ARTICLE
Journal of Cellular Biochemistry 114:1322–1335 (2013)

Journal of Cellular Biochemistry

Differential Effects of Exogenous and Autocrine Growth Hormone on LNCaP Prostate Cancer Cell Proliferation and Survival

Alona O. Nakonechnaya, Holly S. Jefferson, Xiaofei Chen, and Brian M. Shewchuk*

Department of Biochemistry and Molecular Biology, Brody School of Medicine at East Carolina University, 5W-52 Brody Medical Sciences Bldg., 600 Moye Blvd., Greenville, North Carolina 27834

ABSTRACT

The prostate gland is regulated by multiple hormones and growth factors that may also affect prostate tumorigenesis. Growth hormone (GH) contributes to prostate development and function, but the direct effects of GH on prostate cancer cells are not well understood. The expression of endogenous GH in prostate cancer cell lines has also been observed, suggesting the potential for an effect of autocrine GH. In the present study, we measure the levels of GH and GH receptor (GHR) mRNA in multiple prostate cancer and normal prostate-derived cell lines, and compare the effects of exogenous and autocrine GH on LNCaP prostate cancer cell proliferation and apoptosis, and the associated signal transduction pathways. We found that GHR and GH expression were higher in the prostate cancer cell lines, and that exogenous GH increased LNCaP cell proliferation, but had no effect on apoptosis. In contrast, autocrine GH overexpression reduced LNCaP cell proliferation and increased apoptosis. The distinct actions of exogenous and autocrine GH were accompanied by differences in the involvement of GHR-associated signal transduction pathways, and were paralleled by an alteration in the subcellular localization of GHR, in which autocrine GH appeared to sequester GHR in the Golgi and endoplasmic reticulum. This alteration of GHR trafficking may underlie a distinct mode of GH-mediated signaling associated with the effect of autocrine GH. These findings clarify the potential effects of GH on prostate cancer cell function, and indicate that the activity of autocrine GH may be distinct from that of endocrine GH in prostate cancer cells. J. Cell. Biochem. 114: 1322–1335, 2013.

KEY WORDS: GROWTH HORMONE; PROSTATE CANCER; SIGNAL TRANSDUCTION

The development, growth, and function of the prostate gland are regulated by the actions of multiple hormones and growth factors. Elucidating the cellular effects and signaling pathways mediated by these factors is thus important for understanding the mechanisms underlying prostate tumorigenesis and cancer progression. Androgens play a dominant role in these processes and are a primary therapeutic target for controlling tumor progression in prostate cancer [Galbraith and Duchesne, 1997; Taichman et al., 2007]. Androgen depletion induces apoptosis in prostate epithelial cells, and androgen ablation therapy is an effective treatment for early stage prostate cancer. However, the effectiveness of anti-androgenic treatments is limited, as the hallmark of prostate cancer progression is the reemergence of castration-resistant tumor cells that lead to a more virulent and disseminated cancer [Wilson and Crawford, 2006; Tzelepi et al., 2011]. Thus, the actions of androgens alone are insufficient to explain all aspects of prostate tumorigenesis. Both in vitro and in vivo evidence suggest that multiple non-androgenic steroids, peptide hormones, and growth factors also affect prostate cell proliferation, function, and survival and may be involved in the progression of prostate cancer [Evangelou et al., 2004]. One class of factors that may affect prostate tumorigenesis are the multiple pituitary peptide hormones that contribute to prostate development, growth, and function, including prolactin (PRL), luteinizing hormone (LH), follicle stimulating hormone (FSH), and growth hormone (GH) [Reiter et al., 1995, 1999; Dirnhofer et al., 1998]. GH in particular is required by the male reproductive system for sexual differentiation, pubertal maturation, and normal prostate organogenesis with respect to gland lobular architecture and mass

Grant sponsor: Leo W. Jenkins Cancer Center; Grant sponsor: Brody Brothers Medical Foundation; Grant number: MT7749.

*Correspondence to: Dr. Brian M. Shewchuk, Department of Biochemistry and Molecular Biology, Brody School of Medicine at East Carolina University, 5W-52 Brody Medical Sciences Bldg., 600 Moye Blvd., Greenville, NC 27834. E-mail: shewchukb@ecu.edu

Manuscript Received: 2 August 2012; Manuscript Accepted: 27 November 2012

Accepted manuscript online in Wiley Online Library (wileyonlinelibrary.com): 13 December 2012

DOI 10.1002/jcb.24473 • © 2012 Wiley Periodicals, Inc.

[Ruan et al., 1999; Kleinberg et al., 2007], and the extensive distribution of the GH receptor (GHR) throughout the male reproductive system suggests that the effects of GH on the prostate may be direct [Lobie et al., 1990; Reiter et al., 1992, 1995; Lincoln et al., 1998].

Several lines of evidence suggest the potential for endocrine GH to affect prostate tumorigenesis in addition to normal prostate development and function. GH is the principal mediator of insulin-like growth factor-1 (IGF-1) production by the liver and peripheral tissues. IGFs have a growth stimulatory role in the prostate, and plasma IGF-1 levels may be an indicator of prostate cancer risk [Chan et al., 1998; Ozkan, 2011]. An increase in the incidence of benign prostate hyperplasia (BPH) is associated with increased GH concentrations in individuals with acromegaly [Jenkins and Besser, 2001; Clemmons, 2002], and increased endogenous GH expression was observed in prostate tissue biopsies taken from individuals with BPH that ultimately progressed to prostate cancer [Slater and Murphy, 2006]. An inverse correlation between serum GH concentration and prostate cancer incidence has also been reported [Fuhrman et al., 2005]. In direct tests of the role of GH in prostate cancer, the absence of GH signaling due to a genetic GHR deficiency abrogated prostate tumorigenesis in rat and mouse models of prostate cancer susceptibility, consistent with a contribution of the GH/IGF-1 axis to prostate tumorigenesis [Wang et al., 2005, 2008]. Taken together, these findings indicate that endocrine GH may have the potential to influence prostate cancer, but the direct effects of GH on prostate cancer cell function are not well understood. The binding of exogenous GH to GHR in the human prostate cancer cell line LNCaP and the concomitant activation of GHR-associated signal transduction pathways has been reported, but no effect of exogenous GH on LNCaP cell proliferation was observed [Weiss-Messer et al., 2004]. This report contrasted with an earlier study that demonstrated an increase in LNCaP cell proliferation upon treatment with exogenous GH [Untergasser et al., 1999], illustrating the need for clarification of the role of GH in prostate cancer cell function. The ectopic expression of GH has also been observed in prostate cancer cell lines and tissue, indicating the possibility of autocrine signaling in prostate tumors that could affect tumor progression [Chopin et al., 2002; Slater and Murphy, 2006]. However, the functional significance of autocrine GH in prostate cancer cells and the possible mechanistic distinction from the effects of endocrine GH are not clear.

In the present study, we compare the expression of GH and GHR in multiple prostate cancer cell lines with lines derived from normal prostate epithelial cells, and address the effects of exogenous hGH on the proliferation and survival of the representative GHR-expressing prostate cancer cell line LNCaP. We also test the effects of the endogenous overexpression of autocrine hGH, and resolve the signaling pathways involved in the actions of exogenous and autocrine hGH. The results described confirm the potential for endocrine hGH to function as a growth and survival factor in this cell line, but surprisingly indicate that autocrine hGH appears to have a distinct effect, which may be due to an alteration of GHR trafficking and signal transduction upon endogenous overexpression of hGH.

MATERIALS and METHODS

CELL CULTURE

RWPE-1, RWPE-2, LNCaP, 22Rv-I, and PC3 cells were purchased from American Type Culture Collection (ATCC). LNCaP, 22Rv-I, and PC3 cells were maintained in RPMI 1640 (Cellgro) supplemented with 10% FBS (Gemini Bio Products) and 1% penicillin/streptomycin/amphotericin B solution (Cellgro). RWPE-1 (normal prostate epithelial cell line) and RWPE-2 (Ki-ras transformation of RWPE-1) were maintained in Keratinocyte SFM (serum-free media, Gibco) supplemented with the supplied 50 μg/ml bovine pituitary extract and 5 ng/ml recombinant epidermal growth factor (EGF). All cell lines were incubated in humidified conditions at 37°C and 5% CO_2.

WESTERN BLOT OF hGHR AND hGH

Whole cell lysates were prepared with radioimmunoprecipitation assay (RIPA) buffer (20 mM Tris–HCl, pH 7.5, 150 mM NaCl, 1% Triton X-100, 1 mM EDTA, 1 mM EGTA) supplemented with a protease inhibitor cocktail (PIC, 1:1,000 dilution) (Sigma), followed by sonication with a Sonicator 3000 (Misonix) (process time 3 min, pulsar on 30 s, pulsar off 1 min power level 1.0) and clearing by centrifugation at 14,000 rpm for 15 min at 4°C. Twenty-five micrograms lysate was resolved on a 10% polyacrylamide (37.5:1 acrylamide:bis-acrylamide) minigel, and transferred to a PVDF membrane (Millipore) by wet electroblotting using standard techniques. The membrane was blocked with 5% nonfat dry milk in TBST (50 mM Tris–HCl, pH 7.6, 150 mM NaCl, 0.05% Tween 20), and incubated with rabbit anti-hGHR antibody raised against the intracellular domain (Santa Cruz sc-74051) in the same buffer at a 1:2,000 dilution overnight at 4°C. For hGH immunodetection, a rabbit anti-hGH antibody (provided by Gary Parlow, National Hormone and Peptide Program) was used at 1:2,000. After multiple washes in TBST, the membrane was incubated with a horseradish peroxidase-conjugated goat anti-rabbit secondary antibody (Pierce) at a 1:4,000 dilution in 5% nonfat dry milk/TBST for 2 h at room temperature. After multiple washes with TBST, immune complexes were detected by incubation with a chemiluminescent substrate (Pierce Super Signal) and exposure to autoradiographic film and phosphorimager (molecular dynamics typhoon). To control for protein loading, blots were stripped with 25 mM glycine–HCl, pH 2, 1% (w/v) SDS, washed with PBS and incubated with a mouse anti-β tubulin antibody (a gift of Fred Bertrand, East Carolina University), and the immune complexes detected with a horseradish peroxidase-conjugated goat anti-mouse secondary antibody (Pierce).

