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Article

Growth Hormone Receptor Antagonist Markedly Improves Gemcitabine Response in a Mouse Xenograft Model of Human Pancreatic Cancer

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Abstract: Chemotherapy treatment against pancreatic ductal adenocarcinoma (PDAC) is thwarted by tumoral activation of multiple therapy-resistance pathways. The growth hormone (GH) – GH receptor (GHR) pair is a covert driver of multimodal therapy resistance in cancer, and is overexpressed in PDAC tumors, yet the therapeutic potential of targeting the same has not been explored. Here, we report that GHR expression is a negative prognostic factor in patients with PDAC. Combinations of gemcitabine with different GHR antagonists (GHRAs) markedly improve therapeutic outcomes in nude mice xenografts. Employing cultured cells, mouse xenografts, and analyses of the human PDAC transcriptome, we identified that attenuation of the multidrug transporter and epithelial-to-mesenchymal transition programs in the tumors underlie the observed augmentation of chemotherapy efficacy by GHRAs. Moreover, in human PDAC patients, GHR expression strongly correlates with a gene signature of tumor-promotion and immune-evasion, which corroborate with that in syngeneic tumors in wild-type vs. GH transgenic mice. Overall, we found that GH action in PDAC promoted a therapy-refractory gene signature *in vivo*, which can be effectively attenuated by GHR antagonism. Our results collectively present a proof of concept towards considering GHR antagonist to improve chemotherapeutic outcome in the highly chemoresistant PDAC.

Keywords: pancreatic cancer; pancreatic ductal adenocarcinoma; gemcitabine; growth hormone (GH); GH receptor (GHR); GHR antagonist; insulin-like growth factor 1 (IGF1); adjuvant; chemotherapy; chemoresistance

1. Introduction

Pancreatic adenocarcinoma (PAAD) is responsible for >50,000 cancer-related deaths (8% of all cancers) annually in the US, with a five-year survival rate of 12.5% (<https://seer.cancer.gov/statfacts/html/pancreas.html>). Currently, it is one of the most therapy-resistant solid cancers in humans [1]. Chemotherapy, including FOLFIRINOX, gemcitabine, and nab-paclitaxel, is a major component of the current multimodal chemotherapeutic approach for pancreatic ductal adenocarcinoma (PDAC), which constitutes >90% of pancreatic cancer cases [2]. However, PDAC is extremely chemoresistant, leading to limited therapeutic efficacy and high relapse and mortality rates, emphasizing the need for better treatment approaches [3]. Factors that endow

chemoresistance in PDAC include, but are not limited to, a desmoplastic tumor microenvironment (TME), upregulated drug metabolism and efflux, epithelial-to-mesenchymal transition (EMT)-driven phenotype plasticity, and extensive metabolic reprogramming [4–7]. Therefore, it is challenging to identify a single therapeutic target which can attenuate this multifactorial treatment resistance in cancer. Over the last decade, abrogation of growth hormone (GH) action by targeting the GH receptor (GHR) to specifically curb cancer chemoresistance has shown promising *in vivo* results in multiple human cancer models [8]. Moreover, the GH-GHR pair and its downstream effectors are abundantly expressed in several non-tumor cell-types in the tumor microenvironment. Current knowledge of GH action in these cells indicate several putative tumor promoting and immune suppressing effects, which can be expected to be emphasized by autocrine/paracrine GH action in the TME [9–11]. Therefore, we postulated that antagonizing the GHR can in turn improve chemotherapeutic efficacy in the highly chemorefractory PDAC.

GH is an anterior pituitary hormone with postnatal whole-body endocrine actions that regulates longitudinal growth, organ development, metabolic homeostasis, and hepatic production of >70% of circulating insulin-like growth factor 1 (IGF1), another well-studied tumor-promoting factor. GH also exerts distinct autocrine/paracrine actions via multiple non-pituitary sources of origin [12,13], including the TME [14]. Following adulthood, pituitary GH level decreases steadily with age, while non-pituitary GH production often increases [13] and drives tumor development in the colon [12,15] and breast [10,11,16]. Systemically, GH action drives insulin resistance[9] and, again, IGF1 production, both of which are detrimental factors in cancer prognosis[17]. Recently, we and others have described the covert role of GH in driving cancer chemotherapy resistance [8,10] in addition to its direct tumor-promoting effects. The intracellular signaling cascade (viz. JAK2-STAT5, SRC-MAPK, PI3K-AKT-mTOR) activated following the GH/GHR binding in the TME signals induction of the EMT program, senescence, cancer stem cells, upregulation of multidrug efflux transporters, and downregulation of apoptosis [8]. Moreover, human GH is also an activating ligand of prolactin receptor (PRLR), while GH-induced hepatic and tissue IGF1 activates IGF1 receptors (IGF1R) and insulin receptors (IR) abundant in the TME. Both occurrences exert overlapping and multiple tumor-promoting effects [10]. We and others have recently shown that in cancers of the liver, breast, endometrium, and melanoma, GHR attenuation markedly improves chemotherapeutic efficacy in preclinical models [10]. GH and GHR are expressed in the mammalian pancreatic tissue in endothelial, epithelial, immune, and acinar cells (Figure S1), as well as in pancreatic tumor samples (Figure S2). Therefore, it is provocative to verify whether GHR antagonism as an adjuvant can improve chemotherapeutic efficacy in PDAC. Here, we report that increased GHR expression has a significant negative correlation with disease-free survival in both male and female patients with PDAC. Thereafter, we performed limited but focused *in vitro* and *in vivo* assessment of the extent of GH action in PDAC in the context of tumor chemoresistance to identify some of the underlying factors. We also identified the differentially expressed genes and related pathways in patients with high or low tumoral GHR expression (above or below the mean GHR expression) in available PDAC patient databases. The findings were then corroborated at RNA level in tumors grown in a syngeneic GH transgenic mouse model. Collectively, our results indicate that GH action indeed promotes “broad-spectrum” therapy resistance in PDAC. The GHR antagonists (GHRAs) can effectively reduce tumor growth but more importantly does improve treatment efficacy of anti-cancer compounds in both male and female mouse xenograft models. , Thus, GHRA in combination with anti-cancer chemotherapy posits a promising application to treat PDAC in human patients.

2. Results

GHR Expression in PDAC and Correlation with Patient Survival:

GHR is expressed in several different types of cells in the pancreas. The cell-type specific expression varies from 11% (of ductal cells) to 92% (of beta cells) in the mouse pancreas (*Tabula muris* [18], Figure S1). The expression patterns of deactivators and markers of GH signaling, CISH, and SOCS2, closely follow that of GHR expression (Figure S1). PDAC originate as transformed pancreatic

acinar and ductal cells, where the GHR expression level is the lowest in normal pancreas. However, in human PDAC patients (179 samples, TCGA database), mean tumor GHR expression is 4-fold higher than untransformed ductal and acinar cells and comparable to the mean GHR expression of normal whole pancreas (171 samples, TCGA and GTEx databases, Figure S2). This indicates a possible increase / overexpression of GHR in transformed compared to non-transformed pancreatic acinar and ductal cell type. Tumoral GHR expression remains stable throughout stages I to IV in the human PDAC database (Figure S2). Disease-free survival (DFS) is a reliable surrogate for overall survival in pancreatic cancer [19]. Querying one of the largest pancreatic cancer patient transcriptome profiles, which includes 1640 samples combined with 16 available GEO datasets [20], we observed that increased GHR expression significantly and negatively correlated with DFS [hazard ratio (HR) = 2.16, $p=0.0003$] and survival (9 vs. 17 months) in human PDAC patients (Figure 1A, Figure S3). Herein, GHR related hazard ratio was markedly higher in male (HR=3.16, $p=0.0004$, 10 vs. 19 months, Figure S3) than in female (HR=1.66, $p=0.07$, 9 vs. 15 months, Figure S3) patients. GH is also a potent ligand and activator of PRLR (21), and several subsets of patients with PDAC display upregulated PRLR expression [22]. In the PDAC dataset, PRLR expression also significantly correlates with increased risk (HR=1.66, $p=0.0179$) and poorer survival (10 vs. 15 months, Figure 1B), preferentially in male (HR=2.8, $p=0.003$, 10 vs. 24 months, Figure S3) than in female (HR=0.77, $p=0.320$) patients. Interestingly, tumoral IGF1R expression correlates with significantly increased HR and poorer overall survival, also preferentially in male patients with PDAC (HR=1.55, log-rank $p=0.012$, Figure S4). Additionally, we observe that in the healthy human pancreas, RNA expression of *GHR* and *IGF1* is positively correlated (Spearman correlation coefficient $R = 0.43$, $p<0.001$) and the correlation further increases in pancreatic tumors ($R=0.71$, $p<0.001$) (Figure 1C). These results indicate probable GH-regulated IGF1 production in the PDAC TME. Moreover, despite no correlation in the normal pancreas ($R=0.07$, $p=0.39$) between *GHR* and *IGF2* (a potent activator of IGF1R), a much higher positive correlation in pancreatic tumors is observed ($R=0.37$, $p<0.001$) for the same (Figure 1C).

