Androgen Receptor Regulation of Local Growth Hormone in Prostate Cancer Cells

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Prostate cancer (PCa) growth is mainly driven by androgen receptor (AR), and tumors that initially respond to androgen deprivation therapy (ADT) or AR inhibition usually relapse into a more aggressive, castration-resistant PCa (CRPC) stage. Circulating growth hormone (GH) has a permissive role in PCa development in animal models and in human PCa xenograft growth. As GH and GH receptor (GHR) are both expressed in PCa cells, we assessed whether prostatic GH production is linked to AR activity and whether GH contributes to the castration-resistant phenotype. Using online datasets, we found that GH is highly expressed in human CRPC. We observed increased GH expression in castration-resistant C4-2 compared with castration-sensitive LNCaP cells as well as in enzalutamide (MDV3100)-resistant (MDVR) C4-2B (C4-2B MDVR) cells compared with parental C4-2B. We describe a negative regulation of locally produced GH by androgens/AR in PCa cells following treatment with AR agonists (R1881) and antagonists (enzalutamide, bicalutamide). We also show that GH enhances invasive behavior of CRPC 22Rv1 cells, as reflected by increased migration, invasion, and anchorage-independent growth, as well as expression of matrix metalloproteases. Moreover, GH induces expression of the AR splice variant 7, which correlates with antiandrogen resistance, and also induces insulinlike growth factor 1, which is implicated in PCa progression and ligand-independent AR activation. In contrast, blockade of GH action with the GHR antagonist pegvisomant reverses these effects both *in vitro* and *in vivo*. GH induction following ADT or AR inhibition may contribute to CRPC progression by bypassing androgen growth requirements. (***Endocrinology*** **158: 2255–2268, 2017)**

Prostate cancer (PCa) is the most common malignancy in men and the second leading cause of cancer-related death in men in Western societies (1). Androgens and androgen receptor (AR) signaling play a central role in PCa development and progression. Androgen deprivation therapy (ADT), the first-line treatment of advanced PCa, is initially effective, but most tumors eventually progress to a more aggressive, castration-resistant PCa (CRPC) stage in which cancer growth becomes insensitive to androgen withdrawal and/or AR inhibition (2, 3).

CRPC growth remains dependent on AR activation even in the presence of very low circulating androgen levels achieved with ADT and AR inhibition. Several mechanisms underlying resistance have been proposed (4, 5), including AR gene amplification (6), intratumoral androgen production (7), expression of constitutively active AR splicing variants that lack the ligand binding

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Abbreviations: ADT, androgen deprivation therapy; AKT, AKT serine/threonine kinase; AR, androgen receptor; ARv7, androgen receptor splice variant 7; CRPC, castration-resistant prostate cancer; CS-FBS, charcoal-stripped fetal bovine serum; EMT, epithelial-mesenchymal transition; FBS, fetal bovine serum; GH, growth hormone; GHR, growth hormone receptor; GS, Gleason score; IGF-1, insulinlike growth factor 1; JAK, Janus kinase; MAPK, mitogen-activated protein kinase; MDVR, MDV3100-resistant; MMP, matrix metalloprotease; mRNA, messenger RNA; PCa, prostate cancer; PSA, prostate-specific antigen; qPCR, quantitative polymerase chain reaction; RRID, research resource identifier; STAT, signal transducer and activator of transcription.

domain (8, 9), and activation of alternative growth factor and cytokine signaling cascades, such as phosphoinositide 3-kinase/AKT serine/threonine kinase (AKT), mitogen-activated protein kinase (MAPK), and Janus kinase (JAK)/signal transducer and activator of transcription (STAT) (10, 11).

Induction of growth hormone (GH) and insulinlike growth factor 1 (IGF-1) axis signaling has been linked to increased PCa risk in several animal studies. In the spontaneous dwarf rat or in GH receptor (GHR) knockout mice, disrupted GH signaling retards prostatic tumorigenesis (12, 13), and human PCa xenografts grow more slowly in mice carrying a mutation in the GH-releasing hormone receptor (14). In humans, there is no clear correlation between serum GH levels and PCa risk (15, 16). However, acromegaly patients harboring a GH-secreting adenoma commonly present with enlarged prostate glands and prostatic disorders (17, 18), and Laron dwarfism syndrome patients harboring an inactivating GHR mutation do not develop PCa (19).

GH is secreted into circulation by pituitary somatotroph cells. Circulating GH induces hepatic IGF-1 production, which, in turn, mediates most GH functions in target tissues. Nonpituitary GH is expressed in several tissues, including prostate, breast, and colon (20, 21), where it activates GHR in an autocrine or intracrine fashion via JAK2/STAT5 signaling (22, 23).

Autocrine GH action is associated with malignant cell transformation, chemoresistance, increased angiogenesis, and induction of epithelial-mesenchymal transition (EMT) in mammary and endometrial cancers (24–27).

GH and GHR messenger RNA (mRNA) and protein expression have been found in both androgen-sensitive and androgen-insensitive human PCa cell lines (LNCaP, 22Rv1, PC3), with expression levels higher when compared with normal prostate cells (28–30). GH expression has also been detected in human PCa biopsy specimens (31), and GHR is also highly expressed in human PCa tissue (30).

The GHR is functional in PCa cells, and GH activation of JAK2/STAT5, p42/p44 MAPK, and Akt signaling in LNCaP cells has been described (30). STAT5 activity is increased in PCa, and synergizes with AR signaling to contribute to PCa growth and progression (32–34). In addition, AR activation induces the suppressor of cytokine signaling 2, a known inhibitor of GHR intracellular signaling and a proposed tumor suppressor and mediator of crosstalk between AR and GH signaling in PCa (35). These observations suggest a potential role of GH in the pathogenesis of CRPC and increased sensitivity of CRPC to GH action.

We sought to examine links between prostatic GH production on AR activity and assess the contribution of GH to the castration resistant phenotype in PCa. We show that GH is highly expressed in human CRPC samples and in CRPC cell lines, and describe a novel negative regulation of locally produced GH by androgens/AR in PCa cells. We also show that GH induces expression of AR splice variant 7 (ARv7), which correlates with resistance to ADT such as, enzalutamide and abiraterone. GH also induces IGF-1, implicated in PCa progression and ligand independent AR activation, and blockade of GH action reverses these effects. Our results suggest that GH induction following ADT or AR inhibition might contribute to CRPC progression by activating pathways that bypass androgen growth requirements.

Materials and Methods

Cell lines and reagents

LNCaP and 22Rv1 PCa cell lines were obtained from the American Type Culture Collection (Manassas, VA), and C4-2 cell line was established and characterized in the laboratory of Dr. L. W. Chung. Parental C4-2B and enzalutamide (MDV3100)-resistant (MDVR) C4-2B (C4-2B MDVR) (36) were generated by Dr. Allen Gao (University of California Davis Medical Center, Sacramento, CA). The cell lines were authenticated by the University of Texas M. D. Anderson Cancer Center (Houston, TX) and Novocure Laboratory (Birmingham, AL). Cells were cultured in RPMI 1640 media supplemented with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin. Androgen deprivation experiments and AR inhibitor treatments were performed in phenol red free RPMI 1640 supplemented with 5% charcoal-stripped FBS (CS-FBS). All cells were maintained at 37°C in a humidified incubator with 5% carbon dioxide.

Reagents used for *in vitro* and *in vivo* experiments included R1881 (Sigma-Aldrich, St. Louis, MO); bicalutamide, enzalutamide, abiraterone acetate, and AZD1480 (Selleckchem, Houston, TX); recombinant human GH (Bio Vision, Milpitas, CA); and STAT5 inhibitor (Calbiochem-Millipore, Billerica, MA). Pegvisomant (Somavert) was kindly provided by Pfizer (New York, NY).

