Human Prostate Cancer Xenografts in *lit*/*lit* Mice Exhibit Reduced Growth and Androgen-Independent Progression

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BACKGROUND. The growth hormone/insulin-like growth factor I (GH/IGF-I) axis has been linked to prostate cancer (PCa) risk. Although previous studies indicate that human breast cancers and a murine PCa model develop more slowly in murine hosts homozygous for a missense mutation in the GH-releasing hormone receptor (*lit*/*lit*) whose ''*little*'' dwarfed phenotype is caused by suppressed GH and IGF-I production, the role of these two hormones remains controversial.

METHODS. To assess how the GH/IGF-I axis influences androgen-responsive, castration-resistant (CR), and androgen-independent (AI) growth of human PCa, we compared xenograft growth of the androgen-responsive human PCa cells, LNCaP, and AI human PCa cells, PC3, in intact and castrate Nod/SCID *lit*/*lit* and *lit*/+ mice, and in vitro growth of these cell lines in *lit*/*lit* and *lit*/+ serum-containing media supplemented with GH or IGF-I.

RESULTS. Tumor growth and PSA accumulation rates were suppressed in LNCaP tumor-bearing *lit*/*lit* mice pre- and post-castration. Growth of PC3 xenografts in *lit*/*lit* mice was also suppressed. In vitro proliferation of LNCaP and PC3 cells cultured in media containing *lit*/*lit* mouse serum was decreased as compared to growth in media containing *lit*/+ serum. Suppressed growth in *lit*/*lit* serum could be restored by the addition of IGF-I, and to a lesser extent, GH. Differences in growth correlated with differences in steady-state AKT and ERK1/2 activation.

CONCLUSIONS. This study demonstrates that circulating GH and IGF-I can promote androgen-responsive growth, CR progression, and AI expansion of PTEN-deficient human PCa cell xenografts and indicates that IGF-I can promote PCa growth in a suppressed GH environment.

KEY WORDS: **prostate cancer; insulin-like growth factor; growth hormone; androgen-independent progression; Ghrhr^{lit} mice**

INTRODUCTION

Prostate cancer (PCa) is the most commonly diagnosed solid tumor malignancy in men in Western countries and a major and escalating international health problem [1]. For patients with advanced, extracapsular disease, androgen-deprivation therapy (ADT) is the primary disease management option. Although 80% of the patients initially respond to ADT, remission generally lasts only 18–36 months and is followed by castration-resistant (CR) disease

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progression [2]. CR progression is a complex process by which cells acquire the ability to survive and proliferate in the absence of gonadal androgens and involves mechanisms attributed to re-activation of the androgen receptor axis and activation of alternative growth factor and cytokine signaling cascades [3–5].

The insulin-like growth factor (IGF) axis is one such alternative growth factor pathway with established roles in PCa biology [4,6–8]. Ligand activation of the type 1 IGF tyrosine kinase receptor (IGF-1R) stimulates the Ras/extracellular signal-regulated protein kinase 1/2 (ERK1/2) and phosphatidylinositol 3′-kinase (PI3K)/AKT downstream signaling pathways. These signaling events are important regulators of cell growth, survival, and metastatic potential in a variety of malignancies and are strongly implicated in PCa etiology [9,10] and progression [11,12]. Physiologically, IGF-I production is regulated by growth hormone (GH). Mice homozygous for the ''*little*'' mutation, a D60G missense mutation in the GH-releasing hormone receptor ($Ghrhr^{lit}$) resulting in suppression of GH and IGF-I production [13], but does not affect androgen levels [14]. These mice have been used to demonstrate that low GH and IGF-I levels decrease growth of sarcoma and breast cancer xenografts [15,16] and suppress development of prostatic malignancy in the ''TRAMP'' SV40 T-Ag-driven PCa model [6].

PI3K/AKT signaling plays a critical role in several human cancers including: PCa, breast cancer, glioblastoma, endometrial cancer, and others [17,18]. Perturbations in PI3K/AKT pathway occur through altered cell adhesion and peptide growth factor-mediated signaling and are often complemented by loss-of-function mutations in the tumor suppressor gene, phosphatase and tensin homolog (PTEN), and activating mutations in the catalytic subunit of PI3Kα, p110. A signature event impacting PI3K signaling in ∼50% of advanced PCa is homozygous loss of PTEN, and among those patients who are not PTEN null, many exhibit loss of one PTEN allele [19,20]. Experimentally, the importance of PTEN in PCa initiation and progression is demonstrated by development of prostatic malignancy in prostate-specific PTEN knockout mice [21] and by reduced PCa cell growth, and induction of apoptosis by ectopic expression of PTEN [22–24]. Furthermore, in murine models of PCa, PTEN can also synergize with putative PCa oncogenes: Nkx3.1, p27, p53, and ERG to promote carcinogenesis and CR progression [25–29]. This latter observation corroborates epidemiologic indication that in tumors harboring TMPRSS2: ERG gene rearrangements, hemizygous PTEN loss increases risk of biochemical progression [30].

PTEN is a PIP3 3′ phosphatase that acts as a negative regulator of PI3K/AKT signaling; whose loss sensitizes cells to a variety of PI3K-activating stimuli. While loss of PTEN appears to be a prominent means by which PCa is initiated and promoted, which and how selection for hyperactivated PI3K signaling is invoked remains to be elucidated. In particular, how IGF availability and loss of PTEN are mechanistically linked to PCa initiation and progression remains to be resolved. We hypothesized that loss of PTEN promotes prostatic malignant transformation due to sensitization of prostatic epithelia to upstream PI3K activating events and that the GH/IGF-I axis is an important contributor to PI3K-dependent survival signaling in androgen-responsive growth, CR progression, and androgen-independent (AI) expansion.

In this study, immunodeficient ($Prkdc^{scid/scid}$) mice segregated for the little ($Ghrhr^{lit}$) mutation [16] as either $Ghrhr^{lit/lit}$ (*lit*/*lit*: GH/IGF-I deficient) or $Ghrhr^{lit/+}$ (*lit*/+: GH/IGF-I replete) were used as hosts for xenografts of the androgen-responsive human PCa cell line, LNCaP, and the AI human PCa cell line, PC3. Both androgen-responsive and CR growth of LNCaP xenografts were significantly reduced in *lit*/*lit* mice as compared to *lit*/+ mice and growth of AI PC3 xenografts was significantly repressed in *lit*/*lit* mice as compared to *lit*/+ mice. Furthermore, LNCaP and PC3 cells grown in vitro with serum from *lit*/*lit* mice showed decreased proliferation as compared with serum from *lit*/+ mice, and addition of IGF-I, and to a lesser extent GH, rescued suppressed growth in *lit*/*lit* serum. Differences in growth correlated with differences in AKT and ERK1/2 activation. These results demonstrate that circulating IGF-I, and GH, significantly contributes to androgen-responsive growth, CR progression, and AI expansion of human PCa cell xenografts and support development of novel hormonal treatment strategies that target the GH/IGF-I axis for patients with PTEN-deficient PCa.

