMOR GROWTH BY IGF-DEPENDENT AND IGF-INDEPENDENT MECHANISMS *Endocrinology* 147: 2112-2121
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Meeting Abstracts:

Dodd, J.G., Sheppard, P.C., Schwartz, J., Leckstrom, A., Silha, J.V., and L.J. Murphy
INSULIN-LIKE GROWTH FACTOR BINDING PROTEIN-3 OVEREXPRESSION INHIBITS
TUMOR PROGRESSION IN VIVO IN IGFBP-3/ LARGE T ANTIGEN DOUBLE
TRANSGENIC MICE
Society for Basic Urologic Research, Fall Meeting, December 2004, Savannah, GA

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IGFBP-3 SUPPRESSES PROSTATE TUMOR GROWTH IN BOTH AN IGF-DEPENDENT
AND IGF-INDEPENDENT MANNER.
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Insulin-Like Growth Factor (IGF) Binding Protein-3 Attenuates Prostate Tumor Growth by IGF-Dependent and IGF-Independent Mechanisms

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IGF binding protein (IGFBP)-3 inhibits cell growth and promotes apoptosis by sequestering free IGFs. In addition IGFBP-3 has IGF-independent, proapoptotic, antiproliferative effects on prostate cancer cells *in vitro*. Expression of the large T-antigen (Tag) under the long probasin promoter (LPB) in LPB-Tag mice results in prostate tumorigenesis. To investigate the IGF-dependent and IGF-independent effects of IGFBP-3 on prostate tumor growth, we crossed LPB-Tag mice with cytomegalovirus (CMVBP-3) and phosphoglycerate kinase (PGKBP-3) mice that overexpress IGFBP-3 under the cytomegalovirus promoter and the phosphoglycerate kinase promoter, respectively, and also I56G/L80G/L81G-mutant IGFBP-3 (PGKmbBP-3) mice that express I56G/L80G/L81G-IGFBP-3, a mutant, that does not bind IGF-I but retains IGF-independent proapoptotic effects *in vitro*. Prostate tumor size and the steady-state level of p53 were attenuated in LPB-Tag/CMVBP-3 and LPB-Tag/PGKBP-3 mice, compared with LPB-

Tag/wild-type (Wt) mice. A more marked effect was observed in LPB-Tag/CMVBP-3, compared with LPB-Tag/PGKBP-3, reflecting increased levels of transgene expression in CMVBP-3 prostate tissue. No attenuation of tumor growth was observed in LPB-Tag/PGKmbBP-3 mice during the early tumor development, indicating that the inhibitory effects of IGFBP-3 were most likely IGF dependent during the initiation of tumorigenesis. At 15 wk of age, epidermal growth factor receptor expression was increased in LPB-Tag/Wt and LPB-Tag/PGKmbBP-3 tissue, compared with LPB-Tag/PGKBP-3. IGF receptor was increased in all transgenic mice, but pAkt expression, a marker of downstream IGF-I action, was increased only in LPB-Tag/Wt and LPB-Tag/PGKmbBP-3. After 15 wk of age, a marked reduction in tumor growth was apparent in LPB-Tag/PGKmbBP-3 mice, indicating that the IGF-independent effects of IGFBP-3 may be important in inhibiting tumor progression. (*Endocrinology* 147: 2112–2121, 2006)

EPIDEMIOLOGICAL STUDIES have demonstrated that high plasma levels of IGF-I and low IGF binding protein (IGFBP)-3 concentrations are associated with increased risk of prostate cancer (1, 2). Such a combination in cell culture would likely favor cell proliferation. IGFBP-3 functions both to inhibit the actions of IGF-I and -II and also in an IGF-independent manner to promote apoptosis and inhibit cellular proliferation of variety of cell lines including human prostate cancer cells (3–5). In addition, in a recent report, IGFBP-3 in combination with retinoid X receptor ligand has been shown to inhibit prostate cancer cell xenografts (6). High plasma IGF-I levels are also a risk factor for benign prostatic hyperplasia, a condition associated with increase prostate cancer risk (7). IGF-I is mitogenic for prostate cancer cells in culture (8). Furthermore, the type 1 IGF receptor is up-regulated in primary prostate cancer and overexpression

of this receptor is associated with progression to androgen independence in culture LAPC-9 and LNCaP cells (9).

Low-plasma IGFBP-3 levels have been reported to have predictive value in identifying individuals with advanced-stage prostate cancer (2). Low-plasma IGFBP-3 could increase the availability of free IGF-I and -II in the prostate and thereby favor the initiation and progression to prostate cancer. In addition, low IGFBP-3, by reducing the IGF-independent, antiproliferative, and proapoptotic effects of IGFBP-3 on prostate epithelium, could also potentially favor the development and progression of prostate cancer.

The epidemiological literature together with the *in vitro* observations provide strong support for the concept that the IGF system, in particular IGF-I and IGFBP-3, is important in prostate cancer. However a direct demonstration for a causal relationship to explain the associations between the IGF system and prostate cancer progression in patients is lacking. As yet there are few *in vivo* data to support the *in vitro* observations with cultured prostate cancer cells. Furthermore, to date, the IGF-independent effects of IGFBP-3 on prostate cancer have been demonstrated only *in vitro*.

The proapoptotic effects of IGFBP-3 are both dependent on and independent of p53 (10, 11). Impaired function of the tumor suppressor protein p53 is involved in the pathogenesis of prostate cancer (12), and increased IGFBP-3 expression is an important downstream mediator of p53 action in prostate and other cancer cells (11, 13). In mouse models of cancer that

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Abbreviations: CMVBP-3, LPB-Tag mice crossed with cytomegalovirus BP-3 mice; DLP, dorsolateral protein; EGF-R, epidermal growth factor receptor; h, human; IGFBP, IGF binding protein; IGF-IR, IGF receptor; LPB, long probasin promoter; PCNA, proliferating cell nuclear antigen; PGKBP-3, LPB-Tag mice crossed with phosphoglycerate kinase BP-3 mice; PGKmbBP-3, I56G/L80G/L81G-mutant IGFBP-3; PIN, prostatic intraepithelial neoplasia; RT, room temperature; SV40, simian virus 40; Tag, T-antigen; Wt, wild type.

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use the simian virus 40 (SV40) large T-antigen (Tag) as the oncogenic transgene, the cellular tumor suppressor p53 is inactivated. In the case of the long probasin promoter (LPB)-Tag mouse model of prostate cancer, transgene expression is targeted specifically to the prostate (14, 15).

The N-terminal domain of IGFBP-3 appears to be most important for binding to IGF-I and -II with high affinity. Mutations in the N terminal of IGFBP-3 result in molecules that do not bind IGF-I or -II (16). Six residues, Ile⁵⁶, Tyr⁵⁷, Arg⁷⁵, Leu⁷⁷, Leu⁸⁰, and Leu⁸¹, have been identified as important in high-affinity binding of IGF to IGFBP-3. Of these, Ile⁵⁶, Leu⁸⁰, and Leu⁸¹ are most important and substitution with glycine or alanine results in a mutant IGFBP-3 that lacks the ability to bind IGF-I or IGF-II but retains the ability to bind plasma membranes (5) and promote apoptosis and inhibit proliferation in prostate and breast cancer cell lines (5, 17, 18).

We previously generated transgenic mice that overexpress human IGFBP-3 using the phosphoglycerate kinase (PGKBP-3) and cytomegalovirus (CMVBP-3) promoters (19). These mice demonstrate fetal and postnatal growth retardation. More recently we generated transgenic mice that overexpress the I56G/L80G/L81G-mutant IGFBP-3 (PGKmbp-3) (20). The PGKmbp-3 transgenic mice do not have a growth-retarded phenotype.

In this report we tested the hypothesis that IGFBP-3 inhibits prostate cancer growth by examining the progression from epithelial hyperplasia to dysplasia and carcinoma in F1 crosses of PGKBP-3, CMVBP-3, and PGKmbp-3 transgenic mice that ubiquitously overexpress IGFBP-3 or PGKmbp-3 with LPB-Tag mice (14).

Materials and Methods

Transgenic mice

The generation and characterization of PGKBP-3 and CMVBP-3 mice have been previously reported (19). The I56G/L80G/L81G-mutant IGFBP-3 plasmid was generated by site-directed mutagenesis and subcloned downstream of the phosphoglycerate promoter in the same plasmid used for the generation of PGKBP-3 mice. Characterization of the PGKmbp-3 transgenic mice has been reported elsewhere (20). The 12T-5 strain of LPB-Tag transgenic mice that express the SV40 Tag under the prostate-specific probasin promoter was used for these studies (14). The 12T-5 strain of LPB-Tag mice develop palpable prostate tumors starting approximately 2 months of age. The Prostate Pathology Committee of the Mouse Models of Human Cancer Consortium (National Cancer Institute, National Institutes of Health) has identified the strain as manifesting mouse prostatic intraepithelial neoplasia (PIN) that progresses to adenocarcinoma (15). All transgenic mice were generated in the same CD-1 genetic background. Homozygous male PGKBP-3, CMVBP-3, or PGKmbp-3 mice and normal wild-type male CD-1 mice were bred with heterozygous LPB-Tag female mice. Male F1 offspring were genotyped and approximately 25% of all the offspring were double-transgenic male animals. These were killed at various ages for determination of prostate size and histology. The presence of the various transgenes was detected either by Southern blot analysis (IGFBP-3) or PCR using tail DNA as previously described (14, 19, 20). All experiments were performed in accordance with protocols approved by the Animal Care Committee of the Faculty of Medicine (University of Manitoba).

