

## Molecular Alterations in Prostate Carcinomas that Associate with *In vivo* Exposure to Chemotherapy: Identification of a Cytoprotective Mechanism Involving Growth Differentiation Factor 15

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**Abstract Purpose:** To identify molecular alterations associating with *in vivo* exposure of prostate carcinoma to chemotherapy and assess functional roles modulating tumor response and resistance.

**Experimental Design:** Patients with high-risk localized prostate cancer (tumor-node-metastasis  $\geq T_{2b}$  or prostate-specific antigen  $\geq 15$  ng/mL or Gleason grade  $\geq 4+3$ ) were enrolled into a phase II clinical trial of neoadjuvant chemotherapy with docetaxel and mitoxantrone followed by prostatectomy. Pretreatment prostate tissue was acquired by needle biopsy and posttreatment tissue was acquired by prostatectomy. Prostate epithelium was captured by microdissection, and transcript levels were quantitated by cDNA microarray hybridization. Gene expression changes associated with chemotherapy were determined by a random variance *t* test. Several were verified by quantitative reverse transcription PCR. *In vitro* analyses determining the influence of growth differentiation factor 15 (GDF15) on chemotherapy resistance were done.

**Results:** Gene expression changes after chemotherapy were measured in 31 patients who completed four cycles of neoadjuvant chemotherapy. After excluding genes shown previously to be influenced by the radical prostatectomy procedure, we identified 51 genes with significant transcript level alterations following chemotherapy. This group included several cytokines, including GDF15, chemokine (C-X-C motif) ligand 10, and interleukin receptor 1 $\beta$ . Overexpression of GDF15 or exposure of prostate cancer cell lines to exogenous recombinant GDF15 conferred resistance to docetaxel and mitoxantrone.

**Conclusions:** Consistent molecular alterations were identified in prostate cancer cells exposed to docetaxel and mitoxantrone chemotherapy. These alterations include transcripts encoding cytokines known to be regulated through the nuclear factor- $\kappa$ B pathway. Chemotherapy-induced cytokines and growth factors, such as GDF15, contribute to tumor cell therapy resistance and may serve as targets to improve responses.

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Two large randomized phase III clinical trials have determined that docetaxel is the most active chemotherapy agent for the treatment of castration-resistant prostate cancer (1, 2). Both trials reported that docetaxel treatment results in improved clinical outcomes based on prostate-specific antigen (PSA) decline (defined as >50% decline in PSA level) and survival, when compared with mitoxantrone chemotherapy. In addition, improved pain control and quality of life were reported by TAX327 investigators and improved time to disease progression was reported by Southwest Oncology Group 9916 investigators. Although mitoxantrone provides palliation in a substantial fraction of castration-resistant prostate cancer patients and produces PSA responses, mitoxantrone has not been shown to improve survival. Although both agents produce significant biochemical and some objective responses, virtually all patients quickly progress to chemotherapy-refractory disease. Thus, defining mechanisms underlying chemotherapy resistance is critical both for selecting patients who may optimally benefit and for designing new therapeutic

strategies that either avoid or specifically target resistance pathways.

Theoretically, treating earlier in the disease course in the setting of micrometastases or locally advanced disease could reduce recurrence rates and eradicate small numbers of disseminated cells that may not have developed the clonal diversity capable of intrinsic resistance to cytotoxic therapies. In this context, adjuvant and neoadjuvant chemotherapy strategies have been routinely used for the treatment of several solid organ tumors, such as cancer of the breast (3) and colon (4). The addition of chemotherapy to surgical approaches for definitive treatment of prostate cancer has yet to show advantages in disease relapse or survival rates. To date, three neoadjuvant treatment studies using docetaxel have been reported in addition to our own (5–7). Remarkably, minimal pathologic responses have been observed in the primary tumor tissues evaluated after therapy, although substantial changes in serum PSA without alterations in serum testosterone concentrations indicate that biological effects are occurring. The specific effects on micrometastasis are unknown. Importantly, the neoadjuvant treatment strategy provides a unique setting for studying changes in molecular signatures before and after chemotherapy in which pretreated and posttreated samples are readily available for analysis.