RNA PURIFICATION AND QUANTITATIVE REVERSE TRANSCRIPTION PCR (qRT-PCR) OF hGH-N AND hGHR mRNA

Total RNA was extracted from 5×10^6 cells with Trizol reagent (Invitrogen) following the manufacturer's protocol. Total RNA was digested with DNase I (New England Biolabs) according to the manufacturer's protocol and repurified by phenol–chloroform extraction and ethanol precipitation. One microgram of DNA-free RNA was reverse transcribed using the iScript cDNA synthesis kit (BioRad). Quantitative PCR was performed in 25 μl reactions using iQ SYBR Green Master Mix (BioRad). The following primers were used: *hGHR*, 5′-GCGAGAGACTTTTTCATGCC-3′ and 5′-TCAGGG-

CATTCTTTCCATTC-3′; *hGH-N*, 5′-CCTAGAGGAAGGCATCCAAA-3′ and 5′GCAGCCCGTAGTTCTTGAGTAG-3′; *hGAPDH*, 5′-GGTGCTAAGCAGTTGGTG-3′ and 5′-GGGTGTGAACCATGAGGA-3′. The qPCR temperature protocol was as follows: (1) 1×, 95°C for 3 min; (2) 40×, 95°C for 10 s followed by 55°C for 45 s; (3) 1×, 95°C for 1 min; and (4) 1×, 55°C for 1 min. Duplicate qPCR reactions yielded threshold cycle (Ct) value averages with a coefficient of variance of <0.5%, calculated following linear transformation of the Ct values. All experiments included template-free (water) and reverse transcriptase-minus controls to ensure that no contaminating templates were introduced. The hGHR and hGH cDNA levels, expressed as the average qPCR Ct value, were normalized to the control GAPDH average Ct value (hGH/hGHR Ct minus GAPDH Ct) to yield ΔCt. Relative cDNA levels were then calculated as ΔΔCt (test ΔCt minus control ΔCt), and expressed as a fold difference using the formula $2^{-\Delta\Delta Ct}$.

MTT ASSAY

Cells were plated at 5,000 cells/well in 96-well plates in 200 μl-supplemented RPMI. The following day media was replaced with 200 μl RPMI 1640 containing the indicated amount of FCS and increasing concentrations of purified hGH (provided by Gary Parlow). For experiments with the stably transfected cell clones no hGH was added. Cells were incubated for the time indicated, 22 μl of 5 mg/ml MTT solution (in PBS) was added, and the cells incubated at 37°C for 2 h. The media was removed, 200 μl DMSO was added to each well, and the absorbance at 562 nm was determined in a plate spectrophotometer.

BROMODEOXYURIDINE (BrdU) INCORPORATION ASSAY

Assays were performed using a BrdU incorporation kit (Invitrogen) according to the manufacturer's protocol. Cells were plated in 96-well plates at 20,000 cells/well in 200 μl supplemented RPMI 1640. The next day, complete media was removed, cells washed with PBS, and serum free media was applied for 24 h. For the assay of WT LNCaP cell proliferation, after 24 h serum starvation, media was removed and serum free media containing 500 ng/ml hGH or PBS together with BrdU reagent was added and the cells incubated at 37°C for indicated times. For LNCaP/EV and LNCaP/hGH cells, after 24 h serum starvation the media was replaced with fresh serum free media containing BrdU and the cells incubated at 37°C for the indicated times. For the proliferation assay with pathway inhibitors, after 24 h serum starvation the media was replaced with fresh serum free media containing the indicated pathway inhibitors for 2 h of pretreatment followed by the addition of 500 ng/ml hGH or PBS and BrdU, and the cells incubated at 37°C for the indicated times. Pathway inhibitors were U0126 (MEK1/2 inhibitor; Selleck Chemicals) at 10 μM, LY294002 (PI3K inhibitor; Selleck Chemicals) at 5 μM, and STAT5 inhibitor (Santa Cruz) at 50 μM. These conditions were verified to not affect cell viability by annexin V/propidium iodide staining and flow cytometry.

ANNEXIN V/PROPIDIUM IODIDE STAINING AND FLOW CYTOMETRY

Cells were plated at 2×10^5 cells/well in supplemented RPMI 1640 in a 6-well plate. The following day, serum free media was applied for 24 h followed by incubation in serum free media containing 500 ng/ml hGH or PBS vehicle for 24 h. FITC-conjugated Annexin V solution (2.5 μl/ml final) (Sigma) and propidium iodide (5 mg/ml final) (Sigma) were added and the plates incubated for 10 min at room temperature in the dark. Cells were harvested, washed with PBS and resuspended in 10 mM Hepes, pH 7.4, 140 mM NaCl, 5 mM $CaCl_2$, and analyzed with a FACScan flow cytometer and Cell Quest software (Beckton Dickinson), collecting data from 10,000 cells. Mock stained cells were processed in parallel to determine background fluorescence and set thresholds.

CONSTRUCTION OF hGH EXPRESSION PLASMID

The phCMV1 eukaryotic expression vector (Gene Therapy Systems) was digested with *Hin*dIII, and a 0.8 kb *Hin*dIII fragment from a plasmid containing the *hGH-N* cDNA (pGEM-hGH, a gift of Norman Eberhardt, Mayo Clinic, Rochester, MN) was inserted. Plasmid sequence was verified by the East Carolina University Genomics Core facility automated DNA sequencing laboratory.

STABLE TRANSFECTION

The phCMV1-hGH or the phCMV1 empty vector was transfected into LNCaP and 22Rv1 cells with Lipofectamine 2000 (Invitrogen) following the manufacturer's protocol. Stably transfected cells were selected and maintained with 500 μg/ml G418 (Gemini Bio Products). Individual clones were isolated and screened for hGH expression by Western blot, using the rat somatotrope cell line GH3 as a control. Multiple LNCaP/hGH and 22Rv1/hGH clones expressing levels of hGH that were no greater than that in GH3 cells were identified for the studies in order to avoid supraphysiological hGH expression. Multiple stable empty vector-transfected clones (LNCaP/EV and 22Rv1/EV) were isolated in parallel as negative controls. Cultures of stably transfected cell clones maintained under drug selection were limited to 25 passages, and hGH expression levels were monitored regularly by Western blot of cell lysates to ensure no changes in expression over time.

hGH ELISA

Secretion of hGH into culture media was measured by an hGH ELISA kit (Roche). LNCaP/hGH clones were plated at 8×10^5 cells/10 cm plate in supplemented RPMI 1640 for attachment overnight. Media was aspirated and cells were washed twice with PBS, 10 ml serum free media added, and plates incubated at 37°C. A 500 μl aliquot of media was withdrawn at 1, 5, 10, 30, and 60 min, then hourly for 8 h. After each withdrawal 500 μl of media was added to the plate to maintain the total volume. A protease inhibitor cocktail (Sigma) was added to the aliquots, which were stored at 4°C until the time course was complete. Samples were diluted 1:10 or 1:100 in serum-free RPMI (after an initial experiment to determine the dilution required to bring hGH levels to within the standard curve) and assayed by ELISA following manufacturer's protocol. Concentrations determined in the diluted samples were corrected for the dilution factor.

WESTERN BLOT ANALYSIS OF JAK2 AND STAT5 ACTIVATION

Cells were seeded at 2×10^6 cells/10 cm plate in supplemented RPMI. The next day, media was removed, cells were washed with PBS, and cells were incubated in serum free RPMI for 24 h. The media was replaced with fresh serum free media containing 500 ng/ml of hGH

and the cells incubated at 37°C. At the indicated times, plates were put on ice, the media aspirated, and the cells were scraped into denaturing lysis buffer (50 mM Tris–HCl, pH 7.5, 150 mM NaCl, 2% SDS) supplemented with protease and phosphatase inhibitor cocktails (Sigma). Lysates were sonicated and cleared by centrifugation at 14,000 rpm for 15 min at 4°C. Western blot of lysates was performed as above. Activated JAK2 was detected with a rabbit anti-phospho-JAK2 (Tyr1007/1008) antibody. A rabbit anti-JAK2 antibody was used as a control. Activated STAT5 was detected with a rabbit anti-phospho-STAT5 (Tyr694) antibody. A rabbit anti-STAT5 antibody was used as a control. Antibodies were from purchased from Cell Signaling and were used at a 1:1,000 dilution.

IMMUNOFLUORESCENCE CONFOCAL MICROSCOPY

Chambered slides were pretreated with 1% nitric acid and 10 μg poly-D-lysine per well. Cells were plated at 4×10^4 cells per chamber, fixed in 3.7% paraformaldehyde or 100% ice-cold methanol for 10 min followed by two PBS washes. Cells were permeabilized with 1% Triton-X 100 or 0.1% Tween-20 in PBS for 10 min. Blocking solution (5% BSA, 10% FBS, and 0.1% Tween-20 in PBS) was applied for 1 h at room temperature. Primary antibodies diluted in blocking buffer were applied and incubated overnight at 4°C. For detection of hGHR alone, rabbit anti-hGHR (Santa Cruz sc-20747) was used at 1:50. For hGH and Golgi co-immunofluorescence, rabbit anti-hGH (Gary Parlow) at 1:200 and mouse anti-58K (Golgi marker, Novus Biologicals) at 1:50 were used. For hGH and hGHR co-immunofluorescence, rabbit anti-hGH at 1:200 and mouse anti-hGHR (Santa Cruz sc-74051) at 1:50 were used. Cells were washed four times with PBS, and secondary antibodies (Alexa Fluor 568-conjugated goat anti-mouse IgG at 1:1,000 and Alexa Fluor 488-conjugated goat anti-rabbit IgG at 1:1,000) in blocking buffer were applied for 2 h at room temperature in the dark. Cells were washed five times with PBS, and cover slips mounted with mounting media (0.05 M Tris pH 7.5, 80% glycerol). A control in which the primary antibody was omitted was included in each experiment, which showed no background staining by the secondary antibodies. Cells were observed with a Zeiss LSM 510 confocal microscope, and images captured with Zeiss LSM software.

CELL MEMBRANE FRACTIONATION BY SUCROSE GRADIENT ULTRACENTRIFUGATION

Cell homogenization and sucrose gradient fractionation of membranes was performed as described [Hu and Kaplan, 2000]. Ten million cells were harvested with enzyme free cell dissociation buffer (Gibco) according to the manufacturer's protocol. Cells were washed twice with PBS containing protease inhibitor cocktail (Sigma) and frozen at −20°C. Cell pellets were thawed on ice, resuspended in homogenizing buffer (0.25 M sucrose, 10 mM Tris pH 7.5, protease inhibitor cocktail), and Dounce homogenized with 40 strokes. The homogenate was centrifuged at 2,500 rpm for 15 min at 4°C and the supernatant collected. An equal volume (about 1.8 ml) of sucrose adjustment buffer (2.55 M sucrose, 10 mM Tris pH 7.5, protease inhibitor cocktail) was added and the sample loaded in a sucrose step gradient in the following order: 920 μl 2 M sucrose, 1.84 ml 1.6 M sucrose, 3.68 ml cell homogenate, 3.68 ml 1.2 M sucrose, and 1.84 ml 0.8 M sucrose. Gradients were centrifuged at 26,000 rpm for 2 h at 4°C in a SW41Ti rotor (Beckman). Five hundred microliters fractions were collected with a Brandel pump, ISCO UA-6 UV/visible absorbance detector and Foxy fraction collector. Three milliliters of imidazole solution was added (25 mM imidazole, 1 mM EDTA, protease inhibitor cocktail) and the fractions centrifuged at 52,011 rpm for 47 min at 4°C in a TLA-100.4 rotor (Beckman). The supernatant was aspirated and pellet resuspended in imidazole solution for subsequent analysis. An alkaline phosphodiesterase assay to identify plasma membrane fractions was performed as described [Gatto et al., 2001]. Briefly, 25 μl of each sucrose gradient fraction was mixed with 575 μl reaction buffer (100 mM Tris pH 7.5, 1 mM thymidine 5-monophosphate *p*-nitrophenyl ester) and incubated for 2.5 h at 37°C. Reactions were terminated with ice-cold 0.5 M glycine, and 0.5 M sodium carbonate. An α-glucosidase assay for the endoplasmic reticulum was also performed as described [Gatto et al., 2001]. Briefly, 30 μl of each fraction was mixed with 70 μl of PBS containing 2.5 mM *p*-nitrophenol-α-glucoside and 1% Triton X-100. Reactions were incubated 5 h at 37°C and terminated with ice-cold 0.2 M Na_2CO_3. An α-mannosidase assay for Golgi membrane fractions was performed as described [Jelinek-Kelly et al., 1985]. Briefly, 25 μl of each fraction was mixed with 225 μl of potassium phosphate buffer (10 mM KH_2PO_4, pH 6.0). Two hundred fifty microliters of 20 mM *p*-nitrophenyl-α-D-mannopyranoside was added and the reactions incubated for 4 h at 37°C followed by termination with ice-cold 0.5 M Na_2CO_3. The ester product of each enzyme assay was measured at 405 nm. The protein concentration of each fraction was measured with Bradford reagent using standard methods. For hGHR detection in the sucrose gradient fractions, 30 μg of each fraction was assayed by a Western blot for hGHR as described above.