Western blotting

Cells were homogenized and lysed in radioimmunoprecipitation assay buffer with 10 μM protease inhibitors (Sigma-Aldrich) and protein concentration determined by Pierce bicinchoninic acid protein assay kit (Thermo Scientific, Rockford, IL). Protein (20 μg) was mixed with 2× Laemmli sample buffer (Bio-Rad, Hercules, CA) and separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, electroblotted onto Trans-Blot Turbo Transfer Pack 0.2 μm polyvinylide fluoride membrane (BioRad), and incubated overnight with indicated antibodies, followed by corresponding horseradish peroxidase–conjugated secondary antibodies (Sigma-Aldrich). Human GH antibodies were obtained from Dr. Albert F. Parlow [National Hormone and Peptide Program, Harbor–University of California, Los Angeles Medical Center, Torrance, CA; Research Resource Identifier (RRID): AB_2631058] or from R&D Systems (Minneapolis, MN; #AF-1067; RRID: AB_354573). Prostate-specific antigen (PSA; #SC-7638; RRID: AB_2134513), AR (N-20; #SC-816; RRID: AB_1563391), and

Table 1. Antibodies Used

Peptide/ Protein Target	Antigen Sequence (if Known)	Name of Antibody	Manufacturer, Catalog #, and/or Name of Individual Providing the Antibody	Species Raised in; Monoclonal or Polyclonal	Dilution Used	RRID
GH		Human GH antibody	Dr. A. F. Parlow at National Hormone and Peptide Program, Harbor-UCLA Medical Center	Rabbit; polyclonal	1/1000 for WB and IHC	AB_2631058
GH		Human GH antibody	R&D, #AF-1067	Goat; polyclonal	1/500	AB_354573
PSA		PSA (C-19) antibody	Santa Cruz Biotechnology, #SC-7638	Goat; polyclonal	1/500	AB_2134513
AR		AR (N20) antibody	Santa Cruz Biotechnology, #SC-816	Rabbit; polyclonal	1/500	AB_1563391
Twist		Twist antibody	Santa Cruz Biotechnology, #SC-81417	Mouse; monoclonal	1/200	AB_1130910
IGF-1		IGF-1 antibody	Abcam, #ab-9572	Rabbit; polyclonal	1/500	AB_308724
β-actin		β-actin antibody	Sigma-Aldrich, #A1978	Mouse; monoclonal	1/10,000	AB_476692
AR variant 7 (ARv7)		anti ARV7	Precision Antibody, #AG10008	Mouse; monoclonal	1/500	AB_2631057

Abbreviation: UCLA, University of California, Los Angeles.

Twist (#sc-81417; RRID: AB_1130910) were obtained from Santa Cruz Biotechnology (Dallas, TX); ARv7 (#AG10008; RRID: AB_2631057) from Precision Antibody (Columbia, MD); IGF-1 (#ab9572; RRID: AB_308724) from Abcam (Cambridge, MA); and β-actin (#A1978; RRID: AB_476692) from Sigma-Aldrich. Detailed antibody descriptions can be found in Table 1.

Immunostaining

Human PCa tissue arrays were purchased from US Biomax (Rockville, MD) and a PCa tissue microarray composed of 21 castration-resistant prostate adenocarcinomas, as confirmed by a pathologist, was kindly provided by Drs. Tzu-Ping Lin and Chin-Chen Pan from Taipei Veterans General Hospital, Taiwan. Slides were stained with GH antibody (RRID: AB_2631058) 1/1000 followed by secondary antibody conjugated with donkey anti-rabbit AlexaFluor 488 (Invitrogen-Thermo Fisher Scientific, Waltham, MA). Antigen retrieval was performed in 10 mM sodium citrate at 95°C, and control reactions without primary antibody staining were performed. Images were obtained with a digital scanner ScanScope AT Turbo Aperio (Leica Biosystems, Buffalo Grove, IL).

Analysis of microarray data sets (Oncomine)

Five PCa DNA microarray data sets (37–41) were downloaded directly from the Oncomine database by licensed access. Microarray data of the Grasso, Taylor 3, and Yu data sets are also publicly available in Gene Expression Omnibus as GSE35988, GSE21034, and GSE6919, respectively.

Real-time PCR

Total RNA was isolated from cells with the RNAeasy mini Kit (Qiagen). Complementary DNA was synthesized from 1 μg total purified RNA by iScript Reverse Transcription Supermix (BioRad, Hercules, CA). Quantitative polymerase chain reaction (qPCR) was performed in 20-μL reactions using Sybr Premix Ex Taq II (Takara, Japan) in BioRad IQ5 iCycler Thermal Cycler (BioRad). Specific validated primers for human GH, matrix metalloprotease (MMP)2 and MMP9 were purchased from SuperArray (Qiagen). All reactions were evaluated in triplicate and relative target gene expression determined by comparing average threshold cycles with that of housekeeping genes by comparative $\Delta\Delta$ threshold cycles.

Anchorage-independent growth assay

22Rv1 cells were stably infected with lentiviral particles expressing human GH (EF1-GH1-IR-GFP) or respective control lentiviral particles, both generated at the Cedars Sinai Viral Core facility. Control and GH expressing cells were resuspended in 5% CS-FBS media containing 0.3% agarose and overlaid on 0.6% agarose layer in six-well plates (2500 cells per well). Colonies were allowed to grow for 15 days, and then stained overnight with 0.5 mg/mL iodonitrotetrazolium chloride (Sigma Aldrich), photographed and counted. Results are expressed as means ± standard error of the mean of triplicate wells within the same experiment.

Migration and invasion assays

22Rv1 cells were transiently transfected with empty vector pIRES2-ZsGreen or GH-pIRES2-ZsGreen (42) by Lipofectamine 2000 Transfection Reagent (ThermoFisher Scientific) according to the manufacturer protocol. After 48 hours, transfected cells were trypsinized and plated (2×10^5 cells/mL) in BD BioCoat growth factor reduced matrigel invasion chambers for invasion assay or in BD control inserts for migration assay (BD Biosciences) in 0.5 mL of serum-free media. After 24 hours, nonmigrated cells on the upper surface of the membrane were removed with a cotton swab. Cells that migrated through membrane pores to the lower membrane surface were fixed with 70% ethanol, washed, and stained with crystal violet. Each experiment was performed three times with three

wells per group per assay. The number of migrating or invading cells was counted in four fields within each transwell.

In vivo CRPC xenograft

$1x10^6$ 22Rv1 cells were mixed 1:1 with matrigel (Corning, Corning, NY) and subcutaneously injected in both flanks of 3-week-old nude male mice (Jackson Laboratories, Sacramento, CA). Once the tumors reached 100 to 200 mm^3 [calculated as length × $(width)^2$/ 2], mice were divided in four groups of eight each and treated with vehicle (0.5% methylcellulose and 0.1% Tween 80, delivered orally in 200 μL and PBS, intraperitoneally in 200 μL), 20 mg/kg enzalutamide (in 0.5% methyl cellulose and 0.1% Tween 80, orally in 200 μL), 100 mg/kg pegvisomant (in PBS, intraperitoneally 200 μL), or a combination of enzalutamide and pegvisomant. Mice were treated three times weekly for 2 weeks. Tumor size was monitored by caliper measurements twice a week. At the end of the experiment, tumors were harvested and snap frozen for posterior Western blotting analysis; blood was collected for measurement of serum PSA by enzyme-linked immunosorbent assay (Genway Biotech, San Diego, CA). Mouse experiments were approved by the Institutional Animal Care and Use Committee (Protocol #6403).

Statistical analysis

Differences between groups were analyzed using two-tailed unpaired Student *t* test. Comparison between more than two groups was analyzed by one-way analysis of variance followed by Tukey multiple comparisons test in GraphPad Prism 7.0 software. Human gene expression data from Oncomine was analyzed by Pearson correlation test. A *P* value of less than 0.05 was considered significant and is indicated with an asterisk in the figures.