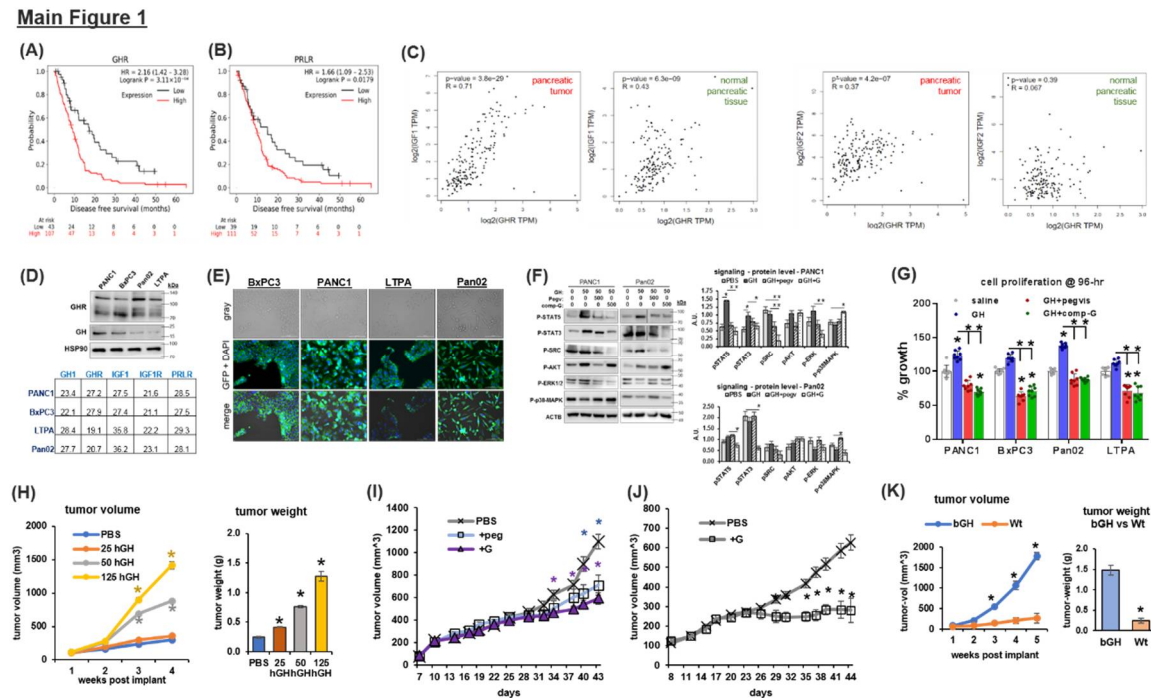


Figure 1. GH action is a driver of pancreatic cancer progression and inversely correlates with disease-free survival in human PDAC patients. (A-B) Kaplan-Meier survival plot depicting correlation of human GHR (A) or PRLR (B) RNA expression with disease-free survival in human pancreatic ductal adenocarcinoma patients [Data re-analyzed using KMplotter from Posta and Gyorffy, *Clinical and Translational Science*, 2023, doi: 10.1111/cts.13563]. (C) Spearman correlation of GHR expression vs IGF1 and IGF2 expression in pancreatic tumor and normal pancreatic tissue in PDAC patients (TCGA and GTEx) [data generated using GEPIA2 portal]. (D) Immunocytochemistry for GHR (green) in

cultured pancreatic cancer cells with nuclei stained in DAPI (cells in grayscale), (E) (top) Western-blot showing protein expression of GH and GHR in cultured pancreatic cancer cell lysates, and (bottom) mean Ct values from quantitative-PCR showing relative RNA expression of GH1, GHR, IGF1, IGF1R, and PRLR in pancreatic cancer cultured cells, (F) Western-blot of GH downstream intracellular signaling mediators - STAT5, STAT3, SRC, AKT, ERK1/2 - in cultured pancreatic cancer cells treated with either of saline, GH (50ng/mL), GH (50ng/mL) + pegvisomant (500nM), or GH (50ng/mL) + compound-G (500nM), (G) Effects of 96-hr treatment with above concentrations of GH and GHR antagonists (pegvisomant and compound-G) on pancreatic cancer cell growth in culture. For the above, Significant difference was compared using two-way ANOVA with Tukey's multiple comparisons test and * signifies $p < 0.05$. (H) increase in tumor volume and final tumor weight in male Nude mice ($n=4$) treated with 25 or 50 or 125 ug/daily of recombinant human GH by intraperitoneal (i.p.) injection, (I-J) Effects of treatment with GHR antagonists (pegvisomant and compound-G) on PANC1 xenograft tumor volume growth in male (I) and female (J) nude mice ($n=6$), (K) Increase in murine Pan02 allograft tumor volume with time and final tumor weight in male syngeneic C57BL6 mice – wild-type (Wt) or transgenic for bovine GH (bGH; high GH, high IGF1) ($n=4$). Significant difference was compared using repeated measures ANOVA combined with Bonferroni's multiple comparison and * signifies $p < 0.05$.

GH Drives Pancreatic Cancer Cell Growth:

In all of our cultured pancreatic cancer cell lines (human – PANC1, BxPC3, mouse – LTPA, and Pan02), RNA and protein expression of GH and GHR are readily detectable (Figure 1D, 1E), indicating an autocrine loop. Moreover, in the cultured cells, the phosphorylation states of specific downstream mediators of GH signaling (STAT5, STAT3, and SRC) are upregulated following GH treatment, whereas GHRAs (either the FDA-approved GHRA, pegvisomant [23], or a novel candidate GHR antagonist, compound G [24]) suppress this effect significantly (compound G > pegvisomant) (Figure 1F, S5a). Interestingly, GHRA treatment in the cell lines exhibits a modest upregulation of the p38-MAPK pathway (Figure 1F, S5a). In this regard, activation of the p38-MAPK pathway enables gemcitabine-induced cytotoxicity in pancreatic cancer [25].

We found that exogenous GH treatment significantly increases proliferation rate in cultured cells, which is blocked by GHRA treatments *in vitro* (Figure 1G). *In vivo*, human PANC1 cell xenografts in male nude mice show a dose-dependent increase in growth rate and final tumor weight with daily intraperitoneal (i.p.) injections of human GH compared to saline-treated controls (Figure 1H). Monotherapy with pegvisomant or compound G effectively reduces the PANC1 xenograft tumor growth rates in male and female mice (Figure 1I, 1J). Additionally, when 100,000 mouse Pan02 cells are implanted in syngeneic (C57BL6) bovine GH transgenic (bGH) mice with supraphysiological levels of GH and IGF1, vs. wild-type (Wt) mice, tumors develop in 100% (4/4) of bGH mice compared to in only 50% (2/4) of WT mice (Figure S6). The bGH tumors are >5-fold higher in volume and weight (Figure 1K) compared to their WT counterparts. In human PDAC patient tumor samples, *GHR* RNA expression is positively and significantly correlated with that of several markers of GH downstream signaling, including *IGF1*, *STAT5B*, *SOCS2*, and *JAK2* (Pearson's correlation coefficient = 0.84, 0.74, 0.73, and 0.49, respectively, false discovery rate (FDR) < 0.05; Figure S7). Gene-set enrichment analysis (GSEA) of genes correlating significantly (FDR < 0.05) with GHR also show positive enrichment of multiple members of JAK/STAT signaling (Figure S8, KEGG pathway). Collectively, the data show a net definite tumor-promoting action of GH in cultured pancreatic cancer cells, xenograft samples, and human patients.

GHR Antagonism Markedly Improves Chemotherapeutic Efficacy against PDAC:

It is currently untested whether GHRA can be administered as an adjuvant with chemotherapy in any human cancer model. We report that in cultured human and mouse pancreatic cancer cells, exogenous GH treatment partially rescue tumor cells from the cytotoxic effects of chemotherapies - gemcitabine, doxorubicin, and the EGFR-inhibitor erlotinib (Figure S9). Both GHRAs, pegvisomant and compound-G, attenuate these effects of GH and markedly restore the cytotoxic effects of the

chemotherapies (Figure S9). For human PANC1 cells xenografted in male nude mice, both high and low physiologic doses of gemcitabine monotherapy markedly reduce the xenograft growth rate. But over the course of treatment, the chemotherapy effect reduces to either a cytostatic (at high dose) or no effect (at low dose) (Figure 2A). In low-dose gemcitabine monotherapy, an increase in tumor volume from day-40 (23-days post-treatment) indicates a possible onset of chemoresistance (Figure 2A). Remarkably, either pegvisomant or compound G combinations restore the gemcitabine cytotoxicity, observable from day-28 (11-days post-treatment). The combination of low-dose gemcitabine and either of the GHR antagonists is significantly more efficacious than both low- and high-dose gemcitabine monotherapy (Figure 2A). The maximum therapeutic efficacy is observed in the combination regimen of compound G and high-dose gemcitabine, where 2/6 male mice in the group are tumor-free by day-43 (26-days post-treatment). In the compound G and low-dose gemcitabine combination, 1 of 6 mice is tumor-free, while no mice in the high or low gemcitabine monotherapy treatment groups achieve tumor clearance. Similar results are obtained in female nude mice (Figure 2B; *tumor growth is slower than in male mice*), where either dose of gemcitabine markedly lower tumor growth rates but fail to reverse the increase in tumor volume or achieve tumor clearance. In low-dose gemcitabine treatment, the tumor volume starts increasing by day-35 (18-days post-treatment), indicating a possible onset of chemoresistance. The co-administration of compound G (*pegvisomant combination not tested in female mice*) restores tumor sensitivity to gemcitabine. Compound G and low-dose gemcitabine combination is comparable to high-dose gemcitabine monotherapy in controlling tumor volume increase, whereas in the compound G and high-dose gemcitabine combination group, 3 of 7 mice are tumor-free by day-44 (27-days post-treatment) (Figure 2B). The post-mortem final tumor weights corroborate the tumor volume measurements (Figure 2C, 2D). No significant differences in body weight, body composition, glucose levels, or serum IGF1 are observed between the nude mice groups (Figs S10, S11). Xenograft tumors in female mice grow significantly smaller than in male mice, which could be a function of lower IGF1 levels (Figure S10) as well as GH antagonizing effects of estrogen in the former but was not confirmed here. In xenograft tumors, querying GH downstream intracellular signaling intermediates predicted from PDAC patient tumor analysis (Figs S7, S8), show that GHRA-treated groups have significant and consistent reductions in the phosphorylation states of STAT5, STAT3, as well as in SRC and AKT, in both male (Figure 2E, 2F) and female mice (Figure 2G). ERK1/2 activation is not significantly affected in the tumors (Figure 2E, 2F). Interestingly, a distinct and significant increase in phosphorylation of p38-MAPK with GHRA treatments is also observed in the male but not in female nude mice (Figure 2E-2G). In cultured human and mouse cells, both GHRAs exhibit similar concomitant suppression of STAT5 and an increase in p38-MAPK phosphorylation states, which is more pronounced in the presence of gemcitabine (Figure S12). Notably, in pancreatic cancer, gemcitabine efficacy necessitates p38-MAPK activation [25], while gemcitabine resistance is augmented by the activation of STAT5 [26], SRC [27,28], and STAT3 [29]. Altogether, our data validates our hypothesis that GHRA combination improves chemotherapy efficacy in part by attenuating the signaling pathways responsible for gemcitabine resistance *in vivo*.