IGFBP-3 and IGF-I assays

Human IGFBP-3 was measured using an immunoradiometric assay from Diagnostic Systems Laboratories (Webster, TX). Total plasma IGF-I was measured by a sensitive rat IGF-I RIA kit (Linco Research, Inc., St. Charles, MO).

RNA extraction and RNase protection assays

Total RNA was extracted from prostate tissue and tumors using TRIzol reagent (Invitrogen, Carlsbad, CA). The concentration of RNA was determined spectrophotometrically, and the integrity of the RNA in all samples was documented by visualization of the 18 and 28S ribosomal RNA bands after electrophoresis through a 0.8% formaldehyde/agarose gel. Maxiscript SP6/T7 and RPAIII kits (Ambion, Austin TX) were used for the RNase protection assay. A 267-bp fragment containing the sequence corresponding to the 3'-end of the human IGFBP-3 cDNA and the bovine GH polyadenylation signal was used as a template as previously described (19). A mouse cyclophilin riboprobe was used as the internal standard, and century RNA markers (Ambion) were used to determine the size of the protected fragment. The protected sizes for the transgene-derived RNA and cyclophilin fragments were 267 and 103 bp, respectively.

Immunoblotting

Analysis of proteins was performed using approximately 20 μ g of protein extracted from prostate tissue samples and separated on 10% SDS-PAGE. Proteins were transferred to nitrocellulose or polyvinylidene difluoride membranes followed by blocking with 5% nonfat milk and incubation with the primary antibody. After incubation, membranes were washed three times (5 min each) in 10 mM Tris, 150 mM NaCl, 0.05% Tween 20 (pH 8.0) and incubated with a 1:5000 dilution of antirabbit horseradish peroxidase-conjugate (Santa Cruz Biotechnology, Santa Cruz, CA) for 1 h at room temperature (RT). After washing (3 $\times$ 5 min) in 10 mM Tris, 150 mM NaCl, 0.05% Tween 20 (pH 8.0), membranes were analyzed with enhanced chemiluminescence detection system. Primary antibodies were used as follows: IGFBP-3, 1:500 dilution of rabbit polyclonal antihuman IGFBP-3 (Santa Cruz Biotechnology) antibody for 2 h at RT; p53 and epidermal growth factor receptor (EGF-R), rabbit polyclonal antibodies (Santa Cruz Biotechnology) at a 1:400 dilution; IGF receptor (IGF-IR), rabbit polyclonal antibody against the C terminus of the β -chain of the IGF-IR (Santa Cruz Biotechnology) at a dilution of 1:1,000; pAkt, rabbit polyclonal antibody phospho-AKT (Ser473) antibody (Cell Signaling, Beverly, MA) at a dilution of 1:1,000; dorsolateral proteins (DLP), rabbit polyclonal antibody against dorsolateral proteins (gift from Dr. G. Cunha, University of California, San Francisco, San Francisco, CA) at a dilution of 1:40,000. This antibody recognizes androgen-dependent dorsolateral prostate secretory products and is a marker of differentiated function of the rodent prostate (21).

Ligand blotting

For ligand blotting 20 μ g protein samples (without dithiothreitol) were separated by SDS-PAGE on a 10% gel and transferred to the nitrocellulose membrane as mentioned above. After blocking with 5% nonfat milk, membranes were incubated in 10 mM Tris-HCl buffer (pH 7.4), 150 mM NaCl, 3% Nonidet P-40 containing 400,000 cpm ¹²⁵I-IGF-I for 3 h at RT. After washing (3 $\times$ 15 min each) with the same buffer without radioligand, the membranes were exposed to MR film (Kodak, Rochester, NY) at -70 C.

Immunohistochemistry

Formalin-fixed, paraffin-embedded prostate tissue was assessed by immunohistochemistry for the expression of androgen receptor, DLP, Tag, or proliferating cell nuclear antigen (PCNA). Antigen retrieval on 4- μ m sections was achieved by microwave treatment in either citrate buffer [0.01 M (pH 6.0)] or urea (1 M). Endogenous peroxidase was quenched using hydrogen peroxide (1% in PBS) for 30 min and blocked with normal goat serum. Primary antibodies were applied overnight at the following dilutions: rabbit anti-AR (5 μ g/ml; Santa Cruz), rabbit anti-DLP (1:2500; kindly provided by G. Cunha), mouse anti-Tag (1:20; Oncogene Science, La Jolla, CA) or mouse anti-PCNA (2.5 μ g/ml; Oncogene Science). After washes in PBS, secondary antibodies were applied: either goat antirabbit or goat antimouse (1:100) followed by either rabbit peroxidase-antiperoxidase or mouse clono-peroxidase-antiperoxidase (1:500; Sternberger Monoclonals Inc., Berkeley, CA). The peroxidase complex was detected with 3,3'-diaminobenzidine tetrahydrochloride. Sections were counterstained with hematoxylin.

Apoptosis

Apoptosis in comparable areas of high-grade PIN in prostate tumors from LPB-Tag/wild-type (Wt), LPB-Tag/PGKBP-3, LPB-Tag/CMVBP-3, and LPB-Tag/PGKBP-3 mice was quantified using the terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate nick end labeling assay (ApopTag peroxidase *in situ* apoptosis detection kit, Chemicon, Temecula, CA), at 9, 13, and 17 wk of age. Three to five sections from three or four mice per transgenic strain were analyzed. Approximately 360 cells per section, or approximately 4300 cells per Tg strain, were scored.

Statistical analysis

All data are expressed as the mean $\pm$ SEM. Statistical analysis was initially performed using an ANOVA and followed by the either Tukey honestly significant difference test, in which multiple comparisons were made between groups, or Dunnett's *t* test in which comparisons were made between multiple groups and a single control group, using online statistical software (<http://faculty.vassar.edu/lowry/VassarStats.html>). Comparison was made between LPB-Tag/Wt mice and the other groups of mice for both the whole data set and data from each time point. Prostate weight was log transformed, and least squares regression analysis was used to determine the lines of best fit and their confidence limits. The statistical significance of the difference in the slope and intercept was then determined.

Results

The increase in prostate weight with age in Wt/Wt, LPB-Tag/Wt, LPB-Tag/CMVBP-3, and LPB-Tag/PGKBP-3 mice is shown in Fig. 1. There was no significant difference in prostate weights in Wt/Wt, CMVBP-3/Wt, and PGKBP-3/Wt mice. For the purpose of clarity, only a single curve is shown for Wt/Wt, CMVBP-3/Wt, and PGKBP-3/Wt mice in Fig. 1. Prostate tumorigenesis, as assessed by prostate weight, was markedly attenuated by overexpression of IGFBP-3 under either the CMV or PGK promoters ($P < 0.001$ by ANOVA). This attenuation was more marked in LPB-Tag/CMVBP-3 mice, compared with LPB-Tag/PGKBP-3 mice ($P < 0.05$). Once initiated, prostate tumors grew at a slightly slower rate in LPB-Tag/CMVBP-3 and LPB-Tag/PGKBP-3 mice than LPB-Tag/Wt mice (Fig. 1). Prostate tumor weight was log transformed, and least squares regression analysis was used to obtain the line of best fit for the relationship between prostate weight and time. The slope of this relationship was significantly less in LPB-Tag/CMVBP-3, compared with LPB-Tag/Wt mice ($P < 0.001$), and similar trend was apparent in LPB-Tag/PGKBP-3 mice (Table 1). In LPB-Tag/Wt mice, a prostate weight of 10 g was achieved at approximately 17 wk of age. This weight was achieved after a delay of approximately 2.5 and approximately 5 wk in LPB-Tag/PGKBP-3 and LPB-Tag/CMVBP-3 mice, respectively. Although the PGKBP-3 and CMVBP-3 mice were approximately 10% smaller than Wt mice (19), differences in body weight did not account for the apparent reduction in prostate tumor growth. A significant reduction in relative weight of the prostate gland was still apparent when expressed as a percentage of total body weight. Examination of the different lobes of the prostate gland in various transgenic strains gave similar results to that seen when the whole prostate gland was considered (data not shown).