MATERIALS AND METHODS

Cell Lines

LNCaP cells were provided by Dr. L.W.K. Chung (Emory University) and PC3 cells were purchased from American Type Culture Collection (ATCC, Rockville, MD). LNCaP cells were maintained in RPMI1640 (Life Technologies, Burlington, Ontario) and PC3 cells were maintained in DMEM (Life Technologies). Media were supplemented with 10% fetal bovine serum (FBS) and cultures were grown at 37°C and 5% CO_2.

C57BL/6 $Prkdc^{scid/scid}$ $Ghrhr^{lit/lit}$ and $Ghrhr^{lit/+}$ Characterization

Male mice averaging 18 weeks of age were used for xenograft studies in accordance with the guidelines of the Canadian Council on Animal Care and with

appropriate institutional certification. Mice were genotyped to confirm *lit* hetero- and homozygosity from tail clip DNA extracted using DirectPCR Lysis Reagent (Viagen Biotech, CA). The *Ghrhr* allele was PCR amplified using the primers: F: 5′-gacttcatcactcagctga-3′, R: 5′-cagcatgtggttccttctgg-3′ and sequenced using the nested primers: F: 5′-ggatgtccctggtcctgac-3′, R: 5′-ctcacacttcggctcagacaca-3′. Each animal's body weight was monitored twice weekly. Serum IGF-I and GH levels of adult male *lit/lit* and *lit/+* mice were performed once before and once at 2 weeks after castration using a mouse/rat IGF-I immunoassay (R&D Systems, Minneapolis, MN) and a mouse/rat GH ELISA (Millipore, Billerica, MA) following manufacturer's instructions.

PCa Xenograft Growth

1×10^6 LNCaP or PC-3 cells were suspended in 0.1 ml Matrigel (Becton Dickinson Labware, Franklin Lakes, NJ) and were inoculated subcutaneously in the flank region of adult male C57BL/6 $Prkdc^{scid/scid}$ $Ghrhr^{lit/lit}$ and $Ghrhr^{lit/+}$ littermates. To assess whether suppressed GH/IGF-I levels affected androgen-responsive growth of LNCaP xenografts, we monitored tumor size and serum PSA levels of tumor-bearing *lit/lit* and *lit/+* mice for up to 7 weeks after xenografts were established ($>100\ mm^3$). To assess the impact of GH/IGF-I levels on CR growth, once serum PSA levels of LNCaP xenograft-bearing mice exceeded 50 ng/ml, mice were castrated and subsequent CR growth and serum PSA accumulation were monitored for up to 6 weeks post-castration. To assess whether suppressed GH/IGF-I levels impacted growth of AI PC3 xenografts, tumor size was measured for up to 4.5 weeks post-inoculation.

Tumor volume measurements were performed twice weekly and calculated by the formula: length $\times$ width $\times$ depth $\times$ 0.5236. Tumor growth rate was calculated for each animal by linear regression analysis. In parallel, serum PSA measurements were performed in LNCaP tumor-bearing *lit/lit* and *lit/+* mice by enzymatic immunoassay (Abbott IMX, Montreal, Quebec, Canada) from blood samples collected from tail vein incisions and the serum PSA accumulation rates were calculated for each animal by linear regression. While serum PSA levels are typically proportional to LNCaP tumor load [31], male *lit/lit* mice are less than half the size of their heterozygous littermates (Fig. 1A). In mice ''giant'' and ''dwarf'' due to gain and loss of GH activity, circulatory volume is proportional to body mass [32]. Therefore, circulating PSA levels were normalized to animal mass as a surrogate for differences in circulatory system volume between *lit/lit* and *lit/+* mice.

In Vitro Cell Proliferation Assay

To assess PCa growth in *lit/lit* and *lit/+* serum, 2.5×10^4 LNCaP cells were seeded in 24-well plates in RPMI + 10% FBS and 2×10^3 PC3 cells were seeded in 96-well plates in DMEM + 10% FBS and allowed to adhere for 24 hr. Respective media were then changed to that supplemented with 1% serum from *lit/lit* or *lit/+* mice, and the cells were cultured for 2, 3, and 4 days. Growth factor rescue was assessed by determination of mitotic activity of cells cultured for 3 days in their respective media + 1% *lit/lit* mouse serum supplemented with 1, 10, and 50 ng/ml recombinant GH (R&D Systems) or 5 and 50 ng/ml recombinant IGF-I (Calbiochem, San Diego, CA). Briefly, cells were pulse-labeled for 2 hr with 10 μM BrdU at the end of each time point. BrdU incorporation was measured by labeling denatured DNA in fixed cells with a peroxidase-coupled anti-BrdU-antibody, and incubation with peroxidase substrate (tetramethylbenzidine) for 30 min. Optical density at 370 nm was measured on a microculture plate reader (Bio-Tek Instruments, Inc., Winooski, VT). Each assay was performed in triplicate.

Western Blot Analysis

In order to assess the impact of culturing in *lit/lit* and *lit/+* mouse serum on steady-state IGF-1R, AKT, and ERK1/2 signaling, 2.4×10^6 LNCaP cells and 1.8×10^6 PC3 cells were seeded in 10 cm dishes in standard culture media and allowed to attach overnight. Cells were then cultured in 1% *lit/lit* or 1% *lit/+* mouse serum for 3 days (LNCaP) or 4 days (PC3). To assess the impact of GH and IGF-I treatment on AKT and ERK1/2 signaling in cells cultured in 1% *lit/lit* mouse serum, cells seeded as above were cultured 24 hr in serum-free media, washed, and then stimulated with media containing 1% *lit/lit* serum $\pm$ 1 and 10 ng/ml recombinant GH, or $\pm$ 5 and 50 ng/ml recombinant IGF-I for 20 min. Whole cell protein lysates were prepared in RIPA buffer (50 mM Tris–HCl, pH 7.2, 1% NP-40, 0.1% deoxycholate, 0.1% SDS, 100 mM NaCl, 1× Roche complete protease inhibitor cocktail), and quantified using a BCA assay (Pierce, Rockford, IL).

Samples containing equal amounts of whole cell lysate protein (30 μg) were subjected to SDS–PAGE, transferred to nitrocellulose filters, blocked in PBS containing 5% nonfat milk powder for 1 hr, and incubated at 4°C overnight with primary antibody (Ab): anti-IGF-1Rβ subunit rabbit monoclonal antibody (MAb), anti-phospho-IGF-1Rβ subunit rabbit MAb, anti-vinculin mouse MAb antibody (Sigma Chemical Co., St. Louis, MO), anti-phospho-AKT (S473) rabbit polyclonal antibody (PAb), anti-AKT rabbit PAb, anti-phospho-ERK1/2 (T202/Y204) rabbit PAb, anti-ERK1/2 rabbit PAb. All antibodies were from Cell Signaling