Main Figure 2

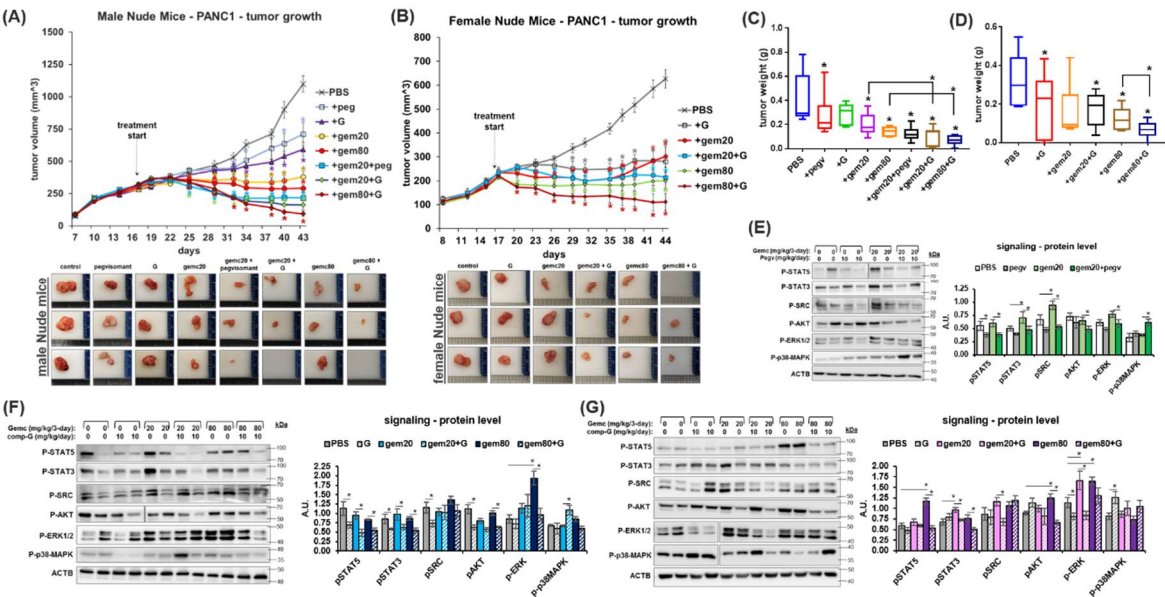


Figure 2. GHR antagonism improves chemotherapeutic efficacy in pancreatic cancer xenografts. (A) Changes in tumor volume over time and treatments with GHRA (pegvisomant, compound-G) or gemcitabine (@20 or 80 mg/kg-BW) or combinations of GHRA + gemcitabine in male (A) and female (B) Nude mice with human PANC1 subcutaneous xenografts. Representative tumors at end-of-study from 2A and 2B are shown below the graphs. (C-D) End of study tumor weights in PANC1 xenograft Nude male (C) or female (D) mice treated with GHRA (pegvisomant, compound-G) or gemcitabine (@20 or 80 mg/kg-BW) or combinations of GHRA + gemcitabine [doxo= doxorubicin, erlo = erlotinib, gemc = gemcitabine, gem20 = gemcitabine @ 20mg/kg/3-dy, gem80 = gemcitabine @ 80mg/kg/3-dy, G= compound-G (GHRA), peg = pegvisomant, control = saline]. (E-G) Western blots showing activation states of intracellular signaling mediators (STAT5, STAT3, SRC, AKT, ERK1/2 and p38-MAPK) downstream of GHR from tumor lysates in pegvisomant-gemcitabine treated male mice (E), compound-G-gemcitabine treated male (F) and female (G) mice (n=6). Significant difference was compared using repeated measures ANOVA combined with Bonferroni's multiple comparison and * signifies p<0.05.

Combination of Chemotherapy and GHR Antagonism Suppresses ABC Multidrug Transporter Expression and Drug Efflux Activity in PDAC Cells and Xenografts

ATP-binding cassettes containing multidrug transporters (ABC transporters), support chemoresistance in cancer by active efflux of numerous antineoplastic agents. In pancreatic cancer, ABCG2 [30], ABCC1, ABCC5 [31], and ABCA8 [32] have been specifically reported to be associated with gemcitabine resistance, whereas additional ABC transporters are widely expressed in chemoresistant PDAC cells [33]. Given that GH action drives overexpression of multiple ABC transporters in melanoma, liver cancer, and breast cancer [10], and our observed GHR associated enrichment of ABC transporter pathway in PDAC patient samples (Figure S8), here we test and observe a strong positive correlation between RNA expression of GHR and 15 ABC multidrug transporters in PDAC patients (FDR<0.05, TCGA) (Figure 3A). Well known mediators of gemcitabine import (SLC28A1, SLC28A3, SLC29A1, SLC29A2), activation (DCK, CMPK1, NME4), or deactivation (DCTD, CDA, NT5C) are not consistently correlated with GHR expression in the TCGA dataset (Figure S13), and also the status of gemcitabine therapy in these patients were not considered. At the protein level, in cultured cells, although exogenous GH treatment did not have a consistent effect on ABC transporters, each of the GHRA robustly suppress the expression of ABCA8, ABCB1, ABCC1, ABCC2, and ABCG2 (Figure 3B, S5b), further supporting the presence of an autocrine/paracrine GH action (Figure 1D) and the ability of GHRA to abrogate the same. Also, gemcitabine treatment itself strongly induces the expression of multiple ABC transporter proteins (ABCA8, ABCB1, ABCG2), and

a distinct attenuation of this effect is observed in the presence of GHRAs (Figure S14). Moreover, pancreatic cancer cells in culture show a >2-fold increase in the rate of drug efflux and a reduction in the amount of intracellular drug surrogate and ABC transporter substrate [DiOC(2)3] retention following GH treatment, whereas both GHRAs completely suppress the same and show increased retention of the fluorescent compound (Figs 3C, S15). Several of the top positively correlated genes from Figure 3A, are tested with RT-qPCR and are found to be differentially upregulated by GH and chemotherapy treatments in xenograft tumors from nude mice (male and female) and also in male bGH vs. WT mice (Figure 3D, S16). Interestingly, GHRA monotherapy increases *GH1* and *PRLR* expression most significantly in the tumor of male mice (Figure S16), whereas in the presence of low-dose gemcitabine, *GH1*, *GHR*, *IGF1*, *IGF1R*, and *PRLR* expression are increased in both male and female mice (Figure S16). Monotherapy with either GHRA reduces RNA expression of *ABCA6*, *ABCA8*, *ABCA9*, *ABCB1*, *ABCG2*, *ABCC1*, *ABCC2*, *ABCC4*, *ABCC5*, and *ABCC9* in the nude mice, wherein compound G shows a higher suppressive effect than pegvisomant (Figure 3D). Additionally, gemcitabine induces the upregulation of ABC transporter RNA expression but not in presence of GHRA. During combination treatment with gemcitabine, compound-G is effective in suppressing the increase in chemotherapy-induced ABC transporter expression in both sexes (Figure 3D, *pegvisomant is tested only in male and not in female mice*). Moreover, as postulated the RNA levels of most ABC transporters assessed are increased in the tumors of bGH mice compared to those of WT mice, showing that even in the absence of chemotherapy, tumors exposed to high GH and IGF1 levels exhibit intrinsic chemoresistance (Figure 3D). In male and female nude mice xenografts treated with GHRAs, western-blots further confirm a marked and consistent suppression of ABC transporter proteins (*ABCA6*, *ABCA8*, *ABCB1*, *ABCC1*, and *ABCG2*) implicated in gemcitabine resistance in human patients and with a strong positive correlation with tumoral GHR expression (Figure 3E-3G). Protein levels of *ABCB1*, *ABCA8*, and *ABCG2* are also significantly upregulated in bGH mouse tumors compared to those in WT mice (Figure 3H). Collectively, our data show that GH action upregulates chemoresistance in PDAC as a function of increased ABC transporter expression and drug efflux, and GHRA treatments can reverse this outcome and appears to be one of the factors underlying our *in vivo* observation in restoring chemosensitivity in PDAC.