Tissue gene expression profiles that reflect chemotherapy exposure as determined by comparing pretreated and posttreated samples may reveal both general and unique patient-specific chemotherapy resistance and response pathways. Correlating the induced profiles with clinical outcomes such as changes in tumor markers, pathologic response, and disease-free survival may also uncover intrinsic differences in tumor susceptibility to cytotoxic drugs and thus identify predictors of chemotherapy susceptibility for individual patients. In this context, studies of breast carcinoma are particularly relevant in view of the common use of neoadjuvant chemotherapy approaches as standard treatment. Perou et al. (8) used cDNA microarray analysis to measure gene expression profiles of primary breast tumors before and after treatment with doxorubicin therapy and identified signatures associated with treatment. A follow-up study using the same data set but different statistical methods to evaluate paired specimens from the same patient before and after chemotherapy identified additional molecular alterations that reflected exposure to cytotoxic drugs (9). Sotiriou et al. (10) reported a small study of 10 patients where gene expression profiles of tumor cells acquired from fine-needle aspirates of breast carcinoma before and after systemic chemotherapy were correlated with clinical responses. A study by Modlich et al. (11) measured gene expression changes in breast tumors before and 24 h after the administration of cytotoxic chemotherapy. Comparative analyses of isogenic matched tumor tissue provided a measure of gene expression alterations in the context of pharmacodynamic drug activity. Together, these data sets serve as rich resources for exploiting both common and unique determinants of tumor response and resistance. To date, comparable systematic analyses delineating the effects of chemotherapy on molecular features of localized or disseminated prostate cancer within individual patients have not been reported.

In this study, we used tissue, sera, and clinical data from a prospective phase I-II clinical trial of neoadjuvant chemotherapy with docetaxel and mitoxantrone in patients with high-risk

localized prostate adenocarcinoma (12) to identify molecular alterations associated with chemotherapy. We directly compared the expression alterations between paired pretreated and posttreated specimens from the same patient. We anticipated that this strategy would optimize the ability to detect chemotherapy-specific gene expression changes by comparing isogenic tissue samples rather than differences attributed with sample heterogeneity across individuals. Cancer cells surviving through docetaxel and mitoxantrone treatment are presumably enriched for resistant clones with molecular pathways contributing to cell survival. We identified consistent molecular alterations potentially representing chemoresistance pathways and evaluated the ability of one frequently altered gene product, growth differentiation factor 15 (GDF15), to influence prostate tumor cell resistance to docetaxel and mitoxantrone.

## Materials and Methods

**Patients and study description.** Between January 2001 and November 2004, 57 patients with high-risk localized prostate cancer (defined as tumor-node-metastasis > cT<sub>2b</sub> or T<sub>3a</sub> or PSA ≥ 15 ng/mL or Gleason grade ≥ 4+3) were recruited for a phase I-II clinical trial of neoadjuvant chemotherapy. The design of the clinical trial has been described previously (12, 13). The study was approved by the Institutional Review Boards of the Oregon Health and Science University, Portland Veterans Affairs Medical Center, Kaiser Permanente Northwest Region, Legacy Health System, and the University of Washington. All patients provided signed informed consent.