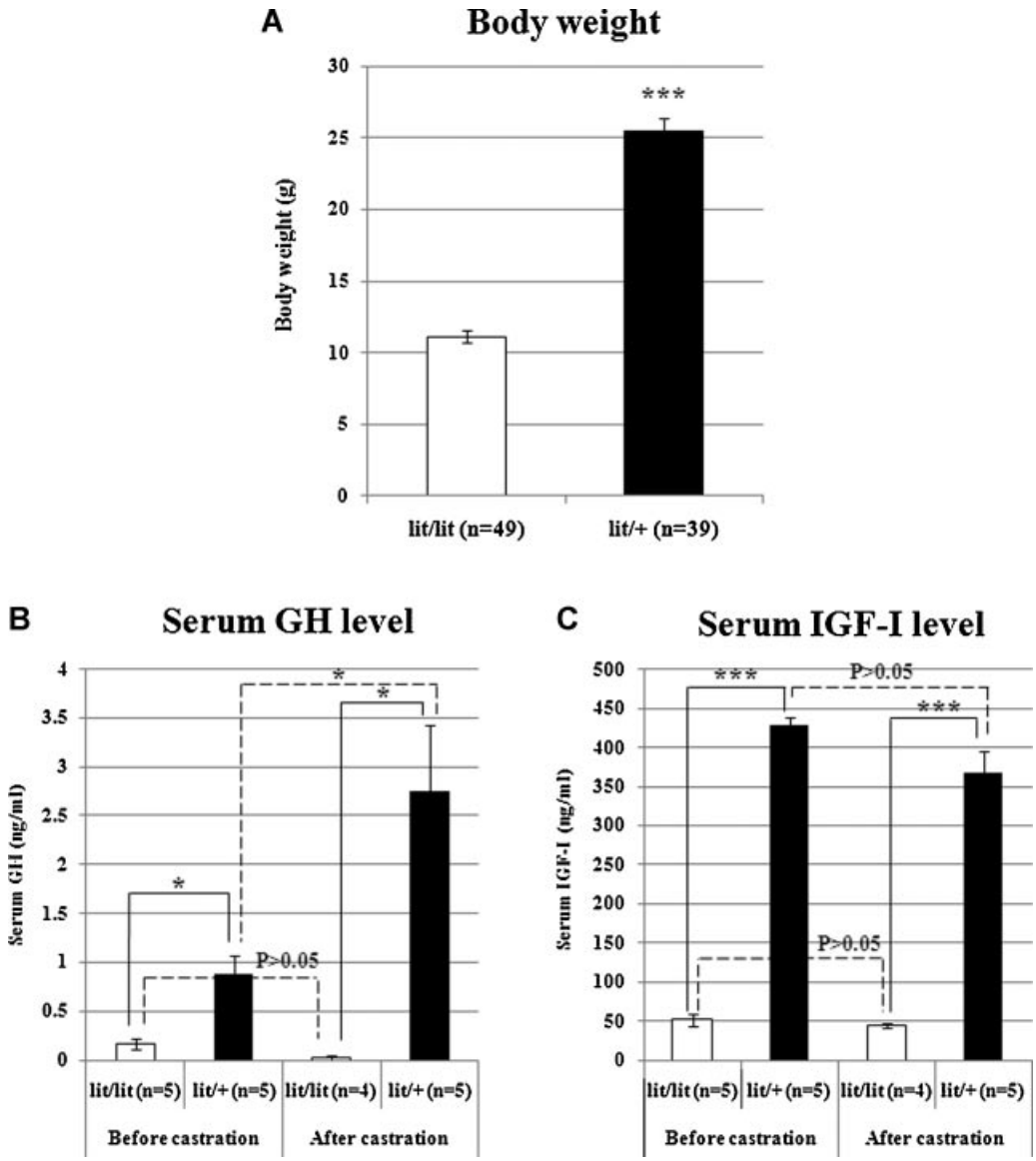

Fig. 1. Comparison of *lit*/*lit* and *lit*/+ male mouse body weight, and serum GH and IGF-I level before and after castration. Body weight of the mice over the course of the xenograft studies did not vary significantly. The average weight of each mouse was included once in the calculation of mean ± SEM for 49 *lit*/*lit* (open bar) and 39 *lit*/+ (filled bar) mice used in xenograft studies (**A**). Serum GH (**B**) and IGF-I (**C**) level were measured in *lit*/*lit* (open bar) and *lit*/+ (filled bar) mice before castration and again 2 weeks after castration (mean ± SEM; $*P < 0.05$ and $***P < 0.001$ by Student's *t*-test).

Technology (Danvers, MA) unless otherwise indicated. The filters were then incubated for 1 hr with horseradish peroxide-conjugated anti-rabbit or anti-mouse IgG antibody (Santa Cruz), and immunoreactive proteins were detected using an enhanced chemiluminescence Western blotting analysis system (Amersham Life Science, Arlington Heights, IL). Band intensities were integrated for at least duplicate immunoblot assays and the phospho/total ratio of each antibody pair was calculated to assess difference in activation state of the respective intracellular signaling factors of cells in the culture conditions described in the legends for Figures 3D and 4C,D.

Statistical Analysis

Statistical comparison of results was performed by Student's *t*-test as indicated in the figure legends.

RESULTS

Body Weights, Serum GH, and IGF-I Level Are Reduced in Male *lit*/*lit* Compared to *lit*/+ SCID mice

Due to deficiency in Ghrhr, *lit*/*lit* mice are less than half the size of their *lit*/+ littermates (Fig. 1A). The Ghrhr deficiency also severely suppresses GH and IGF-I

production. Serum GH level of *lit/lit* mice was 23% and 1.8% than that observed of *lit/+* mice pre- and post-castration, respectively [serum GH ng/ml (mean ± SEM) 0.2 ± 0.04 vs. 0.87 ± 0.19, respectively (pre-castration), 0.05 ± 0.03 vs. 2.83 ± 0.64, respectively (post-castration), $P < 0.05$] (Fig. 1B). This difference was due primarily to the significant elevation in serum GH levels in *lit/+* mice post-castration ($P < 0.05$). This change in GH level in castrated *lit/+* mice did not appear to influence serum IGF-I levels as in both pre- and post-castrated cohorts, serum IGF-I level of *lit/lit* mice was ~12% than that observed of *lit/+* mice [serum IGF-I ng/ml (mean ± SEM) 51.8 ± 7.7 vs. 427.8 ± 11.7, respectively (pre-castration), 44.5 ± 3.2 vs. 366.8 ± 27.5, respectively (post-castration), $P < 0.001$] (Fig. 1C). While castration resulted in an apparent 14% decrease in serum IGF-I levels in both *lit/lit* mice and *lit/+* mice, this variance was not deemed statistically significant ($P > 0.05$). It is also noted that circulating androgen levels are indistinguishable in male *lit/lit* and *lit/+* littermates [14]. These features make this mouse strain ideal for determining whether androgen-responsive growth, CR progression, and AI expansion of PCa can be influenced by host GH/IGF-I levels. We therefore assessed xenograft growth of the androgen-responsive human PCa model, LNCaP, in intact *lit/lit* mice and compared their androgen-responsive growth rates to that in *lit/+* littermates.