Results

GH is highly expressed in human prostate cancer tissue and in cell lines

Previous studies have reported GH expression in normal prostate epithelial cells and in several human PCa cell lines (28). Immunoreactive GH expression was reported in high Gleason score (GS) human PCa samples (31). We confirmed GH immunoreactivity in human PCa tissue by immunofluorescence staining in tissue arrays of human PCa samples (Biomax) and in a tissue array of 21 human CRPC samples. GH immunopositivity was observed in 19 of 38 (50%) prostate adenocarcinoma samples and in 1 of 5 (20%) prostatic hyperplasia samples [Fig. 1(a)]. Among the prostate adenocarcinoma samples evaluated, we found positive GH staining in three of nine (33.3%) low-grade tumors (GS of 2 to 4), in eight out of 12 (66.6%) tumors with GS of 5 to 6, and in eight out of 17 tumors with a high GS between 7 and 10. Among the CRPC cases, 14 of 21 (66.6%) were immunopositive for GH staining.

To further evaluate GH expression in human PCa, we analyzed human gene expression datasets from the Oncomine repository. Relative GH gene expression was significantly increased in PCa (n = 150) vs normal prostate tissue samples (n = 29) in the Taylor 3 dataset [$P = 0.0477$; Fig. 1(b)] (40), consistent with previous *in vitro* observations in normal vs cancer cell lines (28). Enhanced GH expression was also observed in tumors with high vs low GS (7 to 9, n = 80 vs GS 5 to 6, n = 69; $P = 0{,}041$, Fig. 1(c)]. Furthermore, we observed higher GH expression in metastatic CRPC (mCRPC, n = 34) than in localized PCa human samples [n = 59, $P < 0.0001$; Fig. 1(d)] in the Grasso dataset (38). These results suggest a role for GH in CRPC. We next sought to recapitulate these results in androgen-dependent and CRPC cell lines. GH mRNA and protein expression levels were 3.4- and 2.5-fold increased, respectively, in castration resistant C4-2 cells vs isogenic parental, androgen-dependent LNCaP cells [$P = 0.009$ and $P = 0.005$, respectively; Fig. 1(e) and 1(f)].

CRPC is characterized by persistent tumor growth despite ADT and/or AR inhibition; indeed, resistance to the second-generation AR inhibitor enzalutamide (formerly MDV3100) is common after several months of treatment in CRPC patients (43). The C4-2B MDVR cell line was generated by culture of C4-2B cells in media supplemented with 20 μM enzalutamide (36) and is nonresponsive to enzalutamide treatment both *in vitro* and *in vivo* (44). Consistent with a potential role for GH in CRPC, we observed elevated GH mRNA and protein expression levels in enzalutamide resistant C4-2B MDVR cells compared with parental C4-2B cells [$P = 0.01$ and $P < 0.001$, respectively; Fig. 1(g) and 1(h)].

Androgens negatively regulate GH expression by AR in prostate cancer cells

GH induction observed in castration- and enzalutamide-resistant cells led us to hypothesize that GH expression might be locally regulated by androgens and AR signaling in CRPC. We first evaluated whether GH expression was affected in AR responsive LNCaP cells grown under androgen deprivation conditions. GH expression was induced after 72 hours of growth in media supplemented with 5% CS-FBS compared with regular 5% FBS media [Fig. 2(a)]. Moreover, addition of the AR ligand R1881 (synthetic dihydrotestosterone analog) fully reversed the increase in GH mRNA levels, indicating a specific action of androgens inhibiting GH expression [Fig. 2(b)]. To further confirm AR regulation of GH, LNCaP cells were exposed to vehicle, R1881, or a combination of R1881 and the AR antagonist bicalutamide. GH mRNA and protein expression levels were attenuated after 24 hours and 48 hours of 1 nM R1881 treatment, and cotreatment with 10 μM bicalutamide was sufficient to fully reverse this effect [Fig. 2(c) and 2(d)]. These results indicate a specific and reversible effect

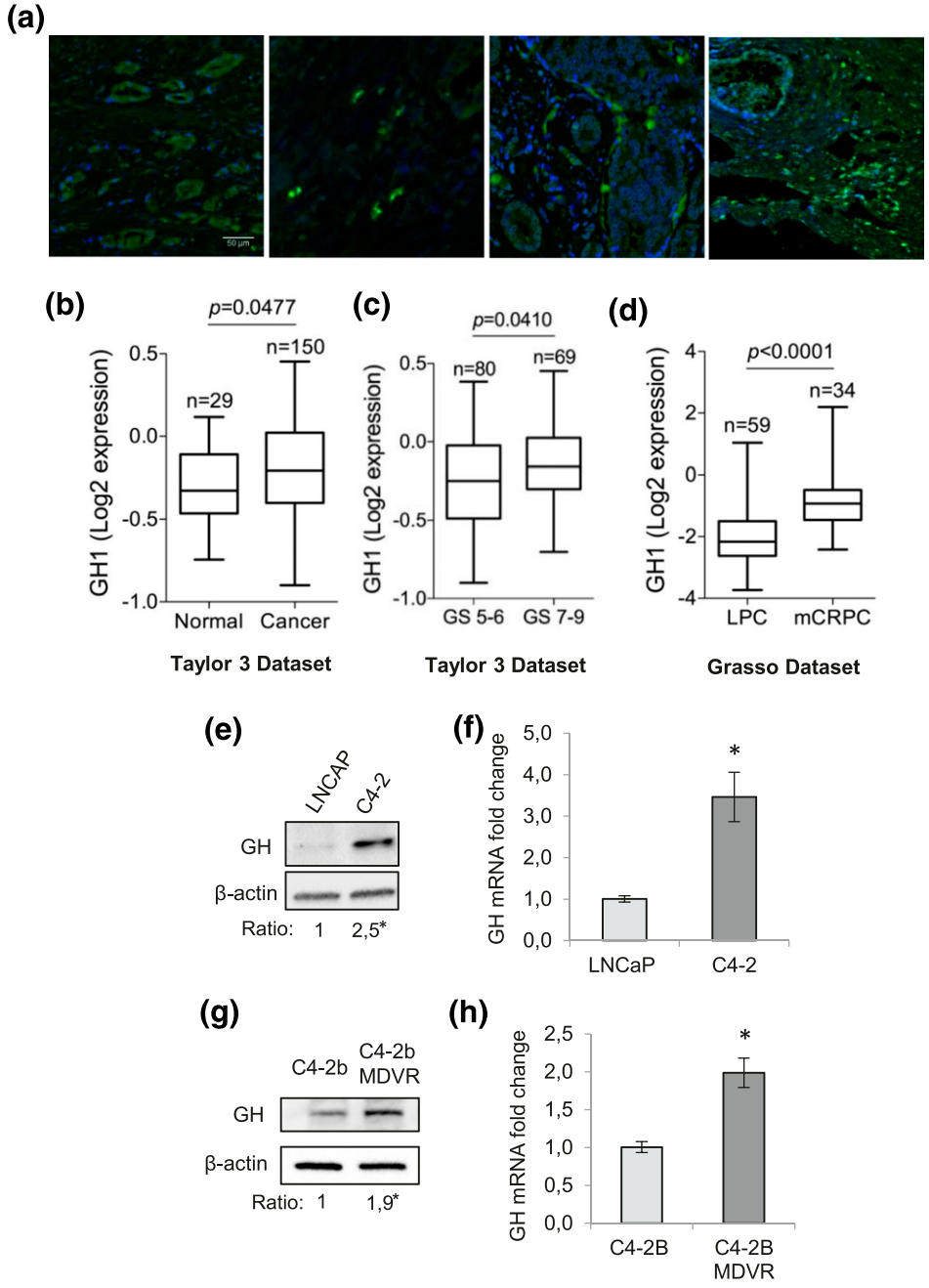

Figure 1. GH expression in human tissue and PCa cell lines. (a) Representative images of GH immunofluorescence staining of human PCa specimens (from left to right: GS 5 tumor; GS 9 tumor; GS 7 tumor and castration resistant PCa) (b–d) Relative GH gene expression in human normal prostate vs PCa; in low GS (GS 5 to 6) vs high GS (GS 7 to 10) tumors; and in localized PCa (LPC) vs metastatic CRPC (mCRPC) obtained from (b, c) Oncomine datasets Taylor 3 and (d) Grasso, respectively. (e) GH protein and (f) mRNA expression levels compared in androgen-dependent LNCaP and isogenic CRPC C4-2 cells. (g) GH protein and (h) mRNA expression levels in C4-2B parental and C4-2B enzalutamide-resistant MDVR cells by Western blot of whole cell lysates and by qPCR after total RNA extraction. β-actin expression was used for normalization. Experiments presented in (d–g) were repeated three times. Differences among groups were compared by two-tailed unpaired Student *t* test. $^*P < 0.05$.