In an attempt to understand the differences in prostate tumor growth in LPB-Tag/CMVBP-3 and LPB-Tag/

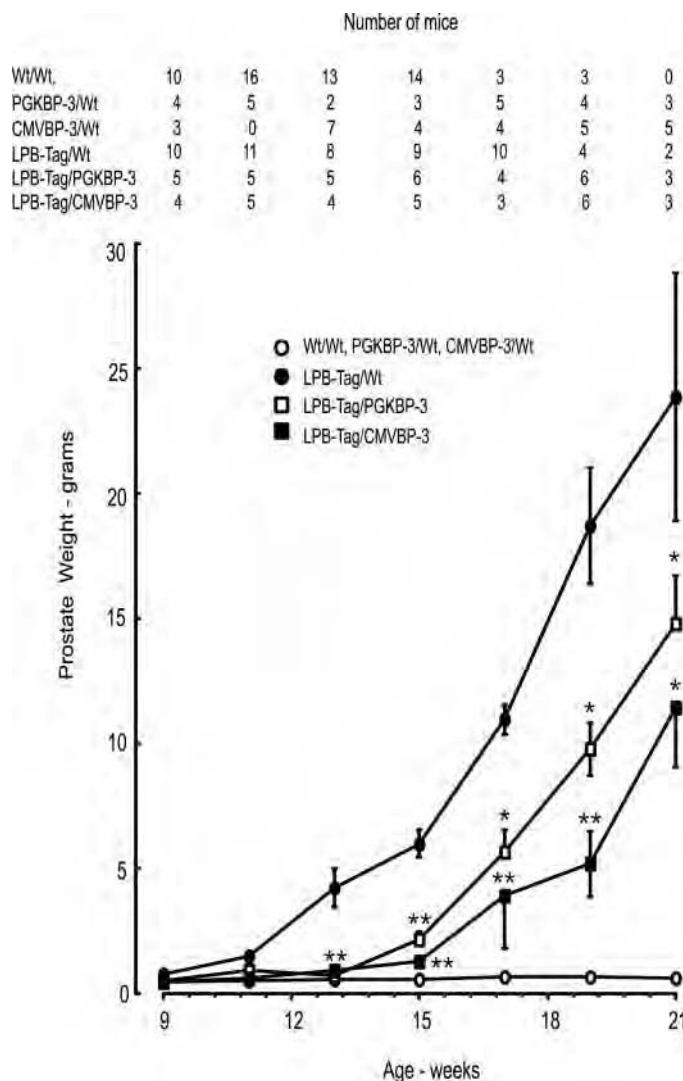


FIG. 1. IGFBP-3 overexpression attenuates prostate tumor development. Prostate weight was assessed in the various mice at different ages. The data represent the mean $\pm$ SEM. The number of mice killed at each time point is shown above. For simplicity only a single line has been used to depict data for Wt/Wt, PGKBP-3/Wt, and CMVBP-3/Wt mice that did not differ significantly from each other. * and **, $P < 0.05$ and $P < 0.01$, respectively, for the difference between the double-transgenic mice and LPB-Tag/Wt mice as determined by ANOVA and Tukey honestly significant difference test.

PGKBP-3 mice, we examined plasma levels of IGF-I and human IGFBP-3 transgene and the abundance of the transgene-derived mRNA in prostate tissue from CMVBP-3 and PGKBP-3 mice. Plasma levels of the transgene-derived IGFBP-3 were similar in CMVBP-3/Wt and PGKBP-3/Wt mice and were also similar to that seen in LPB-Tag/PGKBP-3 (Fig. 2A). The same was true for IGF-I, which was significantly increased in CMVBP-3/Wt, PGKBP-3/Wt, and LPB-Tag/PGKBP-3 mice, compared with Wt/Wt control mice of similar age, reflecting the increased IGF-I binding capacity in the serum. We have previously shown that the presence of the transgene derived human IGFBP-3 has negligible effects on the abundance of murine IGFBP-3 or other IGFbps in CMVBP-3, PGKBP-3, and LPB-Tag/PGKBP-3 mice (19, 20).

TABLE 1. Regression analysis of prostate weight vs. age in transgenic mouse strains

	LPB-Tag/Wt	LPB-Tag/PGKBP-3	LPB-Tag/CMVBP-3
Slope	0.131 ± 0.004	0.126 ± 0.006	0.099 ± 0.007 ^a
Y-intercept	−1.269 ± 0.061	−1.499 ± 0.082 ^b	−1.274 ± 0.099
Correlation coefficient	R = 0.971	R = 0.945	R = 0.896
Doubling time at 10 g, wk	2.29 ± 0.07	2.39 ± 0.12	3.07 ± 0.22 ^b

^a $P < 0.001$ for the difference between double-transgenic and LPB-Tag/Wt mice.

^b $P < 0.05$ for the difference between double-transgenic and LPB-Tag/Wt mice.

However, transgene expression was markedly increased in prostate tissue from CMVBP-3/Wt, compared with PGKBP-3/Wt mice (Fig. 2B). The RNase protection assay is specific for the transgene and does not detect murine IGFBP-3 (20), hence the absence of signal in the lanes containing prostate RNA from Wt/Wt mice. The abundance of human (h)IGFBP-3 mRNA in prostate tissue from CMVBP-3/Wt mice was increased 5.6 ± 0.9 -fold, compared with PGKBP-3/Wt mice ($P < 0.001$).

The phenotypic manifestations of overexpression of mutant IGFBP-3 in PGKBP-3 mice have been previously reported (20). These mice do not demonstrate growth retardation and have slightly higher levels of IGF-I and murine IGFBP-3 than Wt mice, possibly reflecting compensation for the IGF-independent growth-inhibiting effects of mutant IGFBP-3 (20). There was no significant difference in prostate tumor growth in LPB-Tag/Wt and LPB-Tag/PGKBP-3 mice for the first 15 wk of life (Fig. 3). However, a marked reduction in tumor growth was observed in LPB-Tag/PGKBP-3 mice after 15 wk of age, and at subsequent time points,

there was no significant difference in prostate tumor size in LPB-Tag/PGKBP-3 and double-transgenic mice expressing the intact IGFBP-3 driven by the same promoter. A total of 43 LPB-Tag/PGKBP-3 mice were examined from three different PGKBP-3 stud male mice. Because the different stud males contributed different numbers of offspring to

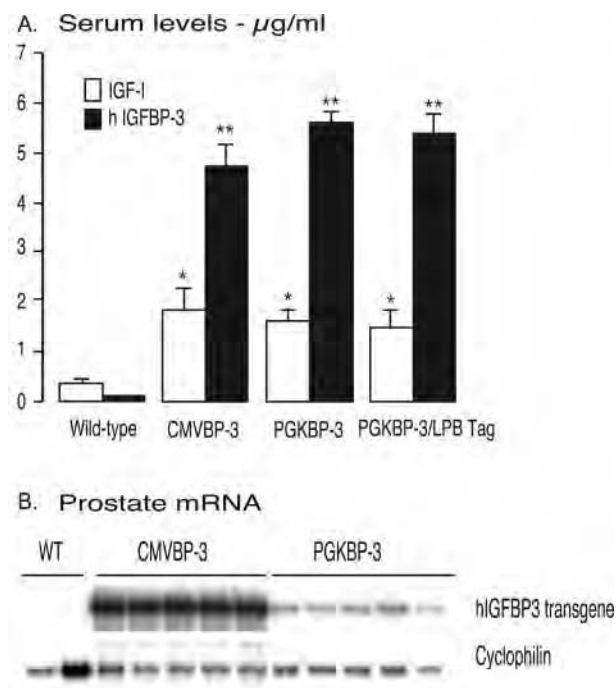


FIG. 2. Serum IGF-I and hIGFBP-3 levels and prostate transgene-derived mRNA levels in PGKBP-3/Wt and CMVBP-3 mice. **A**, Data represent the mean $\pm$ SEM levels for $n = 5$ or more mice per group at approximately 4 months of age. * and **, $P < 0.01$ and $P < 0.001$, respectively, for the difference between the transgenic and wild-type mice. **B**, RNase protection assay using a hIGFBP-3-specific probe. Cyclophilin is included as an internal control.