LNCaP Xenografts Exhibit Reduced Growth and CR Progression in *lit/lit* Mice

Tumor size and serum PSA levels in intact tumor-bearing mice were monitored to compare growth properties in *lit/lit* and *lit/+* mice. In a second cohort, CR LNCaP xenograft growth was assessed. Once serum PSA levels exceeded 50 ng/ml, mice were castrated and subsequent CR tumor growth and serum PSA accumulation were monitored for up to 6 weeks after castration. Primary data for actual tumor size and serum PSA levels of LNCaP xenografts in intact *lit/lit* mice and *lit/+* mice and calculation of tumor growth and PSA accumulation rates for each animal of the LNCaP xenograft cohorts are presented in the Supplemental Data (Fig. S1A,B and Tables S1A, S2A, and S3A, respectively). In intact mice, the averaged androgen-responsive LNCaP xenograft growth was linear in *lit/lit* and *lit/+* mice ($r^2 = 0.949$ and 0.9027, respectively); however, the growth rate of LNCaP xenografts in *lit/lit* mice was reduced by 40% as compared to that in *lit/+* mice [tumor growth rate, mm^3/week (mean ± SEM) 56.0 ± 8.5 vs. 93.2 ± 16.3, $P < 0.05$, respectively] (Fig. 2A). Similarly, when normalized to animal mass as a surrogate for differences in circulatory system volume, serum PSA accumulation rates in LNCaP-xenograft bearing *lit/lit* mice was significantly lower than in the *lit/+* cohort [serum PSA accumulation, ng/week (mean ± SEM) 366.5 ± 96.7 vs. 1592.7 ± 687.3, $P < 0.05$, respectively] (Fig. 2B).

In order to assess if CR progression of LNCaP xenografts is also affected in *lit/lit* mice, tumor growth and serum PSA accumulation rates were monitored following castration. Actual tumor size (mm^3) of LNCaP xenografts in castrated *lit/lit* mice and *lit/+* mice is provided in the Supplemental Data Figure S1C,D. Tumor growth rate (mm^3/week) of each animal of LNCaP xenografts in castrated mice, serum PSA level (ng/ml) of LNCaP xenografts in castrated mice, and serum PSA accumulation in castrated mice are presented in the Supplemental Data (Tables S1B, S2B, and S3B, respectively). Growth rate of LNCaP xenografts in castrated *lit/lit* mice was 25% to that observed in *lit/+* mice [tumor growth rate, mm^3/week (mean ± SEM) 41.0 ± 10.3 vs. 167.8 ± 59.9, $P < 0.05$, respectively] (Fig. 2C). This dramatic repression of CR growth in *lit/lit* mice was paralleled by a similar decrease in serum PSA accumulation rate which in *lit/lit* mice, was 13% of that observed in *lit/+* mice [serum PSA accumulation, ng/week (mean ± SEM) 110.1 ± 33.4 vs. 871.8 ± 370.6, $P < 0.05$, respectively] (Fig. 2D). These results indicate that the GH/IGF-I axis is an important stimulator of both androgen-responsive tumor growth and CR progression of LNCaP xenografts.

PC3 Xenografts Exhibit Reduced Growth in *lit/lit* mice

To determine whether suppressed CR growth of LNCaP xenografts was mirrored by another PTEN-deficient, AI xenograft model, we assessed xenograft growth of the androgen receptor-negative PCa cell line, PC3, in *lit/lit* and *lit/+* mice. Actual tumor size of PC3 xenografts in *lit/lit* mice and *lit/+* mice is presented in the Supplemental Data (Fig. S1E,F). Calculation of tumor growth rate (mm^3/week) for each PC3 xenograft is presented in the Supplemental Data (Table S1C). Subcutaneous growth of the averaged PC3 xenografts was linear in *lit/lit* and *lit/+* mice ($r^2 = 0.9643$ and 0.9226, respectively); however, the growth rate of PC3 xenografts in *lit/lit* mice was approximately half that of observed in *lit/+* mice [tumor growth rate, mm^3/week (mean ± SEM) 234.5 ± 24.1 vs. 441.6 ± 42.4, $P < 0.001$, respectively] (Fig. 2E). These results corroborate the result of LNCaP xenograft experiments after castration and demonstrate that GH/IGF-I axis is an important contributor to CR progression and AI expansion of PTEN-null PCa.