STATISTICAL ANALYSIS

Statistical significance of differences between two means was determined by a two-tailed Student's *t*-test. Comparison of greater than two means was performed by ANOVA, followed by a Tukey–Kramer multiple comparison test ($\alpha = 0.05$).

RESULTS

INCREASED EXPRESSION OF hGH AND hGHR IN PROSTATE CANCER CELL LINES

Multiple prostate cancer cell lines have been shown to express transcripts for both hGHR and hGH, suggesting the potential for direct hGH-responsiveness and a possible autocrine mechanism of hGH-hGHR signaling in prostate cancer cells [Untergasser et al., 1999; Ballesteros et al., 2000; Chopin et al., 2002; van Garderen and Schalken, 2002; Weiss-Messer et al., 2004], but the levels of hGH and hGHR expression relative to normal prostate epithelial cells are unclear. We first determined whether established prostate cancer cell lines (LNCaP, 22Rv1, and PC3) overexpress hGH and hGHR compared to the normal prostate epithelial cell-derived line RWPE-1 [Bello et al., 1997]. The qRT-PCR assay showed significantly more hGHR mRNA in prostate cancer cell lines compared to RWPE-1 cells. RWPE-2 cells, a tumorigenic Ki-Ras2-transformed derivative of RWPE-1 [Bello et al., 1997], also showed increased hGHR expression

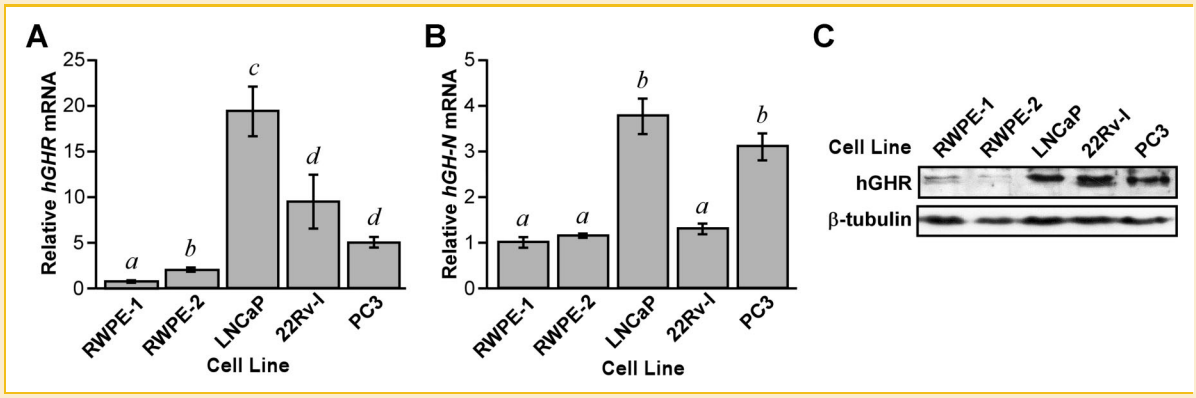

Fig. 1. Prostate cancer cell lines overexpress both hGHR and hGH compared to normal prostate epithelial cells. qRT-PCR for (A) hGHR and (B) hGH-N mRNA in prostate cancer cell lines. Results represent mRNA levels relative to the normal prostate epithelial cell line RWPE-1, normalized to hGAPDH mRNA by the comparative Ct ($\Delta\Delta$Ct) method. The data represent the mean ± SD of three independent experiments. ANOVA indicated significant differences in mRNA levels among the cell lines ($P < 0.0001$). Levels not connected by the same letter are significantly different as determined by a Tukey–Kramer multiple comparison test ($P < 0.05$). C: Western blot for hGHR from whole cell lysates. A β-tubulin Western blot serves as a loading control.

(Fig. 1A). LNCaP and PC3 cells also displayed significantly increased hGH mRNA (Fig. 1B). Immunoblot analysis of lysates from these cell lines indicated that hGHR protein is readily detectable (Fig. 1C). However, while hGH-N mRNA was detected in the cell lines by qRT-PCR, hGH protein levels were below detection by immunoblot (Fig. 3A). Overall, prostate cancer cells appear to express hGHR to a greater extent than normal prostate-derived cells, indicating the possibility of increased sensitivity to exogenous hGH. The LNCaP cell line displayed the highest hGHR expression, and previous studies have employed this cell line as a model system to address hGH effects in prostate cancer cells [Untergasser et al., 1999; Weiss-Messer et al., 2004]. Thus, we chose this cell line to test the effects of exogenous and autocrine hGH on cell proliferation and survival.

EXOGENOUS hGH INCREASES LNCaP CELL PROLIFERATION

The expression of hGHR by LNCaP cells suggests the ability to respond to hGH. However, the ability of hGH to affect LNCaP cell function has not been resolved due to limited and conflicting

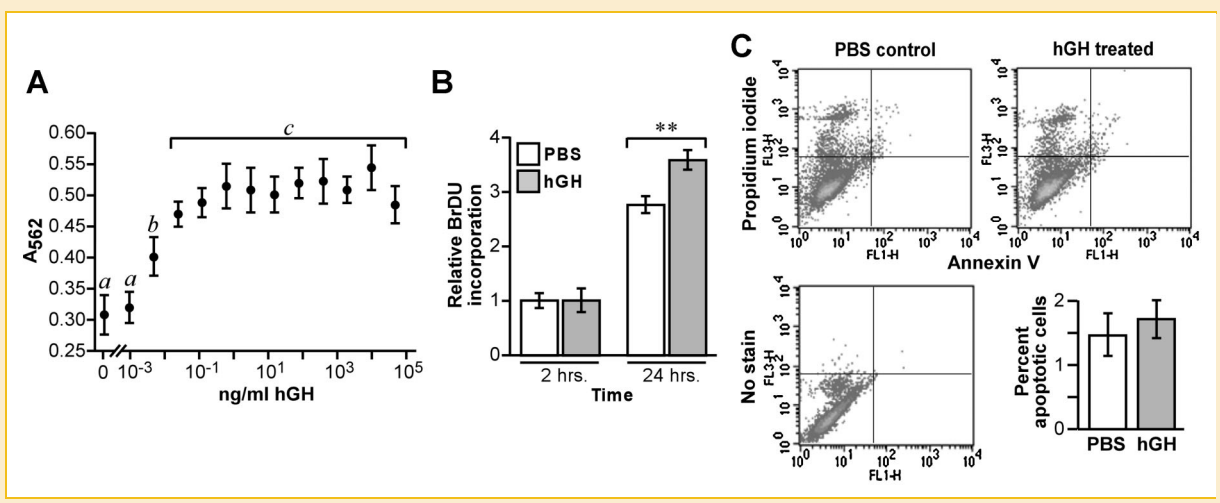

Fig. 2. Exogenous hGH increases LNCaP cell proliferation and has no effect on apoptosis. A: MTT assay of LNCaP cell culture growth. Cells grown in 1% FCS were treated with an hGH serial dilution series (50 μg/ml to 1 pg/ml) for 4 days, followed by MTT assay of cell density. The absorbance (A_{562}) is proportional to viable cell number. The data represent the mean ± SD of five independent cell cultures. The *x*-axis is presented as a log scale, and an hGH-free negative control is also shown. ANOVA indicated significant differences in absorbance among the hGH concentrations ($P < 0.0001$). Levels not connected by the same letter are significantly different as determined by a Tukey–Kramer multiple comparison test ($P < 0.05$). B: BrdU incorporation assay of WT LNCaP cells upon stimulation with 500 ng/ml exogenous hGH or vehicle control (PBS) for 24 h in serum-free media. Data are normalized to the 2 h time point, and represent mean ± SD of three independent experiments. Significance was determined by a two-tailed Student's *t*-test; $^{**}P < 0.01$. C: Example of flow cytometry following annexin V/propidium iodide staining of WT LNCaP cells treated with vehicle (PBS) or 500 ng/ml hGH. A mock stained control cell sample was used to determine the background fluorescence and to set gates. Fluorescent cells in the lower right (annexin V^+/PI^-) and upper right (annexin V^+/PI^+) quadrants represent early and late apoptotic cells, respectively. Cells in the upper left quadrant (annexin V^-/PI^+) represent damaged and necrotic cells and were not counted as apoptotic. Quantification of flow cytometry results is shown in the graph. The data represent mean ± SD of three independent experiments.