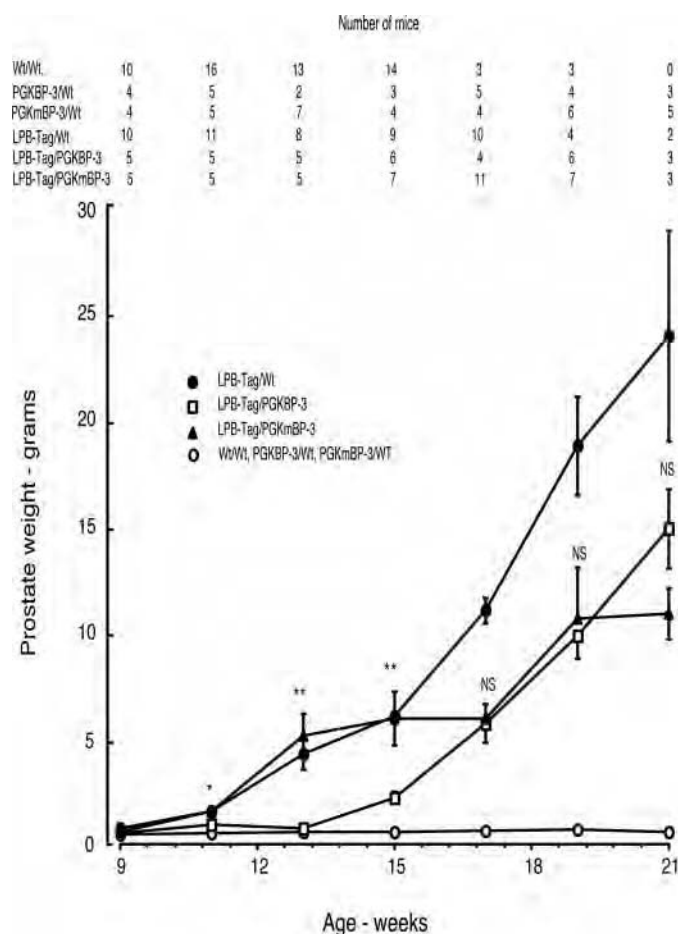


FIG. 3. Mutant IGFBP-3 overexpression attenuates prostate tumor development at the later time points. Prostate weight was assessed in the various strains of mice at different ages. The data represent the mean $\pm$ SEM. The number of mice killed at each time point is shown above. For simplicity only a single line has been used to depict data for Wt/Wt, PGKBP-3/Wt, and PGKBP-3/LPB Tag mice that did not differ significantly from each other. * and **, $P < 0.05$ and $P < 0.01$, respectively, for the difference between the prostate weight in LPB-Tag/PGKBP-3 and LPB-Tag/Wt mice as determined by ANOVA followed by the Tukey honestly significant difference test. N.S., No significant difference between LPB-Tag/PGKBP-3 and LPB-Tag/CMVBP-3 mice. #, $P < 0.001$ for the difference between LPB-Tag/PGKBP-3 and LPB-Tag/Wt for the data from 17, 19, and 21 wk combined.

each point, we investigated whether there was any difference in prostate weight in offspring of different stud males at 15 and 17 wk in which there was adequate representation of offspring from all three. When prostate weight for individual mice was expressed as a percentage of mean prostate weight for the whole group at each time point, there was no significant difference in prostate weight of the offspring of different PGKmbp-3 stud males.

Immunoblotting and Western ligand blotting were used to investigate the presence of the transgene-derived protein product in prostate tissue from the various transgenic strains. Using antibody specific for hIGFBP-3, an intense signal was apparent in lanes containing prostate extract from LPB-Tag/CMVBP-3 mice (Fig. 4A). Both intact hIGFBP-3 of approximately 40 kDa and a less abundant approximately 19 kDa IGFBP-3 proteolytic fragment, previously reported in other tissue extracts (22), were apparent. hIGFBP-3 was also detected, but less abundant, in prostate extracts from LPB-Tag/PGKBP-3 mice. As anticipated, no signal was observed in extracts from LPB-Tag/Wt or Wt/Wt mice. Weak immunoreactivity was apparent in lanes containing extracts from

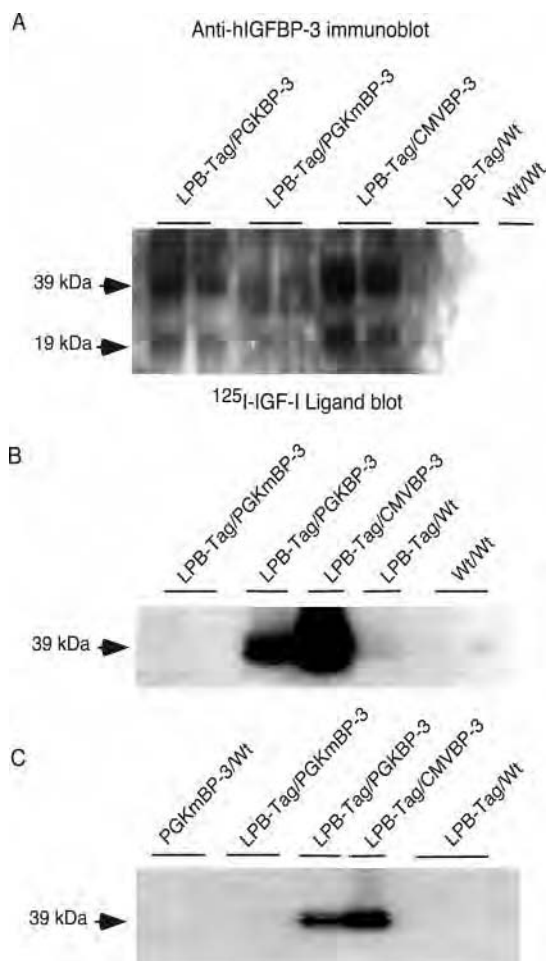


FIG. 4. Expression of IGFBP-3 in prostate tumors. A, Immunoblot of prostate extracts from various mouse strains at 15 wk of age using a human IGFBP-3-specific antibody. B, Western ligand blot using 125 I-IGF-I of the same gel. C, Western ligand blot of prostate tissue extracts at 21 wk of age.

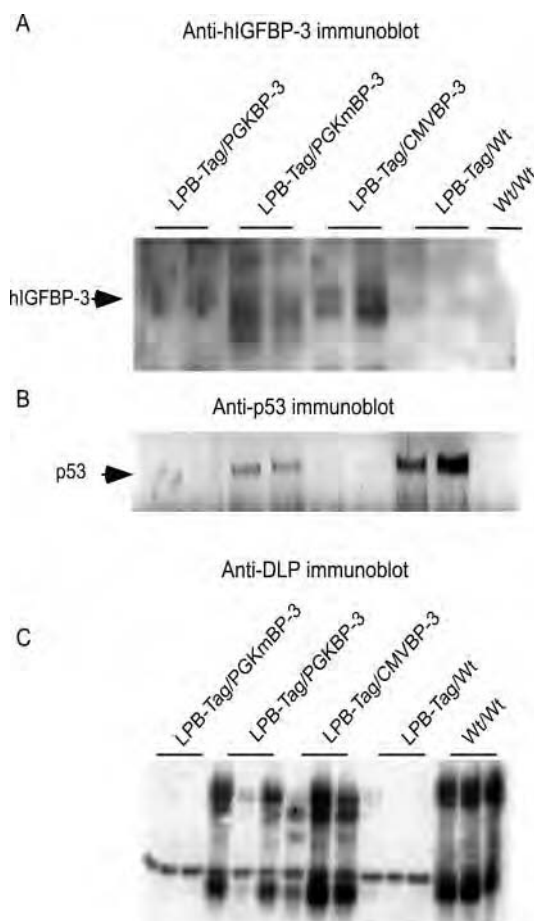


FIG. 5. Expression of p53 and loss of expression of dorsolateral protein in prostate tumors. Prostate extracts from 15-wk-old mice were analyzed by immunoblotting with antihuman IGFBP-3 (A). The same filter was subsequently reprobed with anti-p53 antibody (B). C, A separate filter was probed with antibody against dorsolateral protein.

LPB-Tag/PGKmbp-3 mice (Figs. 4A and 5A). In extracts from these mice, the approximately 40-kDa hIGFBP-3 immunoreactivity was present as a smear and the approximately 19-kDa fragment was not seen, suggesting the possibility of extensive degradation of the non-IGF binding mutant IGFBP-3 consistent with our previous report (20).

Western ligand blotting with 125 I-IGF-I confirmed the higher level of expression of the transgene in prostate tissue of LPB-Tag/CMVBP-3 mice, compared with LPB-Tag/PGKBP-3 mice (Fig. 4B). No binding was observed in lanes containing prostate extract from LPB-Tag/PGKmbp-3 mice because this mutant IGFBP-3 does not bind IGF-I (20). Radioactivity was also not detected in lanes containing extracts from LPB-Tag/Wt and Wt/Wt mice, probably because of the low sensitivity of this technique in detecting endogenous murine IGFBPs in prostate tissue extracts under these conditions. The data shown in Fig. 4B are from prostate tissue obtained at 15 wk of age. To ensure that the LPB-Tag/PGKmbp-3 continued to carry a mutant IGFBP-3 at the later time points, we used Western ligand blotting to analyze samples collected at 21 wk of age. Similar results were obtained with these tissues (Fig. 4C). 125 I-IGF-I binding was observed in lanes containing extracts from LPB-Tag/

CMVBP-3 and LPB-Tag/PGKBP-3 mice but not in lanes with extracts from LPB-Tag/PGKMBP-3 mice. The difference in signal intensity between Fig. 4B and Fig. 4C results from differences in decay in radiolabel and autoradiography exposure time, and no meaningful conclusions can be drawn concerning the abundance of transgene expression at the two time points.