Main Figure 3

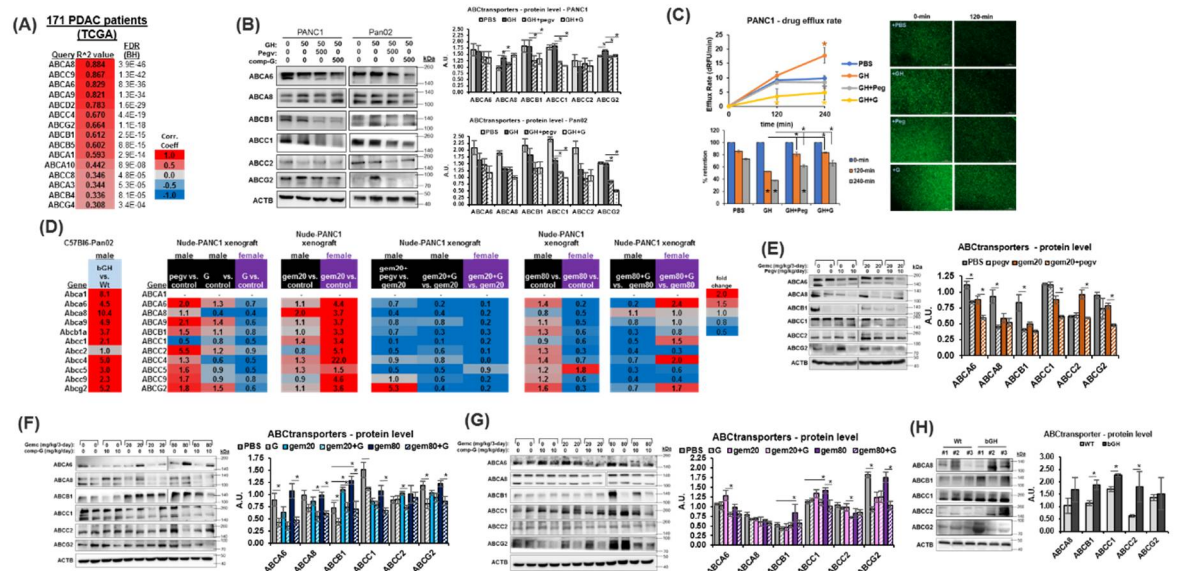


Figure 3. GH upregulates ABC multidrug transporter expression in PDAC: (A) List of top 15 ABC transporter expression correlations with GHR expression in 171 human PDAC patients (TCGA database). (B) Changes in protein levels of ABC-transporters following treatment with either GH or GH + pegvisomant or GH + compound-G across all four pancreatic cancer cell lines. (C) (top) Cellular drug efflux rate and (bottom) cellular retention of fluorescent DiOC2 (ABC transporter substrate) in pancreatic cancer cells treated with either GH or GH + pegvisomant or GH + compound-G. (D) GHRA monotherapy increases *GH1* and *PRLR* expression most significantly in the tumor of male mice (Figure S16), whereas in the presence of low-dose gemcitabine, *GH1*, *GHR*, *IGF1*, *IGF1R*, and *PRLR* expression are increased in both male and female mice (Figure S16). Monotherapy with either GHRA reduces RNA expression of *ABCA6*, *ABCA8*, *ABCA9*, *ABCB1*, *ABCG2*, *ABCC1*, *ABCC2*, *ABCC4*, *ABCC5*, and *ABCC9* in the nude mice, wherein compound G shows a higher suppressive effect than pegvisomant (Figure 3D). Additionally, gemcitabine induces the upregulation of ABC transporter RNA expression but not in presence of GHRA. During combination treatment with gemcitabine, compound-G is effective in suppressing the increase in chemotherapy-induced ABC transporter expression in both sexes (Figure 3D, *pegvisomant is tested only in male and not in female mice*). Moreover, as postulated the RNA levels of most ABC transporters assessed are increased in the tumors of bGH mice compared to those of WT mice, showing that even in the absence of chemotherapy, tumors exposed to high GH and IGF1 levels exhibit intrinsic chemoresistance (Figure 3D). In male and female nude mice xenografts treated with GHRAs, western-blots further confirm a marked and consistent suppression of ABC transporter proteins (*ABCA6*, *ABCA8*, *ABCB1*, *ABCC1*, and *ABCG2*) implicated in gemcitabine resistance in human patients and with a strong positive correlation with tumoral GHR expression (Figure 3E-3G). Protein levels of *ABCB1*, *ABCA8*, and *ABCG2* are also significantly upregulated in bGH mouse tumors compared to those in WT mice (Figure 3H). Collectively, our data show that GH action upregulates chemoresistance in PDAC as a function of increased ABC transporter expression and drug efflux, and GHRA treatments can reverse this outcome and appears to be one of the factors underlying our *in vivo* observation in restoring chemosensitivity in PDAC.

Reverse transcription and real-time quantitative PCR of RNA from xenograft tumors for relative RNA levels and Fold-changes in xenograft tumors in bGH vs WT, and male and female Nude mice with PANC1 tumors and treated with gemcitabine or GHRAs alone or in combination. (E-H) Changes in tumor protein levels of ABC transporters in (E) PANC1 tumors in male Nude mice treated with pegvisomant –gemcitabine combination, (F) PANC1 tumors in male Nude mice treated with compound-G –gemcitabine combination, (G) PANC1 tumors in female Nude mice treated with compound-G–gemcitabine combination, or (H) Pan02 tumors from bGH and Wt mice. Significant difference was compared using repeated measures ANOVA combined with Bonferroni's multiple comparison and * signifies $p < 0.05$.

Combination of Chemotherapy and GHR Antagonism Suppresses EMT Induction in PDAC Cells and Xenografts

Commitment to the EMT program is central to gemcitabine chemoresistance in pancreatic cancer [34,35]. GH is a potent driver of EMT in normal and transformed cells [37], and has also been shown to promote EMT in pancreatic cancer cells *in vitro* [37]. In PDAC patient transcriptomes, we observe a strong correlation of GHR expression with multiple EMT transcription factors and markers, including *ZEB1*, *ZEB2*, *Snail* (*SNAI1*), and activating components of the EMT-inducing TGF β signaling pathway (*TGFB3*, *TGFBR1*, *TGFBR2*, *TGFBR3*) (Figure 4A). In cultured cells, GH consistently upregulates the protein expression of EMT transcription factors *ZEB1* and *Snail* (*SNAI1*) (Figure 4B, S5c). However, in all cases, GHRA treatment suppresses EMT markers to below baseline levels, indicating the suppression of the autocrine/paracrine GH action (Figure 4B, S5c). Accordingly, GH increases the basement membrane invasion capacity which is suppressed by both pegvisomant and compound G (Figure 4C). The number of colony forming units following either doxorubicin or gemcitabine treatment is also upregulated by GH and suppressed by pegvisomant (Figure 4D). Gemcitabine treatment alone strongly induced the expression of EMT transcription factor protein expression, but in presence of GHRAs this effect is attenuated (Figure S17). Next, in xenograft tumors, we assessed the RNA expression of EMT mediators that are positively correlated with GHR expression in human tumor samples (Figure 4A). In bGH mouse tumors, multiple EMT factors, similar to that observed in human patients (in Figure 4A), are upregulated by >2-fold compared to their WT counterparts even in the absence of chemotherapy (Figure 4E). In the case of GHRA monotherapy in nude mouse model, compound G is more effective than pegvisomant in suppressing the increase in mesenchymal marker RNAs for *CDH2*, *SNAI2*, *TGFB3*, *TWIST* (1 and 2), and *ZEB1* in male mice (Figure 4E). Gemcitabine treatment induces a strong increase in all EMT markers tested, which is attenuated by presence of GHRA in both sexes (Figure 4E). Furthermore, both GHRAs show consistent suppression of the protein (Figure 4F-4H) expression of mesenchymal markers *ZEB1*, *SNAI1*, *CDH2*, and *VIM* and consistent upregulation of the epithelial marker *CDH1* *in vivo*. On the other hand, bGH mouse tumors show increased expression of EMT transcription factors (*ZEB1*, *SNAI1*, *SNAI2*) and mesenchymal markers (*CDH2*, *VIM*) compared to WT tumors (Figure 4I). Our *in vitro* and *in vivo* analyses validate that targeting GH action effectively attenuates EMT program activation, especially under chemotherapeutic challenge, in pancreatic tumors and supports our hypothesis that GHRA promotes chemo-sensitization in PDAC.