of AR on GH expression. Importantly, we confirmed this finding in human PCa Oncomine databases, where we observed a negative correlation between GH and PSA expression, a marker of AR activity in four independent datasets [Fig. 2(d) and Supplemental Table 1].

AR inhibition by AR antagonists and androgen biosynthesis inhibitors is commonly used in combination with ADT to further block AR activity in advanced PCa and CRPC, but fails to stop CRPC growth (43, 45, 46). Given our finding that AR regulates GH expression and reversibility of this effect by bicalutamide, we tested the effects of antiandrogen therapies on GH expression. Consistent with negative regulation of GH by AR, we found GH expression increased after treatment with

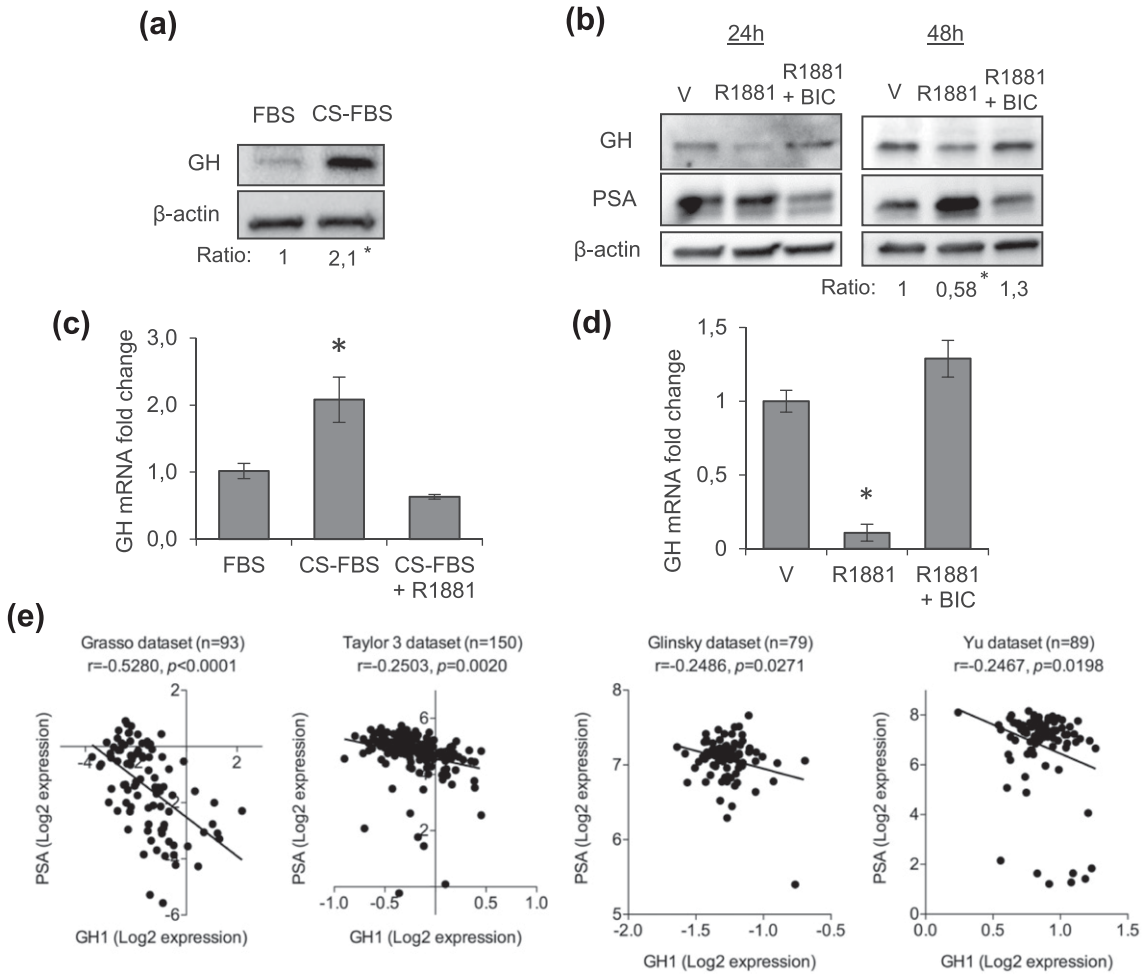

Figure 2. Androgens negatively regulate GH expression by AR in PCa cells. (a) GH expression was evaluated by Western blot in LNCaP cells growing for 72 hours in RPMI 1640 supplemented with 5% FBS or 5% charcoal-stripped FBS (CS-FBS). Quantification of band intensities is shown below each lane as the GH/actin ratio relative to FBS group (mean of three independent experiments). Differences were compared by two-tailed unpaired Student *t* test. $*P < 0.05$. (b) GH mRNA expression was analyzed by qPCR in LNCaP cells grown for 72 hours in RPMI 1640 supplemented with 5% FBS, 5% CS-FBS and CS-FBS + 1 nM R1881. $*P < 0.05$ (FBS vs CS-FBS, and CS-FBS vs R1881). (c, d) LNCaP cells were grown in RPMI 1640 5% CS-FBS for 24 hours before treatment with 1nM R1881 alone or in combination with the AR antagonist bicalutamide (Bic, 10 μM). PSA expression was used as a marker for drug efficacy, and β-actin as protein-loading control. Experiments in (a–d) were repeated three times. Differences between means were compared by one-way analysis of variance, $*P < 0.05$. (e) Correlation of GH and PSA gene expression in human PCa datasets downloaded from Oncomine. Four different datasets were analyzed by Pearson correlation test.

bicalutamide and enzalutamide in a concentration-dependent manner (Fig. 3), confirmed by GH Western blot and qPCR in androgen-sensitive LNCaP cells and in castration-resistant C4-2 and 22Rv1 cells (Fig. 3). Similarly, qPCR showed that inhibition of intratumoral androgen biosynthesis by 5 μM abiraterone acetate induced GH expression approximately twofold in LNCaP, C4-2, and 22Rv1 cells (Fig. 3). Taken together, these results confirm that GH expression is suppressed by AR in PCa cells, and induction of GH expression after ADT suggests a role for locally produced GH in castration resistance.