The presence of immunoreactive p53 was assessed in prostate tissue in various mouse strains at 15 wk of age. In LPB-Tag/Wt mice, the SV40 large Tag stabilizes p53, and p53 immunoreactivity was easily detected in prostate extracts from these mice (Fig. 5). A lower level of p53 protein was also detected in extracts from LPB-Tag/PGKMBP-3 mice. No p53 was apparent in lanes containing extracts from Wt/Wt, LPB-Tag/PGKBP-3 or LPB-Tag/CMVBP-3 mice. Expression of dorsolateral proteins, a marker of differentiated function (21), was lost in prostate tissue from LPB-Tag/Wt and LPB-Tag/PGKMBP-3 mice but relatively preserved in LPB-Tag/CMVBP-3 and LPB-Tag/PGKBP-3 mice (Fig. 5C).

Expression of both EGF-R and IGF-IR were up-regulated in the prostate tumors (Fig. 6). EGF-R was most abundant in LPB-Tag/Wt and LPB-Tag/PGKMBP-3 mice, and the lowest levels of expression were apparent in tissue from LPB-Tag/CMVBP-3 mice. In contrast, IGF-IR was significantly elevated in all transgenic mice carrying the LPB-Tag transgene, compared with Wt mice, and there was no significant difference between those expressing intact or mutant IGFBP-3. Despite increased levels of IGF-IR in LPB-Tag/CMVBP-3 and LPB-Tag/PGKBP-3 mice, the abundance of phospho pAkt(Ser 473) was reduced in these mice, compared with LPB-Tag/Wt and LPB-Tag/PGKMBP-3 mice (Fig. 6, lower panel), suggesting that signaling at the IGF-IR was attenuated in the double-transgenic mice expressing intact IGFBP-3.

Prostate tissue from all four groups of tumor bearing mice showed a similar histological appearance. In LPB-Tag/Wt mice and LPB-Tag/PGKMBP-3, by 9 wk of age, virtually all acina demonstrated hypercellularity typical of high-grade PIN, whereas prostate tissue from LPB-Tag/PGKBP-3 mice at the same stage showed a more heterogeneous pattern with many acina appearing normal or demonstrating low-grade PIN (Fig. 7, upper panel). Tissue from LPB-Tag/CMVBP-3 at 9 wk was similar to that from LPB-Tag/PGKBP-3 mice (data not shown). At later time points, prostate tissue from LPB-Tag/CMVBP-3 and LPB-Tag/PGKBP-3 mice resembled early time points in LPB-Tag/Wt mice with high-grade PIN throughout the entire gland. In general, high-grade PIN was associated with loss of DLP staining, increased nuclear localization of androgen receptor staining, and positive staining for PCNA and Tag (Fig. 7, lower panel). There was no obvious difference in immunostaining or histology between tissues from LPB-Tag/PGKBP-3 and LPB-Tag/PGKMBP-3 mice.

The percentage of apoptotic cells in areas of high-grade PIN was assessed in each of the strains of tumor-bearing mice. Apoptotic cells were most abundant in LPB-Tag/CMVBP-3 mice and least abundant in LPB-Tag/Wt mice (Fig. 8). In LPB-Tag/Wt, LPB-Tag/PGKBP-3, and LPB-Tag/CMVBP-3 mice, the percentage of apoptotic cells was relatively constant from 9 through 17 wk. In contrast, in LPB-Tag/PGKMBP-3 mice, the percentage of apoptotic cells was

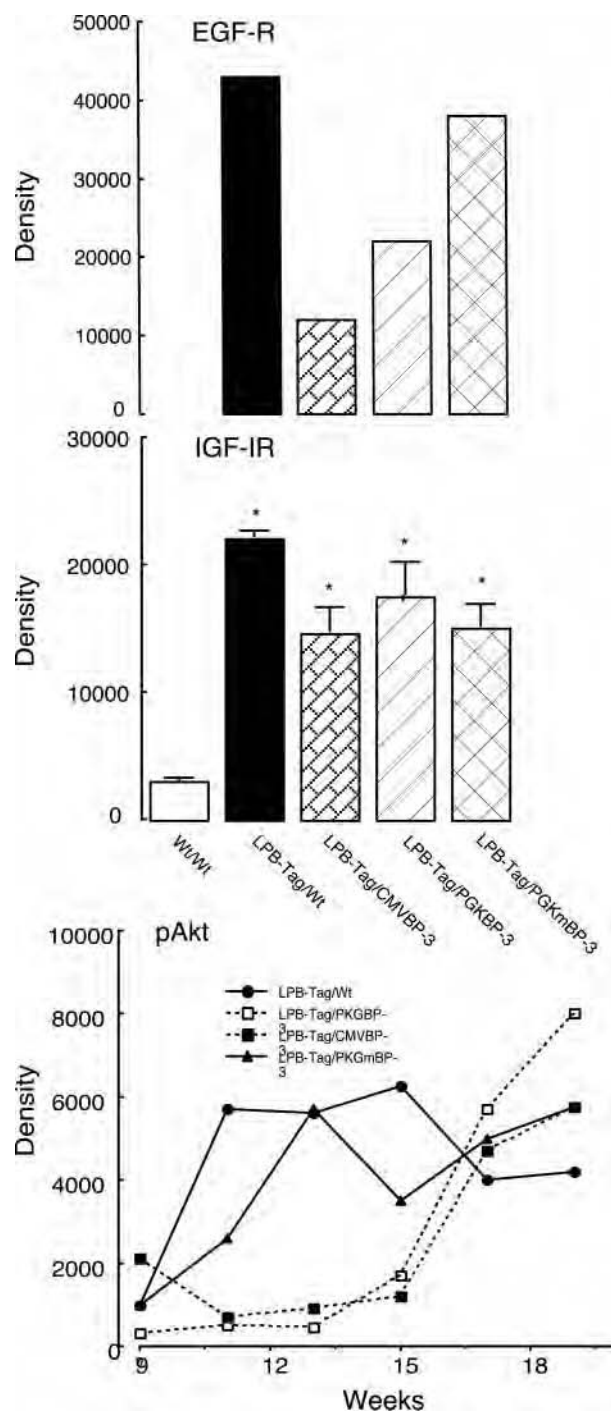


FIG. 6. Expression of EGF-R and IGF-IR and phospho-Akt(Ser473) in prostate tumors. Immunoblots were quantified by densitometry. Data represent two or more samples for each group. *, $P < 0.05$ for the difference between transgenic and wild-type mice.

low at 9 and 13 wk but increased significantly at 17 wk (Fig. 8).

Discussion

Heterozygous LPB-Tag mice carry a genetic predisposition to neoplasia restricted to the prostate because of the tissue specificity of the long probasin promoter (14). The