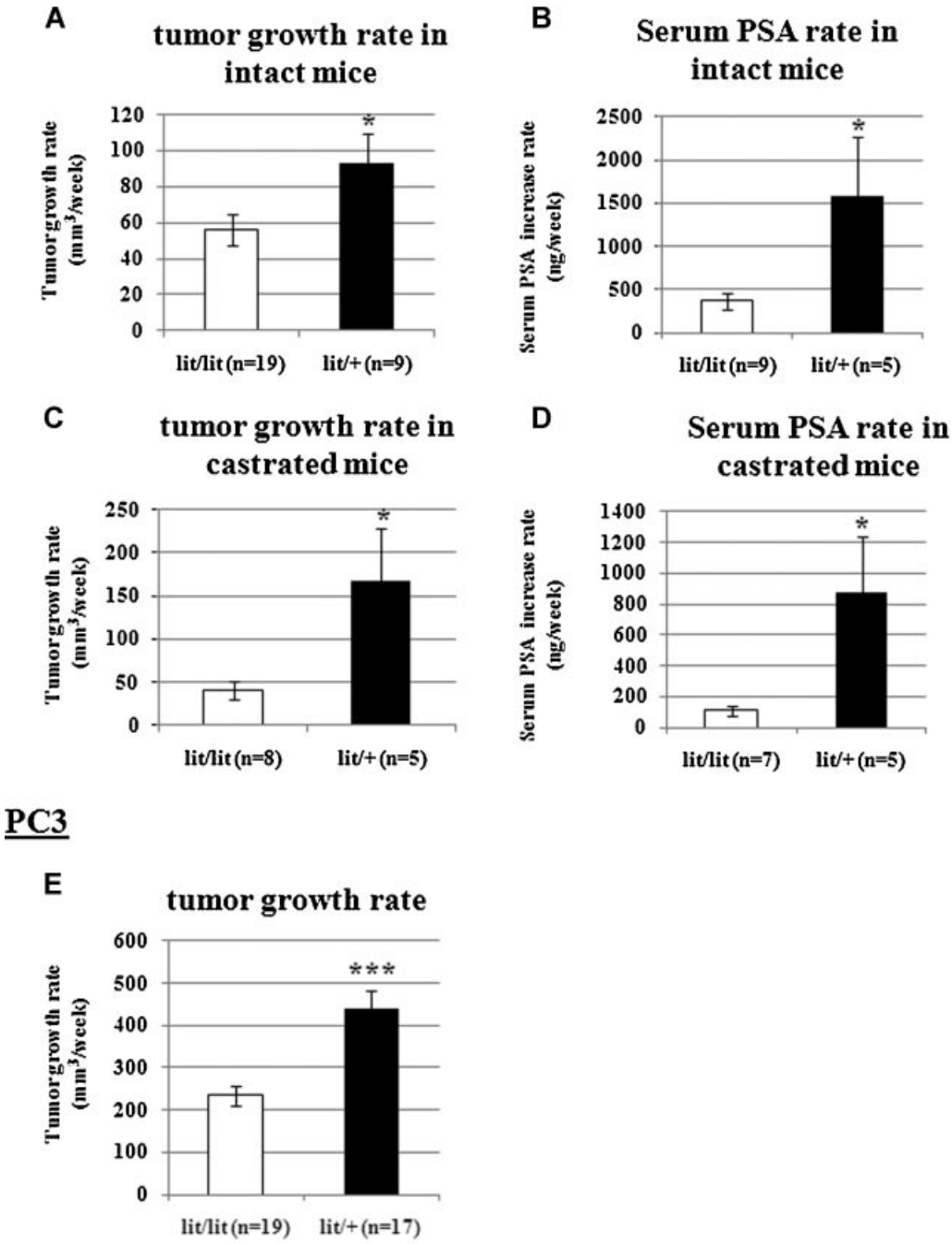

Fig. 2. Tumor growth rate of LNCaP and PC3 xenografts and serum PSA accumulation of LNCaP xenografts in *lit*/+ mice and *lit*/*lit* mice. LNCaP (**A–D**) or PC3 (**E**) xenografts were established in the flanks of *lit*/*lit* (open bars) and *lit*/+ (filled bar) mice. Tumor growth rates and serum PSA accumulation rates were measured in intact (A,B,E) and castrated (C,D) mice as described in Materials and Methods and Supplemental Data Figure S1 and Tables S1–S3. Panels A, C, and E express mean xenograft growth rates (mm^3/week) ± SEM. Panels B and D express mean PSA accumulation rates (ng/mouse/week) ± SEM; $^*P < 0.05$ and $^{***}P < 0.001$ by Student's *t*-test.

In Vitro Growth of LNCaP and PC3 Cells Is Suppressed in Media Supplemented With *lit/lit* Serum and Is Correlated With Decreased Steady-State IGF-IR Signaling

In parallel to the xenograft experiments suggesting that low circulating GH and IGF-I levels significantly suppress PCa growth, we analyzed in vitro growth of LNCaP and PC3 cells in media supplemented with *lit/lit* or *lit/+* mouse serum for 48–96 hr (Fig. 3). Both LNCaP and PC3 cells exhibited slower growth in *lit/lit* serum than in *lit/+* serum. For LNCaP cells, difference in proliferation was observed by day 2 in which cells in *lit/+* serum had expanded ~30% more than in *lit/lit*

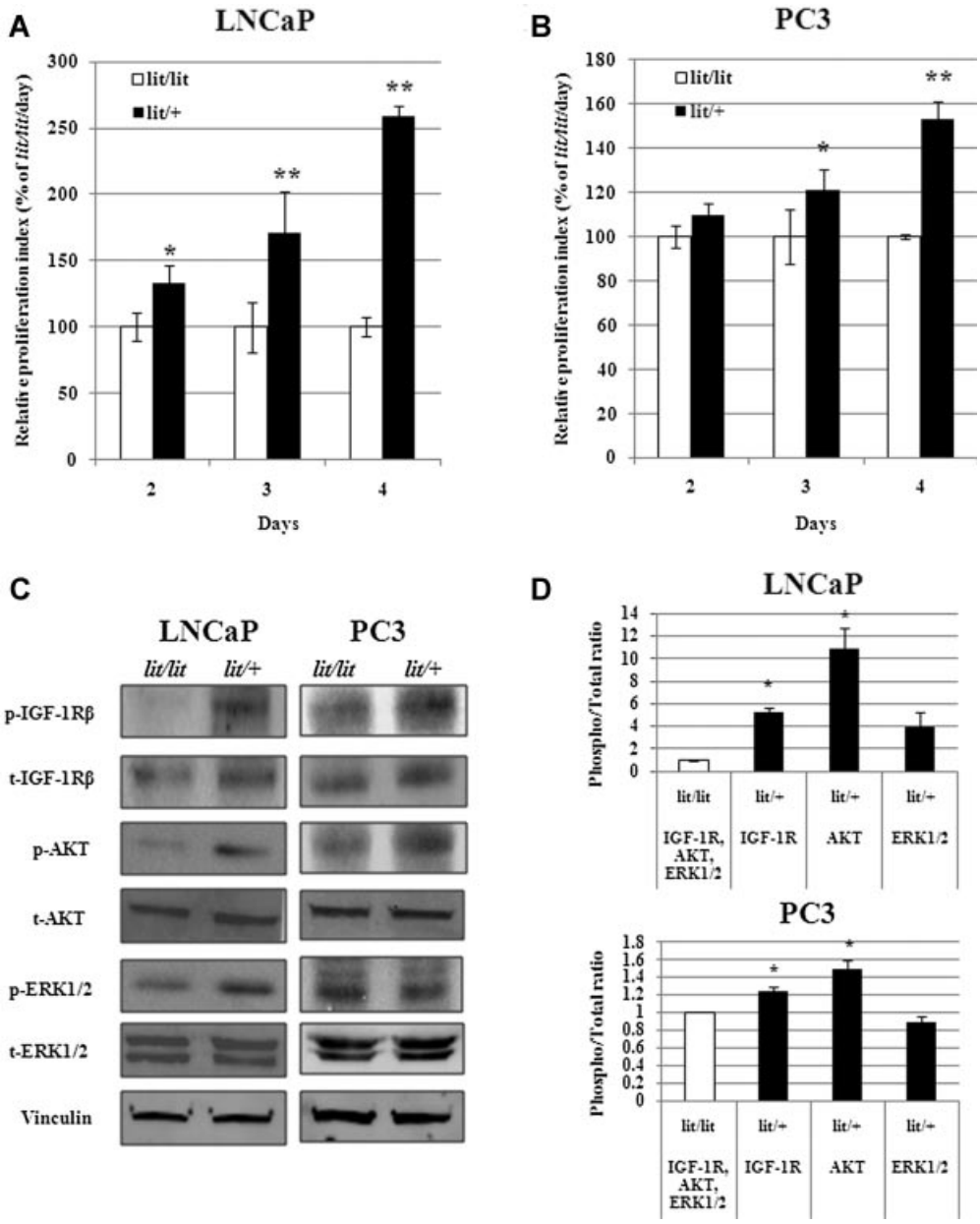

Fig. 3. LNCaP and PC3 cell in vitro proliferation and activation of IGF-I signaling in *lit/+* and *lit/lit* serum. LNCaP cells (**A**) and PC3 cells (**B**) were cultured in basal media + 1% *lit/lit* serum or 1% *lit/+* serum. Relative proliferation was examined by BrdU assay at days 2, 3, and 4 as described in Materials and Methods. Data are expressed as fold-change in proliferation in *lit/+* serum normalized to that in *lit/lit* serum at each day (mean ± SD, n = 3; $^*P < 0.05$ and $^{**}P < 0.01$ by Student's *t*-test). **C**: Whole cell lysates (30 mg) from LNCaP and PC3 cells were collected after culture in 1% *lit/lit* serum or 1% *lit/+* serum for 3 and 4 days, respectively, and subjected to immunoblotting against p-IGF-1Rβ, total IGF-1Rβ, p-AKT, total AKT, p-ERK1/2, total ERK1/2, and vinculin with antibodies as described in the Materials and Methods Section. **D**: Phospho/total ratio of IGF-1R, AKT, and ERK1/2 was calculated by densitometric quantification of three independent immunoblots performed as in C. Data are expressed as fold-change of phospho/total ratio normalized to that in *lit/lit* serum (mean ± SEM; $^*P < 0.05$ by Student's *t*-test).

serum. This difference in proliferation continued to increase such that cells in *lit*/+ serum had expanded ~60% and >100% more than cells in *lit*/*lit* serum at days 3 and 4, respectively (Fig. 3A). For PC3 cells, although differences in proliferation trended higher at day 2, a significant difference in proliferation was not observed until days 3 and 4 where cells cultured in *lit*/+ serum had expanded ~20% and ~50% more than cells in *lit*/*lit* serum (Fig. 3B).