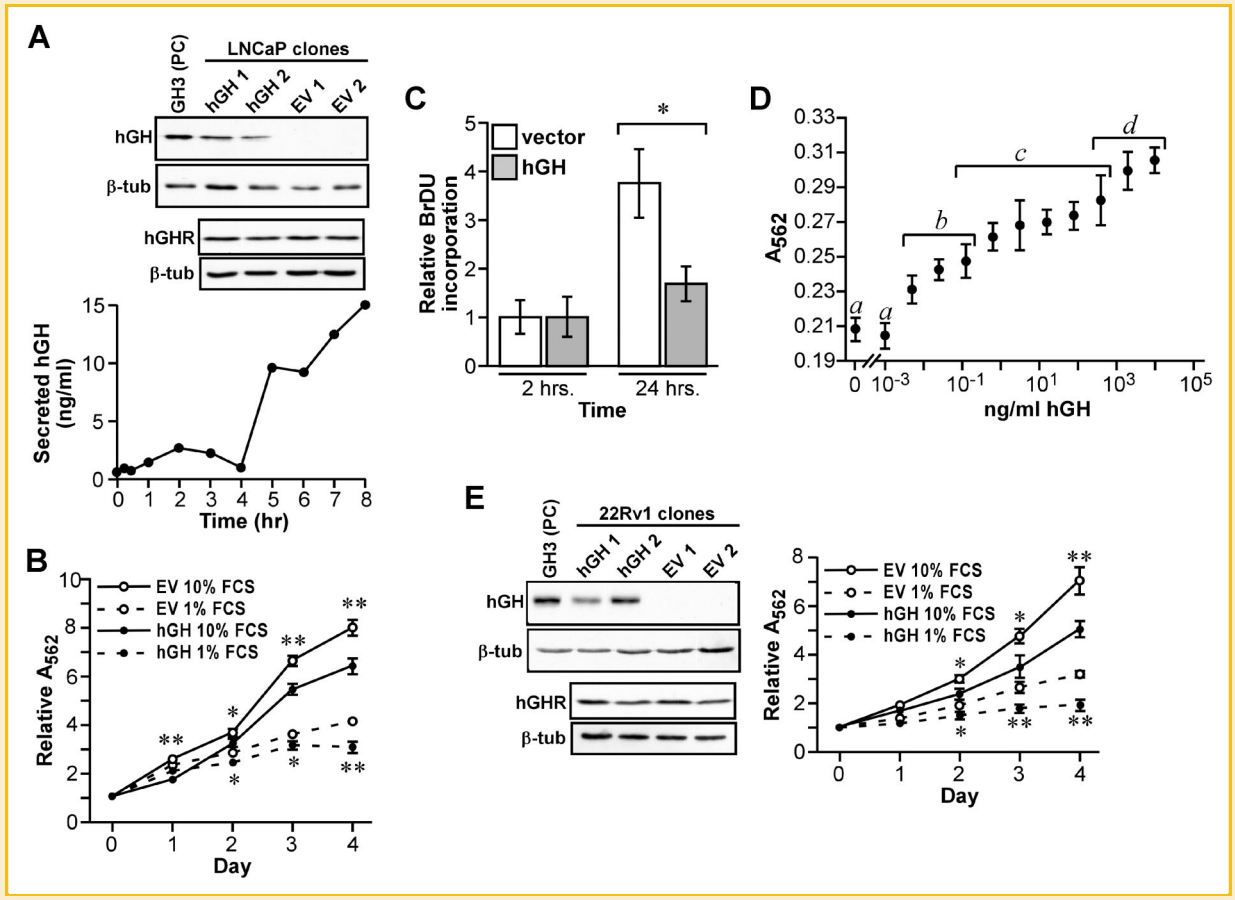

Fig. 3. Autocrine hGH expression decreases LNCaP cell proliferation. A: hGH ELISA of the culture media of LNCaP/hGH clone 1. Immunoblots of hGH and hGHR in LNCaP/hGH and LNCaP/EV cell lysates are shown above the graph. The somatotrope cell line GH3 is included as a GH control (PC). β-tubulin serves as a loading control. B: MTT assay of stably transfected LNCaP cell culture growth. LNCaP/EV and LNCaP/hGH cells were grown in media containing 1% or 10% FCS and MTT assays performed at the indicated times. Data are normalized to the initial cell density (day 0) and represent the mean ± SE of five experiments with two LNCaP/hGH and two LNCaP/EV clones (10 samples total). Note that some error bars are occluded by the data point. The significance of the difference between LNCaP/EV and LNCaP/hGH cells at each time point was determined by a two-tailed Student's *t*-test (upper, 10% FCS; lower, 1% FCS) $^{**}P < 0.01$ and $^{*}P < 0.05$. C: BrdU incorporation assay with LNCap/EV and LNCaP/hGH cells grown in serum free media for 24 h. The data are normalized to the 2 h time point and represent the mean ± SD of three experiments with two LNCaP/EV and two LNCaP/hGH clones (six samples total). Significance was determined by two-tailed Student's *t*-test; $^{*}P < 0.05$. D: MTT assay of 22Rv1 cell culture growth preformed as described for Figure 2A. E: MTT assay of stably transfected 22Rv1 cell culture growth performed as described for panel B. Immunoblots for hGH and hGHR in 22Rv1/hGH and 22Rv1/EV clones are shown to the left of the graph.

observations [Untergasser et al., 1999; Weiss-Messer et al., 2004]. We first determined sensitivity of WT LNCaP cells to exogenous hGH at concentrations that encompassed a physiological range using a colorimetric MTT cell proliferation assay (Fig. 2A). The results show that hGH increased LNCaP cell culture growth in a dose-dependent manner. This effect was observed at pg/ml concentrations of hGH, indicating a high-sensitivity of LNCaP cells to exogenous hGH which reached a plateau at physiological hGH concentrations (~10 ng/ml).

Because the MTT assay measures the amount of viable cells in the culture, which is a product of both proliferation and cell death, we further resolved the basis for the increased cell number in response to exogenous hGH. To determine the direct effect of hGH on LNCaP cell proliferation, BrdU incorporation was measured over 24 h in serum-free media in the presence and absence of hGH, which indicated that hGH increased the rate of LNCaP cell proliferation (Fig. 3B). To determine the effect of exogenous hGH on cell survival, annexin-V/propidium iodide staining of LNCaP cells was performed after 24 h of serum starvation in the presence and absence of hGH to identify apoptotic (annexin V-positive) cells. The results of this assay showed no effect of exogenous hGH on the proportion of apoptotic cells (Fig. 2C). Thus, the increase in LNCaP cell culture growth mediated by exogenous hGH is due primarily to a significant increase in the rate of cell proliferation. This finding indicated that LNCaP prostate cancer cell proliferation could be directly affected by physiological concentrations of hGH, consistent with the potential for hGH to affect prostate tumor growth.

AUTOCRINE hGH DECREASES LNCaP CELL PROLIFERATION AND INCREASES APOPTOSIS

In addition to the effect of exogenous hGH on LNCaP cell proliferation, observation of the expression of *hGH-N* mRNA in

prostate cancer cell lines has led to the hypothesis that autocrine hGH expression in prostate cancer cells may also have effects significant to tumor progression [Chopin et al., 2002]. To test this hypothesis, we increased hGH expression in LNCaP cells by stable transfection with an *hGH-N* cDNA expression vector (LNCaP/hGH cells). We chose clones that expressed a level of GH less than that in the rat somatotrope cell line GH3 to avoid supraphysiological overexpression. LNCaP cells transfected with the empty vector (LNCaP/EV cells) were generated as a negative control. An immunoblot of transfected cell lysates indicated hGH expression mediated by the *hGH-N* expression vector in comparison to the GH3 cell control (Fig. 3A). An immunoblot for hGHR in the LNCaP/hGH and LNCaP/EV clones showed comparable hGHR expression. To further verify the level of expression and secretion of vector-encoded hGH, we measured the concentration of hGH in the culture media of LNCaP/hGH cells, which was readily detectable by hGH ELISA. The concentration of hGH in the culture media rose to ~2 ng/ml within 2 h and accumulated to ~15 ng/ml over the full 8 h time course, representing a normal range of physiological serum concentration (Fig. 3A). Secreted hGH was not detectable under these conditions with wildtype LNCaP cells, indicating that the hGH secreted from LNCaP/hGH cells was vector-encoded.

We first tested the effect of autocrine hGH on LNCaP cell culture growth. Employing an MTT assay, the growth of LNCaP/EV and LNCaP/hGH cells was determined in the presence of low and high serum concentrations, to differentiate the effect of autocrine hGH from any potentiating effects of additional growth factors present in serum. Remarkably, in contrast to the proliferative effect of a comparable concentration of exogenous hGH on wildtype LNCaP cells (Fig. 2A), LNCaP/hGH cells displayed reduced growth compared to LNCaP/EV control cells, independent of serum concentration (Fig. 3B). The growth of both LNCaP/hGH and LNCaP/EV cells was greater in the presence of 10% serum. Consistent with the MTT assay results, a BrdU incorporation assay showed that LNCaP/hGH cells had a significantly reduced rate of proliferation compared to LNCaP/EV control cells (Fig. 3C). Similar contrasting effects of exogenous and autocrine hGH were observed in 22Rv1 cells, which displayed a lower inherent level of hGH and hGHR mRNA than LNCaP cells (Fig. 1B); while exogenous hGH at physiological concentrations increased 22Rv1 cell proliferation (Fig. 3D), autocrine hGH expression in stably transfected 22Rv1/hGH clones reduced cell proliferation (Fig. 3E).

We next determined the effect of autocrine hGH on cell survival upon serum starvation. Annexin-V/propidium iodide staining followed by flow cytometry of cells after 24 h of serum starvation showed that autocrine hGH expression in LNCaP/hGH cells was associated with a significant increase in the proportion of apoptotic cells as compared to LNCaP/EV control cells (Fig. 4). Taken together, these results show that autocrine hGH resulted in decreased LNCaP cell proliferation in association with increased apoptosis. These effects of autocrine hGH are in marked contrast to those of exogenous hGH, which increased LNCaP cell proliferation and had no effect on apoptosis (Fig. 2). These findings counter the hypothesis that endogenously expressed autocrine hGH could potentiate prostate cancer cell growth and survival. Instead, these observations suggest that autocrine hGH, in contrast to endocrine hGH, can decrease prostate cancer cell proliferation and survival.