Main Figure 4

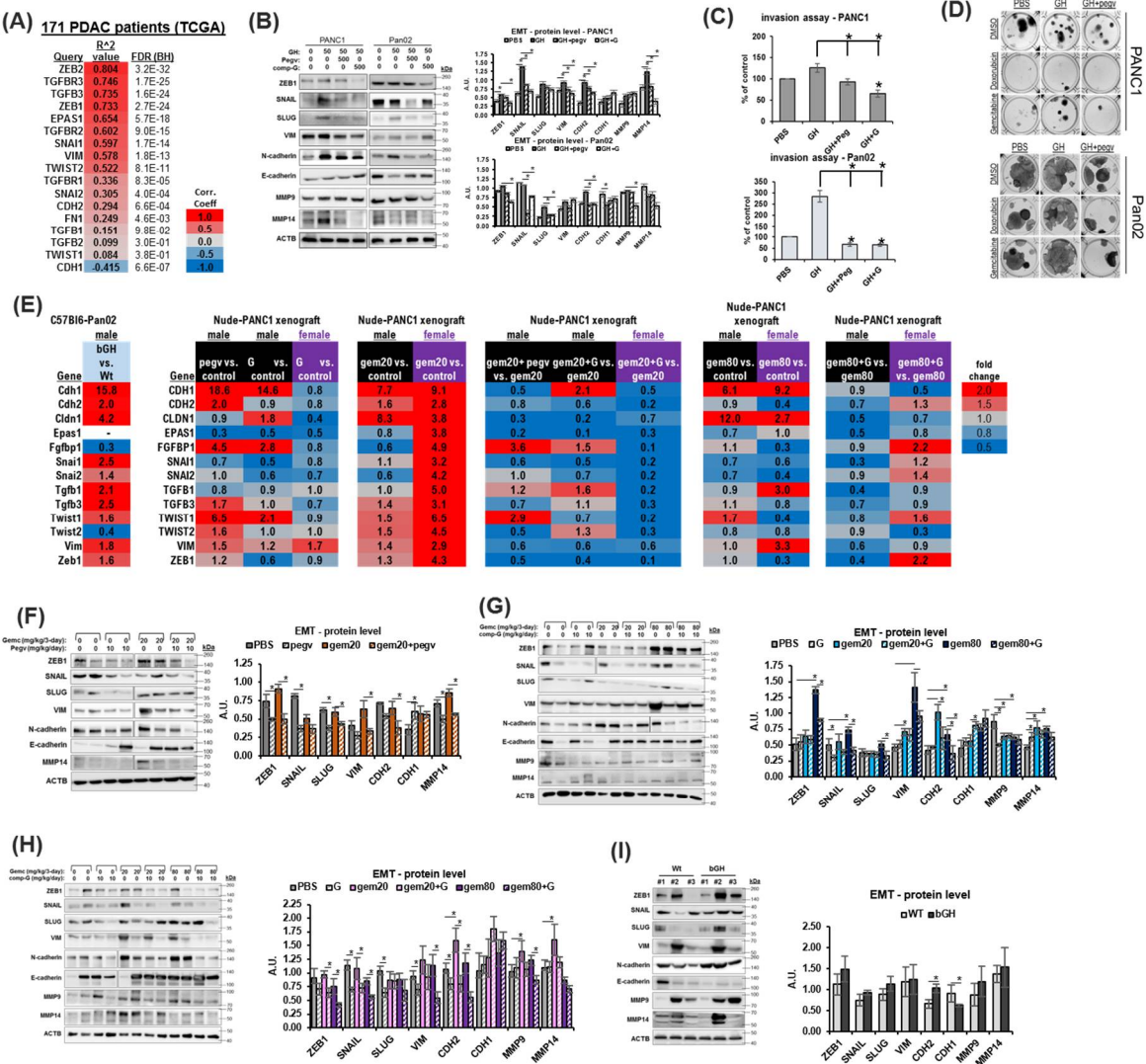


Figure 4. GH induces expression of epithelial-to-mesenchymal transition (EMT) factors in PDAC: (A) List of Pearson correlation coefficients of top 15 EMT related gene with GHR expression in human 171 PDAC patients (TCGA database). (B) Changes in protein levels of EMT factors following treatment with either GH or GH + pegvisomant or GH + compound-G across all four pancreatic cancer cell lines. (C) Effects of treatments with either GH or GH + pegvisomant or GH + compound-G on basement membrane invasion capacity of pancreatic cancer cells in culture, (D) number of viable colonies formed by human and mouse pancreatic cancer cells following treatment with either GH or GH + pegvisomant or GH + compound-G in presence / absence of chemotherapies (doxorubicin or gemcitabine). (E) Reverse transcription and real-time quantitative PCR of RNA from xenograft tumors for relative RNA levels and Fold-changes in xenograft tumors in bGH vs WT, and male and female Nude mice with PANC1 tumors and treated with gemcitabine or GHRAs alone or in combination. (F-I) Changes in tumor protein levels of EMT factors in (F) PANC1 tumors in male Nude mice treated with pegvisomant –gemcitabine combination or (G) PANC1 tumors in male Nude mice treated with compound G –gemcitabine combination or (H) PANC1 tumors in female Nude mice treated with compound G–gemcitabine combination, or (I) Pan02 tumors from bGH and Wt mice. Significant difference was compared using repeated measures ANOVA combined with Bonferroni’s multiple comparison and * signifies $p<0.05$.

The Combination of Chemotherapy and GHR Antagonism Suppresses Markers of Apoptosis, Fibrosis, Lymphangiogenesis, Angiogenesis, Senescence and Stemness in PDAC Cells and Xenografts

PDAC tumors are poorly responsive to several therapeutic modalities owing to a desmoplastic TME, characterized by extensive extracellular matrix (ECM) remodeling and fibrosis [38–40]. Additional factors include suppression of apoptosis [41], dysregulated vascular and lymphatic endothelial action [42,43], induction of senescence and immunosuppressive effects from the senescence-associated secretory phenotype (SASP) [44], induction of stemness [45], and drug-metabolizing enzymes, such as cytochrome P450s [46]. Based on the well-studied effects of GH in enhancing several of these pathways across multiple cancer types [10], we first compared the effect of GH on the expression of corresponding gene modules in the human PDAC patient database (TCGA). Gene sets, representing the established markers of each molecular mechanism, are arranged into individual modules, viz. *Fibrosis*, *Apoptosis*, *Senescence*, *Angiogenesis*, *Lymphangiogenesis*, *Stemness*, and *CYP450s*. Tumors with high GHR (>mean) expression display a strong positive correlation with several marker genes of each of these pathways (Figure 5). ECM remodeling and fibrosis-related genes underlying the desmoplastic TME of PDAC are most strongly positively correlated with GHR expression, indicating that GH may be an important mediator of desmoplasia in the pancreatic TME. Cancer-associated fibrosis promotes therapeutic resistance and immune evasion in pancreatic cancer [40,41,49] and recent evidence implicates GH as a covert driver of multi-tissue fibrosis [8]. Here, firstly we observe a high positive correlation between RNA expression of *GHR* and that of *FAP*, *ACTA2*, and multiple collagen (*COL*), matrix metalloproteinase (*MMPs*), and tissue inhibitor of metalloproteases (*TIMPs*) genes (Figure 5A). Secondly, higher *GHR* expression correlates positively with anti-apoptotic *BCL2* and negatively with several pro-apoptotic mediators (*BIK*, *BAD*, *BID*, *BAX*, *BAK1*, *CASP6*, *CASP7*) in the patient samples (Figure 5B). *GHR* expression in lymphatic endothelial cells is many times higher than that in blood vascular endothelial cells, and GH is known to promote lymphangiogenesis *in vitro* and *in vivo* [49], while forced autocrine GH expression increases vascular and lymphatic micro vessel density in human breast cancer xenografts [11]. In the human PDAC patient tumor transcriptome (TCGA), we identify strong and significant correlations between tumoral *GHR* expression and known lymphangiogenic (*LYVE1*, *FLT4*, *VEGFC*, *PDPN*) [43] and angiogenic (*PDGFRa*, *FGFR1*, *TGFB3*, *TGFB1*, *TGFBR2*, *HIF1a*, *IL6*, *IL1B*) [50] markers (Figure 5C, 5D). Furthermore, chemotherapy accelerates the development of sub-populations of senescent cells in the TME, which promotes widespread immune suppression via the secretion of a cocktail of immunoinhibitory cytokines, known collectively as SASP [44]. In human PDAC patient tumors, we observe that several SASP factors, such as *GH1* [51], *IL10*, *IL1b*, *IL6*, *CCL2*, and *CCL5* which exert primary immune suppression (effector T cell inactivation and regulatory T cell production) are significantly and positively correlated with *GHR* expression (Figure 5E). Moreover, markers of cancer stem cells, *ALDH1* and *CXCR4*, are strongly associated with *GHR* in PDAC (Figure 5F). We also observed that a number of cytochrome P450s (known targets of hepatic GH action and drug modulators) are upregulated in PDAC patient tumors, with *CYP1B1*, *CYP2U1*, and *CYP7B1* displaying the highest and *CYP2W1* having the lowest correlation with *GHR* expression (Figure 5G). Incidentally, *CYP2W1* is important for the activation and response to chemotherapies such as mitotane in cancer [52], whereas high expression of *CYP1B1* is known to drive gemcitabine resistance in pancreatic cancer [46]. We performed a limited verification of these findings from the transcriptome analysis of human PDAC patients, using RNA from Pan02 murine tumor allografts in bGH and WT mice and performed qPCR for selected genes. Despite species differences, treatment-naivety, and differences in tumor genetics, the mouse tumors exposed to supraphysiological levels of GH and IGF1 (as in bGH mice) show increased expression of markers of ECM remodeling and fibrosis (*Timp2*, *Timp3*, *Timp4*, and *Col6a3*), anti-apoptosis (*Bcl2*), lymphangiogenesis (*Flt4*, *Lyve1*, *Pdpn*), angiogenesis (*Vegfa*, *Il1b*, *Pdgfra*, *Tgfb3*, *Tgfb1*), senescence (*Il13*, *Il6*, *Pdl1/Cd274*, *Ccl5*, *Il10*, *Il1b*, *Ccl2*), stemness (*Thy1*), and CYP450s (*Cyp2u1*, *Cyp1b1*) (Figure 5H, S16), confirming the immune-suppressive and therapy-resistant actions of GH in PDAC. Moreover, tumoral *Pdl1* (*Cd274*), but not *Pdl2* (*Pdcd1lg2*), expression was elevated by almost 6-fold in bGH tumors (Figs 5H, S18). In marked corroboration, we also found that in 154 tumor samples from patients with PDAC (TCGA cohort): (i) Geneset enrichment analysis (GSEA) of *GHR* correlated genes show enrichment of ‘cancer immunotherapy by PD-1 blockade’ pathway (Figure S19), (ii) out 30 cancer types, *GHR* is positively

correlated strongly with 17 out of 23 immune-inhibitory cytokine expression in PDAC (Figure S20a), (iii) high tumor infiltration of immune-suppressive lymphocytes (T-reg, MDSCs and macrophages) in GHR enriched PDAC tumors (Figure S20b), and (iv) enrichment of several (13 out of 23 top enriched pathways) immune-related pathways correlated with tumoral GHR expression (Figure S20c). Together our data indicate that in addition to direct chemorefractory effects on the tumor, GH action may promote desmoplasia, and host immune evasion in PDAC.