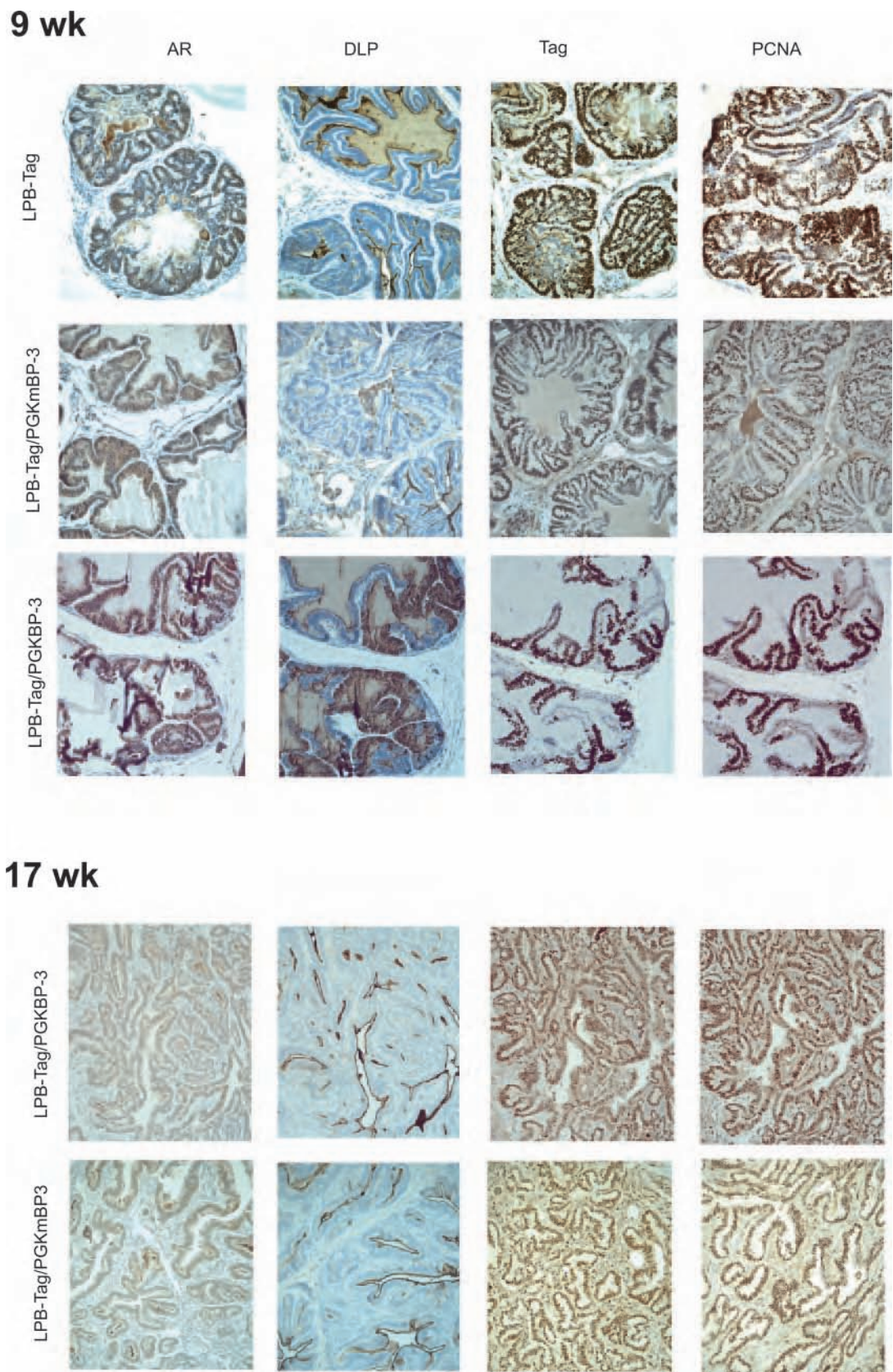


FIG. 7. Immunohistochemical analysis of prostate tumors in LPB-Tag/Wt, LPB-Tag/PGKBP-3, and LPB-Tag/PGKmbBP-3 mice. Tissue from mice at 9 and 17 wk of age were analyzed using antibodies to androgen receptor (AR), DLP, Tag, and PCNA. The magnification is $\times 200$.

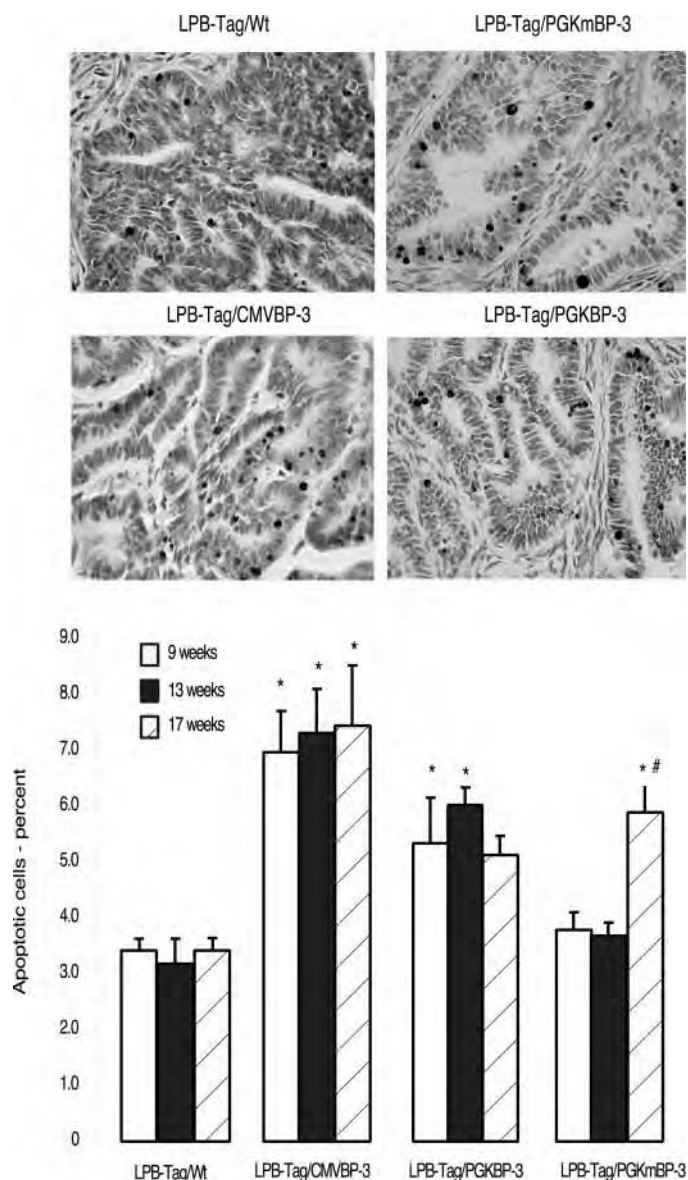


FIG. 8. Apoptosis in prostate tumors in LPB-Tag/Wt, LPB-Tag/PGKBP-3, LPB-Tag/CMVBP-3, and LPB-Tag/PGKMBP-3 mice. *Upper panel*, Representative sections from tumors from 17-wk-old mice. The magnification is $\times 200$. *Lower panel*, The percentage of apoptotic cells in similar high-grade PIN lesions in tumors from the four groups of mice were enumerated at each of the time points. The data represent the mean $\pm$ SEM. *, $P < 0.05$ for the difference LPB-Tag/Wt mice and other mouse strains at each time point; #, $P < 0.05$ for the difference from the 9-wk time point within the one mouse strain.

SV40 large T oncoprotein interferes with the cellular tumor suppressor protein p53 mimicking molecular alterations that occur in human prostate cancer (12, 14). The LPB-Tag mice are an appropriate transgenic model of prostate cancer to study the effect of IGFBP-3 because many features of human prostate cancer are reproduced including androgen dependence of tumor formation and growth; tumor formation in normal hormone- and immune-competent environment; and the expression of biomarkers associated with human PIN that predict progression to invasive carcinoma such as increased PCNA, a marker of proliferation, decreased apopto-

sis, enhanced growth factor receptor expression (erbB family), increased expression, and nuclear localization of the androgen receptor. Most importantly, the histological feature of the putative precursor lesions, mouse PIN, and progression to adenocarcinoma are also apparent in this mouse model (15).

In this manuscript, we report the attenuation of prostate tumor growth in mice that overexpress IGFBP-3. These experiments were performed by generating double-transgenic mice. Both the IGFBP-3 and the LPB-Tag mice were generated in the same CD1 strain, thereby eliminating the potential confounding effects of different genetic backgrounds. These observations represent the first demonstration of attenuated prostate tumor growth *in vivo* by IGFBP-3 overexpression.

The marked reduction of tumor growth seen in both LPB-Tag/PGKBP-3 and LPB-Tag/CMVBP-3 mice was predominantly due to paracrine/autocrine effects in the prostate rather than the result of systemic IGFBP-3 because the effect was more marked in LPB-Tag/CMVBP-3 mice that have higher levels of transgene expression in the prostate but similar levels of circulating IGFBP-3 to LPB-Tag/PGKBP-3 mice. Furthermore, attenuation of prostate tumorigenesis was apparent despite significantly increased levels of IGF-I in the circulation in both these strains of double-transgenic mice (19).

After 15 wk of age, the tumors in LPB-Tag/PGKBP-3 and LPB-Tag/CMVBP-3 grew rapidly, although at a slightly slower rate than that seen in LPB-Tag/Wt mice, suggesting that the predominant effect of overexpression of IGFBP-3 was at the early stages of tumor development. The mechanisms involved in tumor development in LPB-Tag mice are not fully understood, but tumor development is delayed until after sexual maturation in this model and is clearly androgen dependent (14). Both PGKBP-3 and CMVBP-3 male mice are fertile and testosterone levels are not markedly different in these mice and Wt mice (Ref. 19; and Silha, J., and L. J. Murphy, unpublished observations). Although not studied in this LPB-Tag model, prostate cancer development is thought to be IGF-I dependent in the early stages, whereas the tumor progression may be less dependent on IGF-I as the disease progresses (23), possibly as a result of amplification of the IGF-IR and/or downstream signal transduction pathways (24). Our observations in LPB-Tag/PGKBP-3 and LPB-Tag/CMVBP-3 mice would be consistent with this notion. In these mice tumor development was delayed, but once well established, the tumor appeared to grow at a rate approaching that seen in LPB-Tag/Wt mice. Furthermore, prostate histology in LPB-Tag/PGKBP-3, LPB-Tag/CMVBP-3, and LPB-Tag/Wt mice appeared similar.