GH promotes aggressive/invasive behavior in CRPC cells

Previous studies showed that GH-responsive LNCaP cells activate protumorogenic JAK2/STAT5, AKT, and p42/p44 MAPK signaling pathways upon GH stimulation (30), and that GH induces cell motility (35, 47). After showing an ADT-induced GH increase in CRPC cells, we investigated whether GH excess promotes aggressive behavior in CRPC 22Rv1 cells. We infected 22Rv1 cells with lentivirus expressing GH, and evaluated colony-formation ability in soft agar assays. GH-expressing 22Rv1 cells showed 35% enhancement of colony number after 14 days of growth in soft agar under androgen deprived conditions (CS-FBS) compared with cells infected with control lentiviral particles [$P = 0.05$; Fig. 4(a)]. We observed significantly enhanced migration (28%) and invasion through matrigel (46%) in GH-expressing cells compared with control after 24 hours [Fig. 4(b) and 4(c)]. GH treatment also increased expression of MMP2 and MMP9 [Fig. 4(d) and 4(e)], which promote invasive

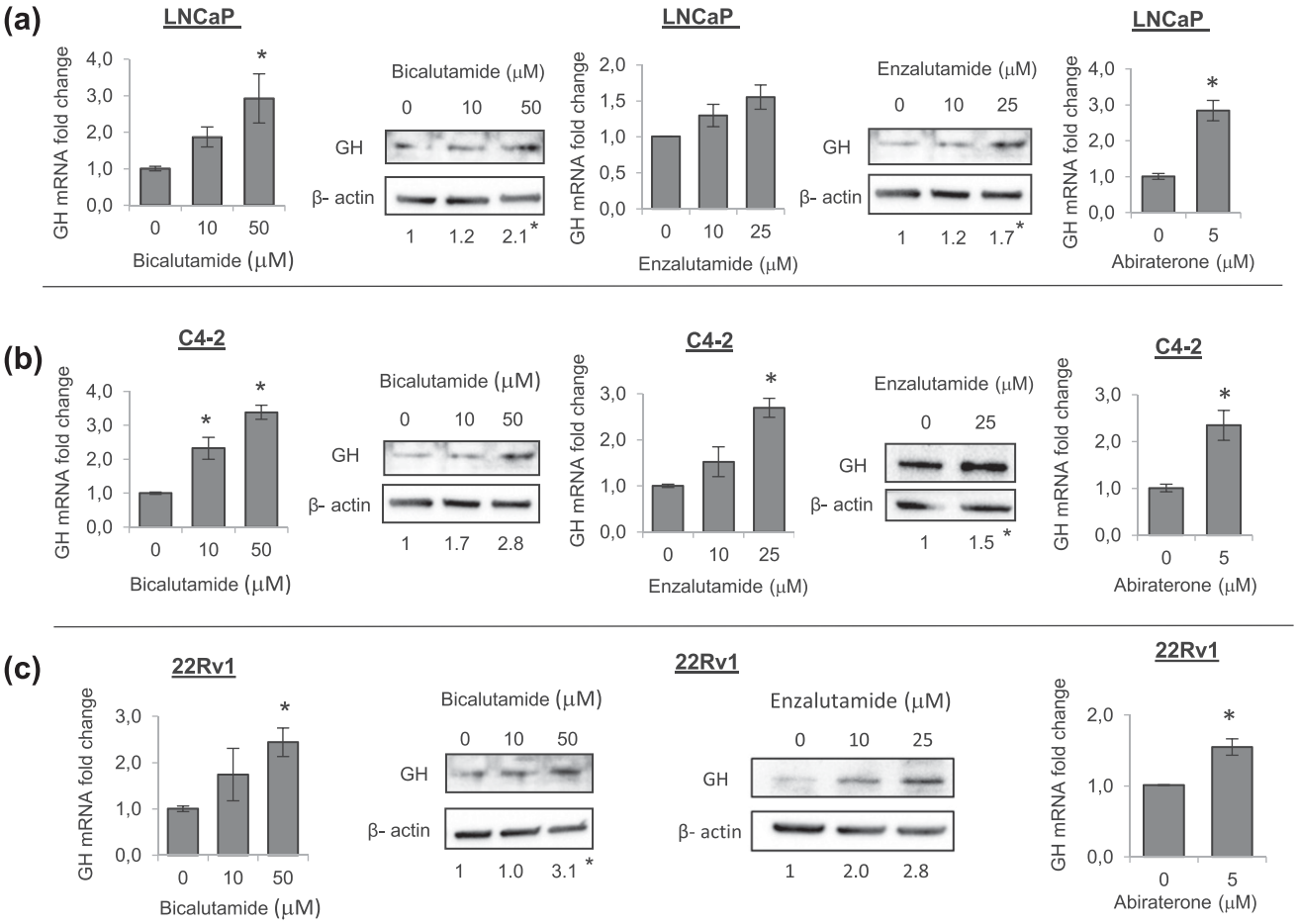

Figure 3. GH is induced by inhibiting AR. (a) LNCaP, (b) C4-2, and (c) 22Rv1 cells were treated with indicated concentrations of bicalutamide, enzalutamide, and abiraterone acetate for 48 hours in RPMI 1640 supplemented with 5% CS-FBS. GH protein expression was evaluated by Western blot in whole cell lysates, and GH mRNA expression levels assessed by qPCR after total RNA extraction. Experiments were repeated three times. Quantification of Western blot band intensities is shown as GH/actin ratio relative to the untreated group. Differences between means were compared by one-way analysis of variance and Student *t* test. $*P < 0.05$.

and metastatic behavior in cancer cells via extracellular matrix remodeling activity (48). On the contrary, treatment with the GHR antagonist pegvisomant inhibited MMP2 and MMP9 mRNA expression [Fig. 4(f) and 4(g)]. Finally, expression levels of the EMT signature transcription factor Twist were up-regulated by GH, while cotreatment with pegvisomant reversed this effect [Fig. 4(h)]. These results suggest that enhanced GH expression in CRPC cells may promote aggressive behavior, as evidenced by increased colony formation ability, cell motility, and MMP and Twist upregulation.

GH induces ARv7 and IGF-1 expression CRPC cells

Because we showed that GH is induced after ADT and enhances invasive behavior of CRPC cells, we explored whether GH might be implicated in promoting resistance to antiandrogen therapies.

Reactivation of the AR pathway is a common feature of CRPC. Thus, we assessed whether locally induced GH by ADT and AR inhibition exerts feedback regulation on AR expression in CRPC cells. Neither exogenous GH treatment nor forced GH overexpression by transient transfection altered full length AR expression levels (110 kD). Nevertheless, the AR band with lower electrophoretic motility (80 kD), attributed to truncated AR splicing variants, was enhanced after both GH treatment and GH transfection [Fig. 5(a) and 5(b)].

AR splicing variants (ARvs) are C-terminal truncated AR isoforms commonly upregulated in CRPC tissue and cell lines and rapidly induced after ADT (49, 50). Because they lack the ligand binding domain, activity of ARvs are thought to underlie resistance to next-generation anti-AR therapies (5). The most well studied and clinically relevant is ARv7 (also known as AR3), which correlates with resistance to enzalutamide and abiraterone in metastatic CRPC patients (51, 52). Remarkably, forced GH expression and exogenous GH treatment both induced ARv7 protein expression levels in 22Rv1 cells, as assessed by Western blot with a specific ARv7 antibody [Fig. 5(a) and 5(b)].