**Specimen collection and processing.** From each patient, 10 standard prostate biopsies (bilateral at the apex, bilateral medial and lateral at midgland, and bilateral medial and lateral at the base of the gland) were obtained under ultrasound guidance and snap frozen in liquid nitrogen before chemotherapy. At the time of radical prostatectomy, cancer-containing tissue samples were snap frozen as ornithine carbamyl transferase-embedded samples were placed into isopentane precooled in liquid nitrogen immediately after prostate removal while preserving pathologic diagnosis. Microscopic evaluation of frozen sections of tissue samples identified the presence of adequate numbers of cancer cells in both pretreatment and posttreatment samples for 31 subjects. Frozen sections (7 μmol/L) were cut from tissue frozen in ornithine carbamyl transferase blocks, stained with Mayer's hematoxylin (Sigma), dehydrated in 100% ethanol and xylene, and used for laser capture microdissection using an Arcturus PixCell IIe microscope (Arcturus, Inc.). To evaluate gene expression alterations after chemotherapy, neoplastic epithelium from pretreated biopsy and posttreated prostatectomy specimens were captured separately (~3,000 cells per sample). The histology of captured cells was verified both by review of an H&E-stained frozen section from each sample and by review of the pre/post-laser capture microdissection images.

**cDNA microarray analysis.** Total RNA was extracted from captured epithelium using a Picopure RNA isolation kit according to the manufacturer's instructions (Arcturus). Extracted RNA underwent two rounds of amplification by a linear T7-RNA polymerase method developed by Eberwine et al. (14) using a messageAMP aRNA kit (Ambion). cDNA was separately synthesized from 3 μg aRNA amplified from pretreated and posttreated specimens as described previously (15). To eliminate potential dye bias, we randomly alternated Cy3 and Cy5 labeling to pretreated and posttreated specimens across 31 samples. Labeled cDNA probes were hybridized in a head-to-head fashion, pretreated versus posttreated from the same individual, simultaneously to custom-made microarrays composed of ~6,800 clones derived from the Prostate Expression DataBase, a public sequence repository of expressed sequence tag data derived from human prostate cDNA libraries (16, 17).

Fluorescent array images were collected for both Cy3 and Cy5 emissions using a GenePix 4000B fluorescent scanner (Axon

Instruments). The image intensity data were gridded and extracted by using GenePix Pro 4.1 software. Preprocessing of array data was done using GeneSpring 7.2 software (Silicon Genetics). To compare the overall expression patterns of all neoplastic samples relative to their patient-matched normal epithelium, log<sub>2</sub> ratio measurements were statistically analyzed using the BRB ArrayTools, version 3.4.0 software.<sup>8</sup> We identified genes that were differentially expressed between the pretreated versus posttreated and good PSA responder to poor PSA responder using a random variance *t* test (18). Genes were considered statistically significant if their *P* values were <0.001.

**Quantitative reverse transcription PCR.** cDNA was synthesized from 1 µg aRNA using 2 µg random hexamers for priming reverse transcription by SuperScript II (200 units per reaction; Invitrogen). Quantitative reverse transcription PCRs (qRT-PCR) were done in duplicate, using ~5 ng cDNA, 0.2 µmol/L of each primer, and SYBR Green PCR Master Mix (Applied Biosystems) in a 20-µL reaction volume and analyzed using an Applied Biosystems 7700 sequence detector. Samples were normalized to the cycle threshold value obtained during the exponential amplification of glyceraldehyde-3-phosphate dehydrogenase (GAPDH). Control reactions with RNA or water as template did not produce significant amplification products. The sequences of primers used in our study were as follows: 5-CCTCAACGACCACCTTTGTC-3 (GAPDH forward), 5-TTACTCCTTG-GAGGCCATGT-3 (GAPDH reverse), 5-ACCTTCCCATCTCCAAGG-3 [chemokine (C-X-C motif) ligand 10 (CXCL10) forward], 5-GGTAGC-CACTGAAAGAATTGG-3 (CXCL10 reverse), 5-TGTCGCTCCAGACC-TATGATGA-3 (GDF15 forward), 5-AATCGGGTGTCTCAGGAACCTT-3 (GDF15 reverse), 5-GTCATTCGCTCCACATTCT-3 [interleukin (IL) 1β (IL1B) forward], and 5-AGCACACCAGTCCAAATTGA-3 (IL1B reverse).