Not unexpectedly prostate tumor development and growth in LPB-Tag/PGKMBP-3 was similar to LPB-Tag/Wt mice during the first 15 wk. Because mutant IGFBP-3 does not bind IGF-I (20), it would be unable to inhibit IGF-I action during the critical early stages of prostate tumorigenesis. We have previously shown that PGKMBP-3 transgenic mice have low levels of human IGFBP-3 in the circulation (~ 0.5 $\mu\text{g/ml}$), compared with PGKBP-3 transgenic mice (~ 5 $\mu\text{g/ml}$), despite identical transgene promoters and similar levels of tissue transgene mRNA (19, 20). We speculated that the mutant IGFBP-3 was more rapidly cleared from the circu-

lation and degraded because mutant IGFBP-3 is unable to bind IGF-I, which appears to be necessary for the formation of stable ternary complexes with the acid-labile subunit (25). Western blotting of prostate extracts confirmed that mutant IGFBP-3 was more degraded than native IGFBP-3. Human prostate tissue contains prostate-specific antigen that can proteolyze IGFBP-3 (26). It is likely that mouse prostate tissue contains similar kallikreins that can degrade IGFBP-3. Immunohistochemical analysis indicated that regions of the prostate demonstrating high-grade PIN had low levels of expression of dorsolateral proteins. We speculate that in these regions IGFBP-3 proteolysis may be reduced.

The unexpected finding in this report was the decline in tumor growth in LPB-Tag/PGKmbp-3 after 15 wk of age. Whereas this most likely represents an IGF-independent effect of IGFBP-3 similar to those reported by other investigators using *in vitro* cultures of prostate and breast cancer cells (5, 11, 17, 18, 22), it is unclear why these IGF-independent effects were not manifested earlier. We carefully excluded the possibility that this was artifactual by reviewing the parentage of each of the mice and also analyzing the tumor extracts from 21-wk-old mice by Western ligand blotting (Fig. 4). The LPB-Tag/PGKmbp-3 offspring used for the age 19- and 21-wk data points were from the same stud PGKmbp-3 male that contributed offspring to earlier time points, and thus, the data from the later time points are not the result of a specific stud male. Consistent with this apparent delayed effect of mutant IGFBP-3 expression, we demonstrate a significant increase in the percentage of apoptotic cells in the high-grade PIN lesion with time in LPB-Tag/PGKmbp-3 mice.

Under *in vitro* conditions, it is possible to demonstrate multiple and opposing effects of IGFBP-3 on cell proliferation and apoptosis. IGFBP-3 has IGF-dependent antiproliferative, proapoptotic effects related to binding IGF-I and restriction of access of IGF-I to the IGF-IR. Under certain conditions, IGF-dependent effects of enhancing cell survival and proliferation, possibly by enhancing delivery of IGF-I to the cell membrane receptor, can also be demonstrated with IGFBP-3 (27). In addition, as discussed above, IGFBP-3 has IGF-independent antiproliferative, proapoptotic effects actions that have been reported *in vitro* in the past. Various potential mechanisms have been proposed to explain these IGF-independent effects of IGFBP-3. These include the interaction of IGFBP-3 with various cell membrane-associated proteins such as the type V TGF β receptor (28) and autocrine motility factor (29) as well as other as-yet-unidentified proteins (30, 31). In addition, IGFBP-3 has been shown to interact with nuclear transcription factors such as the retinoid X receptor- α and stimulate apoptosis via this interaction (32).

This report represents the first demonstration of the IGF-independent antiproliferative effects of IGFBP-3 *in vivo*. IGF-independent effects of IGFBP-3 demonstrated *in vitro* are apparent only under conditions in which IGF-I is absent (5, 11, 16) or in cell lines that are not dependent on IGF-I for growth because they lack IGF-IR (3, 4). It is possible that the IGF-independent effects of IGFBP-3 are inhibited by IGF-I or not apparent in cells in which the IGF-I signal transduction pathway is activated. Thus, early in prostate tumorigenesis in LPB-Tag/PGKmbp-3 mice in which the tumors are grow-

ing in response to IGF-I, these IGF-independent effects of IGFBP-3 may be blocked or masked by IGF-I stimulated mitogenesis.

An alternative explanation for the apparent lack of effect of mutant IGFBP-3 during early prostate cancer growth in this model may be related to the enhanced degradation of mutant IGFBP-3 in prostate tissue. With the loss of markers of differentiation, such as dorsolateral protein as the tumors progress, there may also be a loss of IGFBP-3 protease activity and consequently enhanced levels of mutant IGFBP-3 that could exert a progressively more marked effect with increasing tumor mass. Lack of sufficient quantities of recombinant mutant IGFBP-3 precluded us from directly testing this hypothesis.

Whereas the exact mechanism whereby overexpression of mutant IGFBP-3 exerts its antiproliferative effect requires further investigation, our data clearly demonstrate that local overexpression of IGFBP-3 attenuates prostate tumorigenesis. Recently Majeed *et al.* (33) reported that development and progression of the transgenic adenocarcinoma of mouse prostate (TRAMP) model of prostate cancer is delayed in the *lit/lit* mouse, which has low IGF-I and GH levels as a result of an inactivating mutation in the GH releasing factor receptor. Their data suggest that IGF-I, GH, or both are important in prostate cancer development and progression. Wang *et al.* (34) used a slightly different model in which the GH receptor gene was disrupted to demonstrate the importance of GH/IGF-I signaling for prostate carcinogenesis in the C3(1)/T-antigen mice. Our observations, using a slightly different model of prostate cancer, support an important role of local IGF-I levels in prostate tumor progression. Furthermore, our data also suggest the use of IGFBP-3 and its mutant may be a useful therapeutic strategy in the treatment of prostate cancer.

Acknowledgments

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The content of this manuscript does not necessarily reflect the position or the policy of the Government, and no official endorsement should be inferred. The paper is part of United States Provisional Patent US60/710,893, filed on August 25, 2005, "Methods of attenuating prostate tumour growth by insulin-like growth factor binding protein 3." Applicants were Josef V. Silha, Janice G. Dodd, and Liam J. Murphy. There is no commercial involvement in the research or the patent prosecution at this time. P.C.S., S.M., Y.G., and J.Sc. have nothing to declare.

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SUPPORTING DATA

FIGURES

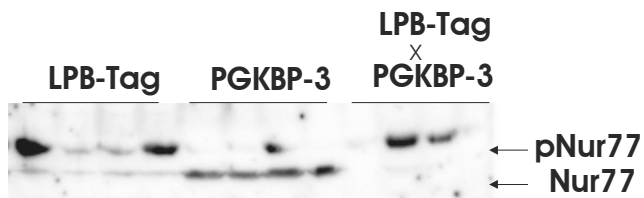


Figure 1 Western blot analysis of phosphorylated and unphosphorylated Nur77 in LPB-Tag mouse model of prostate cancer. In the parental tumor line (LPB-Tag) the predominant isoform is pNur77, while in the parental line overexpressing IGF-BP3 (PGK-BP-3) the predominant form is Nur77. In the double transgenic line (LPB-Tag x PGKBP-3) the predominant form is pNur77.

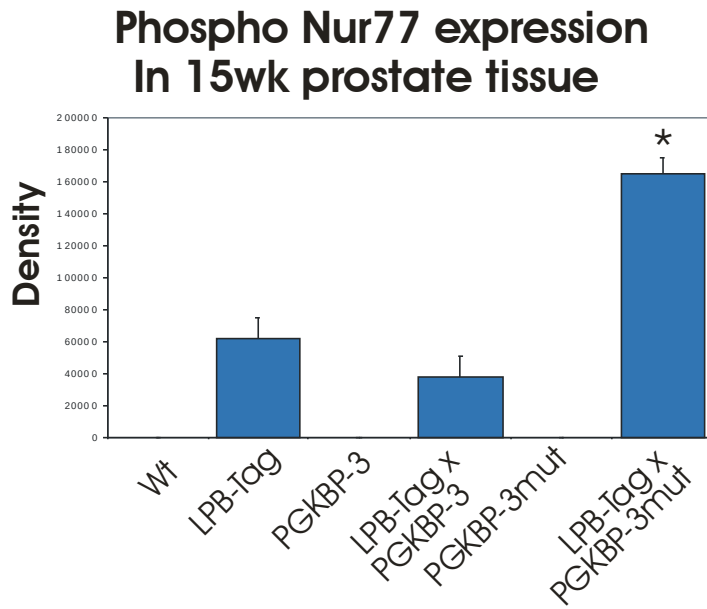


Figure 2 Relative expression of phospho-Nur77 as assessed by immunoblot analyses using anti-pNur77 (Santa Cruz; sc-16992). In tumor mice expressing the mutant form of IGF-BP3, the levels of pNur77 were significantly higher than in mice expressing the normal IGF-BP3 allele ($p < 0.001$) and the parental tumor line ($p < 0.001$). The

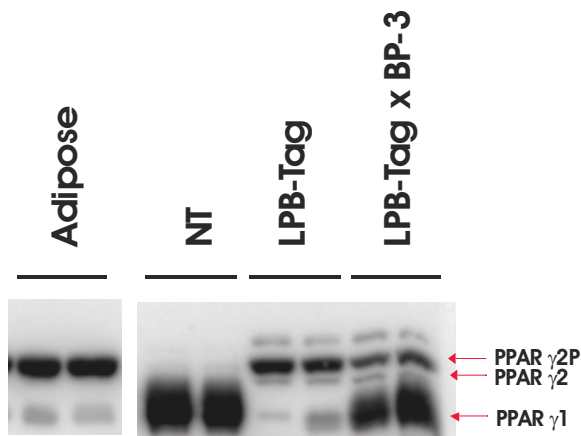


Figure 3 Western blot analysis of PPAR γ in protein extracts of mouse prostate and adipose tissue. PPAR γ 1 is the predominant isoform of PPAR γ expressed in non-adipose tissues including normal prostate. PPAR γ 2 is the predominant isoform expressed in adipose tissue, and in some cancers including human CaP and PIN. In the mouse model non-transgenic (NT) normal mouse prostates expressed the PPAR γ 1 isoform, while LPB-Tag prostate tumors expressed predominantly the PPAR γ 2 isoform. Overexpression of IGFBP-3 in LPB-Tag x BP-3 animals is associated with expression of both PPAR γ isoforms. This may represent the partial restoration of a more differentiated prostatic phenotype, consistent with the delay in tumor progression. Adipose tissue is positive control.