We next assessed whether differences in growth of cells in *lit*/*lit* or *lit*/+ serum correlated with steady-state activation of key IGF axis signaling molecules by immunoblotting for specific activation-state phosphorylation site levels as compared to total cognate protein levels (Fig. 3C). In LNCaP cells, steady-state IGF-1R activation was essentially undetectable when cultured in *lit*/*lit* serum but was substantially elevated when cultured in *lit*/+ serum. Similarly, steady-state activation of AKT and ERK1/2 was low in cells cultured in *lit*/*lit* serum and substantially increased in cells cultured in *lit*/+ serum (Fig. 3C). Phospho/total ratio of IGF-1R and AKT of LNCaP cells cultured in *lit*/+ serum exhibited significantly higher as compared in *lit*/*lit* serum (5.3- and 10.9-fold, respectively; Fig. 3D, upper panel). In contrast, IGF-1R activation and phospho-AKT and phospho-ERK1/2 levels were readily detectable in PC3 cells cultured in *lit*/*lit* serum. While no difference in ERK1/2 activation was observed in PC3 cells cultured in *lit*/+ serum, modest, but significant reproducible increases in steady-state phospho-IGF-1R and phospho-AKT levels were observed (1.25- and 1.5-fold, respectively; Fig. 3C,D, lower panel). Together, these in vitro results support the contention that the GH/IGF axis signaling contributes the proliferation of both androgen-responsive and AI PTEN-null PCa cells through the activation of PI3K/AKT and ERK1/2 pathways.

Suppressed Growth of LNCaP and PC3 Cells in *lit*/*lit* Serum Can Be Rescued by the Addition of GH or IGF-I

The above results indicate that suppressed proliferation of LNCaP and PC3 cells in *lit*/*lit* serum is correlated with decreased IGF-1R signaling. We therefore tested whether suppressed growth in *lit*/*lit* serum could be rescued by reconstitution with GH or IGF-I (Fig. 4). No distinguishable change in proliferation was observed in either cell line when cultured in *lit*/*lit* serum supplemented with 1 ng/ml GH and while LNCaP cell proliferation was almost doubled in *lit*/*lit* serum supplemented with 10 ng/ml GH; PC3 cell proliferation was only enhanced 10% under this condition. At 50 ng/ml, increased LNCaP proliferation was still observed; however, PC3 proliferation was again indistinguishable from the unsupplemented control. Because the increased proliferation ratio of GH-supplemented LNCaP and PC3 cells using 50 ng/ml was less than that observed using 10 ng/ml, we conclude that GH-responsive growth of these cell lines is biphasic with a maximum sensitivity of ~10 ng/ml. In contrast, LNCaP and PC3 cells exhibited dose-dependent increased proliferation when cultured in *lit*/*lit* serum supplemented with IGF-I. In LNCaP cells, IGF-I increased proliferation 2- and 3.4-fold at 5 and 50 ng/ml, respectively, while PC3 cells exhibited more modest increases in proliferation reaching 1.25-fold in *lit*/*lit* serum supplemented with 50 ng/ml IGF-I (Fig. 4A,B).

In order to determine how GH and IGF-I supplementation of *lit*/*lit* serum affected AKT and ERK1/2 activation, we performed immunoblotting of lysates from cells serum starved and acutely stimulated with *lit*/*lit* serum ± GH or IGF-I. Stimulation of LNCaP and PC3 cells with *lit*/*lit* serum supplemented with GH (10 ng/ml) increased AKT activation (2.8- and 1.7-fold, respectively) while no change in ERK1/2 activation was detected. Addition of 50 ng/ml IGF-I to *lit*/*lit* serum increased activation of AKT and ERK1/2 in LNCaP cells (22- and 15-fold, respectively), but only resulted in 1.3-fold increased AKT activation in PC3 cells (Fig. 4C,D). These results indicate that decreased circulating GH and IGF-I are significant contributors to suppressed growth of both androgen-responsive and AI PTEN-null PCa cells in *lit*/*lit* hosts. GH-stimulated growth and AKT activation were only observed at levels 3- to 10-fold above that observed in *lit*/+ circulation post- and pre-castration, respectively. In contrast, IGF-I-stimulated growth and increased AKT and (in LNCaP) ERK1/2 activation at doses equivalent to that observed in *lit*/*lit* serum and well within the concentration observed in *lit*/+ serum. We therefore conclude that decreased IGF-I availability and signaling from IGF-1R to AKT is the primary missing growth stimulatory pathway for PTEN-deficient PCa cells in the *lit*/*lit* host.

DISCUSSION

Numerous studies implicate the GH/IGF-I axis as an important regulator of growth, survival, and metastatic potential in a variety of malignancies and it is strongly implicated in PCa etiology [9,10]. Perturbations in expression of GH/IGF-I axis components are implicated in susceptibility and progression of PCa [33]. IGF-I is a potent mitogen that regulates proliferation and survival of PCa cells. High serum IGF-I levels predispose to PCa [7,11,34,35] and elevated IGF-1R expression is observed in metastatic and CR PCa [4,8,36]. Furthermore, maintaining IGF-I responsive-