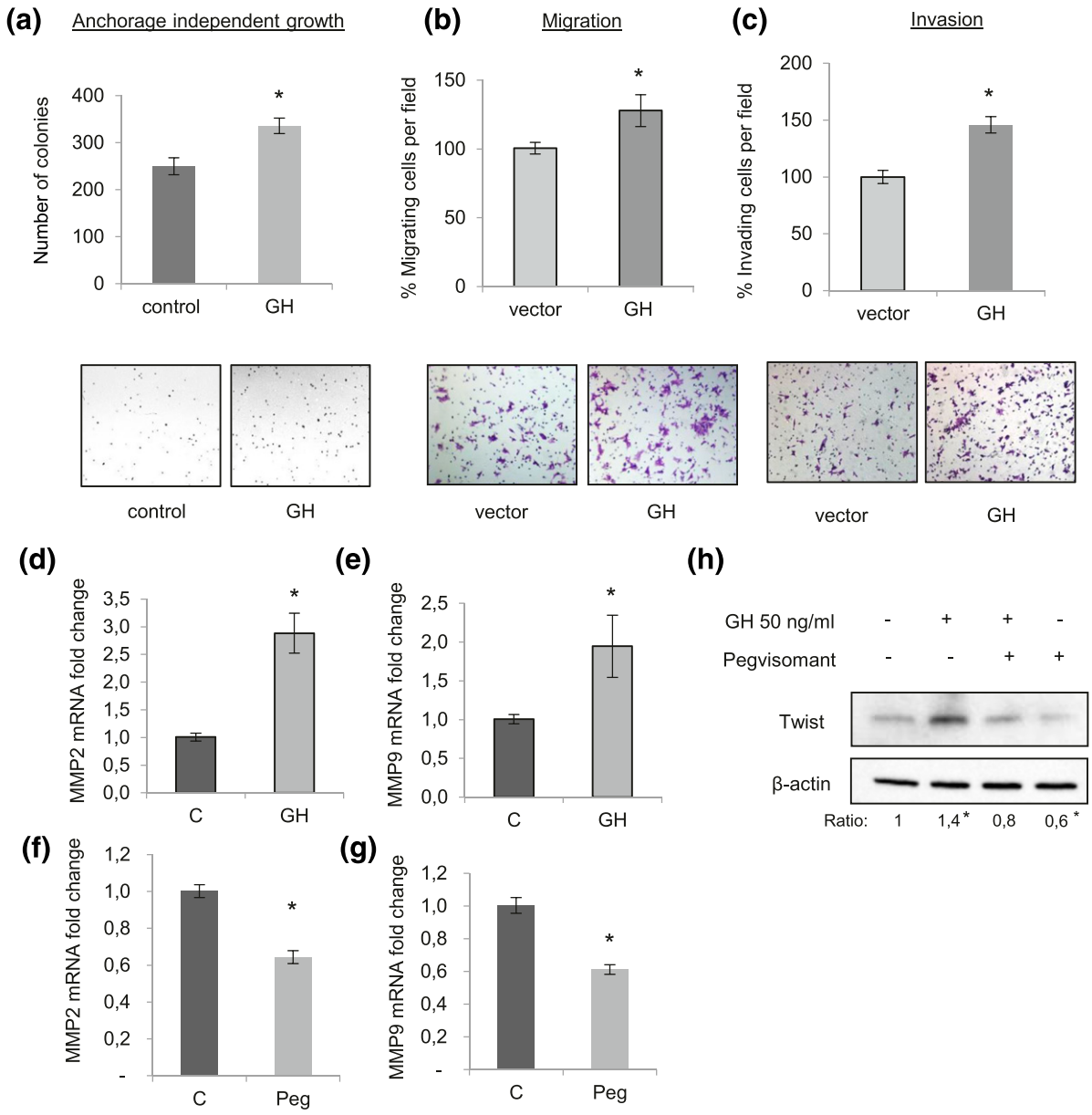

Figure 4. GH promotes aggressive/invasive behavior in castration-resistant PCa cells. (a) Number of colonies formed in soft agar assay by 22Rv1 cells stably infected with lentivirus expressing GH or control. Assay was performed in androgen deprivation condition (5% CS-FBS). (b, c) 22Rv1 cells were transiently transfected with an empty vector (pIRES2-Zs-green) or with a GH expression vector for 48 hours before being transferred to (b) uncoated Boyden chambers to evaluate migration or to (c) Matrigel-coated Boyden chambers for invasion. Results are presented as mean ± standard error of the mean (SEM) of three independent experiments in triplicate wells. *$P < 0.05$. (d, e) MMP2 and MMP9 mRNA expression evaluated by qPCR after 48 hours treatment with 500 ng/mL GH vs control (C, PBS) in 22Rv1 cells. Results are presented as mean ± SEM of three independent experiments by triplicate. *$P < 0.05$. (f, g) MMP2 and MMP9 mRNA expression evaluated by qPCR after 48 hours treatment with 20 μg/mL pegvisomant (Peg) vs control (C, PBS) in 22Rv1 cells. Results are presented as mean ± SEM of three independent experiments by triplicate. *$P < 0.05$. (h) Western blot analysis of Twist protein expression levels after 48 hours treatment with 50 ng/mL GH alone or combined with 20 μg/mL pegvisomant. β-actin was used as loading control, representative blots are shown of three replicated experiments. The fold change of Twist/β-actin ratio is indicated below the Western blot image. *$P < 0.05$ compared with the untreated group.

The mitogenic polypeptide IGF-1 and its receptor IGF-1R have been implicated in PCa progression (53, 54) and ligand-independent AR activation (55). As shown in Fig. 5(a) and 5(b), we found increased IGF-1 protein expression in 22Rv1 cells transfected with GH vector vs empty vector; as well as after 50 ng/mL GH treatment of 48 hours in 22Rv1 cells.

To further confirm GH action in regulating IGF-1 and ARv7 expression, 22Rv1 cells were treated with increasing concentrations of the GHR antagonist pegvisomant for 24 hours. Consistent with a positive GH regulation, pegvisomant treatment inhibited both baseline [Fig. 5(c)] and GH-induced ARv7 and IGF-1 expression levels in 22Rv1 cells [Fig. 5(d)]. Similar results were observed in enzalutamide-resistant C4-2B MDVR cells expressing high levels of ARv7 (36) [Fig. 5(e)], with higher GH levels seen in C4-2B cells MDVR in than parental C4-2B cells [Fig. 1(f) and 1(g)],