DIFFERENTIAL ASSOCIATION OF SIGNAL TRANSDUCTION PATHWAYS WITH THE ACTIONS OF EXOGENOUS AND AUTOCRINE hGH IN LNCaP CELLS

The GHR is a class I cytokine receptor that lacks intracellular kinase activity. GHR signaling is proximately mediated by the activation of JAK2 associated with the GHR intracellular domain, which in turn

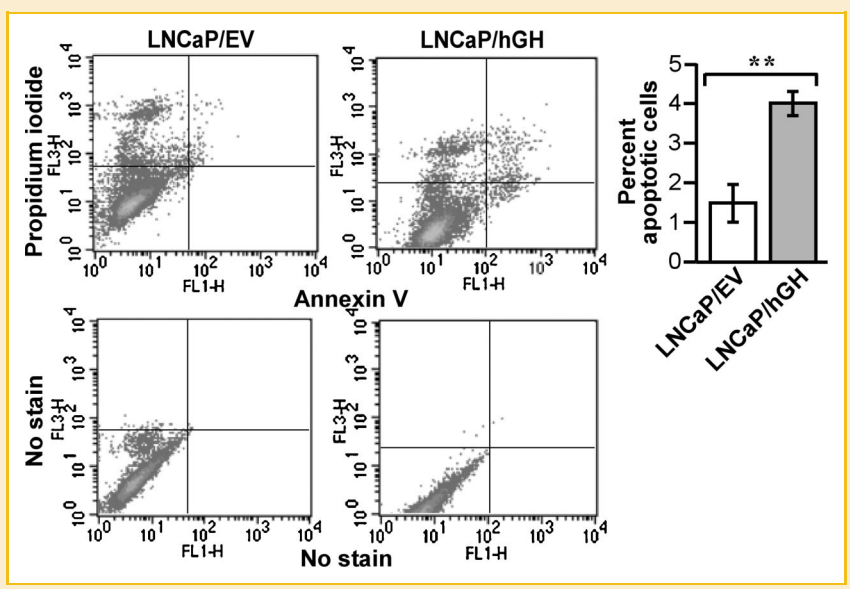

Fig. 4. Autocrine hGH increases LNCaP cell apoptosis. A: Example of flow cytometry of LNCaP/EV and LNCaP/hGH clones stained with annexin V/propidium iodide after 24 h serum starvation. Gates were set using mock-stained cells and apoptotic cells scored as described in Figure 2. Quantification of flow cytometry results from multiple experiments is shown in the graph. The data represent the mean ± SD of three independent experiments. Significance was determined by two-tailed Student's *t*-test; $^{**}P < 0.01$.

can activate at least three downstream signaling pathways (STAT5, MAPK, and PI3K) depending on the cell type, resulting in a range of functional effects [Moutoussamy et al., 1998]. To confirm that exogenous hGH can initiate GHR-mediated signal transduction as evidenced by JAK2 activation, wildtype LNCaP and LNCaP/EV control cells were treated with exogenous hGH, and phosphorylated JAK2 detected by immunoblot of lysates taken at various time points. We observed a rapid and transient activation of JAK2 that peaked at 3 min and declined to baseline within 30 min (Fig. 5A), consistent with a previous report employing the same conditions [Weiss-Messer et al., 2004]. A primary downstream target for phosphorylation by GH-activated JAK2 is the transcription factor STAT5. Thus, to further confirm hGH-mediated GHR activation in LNCaP cells, STAT5 phosphorylation was measured. The results of this assay indicated a transient activation of STAT5 that reached a maximum level at 10–20 min (Fig. 5B), subsequent to the peak of JAK2 phosphorylation. These results confirm that LNCaP cells are responsive to exogenous hGH through canonical GHR-mediated signaling pathways.

Whether autocrine hGH overexpression in LNCaP/hGH cells caused the constitutive activation of JAK2 was determined next. The results indicated that hGH overexpression did not cause constitutive JAK2 activation (time 0, Fig. 5C). Since the endogenously expressed hGH is secreted (Fig. 3A), this finding raised the question of whether LNCaP/hGH cells are refractory to extracellular hGH. Treatment of LNCaP/hGH cells with additional exogenous hGH did not result in JAK2 phosphorylation (Fig. 5C), in contrast with the effect of this treatment on wildtype LNCaP and LNCaP/EV control cells (Fig. 5A). To ensure that a rapid transient JAK2 phosphorylation prior to the 1 min time point was not missed, an assay of lysates taken at 15 and 30 s after the addition of hGH was performed, which also showed no phosphorylated JAK2 (data not shown). These observations suggest an alteration of GHR signaling, or the activation of a pathway for JAK2 dephosphorylation, resulting from autocrine hGH expression. To determine whether the lack of JAK2 activation by autocrine hGH was due to the long-term increased expression of hGH in LNCaP cells, we performed a transient transfection of LNCaP cells with the hGH expression vector, and assayed hGH expression and JAK2 phosphorylation at multiple time points following transfection. We did not observe JAK2 phosphorylation in parallel with the onset of hGH expression, reinforcing the possibility of an altered signaling pathway. However, the activation of a phosphatase activity that

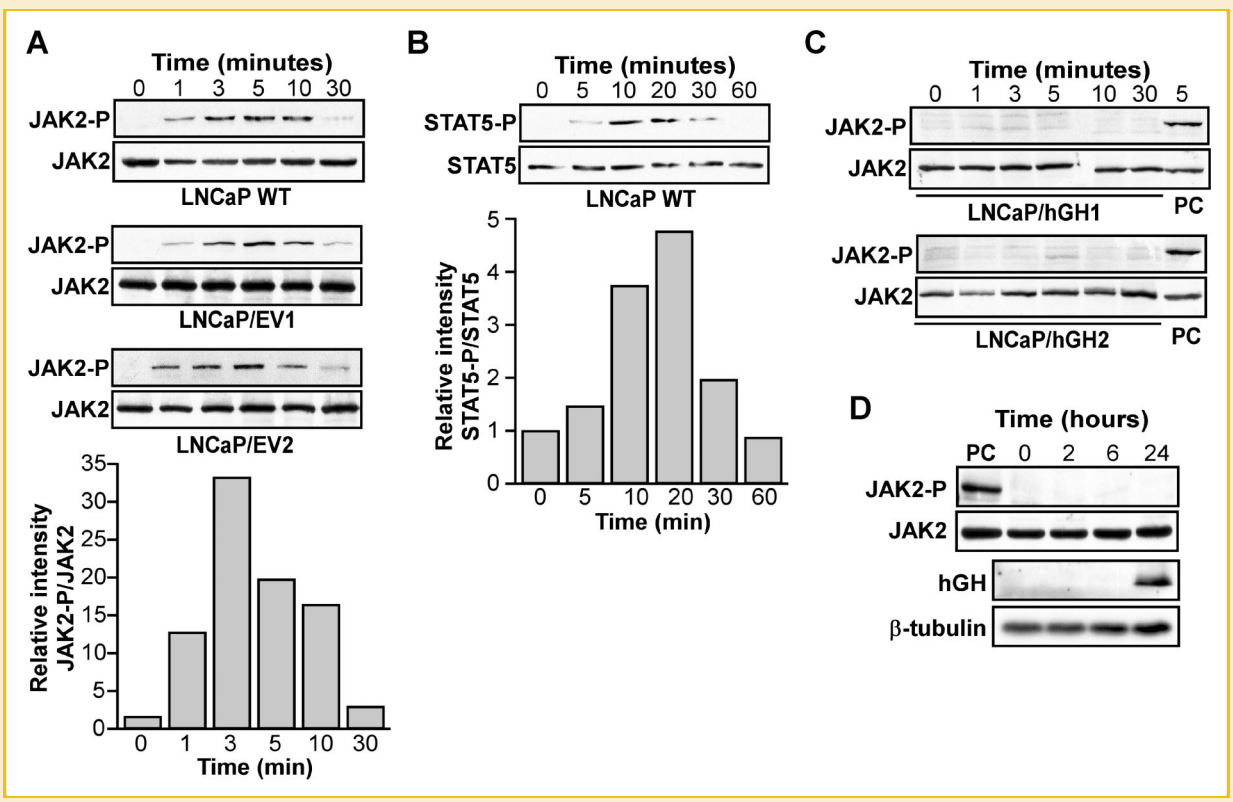

Fig. 5. Activation of JAK2 and STAT5 by exogenous hGH in LNCaP cells. A: Western blot detection of JAK2 phosphorylation in WT LNCaP cells and two LNCaP/EV control cell clones treated with 500 ng/ml hGH for the indicated times. The level of phosphorylated JAK2 (JAK2-P) normalized to total JAK2 in hGH-treated WT LNCaP cells are quantified in the graph as determined by phosphorimager analysis of the immunoblot. B: Western blot detection of phosphorylated STAT5 in WT LNCaP cells treated with 500 ng/ml hGH for the indicated times. The normalized levels of phosphorylated STAT5 are quantified in the adjacent graph. C: Immunoblot detection of JAK2 phosphorylation in two LNCaP/hGH cell clones in the basal state (time 0) and upon treatment with 500 ng/ml hGH for the indicated times. The positive control (PC) is WT LNCaP cells treated with 500 ng/ml hGH for 5 min. D: Western blot of JAK2 phosphorylation and hGH expression at multiple time points following transient transfection of LNCaP cells with the phCMV1-hGH expression vector. The positive control (PC) was generated as for panel C.

prevents the detection of phosphorylated JAK2 within the parameters of our assays cannot be formally ruled out.

To further investigate the potential signaling pathways associated with of the effects of exogenous and endogenous hGH that we observed in LNCaP cells, the role of the three major GHR-linked pathways (STAT5, PI3K, and MAPK) in these effects was tested. To this end, STAT5, PI3K, and MEK1/2 (MAPK kinase) activities were blocked in LNCaP cells using established chemical inhibitors. To first address the effects of exogenous hGH, BrdU incorporation assays were conducted in LNCaP/EV cells with or without exogenous hGH stimulation after pretreatment with a STAT5, PI3K, or MEK1/2 inhibitor. In the absence of exogenous hGH, inhibition of STAT5, PI3K, and MEK1/2 each resulted in decreased cell proliferation, indicating that each of the three signaling pathways are required for basal LNCaP cell proliferation (Fig. 6A). Treatment of LNCaP/EV cells with exogenous hGH increased cell proliferation (compare DMSO-treated samples in Fig. 6A,B), as seen previously (Fig. 2B). However, in LNCaP/EV cells treated with exogenous hGH, only inhibition of PI3K and STAT5 resulted in significantly decreased proliferation (Fig. 6B). This indicated that hGH-stimulated proliferation of LNCaP/EV cells was both PI3K and STAT5-dependent, and that hGH could bypass the anti-proliferative effects of MEK1/2 inhibition, presumably though PI3K and STAT5-mediated mechanisms. The experiment with LNCaP/hGH cells showed that autocrine hGH reduced LNCaP cell proliferation (Fig. 6C, DMSO), as seen previously (Fig. 3B). Treatment of LNCaP/GH cells with MEK1/2 inhibitor had no significant effect on cell proliferation. However, inhibition of both STAT5 and PI3K resulted in a significant increase in cell proliferation (Fig. 6C), in contrast to the effects of these inhibitors on LNCaP/EV cells with or without hGH stimulation. Thus, the PI3K and STAT5 pathways were involved in both the positive effect of exogenous hGH and the negative effect of autocrine hGH on LNCaP cell proliferation.

The interpretation of the results from the JAK2 activation experiment was that autocrine hGH had rendered the LNCaP/hGH cells refractory to exogenous hGH (Fig. 5C). In order to test this further, a BrdU incorporation assay was performed with LNCaP/hGH cells treated with additional exogenous hGH in the presence and absence of the pathway inhibitors (Fig. 6D). In the absence of inhibitors, exogenous hGH did not increase LNCaP/hGH cell proliferation compared to untreated LNCaP/hGH cells (Fig. 6; compare panels C and D, DMSO). In addition, the effects of the pathway inhibitors on the proliferation of LNCaP/hGH cells treated with additional exogenous hGH were comparable to the effects on untreated LNCaP/hGH cells (Fig. 6C), reinforcing the interpretation that these cells are refractory to exogenous hGH.