Main Figure 5

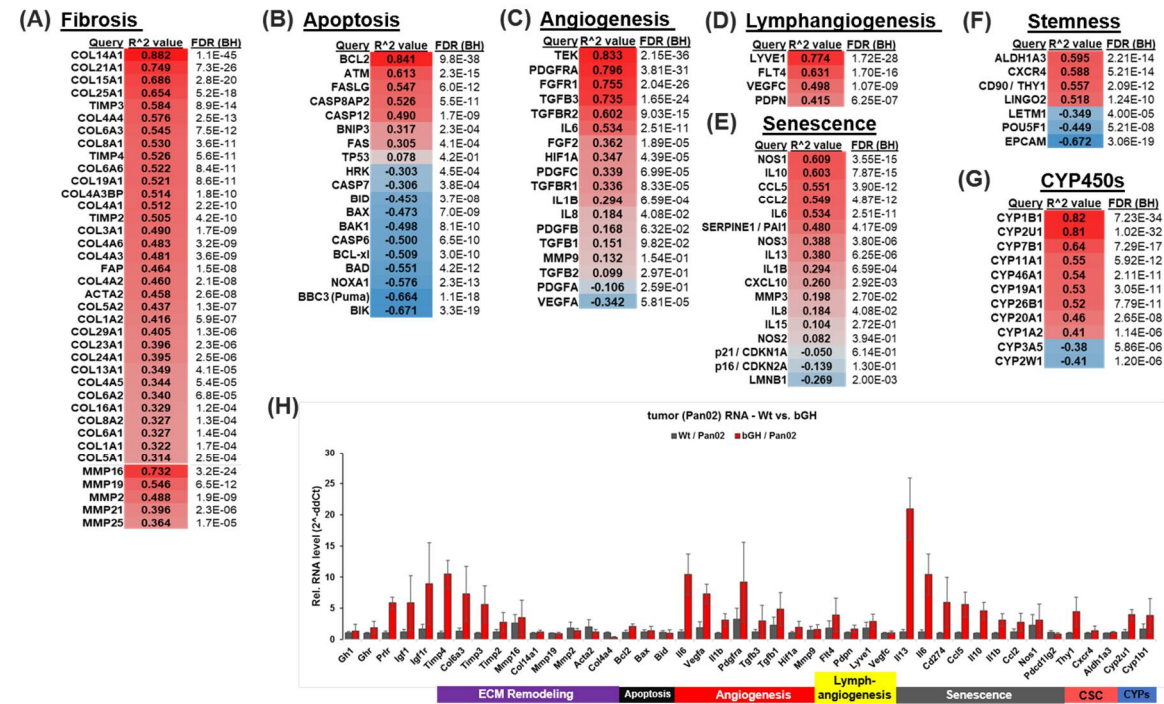


Figure 5. GH induced gene expression have a multi-modal therapy refractory signature in PDAC: (A-F) List of marker genes most significantly (Pearson coefficient > 0.3, FDR<0.05) correlated with GHR expression in human PDAC patients, associated with the therapy resistant processes (modules) of fibrosis (A), apoptosis (B), lymphangiogenesis (C), angiogenesis (D), senescence (E), stemness (F), and cytochrome P450s (G). (H) Heatmap depicting a RT-qPCR cross validation of RNA expression pattern of selected genes from each module (in A-G), in Pan02 tumors from WT vs bGH mice.

3. Discussion

Targeting GHR in multiple types of human cancers has been hypothesized and shown to significantly attenuate tumor resistance to multiple antineoplastic approaches, including radiation therapy, chemotherapy, targeted therapy, and immunotherapy [10]. GHR is overexpressed in human PDAC. The chemotherapy-sensitizing effects of direct GHR antagonism in preclinical models of human melanoma, breast cancer, and liver cancer exhibit a consistent suppression of ABC multidrug transporters and markers of EMT, leading us to hypothesize that targeting GHR in PDAC can reverse the chemoresistant phenotype of this cancer. To date, one isolated study has reported the anti-cancer properties of an RNAi-mediated GHR silencing in cultured human pancreatic cancer cells [37], and no studies have reported the *in vivo* use of GHR antagonism or a combination of GHRA with commonly used chemotherapies to improve therapeutic outcomes in any cancer. Here, we report the first pre-clinical validation of the postulated gemcitabine-sensitizing effect of GHR antagonism using two different GHRAs in cultured pancreatic cancer cells and mouse tumor xenografts. GHR antagonism rescued gemcitabine resistance and improved gemcitabine efficacy *in vivo*, wherein the GHRA treated groups had marked tumor retraction in multiple mice of both sexes. Drug- drug interaction (DDI) measured by Chou-Talalay method (PMID: 20068163) showed that coefficients of DDI at the end of study were 0.875, 0.787, and 0.597 for pegvisomant + 20mg/kg gemcitabine, compound G + 20mg/kg gemcitabine, and compound G + 80mg/kg gemcitabine, respectively in male

mice. In contrast, female mice showed only an additive effect and no synergy for the dose of compound G used. Our analyses of the human PDAC tumor transcriptome showed that increasing GHR expression correlates positively with several gene expression modules, which collectively confer therapy resistance and immunosuppression in patients. Additionally, we compared these results in a syngeneic mouse model of excess versus normal GH expression, and the majority of observations of therapy-refractory gene expression changes were corroborated. Overall, our results indicate GHR antagonist in combination with gemcitabine is a promising modality to significantly potentiate the efficacy of the existing anti-PDAC regimen. This is particularly important, as gemcitabine is one of the most widely used chemotherapies in PDAC[53] and often displays a blunted efficacy in patients[54].

GHR is expressed in all pancreatic cell types, albeit variably, with the highest level (92% cells, mean expression = 4.4) in pancreatic beta cells and the lowest level (11% cells, mean expression = 0.5) in pancreatic ductal cells of the normal pancreas (Figure S1). PDAC originates mainly from transformed ductal cells of the pancreas and displays a GHR expression comparable to the whole pancreas (all cell types combined). This indicates that the GHR expression level is supraphysiological in transformed ductal cells compared with that in normal ductal cells of the pancreas. Notably, *IGF1R* RNA expression, in addition to having a significant inverse correlation with human male PDAC patient survival, closely followed *GHR* expression in the mouse pancreatic cell types (Figure S1) and human PDAC transcriptome, indicating active local IGF1R signaling in the pancreas, which is detrimental to the TME [55]. Additionally, gemcitabine dose-dependent activation of IGF1R drives gemcitabine resistance in pancreatic cancer cells by inducing EMT-mediated phenotype switching [56]. Both IGF1 and IGF2 are potent activating ligands for IGF1R. In relevance, a Pfizer clinical trial showed that 40 mg daily pegvisomant treatment for 14-days caused a sustained reduction of both serum IGF1 and IGF2 by 33% and 35%, respectively, in healthy human subjects [57]. Therefore, appropriate doses of GHRAs that result in IGF1 lowering can be expected to significantly attenuate the tumor-promoting effects mediated by IGF1R activation. IGF1R attenuation is achievable by IGF1R inhibitors, and IGF1 is deemed a provocative target in anti-cancer research, supported by a plethora of studies [58]. However, IGF1R targeting in cancer [59] has proven to be a consistently ineffective strategy in controlling tumor progression, as shown in 183 human clinical trials involving >12,000 patients across several tumor types (2003-2021) at a net cost estimated between \$1.6-2.3 billion [60]. A major underlying factor behind these results is that IGF1R inhibition increases GH production due to de-repression of IGF1 mediated negative feedback loop in the pituitary, as well as potentially allowing free IGF1 and IGF2 to activate the insulin receptor (IR) in the tumors. Therefore, it is provocative to test the use of GHRAs as a case-specific maintenance protocol against tumor relapse or as a preventive regimen in high-risk cases of age-associated tumor development promoted by local GH action in tissues [13] (not tested here).

It is important to note that the GHRA, pegvisomant, does show physiological effects *in vivo*, independent of IGF1 reduction, even at doses five times lower than the dose used here [61]. The GHRA dose in the current study are thus physiologically relevant but lower than most previously used doses of pegvisomant in nude mouse tumor xenograft studies which were aimed at causing significant serum IGF1 reduction in mice. Our current GHRA dose was chosen to not cause any marked suppression of serum IGF1 in the mice, i.e. it did not block the endocrine mouse GH action. Therefore, our results preferentially reflect the effects of blocking IGF1 independent and autocrine/paracrine actions of GH in the tumor milieu. Future studies with higher dosage of the long-acting pegylated GHRAs used in this study can in addition to blocking direct GH actions on the tumor can also suppress systemic IGF1 levels. The effects of such escalated dosage on treatment outcome needs to be ascertained. Focused pre-clinical studies with human PDX models with dose-titration of combinations of GHRAs and selected antineoplastic agents, and subsequent carefully designed human clinical trials, are needed to validate the transformative prognostic potential of targeting GHR in pancreatic cancer. Recent mechanistic combinatorial targeted therapy approaches, such as double inhibition of IGF1R and ERK signaling in organoid models, display increased sensitivity of pancreatic tumors to autophagy inhibitors such as hydroxychloroquine [62] and serve

as a precedence to our concept. Moreover, given the role of PRLR activation in PDAC and human GH as a potent ligand for PRLR, new dual inhibitors of GHR and PRLR [24,63] provide exciting opportunities.