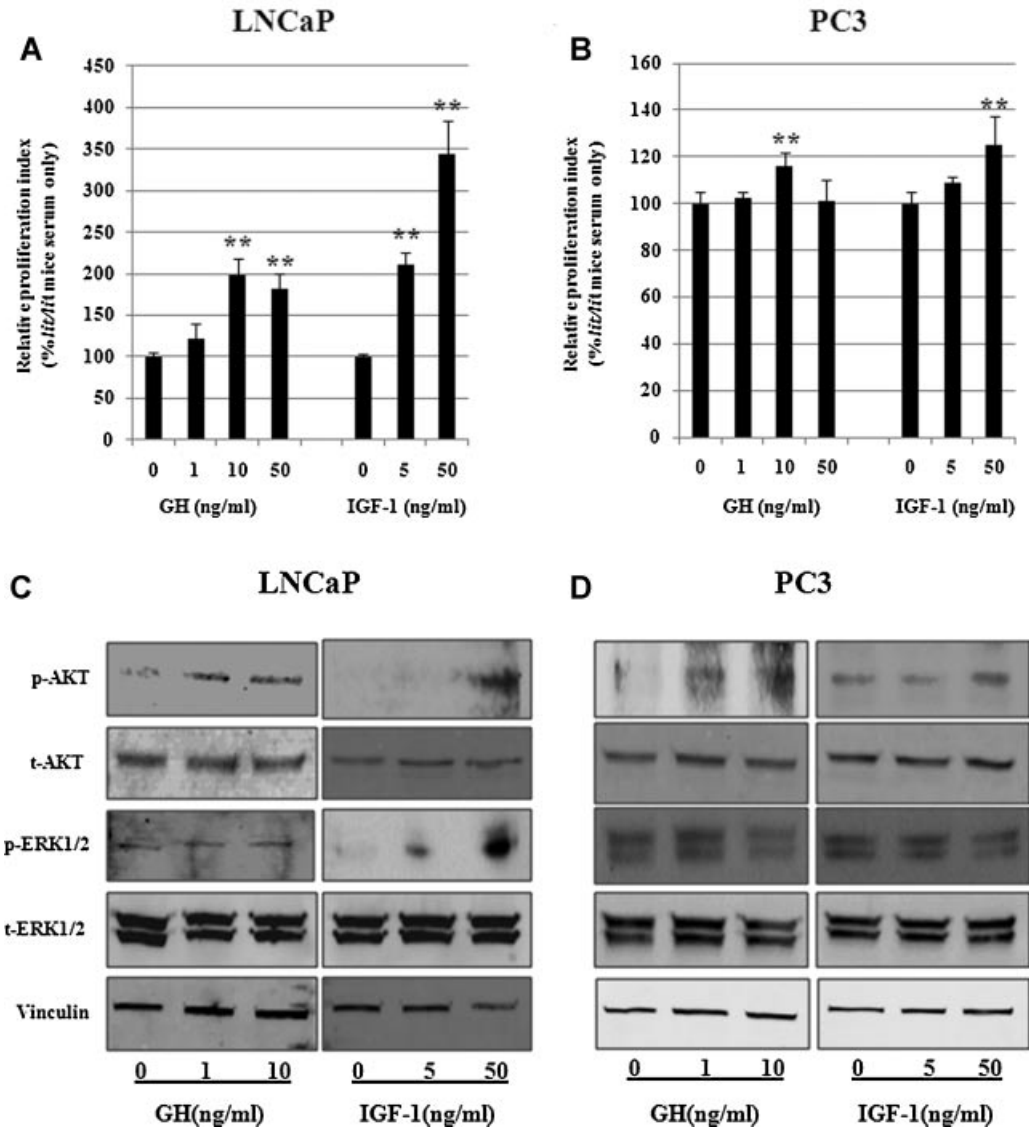

Fig. 4. LNCaP and PC3 cell in vitro proliferation and activation of AKT and ERK1/2 signaling in *lit*/*lit* serum rescued by recombinant GH and IGF-I. LNCaP cells (**A**) and PC3 cells (**B**) were cultured in 1% *lit*/*lit* serum ± recombinant GH (1 or 10 or 50 ng/ml) or recombinant IGF-I (5 or 50 ng/ml) for 3 days. Relative proliferation was measured by BrdU incorporation as in Fig. 3 and expressed relative to that of cells cultured without addition of exogenous growth factors (mean ± SD, n = 3; $^{**}P < 0.01$ by Student's *t*-test). Whole cell lysates (30 mg) from LNCaP (**C**) and PC3 cells (**D**) cultured 24 hr in serum-free media and stimulated with 1% *lit*/*lit* serum + 0, 1, 10 ng/ml GH, or + 0, 5, 50 ng/ml IGF-I for 20 min were subjected to immunoblotting against p-AKT, total AKT, p-ERK1/2, total ERK1/2, and vinculin with antibodies as described in Materials and Methods.

ness facilitates PCa survival and growth and is achieved through androgen-modulated IGF-1R expression [4,37]. However, there are reports in which no association between circulating levels of IGF-I and the risk of PCa were found [38–40]. It therefore remains to be determined to what extent circulating IGF-I affects PCa progression.

The dwarf *little* (*lit*) mouse carries a recessive, autosomally inherited point mutation in Ghrhr analogous to one observed in human dwarfism that results in a growth defect that models the human growth disorder termed, isolated GH deficiency type 1 [41]. Sarcoma and breast cancer xenografts develop more slowly in *little* murine hosts [15,16]. Furthermore, crosses with the TRAMP murine PCa model demonstrated that the *lit* mutation significantly reduced the percentage of the prostate gland showing neoplastic changes at 35 weeks of age [6]. These studies show *lit* as an example of a germline mutation that reduces carcinogenic potential of several malignancies. While these studies implied a role of IGF in carcinogenic potential, Anzo et al. [42] used a liver-deficient IGF-I

mouse model cross with TRAMP to indicate that the direct action of GH might be important for the development of PCa.

To determine whether androgen-responsive growth, CR progression, and AI expansion of human PCa xenografts are influenced by the host GH/IGF-I axis, we carried out in vivo growth studies using Nod/SCID *lit/lit* and *lit/+* mice. In agreement with previous studies examining characteristics of *lit/+* mice and *lit/lit* mice, body weights, serum GH, and IGF-I level were reduced in male *lit/lit* mice and castration did not significantly alter IGF-I levels in *lit/lit* mice. Intriguingly, castration of *lit/+* mice resulted in elevated serum GH levels. Although castration is not generally thought to alter GH production, estradiol and androgens appear to promote GH release [43,44]. Since castration of *lit/+* mice did not significantly alter serum IGF levels, the ~3.5-fold increase in GH level was apparently insufficient to alter systemic IGF-I production in the *lit/+* host. How castration might promote GH production/release in animals with a heterozygous Ghrhr deficiency remains to be determined.

Tumor growth and PSA accumulation rate results from LNCaP xenografts in intact mice indicated that circulating GH and IGF-I significantly contributes to androgen-responsive PCa tumor growth. Furthermore, growth and PSA accumulation in castrated mice harboring LNCaP xenografts strongly implicate circulating GH and IGF-I in CR PCa tumor progression, while curtailed xenograft growth of PC3 cells in *lit/lit* hosts indicates that the GH/IGF-I axis also influences growth of AI PCa tumors. While a recent report indicated that PI3K activation in PTEN-deficient PCa cells can be driven by receptor tyrosine kinase-independent p110β and p110δ activation [45], we conclude from results reported here that in vivo and in vitro growth of these two PTEN-deficient PCa models can be substantially augmented by extrinsic growth factor signaling.

In order to test the conclusion that the GH/IGF-I axis affects PCa progression, we compared the ability of serum from *lit/lit* and *lit/+* mice to support growth of LNCaP and PC3 cells in vitro. Consistent with reduced xenograft growth was our finding that serum from *lit/lit* mice was less mitogenic for LNCaP and PC3 cells than serum from *lit/+* mice and that suppressed growth in *lit/lit* serum could be reversed by adding recombinant GH or recombinant IGF-I. Addition of GH to *lit/lit* serum produced a relatively modest, biphasic increased proliferation of LNCaP and PC3 cells and required a GH concentration 3- to 10-fold higher than that observed in *lit/+* serum post- and pre-castration. In contrast, addition of IGF-I to *lit/lit* serum produced a dose-dependent increase in proliferation rates equivalent to that observed for cells grown in *lit/+* serum. The maximum IGF-I concentration tested (50 ng/ml) is the threshold IGF-I level observed in *lit/lit* serum and 1/8th the concentration observed in *lit/+* serum. These ''rescue trials'' suggested that in the Ghrhr mutant background, circulating IGF-I may be the more important stimulator for PCa progression.