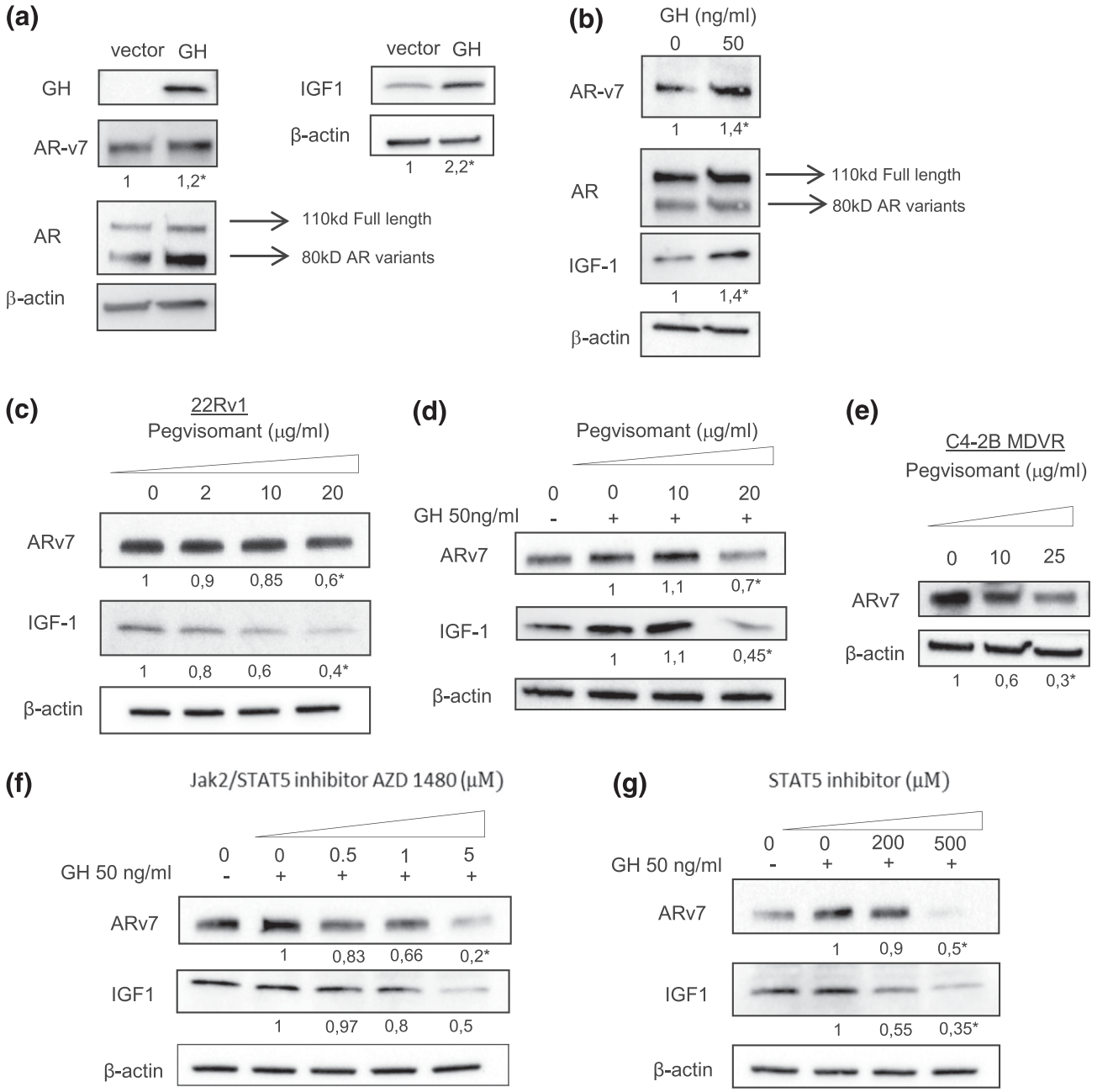

Figure 5. GH induces ARv7 and IGF-1 expression in CRPC cells. Western blot analysis of AR, ARv7, and IGF-1 protein expression levels in (a) 22Rv1 cells transiently transfected with an empty vector (pIRES2-Zs-green) or with GH expression vector for 48 hours, (b) 22Rv1 cells treated with 50 ng/mL GH for 48 hours, (c) 22Rv1 cells treated for 24 hours with increasing concentrations of pegvisomant alone or (d) in combination with 50 ng/mL GH, (e) C4-2B MDVR cells treated with indicated concentrations of pegvisomant for 24 hours, (f, g) 22Rv1 cells treated for 24 hours with increasing concentrations of AZD1480 (JAK2 inhibitor) or specific STAT5 inhibitor (Millipore) in combination with 50 ng/mL GH. Experiments were repeated at least three times; representative blots are shown.

supporting a positive association between GH and ARv7. Analysis of three independent datasets also showed that GH gene expression correlates positively with the ARv7-induced genes UBE2C and CCNA2 (Supplemental Fig. 1 and Supplemental Table 2).

Cellular GH actions are mediated by GHR binding, followed by activation of JAK2/STAT signaling. To determine whether blocking these pathways would also inhibit GH-induced ARv7 and IGF-1 expression, we treated 22Rv1 cells with increasing concentrations of the JAK2/STAT inhibitor AZD 1480 and with a specific STAT5 inhibitor in the presence of 50 ng/mL GH. Both inhibitors of intracellular GH signaling reduced ARv7 and IGF-1 expression in a concentration dependent manner [Fig. 5(f) and 5(g)].

Inhibiting GH action decreases ARv7 and IGF-1 in 22Rv1 xenografted tumors *in vivo*

Finally, we aimed to translate our *in vitro* findings to an *in vivo* xenograft model of CRPC growth by

subcutaneous injection of 22Rv1 cells in 32 nude mice. When xenografted tumors had reached 100 to 200mm^3, mice were separated into 4 groups and treated with vehicle, enzalutamide, pegvisomant, or combination of enzalutamide and pegvisomant for 2 weeks. Although tumor growth and final tumor weight were not affected by any of the treatments (Supplemental Fig. 2), serum PSA was significantly reduced in mice treated with the combination of enzalutamide and pegvisomant [Fig. 6(a)]. Efficacy of pegvisomant treatment was confirmed by observed reduction of liver IGF-1 protein expression levels [Fig. 6(b)]. Consistent with our *in vitro* findings, pegvisomant treatment alone or in combination with enzalutamide significantly reduced ARv7 and IGF-1 tumor expression levels as evidenced by Western blot analysis [Fig. 6(c)].

Discussion

We describe here a novel regulatory link between GH and AR pathways in PCa cells. We showed that GH is repressed by androgens in PCa cells, and that use of ADT and AR inhibitors causes an increment in local GH expression. GH excess, in turn, induces invasive behavior and ARv7 and IGF-1 expression in PCa cells, suggesting that GH signaling may play a key role in treatment resistance.

Higher GH and GHR expression levels in PCa cells vs normal cells has been previously reported (28, 29). We confirmed this in human gene expression databases, and also found that GH expression is significantly higher in human metastatic CRPC samples compared with localized PCa. GH levels are also higher in the CRPC C4-2 cell line vs isogenic androgen-sensitive LNCaP cells, as well as in the enzalutamide-resistant C4-2B MDVR vs parental C4-2B cells, implying a possible role for GH in progression of CRPC and a link with AR activity.

Regulation of local prostatic GH production has not been studied before. We demonstrate here a novel negative regulation of GH expression by androgens/AR, as evidenced by increased GH mRNA and protein expression under androgen deprivation conditions, which was reversed by addition of androgen to the media. Moreover, GH levels were inhibited by the AR agonist R1881 and fully recovered by cotreatment with the AR antagonist bicalutamide, and increased concentrations of the antiandrogens bicalutamide and enzalutamide and the androgen biosynthesis inhibitor abiraterone induced GH expression in LNCaP, C4-2, and 22Rv1 cell lines. Together, these results suggest that GH is an androgen repressed gene, a finding that is further supported by the negative correlation we found between GH expression and PSA in human PCa datasets.

Similar negative regulation of GH expression by androgens was described in mice with targeted deletion of AR gene in mineralizing osteoblasts and osteocytes, in which AR loss induced GH expression (56). Our results are also in agreement with a previous study showing increased serum GH in pubertal males treated with the AR inhibitor flutamide (57), suggesting that AR regulation of GH is not restricted to prostatic tissue.

Counteracting effects of AR signaling on GH action have also been reported in PCa cells, and might be mediated by AR induction of suppressor of cytokine signaling 2, an inhibitor of GH intracellular signaling (35). Furthermore, GH binding to GHR is decreased after long term exposure to mibolerone, a potent androgen synthetic analog (58).

We considered mechanisms underlying AR inhibition of GH expression and did not observe direct AR binding to the GH promoter by chromatin immunoprecipitation assays (data not shown), indicating that other AR regulated genes might mediate regulation of GH expression. Interestingly, ADT induces senescence in PCa cells (59, 60), and we previously showed that GH is a target of the p53/p21 senescence pathway (42), suggesting a possible link between ADT-induced senescence and GH induction in PCa.

Based on our results and others (35, 58), androgens, through AR binding, exert a counteracting effect on local prostatic GH expression and action, and release of this inhibitory effect as a consequence of androgen ablation may facilitate castration-resistant tumor progression. Supporting this hypothesis, we showed that GH excess promotes invasive cell behavior in the absence of androgens, as evidenced by enhanced anchorage-independent colony formation ability, increased migration and invasion, and increased expression of MMPs as well as induction of the EMT signature transcription factor Twist. These results are consistent with previous reports demonstrating oncogenic effects of autocrine GH in mammary and endometrial cells (24, 25, 27).

In contrast, the role of GH in prostatic oncogenic transformation is not clear. Differential actions of endocrine and autocrine GH on cell proliferation and survival have been reported in PCa cells, and these were attributed to intracellular activation of GHR by autocrine GH (28). Nevertheless, both autocrine and exogenous GH stimulate invasive behavior of LNCaP cells (47), in agreement with our observations in CRPC 22Rv1 cells. Our results suggest a moderate proinvasive and proaggressive effect of local GH that may be permissive for CRPC progression in the absence of androgens.