We and others have described a covert action of GH-GHR in cancer, not only in increasing cell proliferation and decreasing tumor cell apoptosis, but also in driving therapy resistance against radiation therapy, chemotherapy, targeted therapy, and immunotherapy [8,10]. This is the first study to extend the validity of our observations to the therapeutically challenging case of PDAC. We showed here that GH drives several therapy-resistance mechanisms in PDAC, focusing mainly on the GH-induced increase in ABC transporter expression and induction of the EMT pathway, which are only part of the molecular mechanisms underlying gemcitabine resistance in cancer. Indeed, in the last five years, multiple ABC transporters have been directly implicated in gemcitabine resistance in PDAC, of which ABCA8 and ABCG2 were consistently suppressed by GHRA. In the present study, we observed a marked suppression of gemcitabine resistance in the presence of GHRAs. We have previously shown that GHR inhibition sensitizes melanoma cells to paclitaxel, as well as platinum-based antineoplastic drugs like cisplatin [64–66], and the pyrimidine analog fluorouracil (5-FU) (70). Therefore, based on our previous and current studies, GHRAs can potentially augment the current anti-PDAC chemotherapeutic combinations such as gemcitabine-paclitaxel, as well as FOLFIRINOX (combination of 5-FU, oxaliplatin, irinotecan, and folinic acid). In addition, several components of the TME express GH and GHR and have well-characterized GH-regulated phenotypes, which are tumor supportive, therapy-refractory, and immune suppressive. Future studies directed at the effects of GHRA treatment in managing these effects are critical and necessary.

Based on the empirically demonstrated role of GH in other cancer studies, we identified that GHR antagonism in PDAC leads to suppression of the gene signature of hallmark cancer processes. Our analyses of human PDAC patient data show that desmoplasia and fibrosis, which constitute major roadblocks in PDAC therapy, correlate strongly with GHR expression. Moreover, a positive correlation of GHR was observed with markers of cancer stem cells, apoptosis inhibition, gemcitabine deactivating CYP450s, increased angiogenesis and lymphangiogenesis, and senescence. Transcriptome analysis of 269 PDAC patients showed that the lymphatic lineage of endothelial cells in the pancreatic TME is maximally associated with poor overall survival (HR=2.91, $p=0.003$) and progression-free survival (HR=4.65, $p<0.001$) [68]. As GH promotes lymphangiogenesis and the corresponding markers in PDAC correlated with GHR expression in our analyses, it can be expected that GHRAs can lower or inhibit tumor lymphangiogenesis to attenuate tumor growth and remains to be tested in PDAC. Lastly, we reveal a provocative association of GH action with cancer immunosuppressive TME. Tumoral GHR expression in patients correlated strongly and positively with SASP components, immunoinhibitory markers, immunosuppressive TILs, and immune-modifying pathways. Some of these findings were validated in the syngeneic tumor bearing bGH mice. Our results align with recent findings of a lack of response to anti-PD1/PDL1 therapy in patients with liver and gastric cancers with high levels of circulating GH [10].

Our observations are limited by bulk RNA estimations of tumor samples and should be treated as a springboard for future validation studies with higher resolution methods. It is imperative to clarify the extent of GH signaling and the effect of GHR antagonism on individual cell types in the TME of PDAC via spatial single-cell transcriptomic and proteomic methods. Moreover, we have not studied longer term post-treatment effects (tumor relapse, survival) in our mice or the details of observed sex-specific differences – which are essential to assess prior to clinical translation. Although not studied in this report, higher serum glycine levels reduced the risk of pancreatic cancer by 70%, as observed in a prospective cohort study in human patients [69]. In this regard, bGH transgenic mice, which develop spontaneous neoplasms at a higher rate than WT mice [70], also have markedly lower glycine levels than their WT counterparts, while the reverse is observed in GHR-knockout (GHRKO) mice, which are protected from cancer [71].

In conclusion, supported by immunodeficient nude and syngeneic mouse tumor models, cell culture, and bioinformatic analyses of human pancreatic cancer patient transcriptome data, this study presents a robust and, to our knowledge, the first validation of combining GHRAs in a chemotherapy

regimen in pancreatic cancer that markedly improved therapy efficacy. We emphasize that although we validated our hypothesis of GHRA mediated chemo-sensitization in this study, GHRAs have direct effects on the non-tumor components of TME in promoting therapy resistance and immune suppression which are yet unknown. Moreover GHRAs are also poised to exert indirect effects by lowering serum IGF1 (and IGF2), the activating ligands for insulin and IGF1 receptors [72], as well as improving insulin sensitivity in patients [73–76]. Rarely can an antitumor agent exert such a versatile effect. Finally, with the existence of an FDA-approved GHR antagonist and several others in active development targeting the full spectrum of GH action [24,63,77], our current study sets course in a feasible paradigm shift in the therapeutic augmentation of pancreatic cancer.

4. Materials and Methods

Cell culture and treatments: Pancreatic cancer cell lines PANC1 (human male, CRL-1469), BxPC3 (human female, CRL-1687), and LTPA (mouse female, CRL-2389) were purchased from ATCC (Manassas, VA, USA), and Pan02 (mouse male) cells were obtained from Charles River (Wilmington, MA, USA). Cells were maintained in RPMI-1640 or DMEM supplemented with 10% fetal bovine serum (FBS) and 1X-penicillin-streptomycin, all purchased from ATCC, in a humidified 5% CO₂ incubator at 37°C. All cell treatments were done in <5-months from purchase of cells and within passage numbers 4-12. No Mycoplasma contamination were detected in the cell cultures, when tested at the beginning and end of study using the Universal Mycoplasma detection kit (ATCC, 30-1012K). Treatment with the indicated concentrations of recombinant human GH (Antibodies Online, Limerick, PA) or chemotherapy [gemcitabine, doxorubicin, etoposide, all purchased from Selleckchem (Houston, TX)], pegvisomant (a gift from Dr. Sebastian Neggers), or compound-G (InfinixBio, Columbus, OH) were performed in 2% FBS-supplemented media for the given time-points as described previously [78]. In the *in vitro* studies, all treatments with anti-cancer drugs were performed at the EC₅₀ values determined at the beginning of the experiment, as shown in Figure S21. All *in vitro* studies were performed using compound G (GGSSG-hGH-G120K-T142C-dPEGA-H151C-dPEGA), and all *in vivo* studies used compound G' (M-hGH-G120K-T142C-dPEGA-H151C-dPEGA) as described previously [24]. Both are G120K substituted versions of human GH with site-specific discrete PEGylation at cysteines substituted at T142 and H151 away from the GH-GHR interaction region)[24]. In this study, both compounds were referred to as compound G.

Mouse xenograft studies: Four million human PANC1 cells in 100uL (1:1 v/v mixture of PBS and Growth Factor Reduced Matrigel Matrix, Corning, #356231, NY) were subcutaneously engrafted on the right flank of 6-months old immunocompromised Nude mice (NU/J, RRID: IMSR_JAX:002019, Jackson Laboratory, Bar Harbor, ME). Following tumor growth stabilization (day-17, after the implanted tumors reached a size of 100-200 mm³), the animals were randomized into groups and treatments initiated with either a low (20 mg/kg-BW/ 3-days) or a high (80 mg/kg-BW/ 3-days) dose of gemcitabine, with or without once-daily administration of either GHRA pegvisomant, or compound-G at 10 mg/kg BW/day. All treatments were by intraperitoneal (i.p.) injections (each injection volume= <200-uL) alternating each day at either the right or left peritoneum. The lengths of the perpendicular tumor diameters were measured every 3-days using a digital caliper, as previously described. Tumor volume was calculated using the following formula: tumor size = 0.5 × (length) × (breadth)². The mice were sacrificed when the tumor volume exceeded 1000 mm³ and excised tumors were either flash frozen in liquid nitrogen and stored at -80C for subsequent RNA and protein extraction or fixed in 10% formalin for 8-hours and transferred to 70% ethanol before being processed for immunohistochemistry (IHC). For Nude mice, there were 6-8 mice per group in each of the male and female mouse groups. For the syngeneic mouse study, 100,000 Pan02 cells in 100uL (1:1 v/v mixture of PBS and Growth Factor Reduced Matrigel Matrix) were subcutaneously engrafted on the right flank of immunocompetent, 6-months old bovine GH transgenic (bGH) mice and their wild-type (WT) counterparts. There were 4 mice per group in the syngeneic mouse study with bGH vs. WT mice. All animal studies were performed in compliance with the policies approved by the Ohio University Institutional Animal Care and Use Committee.

Cell viability assay: This was performed to assess the effects of chemotherapy and/or GH and/or GHRA treatment on cell viability *in vitro*, as described previously [78][79]. Experiments were performed in triplicate. Briefly, cells were plated at 10,000 cells/well in a 96-well plate. After overnight incubation, cells were treated as described for the specified amount of time. Cells were incubated in 2%-FBS supplemented media containing 2.5nM / 50 ng/mL (or otherwise mentioned) recombinant human (for human cells) or bovine (for mouse cells) GH and/or 500nM pegvisomant or compound-G for 72-hr. Finally, the media was replaced with resazurin reagent (Abcam, cat#129732) and incubated for up to 2-hr, and absorbance at 570 nm (reference wavelength 600 nm) was measured using a Spectramax250 (Molecular Devices) spectrophotometer with SoftMaxPro v4.7.1 software. Three individual experiments on different days were performed.