While recombinant IGF-I was more effective at stimulating growth and survival signaling in LNCaP and PC3 cells in *lit/lit* mice serum than was recombinant GH, a role for circulating GH cannot be ruled out. Development of TRAMP tumors in animals with low circulating IGF-I levels but elevated circulating GH levels are indistinguishable from controls [42]. GH receptor (GHR) has been shown to be expressed in normal rat prostate and in several rat and human PCa cell lines, as well as in human prostate tissue [46]. While we did not detect significant expression of GHR in LNCaP and PC3 cells by immunoblotting techniques (data not shown), the recombinant GH ''rescue'' results imply that LNCaP and PC3 can respond to physiologically relevant GH concentrations by enhancing proliferation in *lit/lit* serum. Together, these in vivo and in vitro data strongly implicate a direct association between PCa risk and GH/IGF-I axis, especially circulating GH and IGF-I.

Though multiple pathways concerning cell growth and survival are stimulated by activation of the IGF-1R axis, the Ras/ERK1/2 and PI3K/AKT pathways play the most prominent roles [47]. We have previously reported that increased ERK1/2 activation does not mediate growth and survival responses to IGF-I [4]. Consistent with these previous results, in this study, steady-state AKT activation was much more pronounced in cells grown in *lit/+* versus *lit/lit* mice serum in both LNCaP and PC3 cells than was steady-state ERK1/2 activation. These findings demonstrate that GH/IGF-I axis stimulates PCa progression mainly through PI3K/AKT pathway. Although GH has been shown to induce time- and dose-dependent phosphorylation of AKT and ERK1/2 [46], in studies here using *lit/lit* serum, IGF-I stimulation induced more pronounced activation of AKT and ERK1/2 in LNCaP cells and only activation of AKT in PC3 cells while GH only marginally activated AKT in both cell lines. We therefore conclude that circulating IGF-I strongly contributes to the proliferation of androgen-responsive growth, CR progression, and AI expansion of PCa as compared with circulating GH, and that this effect is mediated mainly through PI3K/AKT activation.

Differences in response of LNCaP and PC3 cells to IGF-I supplemented *lit/lit* serum in vitro are complicated by differences in IGF-II and IGF binding protein (IGFBP) production by these cell lines. PC3 cells produce, and are responsive to, IGF-II [48]. Such autocrine signaling may be responsible for the elevated

steady-state IGF-1R, AKT, and ERK1/2 activation observed in PC3 cells cultured in *lit/lit* serum. Furthermore, physiologically, IGF availability is tightly regulated by IGFBPs. Circulating IGF-I is sequestered in complex with IGFBP-3/ALS and can be captured and delivered to target tissues by locally high concentrations of other IGFBPs, such as IGFBP-2 and -5 and the action of IGFBP proteases [49]. We have quantified conditioned media IGFBP2 and IGFBP5 levels for LNCaP and PC3 cells grown under conditions described for the proliferation assays of Figure 4A,B (Supplemental Data Materials and Methods and Fig. S2A,B). Under these conditions, LNCaP cells produced 446 ng/ml/cell of IGFBP2, while PC3 cells produced 1.7 ng/ml/cell of IGFBP5. Therefore, when grown in *lit/lit* serum supplemented with 50 ng/ml IGF-I, LNCaP cells produced a 1.8-fold molar excess of IGFBP-2, while PC3 cells were subjected to IGF-I at a 128-fold molar excess to IGFBP-5. This difference in presentation of potentially IGFBP-bound versus IGFBP-free IGF-I and the resulting chronic versus acute IGF-I exposure may account for the increased responsiveness of the LNCaP cells to growth in IGF-I-supplemented *lit/lit* serum.

To assess whether androgen stimulates PCa progression via GH/IGF-I axis, we performed in vitro study using 1% *lit/lit* and *lit/+* mice serum ± androgen in LNCaP and PC3 cells (Supplemental Data Fig. S3). LNCaP cells cultured in *lit/lit* and *lit/+* mice serum supplemented with 1 nM R1881 exhibited significantly increased proliferation (2.2- and 1.5-fold, respectively). Androgen-receptor negative, PC3 cells exhibited no difference in growth when cultured in media supplemented with 1 nM R1881. The increased growth of LNCaP cells in 1% mouse serum supplemented with 1 nM R1881 is in agreement with established response of these cells to androgen stimulation in vitro. While we have previously reported that androgens enhance IGF-1R expression to promote protection from apoptotic stress in LNCaP cells [4], since the response to R1881 in *lit/lit* and *lit/+* serum is essentially indistinguishable, and since serum androgen levels in *lit/lit* mice have been reported to be indistinguishable from that of *lit/+* littermates [14], we conclude that androgen-stimulated LNCaP cell growth under these conditions is GH/IGF-I axis independent.

Various tests of in vitro and in vivo hormonal treatments that target the GH/IGF-I axis for PCa have been performed. GHRH antagonists suppress the growth of various human cancer lines as xenografts in immune-compromised mice. A series of GHRH antagonists have been shown to inhibit growth of human AI PC3 and DU145 PCa xenografts [50,51]. These GHRH antagonists are implied to exert their effects directly on tumors by blocking the action of tumoral GHRH. GHRH antagonists might interfere with mechanisms involved in CR progression of PCa and could be used clinically as agents preventing relapse in patients who have PCa and receive androgen-deprivation therapies [50,52]. Several other pituitary hormones including prolactin (PRL), GH, luteinizing hormone, and follicle stimulating hormone have also been shown to play a role in prostate growth, development, and physiological function [53,54]. GH might be involved in regulating prostate function, whether directly via tumor-expressed GHR or indirectly via the IGF-I/IGF-1R system.

IGF/IGF-1R signaling appears to play an important role in promoting PCa malignancies. We have previously reported that ATL1101, a 20-MOE-modified antisense oligonucleotide (ASO) targeting human IGF-1R on both androgen-responsive and AI PCa cell lines resulted in the potent growth inhibition and delayed AI PCa progression in vitro and in vivo [31], and that antisense targeting the IGFBPs can also suppress PCa androgen-responsive and CR growth [55,56]. Additionally, Koyama et al. [57] revealed novel interactions between the IGF system and pomegranate-induced apoptosis. A range of therapeutic approaches including reducing ligand availability by GH antagonist, IGF-I antibodies, and recombinant IGFBPs, reducing IGF-1R expression by antisense and RNA interference, or inhibiting of IGF-1R signaling by IGF-1R antibodies and small molecule tyrosine kinase inhibitors are under investigation in CR PCa. While several of these, particularly the humanized IGF-1R antibodies, are showing great promise, continued research to understand how the IGF axis functions to promote PCa growth and progression and to identify new ways to target the GH/IGF-I axis in PCa should be pursued.

CONCLUSIONS

In summary, our in vivo xenograft study indicates that circulating GH and IGF-I strongly stimulate androgen-responsive growth, CR progression, and AI expansion of PCa xenografts using a dwarf immunocompromised host. Our in vitro experiments using *lit/lit* mice serum and *lit/+* mice serum strongly supported in vivo findings and indicated that in GH/IGF-I axis, IGF-I is the leading factor that contributes to PCa progression and activates mainly the PI3K/AKT pathway and supports the continued development of clinical trials of novel hormonal treatment strategies that target the GH/IGF-I axis for PCa patients.

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