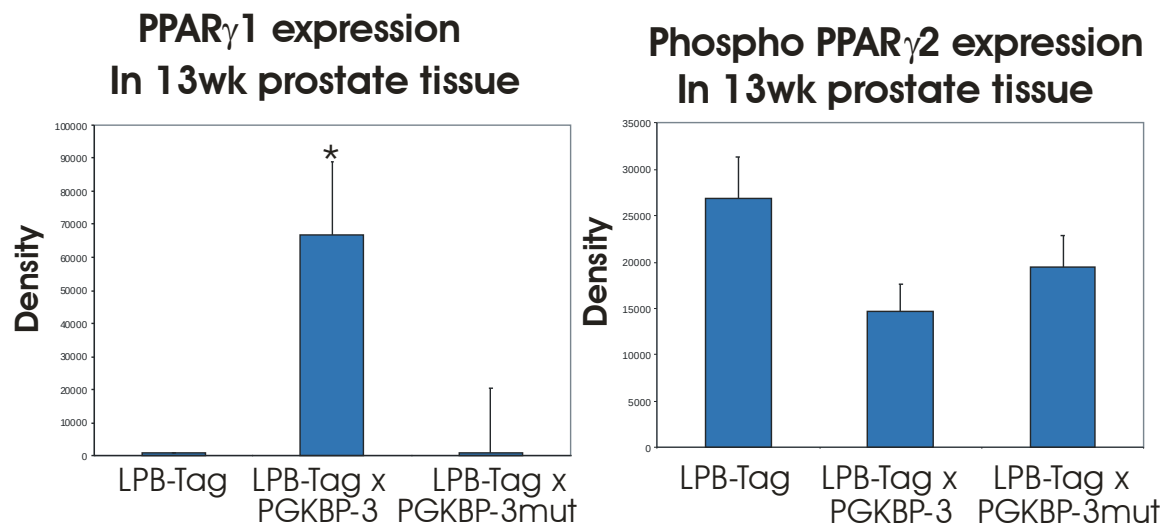


Figure 4 Relative expression of PPAR γ isoforms by immunoblot analysis in the mouse model at 13 weeks of age. Left panel, PPAR γ 1 expression is elevated in the prostate tumors of mice that overexpress the normal IGF-BP3 allele relative to the parental tumor line and the mice carrying the mutant IGF-BP3 ($p < 0.05$). Right panel, PPAR γ 2 expression is reduced in mice expressing the normal IGF-BP3 allele but it does not reach statistical significance.

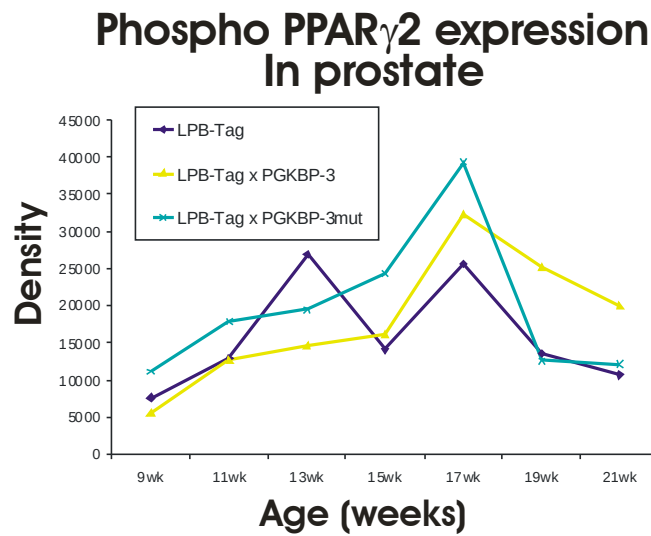


Figure 5 Relative expression of phosphorylated PPAR γ 2 by immunoblot analysis over the time course of tumor progression. Early in progression, the expression of phosphoPPAR γ 2 is lower in the mice overexpressing the normal IGF-BP3 allele (LPB-Tag x PGKBP-3) relative to the parental tumor line LPB-Tag and the mice overexpressing the mutant IGF-BP3 allele. This corresponds to the period in which tumor growth is delayed in the LPB-Tag x PGKBP-3 mice. After 15 weeks, expression of phospho PPAR γ 2 is elevated in all the lines, corresponding to the period in which tumor growth is maximal.

AMF/PGI expression in prostate

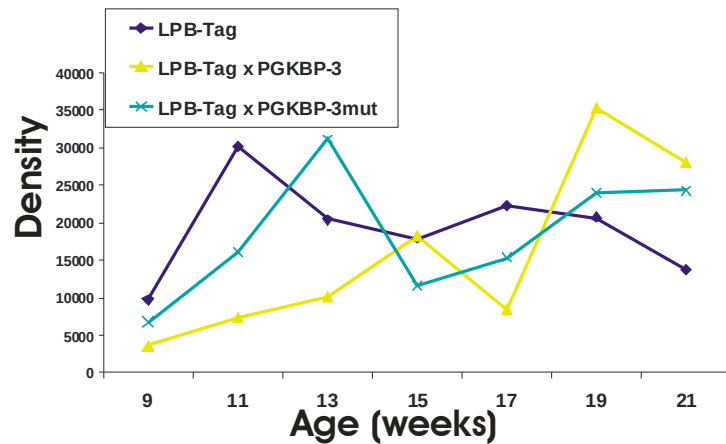


Figure 6 Relative expression of AMF/PGI protein by immunoblot analysis using anti-AMF/PGI (provided by Dr. S. Mishra) in the mouse model during tumor progression. In mice overexpressing the normal IGF-BP-3 allele (yellow curve) the level of AMF/PGI is low until late in progression (after 15 weeks). In contrast, both the parental line (purple line) and the mice overexpressing the mutant form of IGF-BP3 (green line) express higher levels of AMF/PGI early in tumor progression (before 15 weeks). This identifies AMF/PGI expression as an IGF-dependent event in the tumor model.

Phospho Hsp27 expression in prostate Compared to wt mice

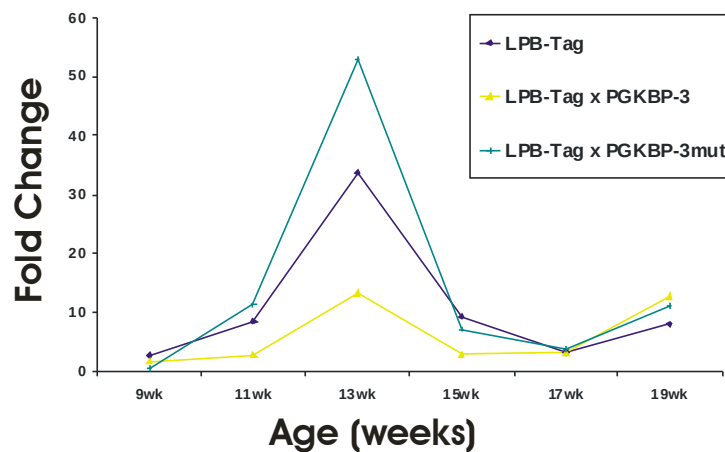


Figure 7 Relative change in expression of phosphorylated HSP27 by immunoblot analyses using anti-pHSP27 (Santa Cruz; pHSP27(ser78): sc-16568) during tumor progression in the mouse model. The comparison is made to normal prostate tissue from wild-type (non-transgenic) age-matched littermates. The peak of pHSP27 expression occurs at 13 weeks in all lines. Levels are lowest in tumor mice overexpressing the normal IGF-BP3 allele (yellow curve), the line with the highest rate of apoptosis. In contrast, the parental tumor line (purple curve) and the tumor mice overexpressing mutant IGF-BP3 (green line) showed higher pHSP27 expression and reduced rates of apoptosis.