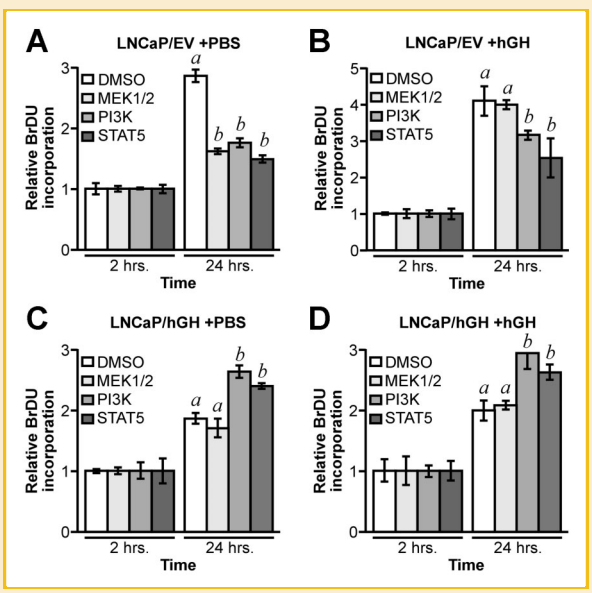

Fig. 6. Effects of signal transduction pathway inhibitors on the actions of exogenous and autocrine hGH in LNCaP cells. Results of BrdU incorporation assays are shown. Cells were pretreated with MEK1/2 (U0126), PI3K (LY294002), or STAT5 inhibitors for 2 h prior to the addition of BrdU and hGH or vehicle. A: LNCaP/EV cells treated with vehicle (PBS). B: LNCaP/EV cells treated with 500 ng/ml hGH. C: LNCaP/hGH cells expressing autocrine hGH treated with vehicle (PBS). D: LNCaP/hGH cells treated with 500 ng/ml hGH. BrdU incorporation at 24 h was normalized to the 2 h time point. The data represent the mean ± SD of three independent experiments. ANOVA indicated significant differences between the treatments ($P < 0.0001$). Levels not connected by the same letter are significantly different as determined by a Tukey–Kramer multiple comparison test ($P < 0.05$).

AUTOCRINE hGH ALTERS THE SUBCELLULAR LOCALIZATION OF hGHR IN LNCaP CELLS

The differential effects of exogenous and autocrine hGH on LNCaP cells raised the question of whether there could be underlying differences in GHR expression. However, a direct comparison of GHR protein levels showed no differences between LNCaP/hGH and LNCaP/EV cells (Fig. 3A). Beside potential effects on GHR expression, it has been reported that autocrine hGH may also alter GHR cellular trafficking. Overexpression of hGH and GHR in ts20 cells (a CHO cell derivative) resulted in their intracellular interaction in the Golgi apparatus and an intracrine mode of signal transduction. This mechanism inhibited the normal trafficking of GHR to the plasma membrane and the bypass of plasma membrane hGH binding and initiation of signaling [van den Eijnden and Strous, 2007]. Considering that the level of GHR expression in LNCaP cells was not influenced by endogenous hGH overexpression, we compared the subcellular distribution GHR in LNCaP/hGH and LNCaP/EV cells by immunofluorescence microscopy. In LNCaP/EV control cells GHR was primarily localized in multiple peripheral patches at the plasma membrane. In contrast, LNCaP/hGH cells showed a distinct and more concentrated cytoplasmic perinuclear GHR staining that suggested a possible localization in intracellular compartments (Fig. 7A). To further test this interpretation, we performed LNCaP/hGH cell membrane fractionation by sucrose gradient ultracentrifugation. Membrane fractions were identified by specific marker enzyme assays. An immunoblot for GHR in the resolved sucrose gradient fractions showed that GHR was predominantly localized to the Golgi (fraction 5) and endoplasmic reticulum (fractions 7–12) with little localization to the primary plasma membrane fraction (fraction 4) consistent with the confocal microscopy observation (Fig. 7B).

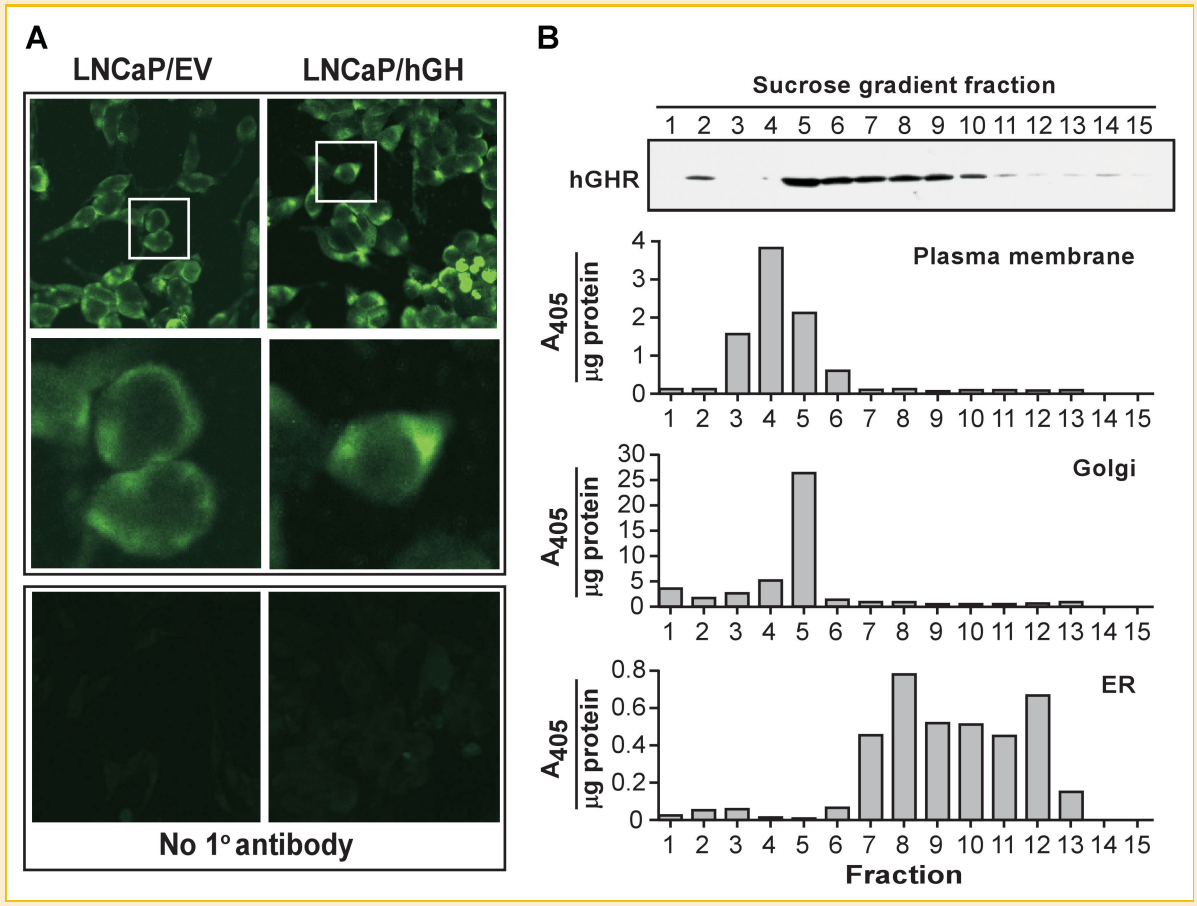

Fig. 7. Autocrine hGH alters the cellular localization of hGHR in LNCaP cells. A: Immunofluorescence confocal microscopy of hGHR in LNCaP/EV and LNCaP/hGH cells. Control images represent fluorescence in the absence of primary antibody. B: Distribution of hGHR between the plasma membrane, endoplasmic reticulum (ER) and Golgi in LNCaP/hGH cells expressing autocrine hGH resolved by sucrose gradient fractionation of subcellular membrane populations. The top panel represents an immunoblot for hGHR in the sucrose gradient fractions. The graphs indicate the resolution of enzyme activities identifying the location of plasma membrane (alkaline phosphodiesterase), Golgi (α-mannosidase) and endoplasmic reticulum (α-glucosidase) membranes in the sucrose gradient fractions.

Taken together, these results suggest that endogenously expressed hGH may sequester GHR in the Golgi and ER in LNCaP cells, resulting in the potential for an intracellular hGH–GHR interaction. To further test this hypothesis, co-immunofluorescence microscopy was performed on LNCaP/hGH cells with hGH, GHR, and Golgi marker antibodies. Consistent with the sucrose gradient results, hGH and GHR appeared to be colocalized in the Golgi in LNCaP/hGH cells, further supporting the possibility of an intracellular interaction (Fig. 8). Thus, the resistance of LNCaP/hGH cells to exogenous hGH and the differential association of signal transudation pathways with the effects of exogenous and autocrine hGH may be due in part to an effect of autocrine hGH on GHR localization and trafficking.

DISCUSSION

The action of GH has been associated with the increased incidence of several types of cancer, including those of epithelial origin. The potential for a role of ectopically expressed autocrine hGH in tumorigenesis has also been proposed [Perry et al., 2006]. Several lines of evidence have suggested a specific correlation between endocrine and autocrine hGH and prostate cancer. However, only a few published studies have directly tested the effect of hGH on prostate cancer cell function, and conflicting findings have been reported. An initial report indicated increased LNCaP cell proliferation in response to hGH [Untergasser et al., 1999]. However, a second study failed to recapitulate this effect, while GHR binding and the activation of the STAT5, MAPK, and PI3K signal transduction pathways by exogenous hGH were observed [Weiss-Messer et al., 2004]. Thus, the role of these pathways in potential hGH-stimulated LNCaP cell proliferation has not been addressed, and the effect of hGH on cancer cell function remained unresolved. In addition, while the detection of autocrine hGH expression in prostate cancer cells has been reported, the activity of autocrine hGH on prostate cancer cell function was not tested [Chopin et al., 2002; Slater and Murphy, 2006]. In the present study, the effects of exogenous and autocrine hGH on prostate cancer cell proliferation

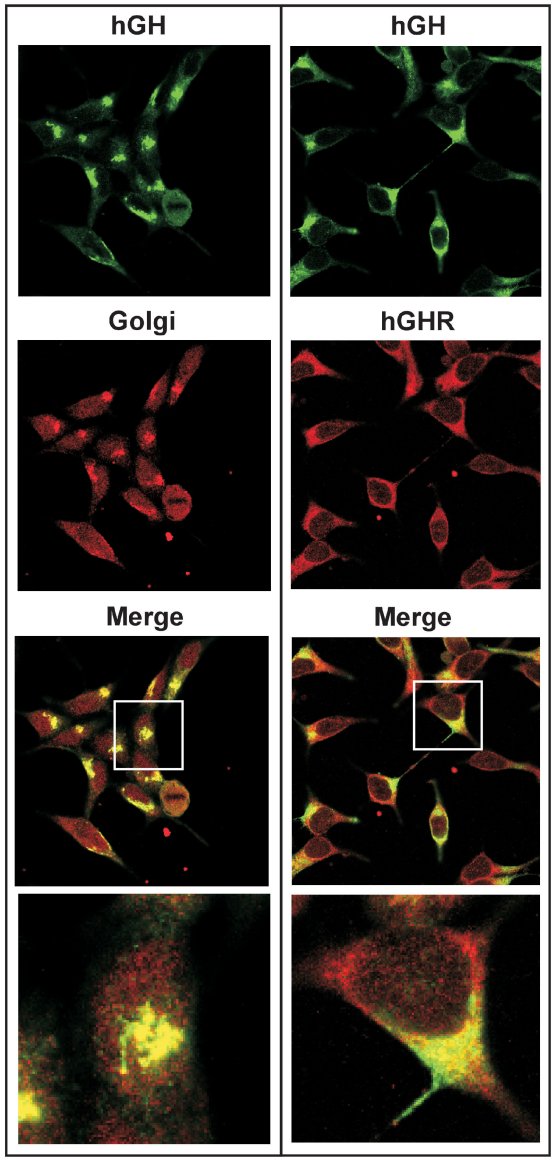

Fig. 8. Colocalization of hGH and hGHR in the Golgi in LNCaP/hGH cells. Co-immunofluorescence confocal microscopy for hGH/Golgi (left) and hGH/hGHR (right). See Materials and Methods Section for technical details.

and survival were investigated, including the associated signal transduction pathways, to test the hypothesis that both exogenous and autocrine hGH could affect prostate cancer cell function in a manner consistent with increased tumorigenic potential.