GH binding to GHR activates the JAK2/STAT5 signaling pathway, which mediates regulation of GH target genes such as the mitogenic growth factor IGF-1. Both STAT5 and IGF-1 have been largely associated with PCa

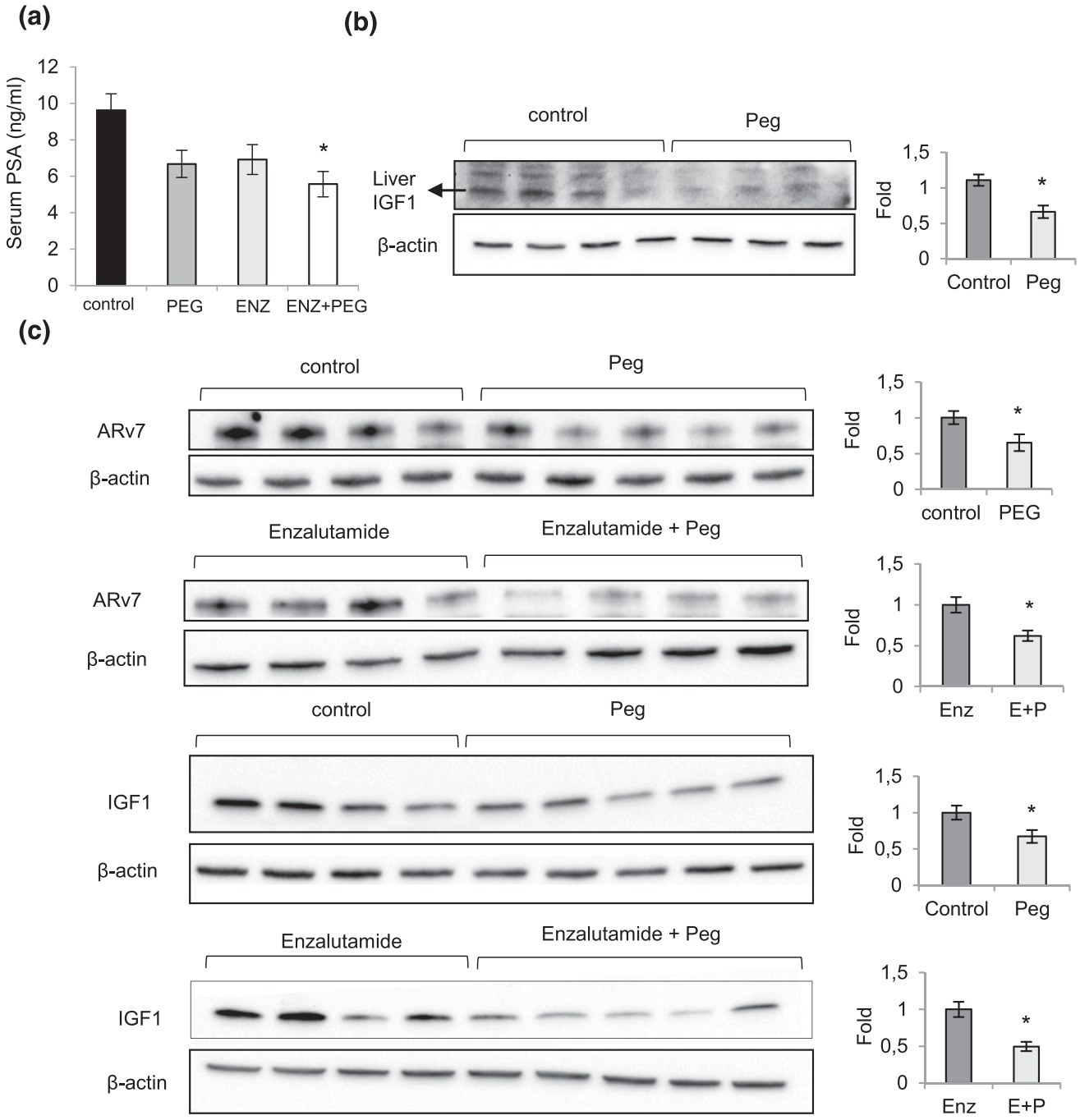

Figure 6. Inhibiting GH action decreases ARv7 and IGF-1 in 22Rv1 xenografted tumors *in vivo*. 22Rv1 cells ($1x10^6$) were subcutaneously injected in both flanks of male athymic nude mice. Once tumors reached 100 to 200 mm^3 volume, mice (n = 8 per group) were treated three times a week with vehicle, 100 mg/kg pegvisomant (Peg, Pfizer), 20 mg/kg enzalutamide (Enz), or both (E+P). (a) Serum PSA levels were measured by enzyme-linked immunosorbent assay. (b) Western blot analysis of IGF-1 in liver extracts derived from control and pegvisomant-treated mice. (c) Western blot analysis of ARv7 and IGF-1 in tumor protein extracts comparing control vs pegvisomant treated mice and enzalutamide vs enzalutamide/pegvisomant treated mice. Representative blots are shown. Intensity of the bands was quantified with ImageJ software and the fold change of ARV7/β-actin or IGF-1/ β-actin is shown in the bar graphs on the right. *$P < 0.05$.

progression (34, 53, 54, 61). Accordingly, we found that GH locally stimulates IGF-1 production in 22Rv1 cells, and pegvisomant treatment inhibits IGF-1 expression both *in vitro* and *in vivo*. IGF-1 mRNA induction upon GH treatment was previously reported in LNCaP cells (62).

Importantly, we also found that GH excess by autocrine forced expression or exogenous treatment increases ARv7 protein expression. ARv7 is upregulated in human CRPC samples and in circulating tumor cells derived from patients who show resistance to enzalutamide and abiraterone. Because of its constitutive activity and nuclear localization, ARv7 is proposed as a mechanism for resistance to anti-AR therapies. Indeed, ARv7 overexpression confers resistance to enzalutamide and

abiraterone both *in vitro* and *in vivo* (63); moreover, ARv7 induces EMT (64) and confers resistance to taxane treatment in PCa cells (65). Targeting ARv7 therefore represents a promising therapeutic approach for CRPC. Previous studies showed that the NFkB pathway regulates ARv7 (66), and the antihelminthic drug niclosamide has been identified as an ARv7 inhibitor in a drug library screening (36). We report here that GH induces ARv7 expression in PCa cells, and that blockade of GHR by pegvisomant significantly reduces ARv7 expression levels *in vitro* and *in vivo* in subcutaneous 22Rv1 xenografts. GH effect on ARv7 expression appears to be mediated by JAK2/STAT5 signaling, as inhibition of this pathway also reverses GH action. Notably, GH effects appear to be specific on ARv7, because we did not observe consistent changes in expression of full length AR (Supplemental Fig. 3). Because AR and ARv7 are transcriptionally coregulated (67), GH could either modulate AR splicing or ARv7 protein stability.

Blocking GHR by pegvisomant has been tested in *in vivo* models of breast and colon cancer, as well as meningioma (68–70). In those studies, pegvisomant effectively reduced subcutaneous xenograft tumor growth. In contrast, in our *in vivo* study, we did not observe tumor growth reduction with pegvisomant alone or in combination with enzalutamide, likely due to the short term treatment (15 days), which was not able to counteract the rapidly growing tumors. However, pegvisomant was effective in reducing serum PSA levels, reflecting a reduction in tumor AR activity, and tumor expression of IGF-1 and ARv7 was significantly reduced with both pegvisomant and pegvisomant/enzalutamide combination compared with vehicle and enzalutamide alone, respectively.

Our results suggest that ADT releases the inhibitory effect of AR on GH expression. In turn, local GH induction might be permissive for persistence of CRPC growth in the absence of androgens by activation of JAK2/STAT5 signaling pathway and induction of IGF-1 and ARv7 expression. As GHR blockade by pegvisomant inhibited both IGF-1 and ARv7 expression *in vivo*, it may represent a new therapeutic strategy to target CRPC progression.

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