Cell migration assay: This assay was performed to assess the effect of GH and/or GHR antagonists on the migration capacity of tumor cells, as described previously [79]. Experiments were performed in triplicate. Cells were seeded at 30,000 cells per well in a 24-well plate and a 200-uL pipette tip was used to streak and clear all cells along the midline of each well. Following a wash with PBS, the cells were incubated in 2%-FBS supplemented media containing 2.5nM / 50 ng/mL (or otherwise mentioned) recombinant human (for human cells) or bovine (for mouse cells) GH and/or 500nM pegvisomant or compound-G for up to 48-hr and imaged every 24-hr. The total uncovered area at the start and end of the assay was imaged using a BioTek Cytation-3 microplate imager (Gen5 v2.09.2 software) and quantified using the ImageJ software (NIH)[80]. Three individual experiments on different days were performed.

Cell invasion assay: This assay was performed to assess the effect of the hGHR antagonist on hGH-induced invasion of cancer cells. Cells were pretreated with GH in DMEM containing 2% FBS-containing DMEM or EMEM for 48-hr. The CytoSelect 96-well Cell Invasion Assay kit (CBA-112, Cell Biolabs, INC) was used according to the manufacturer's instructions. Briefly, on the day of the assay, the cells were trypsinized, counted, and 100,000 thousand cells were seeded per well in the upper chamber of the 96-well invasion assay well coated with basement membrane and incubated with the respective treatments in serum-free media for 24 h. After 24-hr, the cells under the membranes were dislodged, lysed, and stained with CyQuant GR dye solution. The fluorescence intensity correlated with invasive cell number was measured at ex-480 nm/em-520 nm using a fluorescence plate reader. Three individual experiments on different days were performed.

Drug retention assay: This assay was performed to measure the effect of the hGHR antagonist on the hGH-induced drug efflux property of pancreatic cancer cells in culture. Briefly, pancreatic cancer cells were treated for 7-days with 2.5 nM hGH or 2.5 nM hGH + 500 nM hGHR antagonist in 2% FBS-containing growth medium, with the medium being refreshed every 24-h. On the day of the assay, the cells were trypsinized, counted, and suspended in cold DiOC₂(3) dye on ice for 30 min (Chemicon International, #ECM910). The cells were then centrifuged, supernatant was removed, and cell pellets were resuspended in cold efflux buffer and distributed into different Eppendorf tubes under the following conditions: one set of tubes was kept on ice as negative controls, while the other two sets were kept in a 37°C water bath for 30 and 120 min, respectively, allowing the active drug-efflux pumps to drive out the DiOC₂(3) dye. The cells were then washed, resuspended, and cell suspensions were dispensed into the wells of a black-walled 96-well plate, and fluorescence was measured using a fluorescence plate reader at an excitation wavelength of 485 nm and an emission wavelength of 530 nm. Three individual experiments on different days were performed.

RNA extraction, quantification, and RT-qPCR performed as previously described [66,79]. Briefly, after the described treatments, the total RNA from cultured cells was extracted using the IBI Total RNA extraction kit (IBI Scientific) or from tumor tissues using the GeneJET RNA purification kit (Thermo Fisher) following the manufacturer's protocols. From the extracted RNA, mRNA levels were quantified by oligo-dT reverse transcription using a High Capacity cDNA Reverse Transcription Kit (Thermo Fisher), followed by quantitative PCR using Applied Biosystems reagents following the manufacturer's protocols. The primer sequences are provided in the Supplementary section (Figure S22).

Protein extraction, quantification, and western-blot: These procedures were performed as described previously (62, 75). Briefly, lysis of cultured cells or tumor tissues was performed using 1.5 × RIPA buffer (Sigma-Aldrich) supplemented with 1X phosphatase-protease inhibitor cocktail (Cell Signaling Technology). Protein concentration in the lysates was estimated using a Bradford assay and 80ug of protein was loaded onto 4%-16% gradient denaturing gels, transferred to 0.2um PVDF membranes (Thermo Fisher), blocked with freshly prepared 5% non-fat dry milk solution in 1X TBS-T buffer, and probed using target-specific primary and secondary antibodies. A list of antibodies and their sources are provided in the Supplementary section (Figure S23).

Bioinformatic analyses: We quantified the Pearson correlation of GHR RNA expression with that of all other genes in the TCGA dataset of 154 patients with pancreatic ductal adenocarcinoma (HiSeq RNA at UNC; pipeline: Firehose_RSEM_log2) using the Linkedomics platform [81]. Pearson correlation coefficients of groups of genes that were differentially and significantly (FDR <0.05) upregulated with increasing GHR expression are represented in the heat map. We further performed gene set enrichment analysis (GSEA; Broad Institute) [normalized TPM values from TCGA PDAC cohort against the Molecular Signatures Database (v7.1 MSigDB)] to identify pathways and ontologies enriched in the high GHR group of patients. Parameters for enrichment analysis = minimum number of IDs in the category: 5; maximum number of IDs in the category: 2000; Significance Level: Top 25; number of permutations: 1000. KMplotter platform [20] was employed for survival analysis and TISIDB platform [82] was used for tumor-immune interactions.

Statistical analyses: For all *in vitro* experiments that compare one parameter between multiple sets of samples, statistical significance was determined by unpaired two-sided students t test for two groups or by one-way or two-way ANOVA with Tukey's multiple comparisons test for three or more groups, using the GraphPad Prism 6 software. Mouse sample sizes were estimated using the resource equation method. Calculating with the degrees of freedom maximum (at 20) at least 6 animals per group in the Nude mice study was required. We started with 8 animals/group to ensure completing with at least 6 animals/group in order to reach significance, accounting for accidental loss of animals during treatment. According to the previous power calculation based on normal distributions and the magnitude of changes observed in preliminary studies, we calculated that a sample size of 8 in each group will have 90% power ($\alpha < 0.05$) to detect a significant difference in means of the parameters to be studied. For mouse experiments, repeated measures ANOVA combined with Bonferroni's multiple comparison was used to compare tumor volumes across treatment groups at each measured time point. Western-blot experiments were compared using one-way or two-way ANOVA with Tukey's multiple comparisons test using the GraphPad Prism 6 software.

Figure Legends

Supplementary Materials: The following supporting information can be downloaded at the website of this paper posted on Preprints.org, Figure S1: GHR expression in mouse pancreatic cell types; Figure S2: GHR expression in human pancreatic tumors; Figure S3: Correlation of tumoral expression of GHR and PRLR with patient disease-free survival (DFS) in human pancreatic ductal adenocarcinoma; Figure S4: Correlation of tumoral GHR or IGF1R expression with patient median overall survival (OS) in patients with pancreatic cancer; Figure S5: Western blot of lysates of cultured pancreatic cancer cells treated with GH and GHRAs; Figure S6: Pan02 allograft in syngeneic male wild-type of bGH transgenic mice C57BL6 strain; Figure S7: Pearson correlation of RNA level of GHR with that of GH/IGF signaling regulators in human PDAC patients; Figure S8: KEGG pathways enriched by genes which are most significantly correlated with GHR expression in pancreatic adenocarcinoma patients; Figure S9: GHR antagonism improves chemotherapeutic efficacy in pancreatic cancer; Figure S10: Serum IGF1 levels, fasted and fed blood glucose levels at end of study of male and female Nude mice with xenografts; Figure S11: Body-weight of nude mice with xenografts across treatment period; Figure S12: Western blot of signaling intermediates in cultured pancreatic cancer cells treated with GH and GHRAs; Figure S13: Pearson correlation of RNA level of GHR with that of modulators of gemcitabine action in PDAC patients; Figure S14: Western blot of ABC transporters in cultured pancreatic cancer cells treated with GH and GHRAs; Figure S15: Drug

efflux assay of human and mouse pancreatic cancer cells in culture following treatments with either GH or GH+ GHRAs; Figure S16: Reverse transcription and real-time quantitative PCR of RNA from xenograft tumors; Figure S17: Western blot of EMT mediators in cultured pancreatic cancer cells treated with GH and GHRAs; Figure S18: Heatmap showing fold change in specific modules of genes in allograft tumors of bGh vs. Wt mice; Figure S19: Wikipathways enriched by genes which are most significantly correlated with GHR expression in PDAC patients; Figure S20: Bioinformatic analyses of tumor RNA expression and related pathways from patient samples (PDAC, TCGA cohort); Figure S21: EC50s (in nM) of the chemotherapies (doxorubicin, gemcitabine, erlotinib) used in the study in all four human and mouse pancreatic cancer cell lines; Figure S22: List of primer pairs and sequences used for the current study; Figure S23: List of antibodies used for the current study.

Author Contributions: Basu, R: designed and performed the *in vitro* and *in vivo* studies, data analyses, bioinformatic analyses, and wrote and edited the manuscript. Kulkarni, P: *In vivo* and *in vitro* studies. Swegan D: Western blots, RNA extraction, cDNA preparation, and *in vitro* studies. Duran Ortiz, S Biostatistical analyses using GraphPad Prism. Ahmad, A. Western blot and *in vitro* studies. Caggiano, L: western-blots. Davis, E Western blot and *in vitro* studies. Brenya, E Bioinformatic analyses and western blots. Koshal A: RNA extraction, cDNA preps, RT-qPCR, and western blotting. Walsh, C: RNA extraction, cDNA preparation, RT-qPCR. Brody, R, and Sandbhor, U designed, prepared, and provided compound G and edited the manuscript. Neggers, S: pegvisomant and instructions for its use in these studies and manuscript editing. Kopchick J: study design, intellectual input for analysis and presentation of results, manuscript editing, and overall supervision of the project.

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