The expression levels of hGH and GHR in prostate cancer cell lines and cells derived from normal prostate epithelium were assessed, which showed that mRNA for hGH and hGHR tended to be greater in the transformed cell lines (Fig. 1). Using the LNCaP cell line as a model, which showed the greatest GHR expression and has been used as a model for GH-responsive prostate cancer in previous reports [Untergasser et al., 1999; Weiss-Messer et al., 2004], we saw that these cells were responsive to treatment with physiological concentrations of hGH, which resulted in increased proliferation but no effect on apoptosis (Fig. 3). However, we surprisingly discovered that exogenous and autocrine hGH had contrasting effects on LNCaP cell function. While exogenous hGH increased LNCaP cell proliferation and had no effect on cell survival, endogenous autocrine hGH decreased proliferation and increased apoptosis (Figs. 2 and 4). Similar contrasting effects were also observed with the 22Rv1 cell line. The disparate effects of exogenous and autocrine hGH were paralleled by distinctions in the response of canonical GHR-associated signal transduction mechanisms and possible changes in GHR trafficking that may underlie the paradoxical results. While wildtype LNCaP and empty vector-transfected LNCaP/EV control cells were sensitive to exogenous hGH treatment as evidenced by JAK2 activation, autocrine hGH-expressing LNCaP/hGH cells were insensitive to additional exogenous hGH, despite no differences in GHR mRNA expression (Fig. 5). Investigating the potential involvement of specific signal transduction pathways through the use of inhibitors suggested that PI3K and STAT5-dependent pathways were involved in both the proliferative effect of exogenous hGH and the anti-proliferative effect of autocrine hGH (Fig. 6). Consistent with the disparate responses to exogenous and autocrine hGH, GHR appeared to be localized at the plasma membrane in LNCaP/EV cells, but was concentrated predominantly in the ER and Golgi in LNCaP/hGH cells (Figs. 7 and 8). These findings support a model in which endogenous hGH expression results in a sequestering of GHR in the ER and Golgi, which in turn alters the mode of hGH-mediated signaling.

The loss of exogenous hGH-stimulated LNCaP cell proliferation upon PI3K and STAT5 inhibition is consistent with a critical role of these pathways in mediating the effect of exogenous hGH. These results also support the reported ability of exogenous hGH to stimulate Akt phosphorylation in LNCaP cells [Weiss-Messer et al., 2004] despite a degree of constitutive PI3K activity due to a PTEN loss-of-function mutation [Li et al., 1997]. The involvement of STAT5 in hGH-mediated LNCaP cell proliferation also comports with the known STAT5-responsiveness of cyclin D1 expression in LNCaP cells, which is required for cell cycle G1/S transition [Dagvadorj et al., 2008]. Remarkably, we observed a converse connection between the effect of autocrine hGH and these pathways in which inhibition of PI3K or STAT5 could overcome the negative effect autocrine hGH on LNCaP cell proliferation (Fig. 6C), returning it to a wildtype level (Fig. 6A, DMSO), which implies that these pathways are also involved in the growth-inhibiting effect of autocrine hGH. Finally, while MAPK pathway signaling appears to be required for basal LNCaP proliferation, MEK(1/2) inhibition did not alter the effects of exogenous or autocrine hGH, indicating that MAPK pathway signaling is not likely involved in mediating the observed effects of hGH.

The lack of JAK2 activation in LNCaP/hGH cells was surprising in light of the apparent involvement of STAT5 in the anti-proliferative effects of autocrine hGH. This may be the result of negative feedback by hGH-activated STAT5 (which may initially be activated through JAK2), which could trigger suppressor of cytokine signaling (SOCS) protein expression that negatively regulates JAK2 activity [Hansen et al., 1999; Flores-Morales et al., 2006]. Alternatively, autocrine hGH effects could be mediated through distinct GHR docking

molecules or kinases, such as JAK1, JAK3, Tyk2 [Hellgren et al., 1999], Src [Zhu et al., 2002], or Ras [Vanderkuur et al., 1997; Zhu et al., 2002]. In the case of JAK-family kinases, STAT3 can also be involved in GHR signaling, in addition to STAT5 [Sotiropoulos et al., 1996]. Recently, opposing actions of STAT5 and STAT3 on cell function through the differential regulation of partially overlapping sets of genes that are both activators and inhibitors of gene transcription have been proposed [Walker et al., 2009; Yang et al., 2011], which could explain the disparate actions of exogenous and autocrine hGH. In the case of a potential GHR association with Ras, it has been shown that a Ras/MEK/Erk pathway may be activated by intracellular ligand binding within the Golgi [Choy et al., 1999; Chiu et al., 2002], which fits our observation of Golgi GHR localization, but MEK inhibition did not counteract the reduced proliferation of LNCaP/hGH suggesting that this pathway is not involved in the anti-proliferative effect of autocrine hGH. However, it is notable that in the presence of PI3K or STAT5 inhibition, the proliferation rate of LNCaP/hGH cells was greater than that of LNCaP/EV cells in the presence of these inhibitors (compare Fig. 6A–C). This could be indicative of a switch to the activation of a Ras/MEK/Erk pathway by autocrine hGH through engagement of hGH in the Golgi that promotes cell proliferation in this context.

Taken together, the results described support the hypothesis that endocrine hGH could directly increase the proliferation of prostate cancer cells. However, our findings also indicate that endogenously expressed autocrine hGH could have a countervailing activity, decreasing prostate cancer cell proliferation and survival. This result contrasts with a significant body of work in a breast cancer cell model that suggests an oncogenic effect of autocrine hGH [Perry et al., 2006]. Elevated hGH expression was associated with increased mammary epithelial cell proliferation mediated by the MAPK/ERK pathway [Kaulsay et al., 1999], and metastatic carcinoma cells showed the highest levels of endogenous hGH expression [Raccurt et al., 2002]. Autocrine hGH overexpression was sufficient to transform normal mammary epithelial cells, conferring an invasive phenotype associated with changes in γ-catenin, E-cadherin, and matrix metalloprotease expression [Mukhina et al., 2004]. Autocrine hGH also conferred protection from apoptosis, due to the upregulation of HOXA1 and gadd153 expression [Zhang et al., 2003]. Increased TERT (telomerase catalytic subunit) expression was also observed, which is known to immortalize mammary epithelial cells [Emerald et al., 2007]. The contrasting effects of autocrine hGH on prostate cancer cell function presented here suggest that the ability of autocrine hGH to increase cancer cell proliferation and survival may be cell type-specific.

The basis for the contrasting effects of exogenous and autocrine hGH remains to be resolved, but our preliminary results described here suggest that an alteration of the actions of hGH-stimulated STAT5 and PI3K pathways may play a role. The basis for this change in the mode of action of these pathways may relate to differences in the nature of the hGH–GHR interaction between these two contexts, as evidenced by an apparent alteration in GHR trafficking and lack of JAK2 activation by autocrine hGH. The autocrine actions of GH could be mediated by the binding of secreted GH to cell surface GHR. Indeed, we have found that hGH expressed by LNCaP cells is packaged in normal secretory vesicles, and is secreted into the media (Fig. 3B). However, recent studies have indicated that GH/GHR interaction and subsequent signal transduction can occur intracellularly, mediated by the GH/GHR complex in the endoplasmic reticulum and Golgi, circumventing the typical trafficking of GHR to the plasma membrane for binding extracellular GH [van den Eijnden and Strous, 2007]. An additional potential mechanism associated with autocrine GH expression is the ligand-dependent nuclear localization of GHR, where it forms part of a transcriptional coactivator complex that activates genes controlling cell cycle progression and proliferation [Conway-Campbell et al., 2007]. Thus, not only does GHR activate multiple signaling pathways, it can also function in a completely distinct mode similar to a nuclear hormone receptor. In light of these findings, our observations support the potential for a mechanism of action of autocrine hGH in prostate cancer cells that may be distinct from the predicted actions of endocrine hGH. Future studies will continue to address this hypothesis, which may ultimately affect the perspective of the potential oncogenic actions of hGH, and the significance of the ectopic activation of hGH expression in cancer.

ACKNOWLEDGMENTS

This work was supported in part by grants from the Leo W. Jenkins Cancer Center and the Brody Brothers Medical Foundation (#MT7749) to B.M.S.

REFERENCES

Ballesteros M, Leung KC, Ross RJ, Iismaa TP, Ho KK. 2000. Distribution and abundance of messenger ribonucleic acid for growth hormone receptor isoforms in human tissues. J Clin Endocrinol Metab 85:2865–2871.

Bello D, Webber MM, Kleinman HK, Wartinger DD, Rhim JS. 1997. Androgen responsive adult human prostatic epithelial cell lines immortalized by human papillomavirus 18. Carcinogenesis 18:1215–1223.

Chan JM, Stampfer MJ, Giovannucci E, Gann PH, Ma J, Wilkinson P, Hennekens CH, Pollak M. 1998. Plasma insulin-like growth factor-I and prostate cancer risk: A prospective study. Science 279:563–566.

Chiu VK, Bivona T, Hach A, Sajous JB, Silletti J, Wiener H, Johnson RL II, Cox AD, Philips MR. 2002. Ras signalling on the endoplasmic reticulum and the Golgi. Nat Cell Biol 4:343–350.

Chopin LK, Veveris-Lowe TL, Philipps AF, Herington AC. 2002. Co-expression of GH and GHR isoforms in prostate cancer cell lines. Growth Horm IGF Res 12:126–136.

Choy E, Chiu VK, Silletti J, Feoktistov M, Morimoto T, Michaelson D, Ivanov IE, Philips MR. 1999. Endomembrane trafficking of ras: The CAAX motif targets proteins to the ER and Golgi. Cell 98:69–80.

Clemmons DR. 2002. Roles of insulin-like growth factor-I and growth hormone in mediating insulin resistance in acromegaly. Pituitary 5:181–183.

Conway-Campbell BL, Wooh JW, Brooks AJ, Gordon D, Brown RJ, Lichanska AM, Chin HS, Barton CL, Boyle GM, Parsons PG, Jans DA, Waters MJ. 2007. Nuclear targeting of the growth hormone receptor results in dysregulation of cell proliferation and tumorigenesis. Proc Natl Acad Sci USA 104: 13331–13336.

Dagvadorj A, Kirken RA, Leiby B, Karras J, Nevalainen MT. 2008. Transcription factor signal transducer and activator of transcription 5 promotes growth of human prostate cancer cells in vivo. Clin Cancer Res 14: 1317–1324.

Dirnhofer S, Berger C, Hermann M, Steiner G, Madersbacher S, Berger P. 1998. Coexpression of gonadotropic hormones and their corresponding FSH- and LH/CG-receptors in the human prostate. Prostate 35:212–220.

Emerald BS, Chen Y, Zhu T, Zhu Z, Lee KO, Gluckman PD, Lobie PE. 2007. AlphaCP1 mediates stabilization of hTERT mRNA by autocrine human growth hormone. J Biol Chem 282:680–690.

Evangelou AI, Winter SF, Huss WJ, Bok RA, Greenberg NM. 2004. Steroid hormones, polypeptide growth factors, hormone refractory prostate cancer, and the neuroendocrine phenotype. J Cell Biochem 91:671–683.

Flores-Morales A, Greenhalgh CJ, Norstedt G, Rico-Bautista E. 2006. Negative regulation of growth hormone receptor signaling. Mol Endocrinol 20:241–253.

Fuhrman B, Barba M, Schunemann HJ, Hurd T, Quattrin T, Cartagena R, Carruba G, Muti P. 2005. Basal growth hormone concentrations in blood and the risk for prostate cancer: A case–control study. Prostate 64:109–115.

Galbraith SM, Duchesne GM. 1997. Androgens and prostate cancer: Biology, pathology and hormonal therapy. Eur J Cancer 33:545–554.

Gatto C, McLoud SM, Kaplan JH. 2001. Heterologous expression of Na(+)-K(+)-ATPase in insect cells: Intracellular distribution of pump subunits. Am J Physiol Cell Physiol 281:C982–C992.

Hansen JA, Lindberg K, Hilton DJ, Nielsen JH, Billestrup N. 1999. Mechanism of inhibition of growth hormone receptor signaling by suppressor of cytokine signaling proteins. Mol Endocrinol 13:1832–1843.

Hellgren G, Jansson JO, Carlsson LM, Carlsson B. 1999. The growth hormone receptor associates with Jak1, Jak2, and Tyk2 in human liver. Growth Horm IGF Res 9:212–218.

Hu YK, Kaplan JH. 2000. Site-directed chemical labeling of extracellular loops in a membrane protein. The topology of the Na,K-ATPase alpha-subunit. J Biol Chem 275:19185–19191.

Jelinek-Kelly S, Akiyama T, Saunier B, Tkacz JS, Herscovics A. 1985. Characterization of a specific alpha-mannosidase involved in oligosaccharide processing in *Saccharomyces cerevisiae*. J Biol Chem 260:2253–2257.

Jenkins PJ, Besser M. 2001. Clinical perspective: Acromegaly and cancer: A problem. J Clin Endocrinol Metab 86:2935–2941.

Kaulsay KK, Mertani HC, Tornell J, Morel G, Lee KO, Lobie PE. 1999. Autocrine stimulation of human mammary carcinoma cell proliferation by human growth hormone. Exp Cell Res 250:35–50.

Kleinberg DL, Ruan W, Yee D, Kovacs KT, Vidal S. 2007. Insulin-like growth factor (IGF)-I controls prostate fibromuscular development: IGF-I inhibition prevents both fibromuscular and glandular development in eugonadal mice. Endocrinology 148:1080–1088.

Li J, Yen C, Liaw D, Podsypanina K, Bose S, Wang SI, Puc J, Miliaresis C, Rodgers L, McCombie R, Bigner SH, Giovanella BC, Ittmann M, Tycko B, Hibshoosh H, Wigler MH, Parsons R. 1997. PTEN, a putative protein tyrosine phosphatase gene mutated in human brain, breast, and prostate cancer. Science 275:1943–1947.

Lincoln DT, Sinowatz F, Temmim-Baker L, Baker HI, Kolle S, Waters MJ. 1998. Growth hormone receptor expression in the nucleus and cytoplasm of normal and neoplastic cells. Histochem Cell Biol 109:141–159.

Lobie PE, Breipohl W, Aragon JG, Waters MJ. 1990. Cellular localization of the growth hormone receptor/binding protein in the male and female reproductive systems. Endocrinology 126:2214–2221.

Moutoussamy S, Kelly PA, Finidori J. 1998. Growth-hormone-receptor and cytokine-receptor-family signaling. Eur J Biochem 255:1–11.

Mukhina S, Mertani HC, Guo K, Lee KO, Gluckman PD, Lobie PE. 2004. Phenotypic conversion of human mammary carcinoma cells by autocrine human growth hormone. Proc Natl Acad Sci USA 101:15166–15171.

Ozkan EE. 2011. Plasma and tissue insulin-like growth factor-I receptor (IGF-IR) as a prognostic marker for prostate cancer and anti-IGF-IR agents as novel therapeutic strategy for refractory cases: A review. Mol Cell Endocrinol 344:1–24.

Perry JK, Emerald BS, Mertani HC, Lobie PE. 2006. The oncogenic potential of growth hormone. Growth Horm IGF Res 16:277–289.

Raccurt M, Lobie PE, Moudilou E, Garcia-Caballero T, Frappart L, Morel G, Mertani HC. 2002. High stromal and epithelial human gh gene expression is associated with proliferative disorders of the mammary gland. J Endocrinol 175:307–318.

Reiter E, Bonnet P, Sente B, Dombrowicz D, de Leval J, Closset J, Hennen G. 1992. Growth hormone and prolactin stimulate androgen receptor, insulin-like growth factor-I (IGF-I) and IGF-I receptor levels in the prostate of immature rats. Mol Cell Endocrinol 88:77–87.

Reiter E, Hennuy B, Bruyninx M, Cornet A, Klug M, McNamara M, Closset J, Hennen G. 1999. Effects of pituitary hormones on the prostate. Prostate 38:159–165.

Reiter E, Kecha O, Hennuy B, Lardinois S, Klug M, Bruyninx M, Closset J, Hennen G. 1995. Growth hormone directly affects the function of the different lobes of the rat prostate. Endocrinology 136:3338–3345.

Ruan W, Powell-Braxton L, Kopchick JJ, Kleinberg DL. 1999. Evidence that insulin-like growth factor I and growth hormone are required for prostate gland development. Endocrinology 140:1984–1989.

Slater MD, Murphy CR. 2006. Co-expression of interleukin-6 and human growth hormone in apparently normal prostate biopsies that ultimately progress to prostate cancer using low pH, high temperature antigen retrieval. J Mol Histol 37:37–41.

Sotiropoulos A, Moutoussamy S, Renaudie F, Clauss M, Kayser C, Gouilleux F, Kelly PA, Finidori J. 1996. Differential activation of Stat3 and Stat5 by distinct regions of the growth hormone receptor. Mol Endocrinol 10: 998–1009.

Taichman RS, Loberg RD, Mehra R, Pienta KJ. 2007. The evolving biology and treatment of prostate cancer. J Clin Invest 117:2351–2361.

Tzelepi V, Efstathiou E, Wen S, Troncoso P, Karlou M, Pettaway CA, Pisters LL, Hoang A, Logothetis CJ, Pagliaro LC. 2011. Persistent, biologically meaningful prostate cancer after 1 year of androgen ablation and docetaxel treatment. J Clin Oncol 29:2574–2581.

Untergasser G, Rumpold H, Hermann M, Dirnhofer S, Jilg G, Berger P. 1999. Proliferative disorders of the aging human prostate: Involvement of protein hormones and their receptors. Exp Gerontol 34:275–287.

van den Eijnden MJ, Strous GJ. 2007. Autocrine growth hormone: Effects on growth hormone receptor trafficking and signaling. Mol Endocrinol 21:2832–2846.

van Garderen E, Schalken JA. 2002. Morphogenic and tumorigenic potentials of the mammary growth hormone/growth hormone receptor system. Mol Cell Endocrinol 197:153–165.

Vanderkuur JA, Butch ER, Waters SB, Pessin JE, Guan KL, Carter-Su C. 1997. Signaling molecules involved in coupling growth hormone receptor to mitogen-activated protein kinase activation. Endocrinology 138:4301–4307.

Walker SR, Nelson EA, Zou L, Chaudhury M, Signoretti S, Richardson A, Frank DA. 2009. Reciprocal effects of STAT5 and STAT3 in breast cancer. Mol Cancer Res 7:966–976.

Wang Z, Luque RM, Kineman RD, Ray VH, Christov KT, Lantvit DD, Shirai T, Hedayat S, Unterman TG, Bosland MC, Prins GS, Swanson SM. 2008. Disruption of growth hormone signaling retards prostate carcinogenesis in the Probasin/TAg rat. Endocrinology 149:1366–1376.

Wang Z, Prins GS, Coschigano KT, Kopchick JJ, Green JE, Ray VH, Hedayat S, Christov KT, Unterman TG, Swanson SM. 2005. Disruption of growth hormone signaling retards early stages of prostate carcinogenesis in the C3(1)/T antigen mouse. Endocrinology 146:5188–5196.

Weiss-Messer E, Merom O, Adi A, Karry R, Bidosee M, Ber R, Kaploun A, Stein A, Barkey RJ. 2004. Growth hormone (GH) receptors in prostate cancer: Gene expression in human tissues and cell lines and characterization, GH signaling and androgen receptor regulation in LNCaP cells. Mol Cell Endocrinol 220:109–123.

Wilson SS, Crawford ED. 2006. Controversies of androgen ablation therapy for metastatic prostate cancer. Curr Pharm Des 12:799–805.

Yang XP, Ghoreschi K, Steward-Tharp SM, Rodriguez-Canales J, Zhu J, Grainger JR, Hirahara K, Sun HW, Wei L, Vahedi G, Kanno Y, O'Shea JJ, Laurence A. 2011. Opposing regulation of the locus encoding IL-17 through direct, reciprocal actions of STAT3 and STAT5. Nat Immunol 12:247–254.

Zhang X, Zhu T, Chen Y, Mertani HC, Lee KO, Lobie PE. 2003. Human growth hormone-regulated HOXA1 is a human mammary epithelial oncogene. J Biol Chem 278:7580–7590.

Zhu T, Ling L, Lobie PE. 2002. Identification of a JAK2-independent pathway regulating growth hormone (GH)-stimulated p44/42 mitogen-activated protein kinase activity. GH activation of Ral and phospholipase D is Src-dependent. J Biol Chem 277:4592–4603.