**Cell culture and transfections.** DU145 and PC3 cells were cultured in RPMI 1640 supplemented with 10% fetal bovine serum at 37°C and 5% CO<sub>2</sub> until 80% to 100% confluent and routinely subcultured 1:10. Cells were transfected with pIRES2-EGFP-GDF15 plasmid (a kind gift from Dr. Samuel N. Breit, Centre for Immunology, University of New South Wales, Sydney, Australia; ref. 19) and pIRES2-EGFP plasmid (BD Bioscience) using LipofectAMINE 2000 (Invitrogen) according to the recommendations of the manufacturer. DU145 and PC3 cell transfectants were selected with 500 µg/mL geneticin (G418; Life Technologies). Stably transfected clones were picked by cloning disk and grown in standard culture medium with 500 µg/mL geneticin. Expression of GDF15 protein was confirmed by Western blot in conditioned medium from stably transfected cells.

**Western blot analysis.** Confluent cultures of DU145 and PC3 cells transfected with empty vector or GDF15 plasmids were rinsed twice with PBS and incubated in serum-free RPMI 1640 for 72 h. The supernatant was harvested and stored frozen at -80°C. Proteins were concentrated before electrophoresis using Centricon-10 concentrators (Amicon, Inc.), dissolved in loading buffer, reduced by boiling with 1% DTT for 5 min, and separated on a NuPAGE 10% Bis-Tris gel with MES running buffer (Invitrogen). Separated proteins were transferred to polyvinylidene difluoride membranes (Invitrogen) in a trans-blot semidry transfer cell (Bio-Rad). Analysis was done using the Odyssey infrared imaging system (Li-Cor Biosciences). The GDF15 antibody was purchased from R&D Systems. Secondary antibody was IRDye 800 conjugated affinity-purified anti-goat IgG (Rockland Immunochemicals, Inc.).

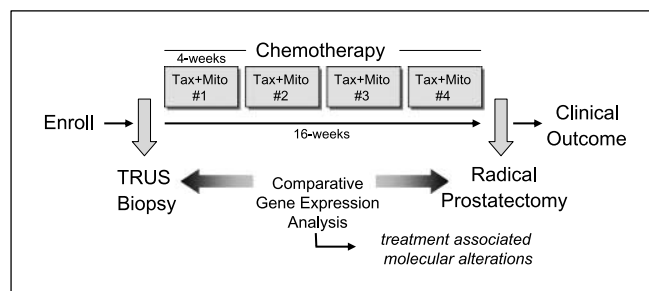
**3-(4,5-Dimethyl-thiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfo-phenyl)-2H-tetrazolium cell viability assay.** DU145 and PC3 cells overexpressing GDF15 or treated with recombinant GDF15 protein (R&D Systems) were incubated in 96-well plates with 0.5% fetal bovine serum RPMI 1640 at  $3 \times 10^3$  cells per well in quadruplicate in each well. The cells were treated with docetaxel (Sanofi-Aventis U.S. LLC) or mitoxantrone (Sigma) for 3 days. 3-(4,5-Dimethyl-thiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfo-phenyl)-2H-tetrazolium (MTS)

cell viability assay was done according to the manufacturer's protocol (Promega). In brief, 20 µL MTS reagent was added to each well (100 µL of medium) on day 3 and incubated at 37°C for 2 h. The absorbance at 490 nm was determined using an ELISA plate reader. The percentage of viable cells was calculated for each experiment by normalizing 490 nm absorbance of each experiment to control (cells without any treatment). Four replicate wells were assayed and all MTS cell viability assays were done in duplicate.

## Results

**Patients characteristics.** Between January 2001 and November 2004, 57 patients with high-risk localized prostate cancer were enrolled in a neoadjuvant treatment protocol of four 28-day cycles of 35 mg/m<sup>2</sup> docetaxel and escalating mitoxantrone doses (phase I) to a maximum of 4 mg/m<sup>2</sup> administered as 3 weekly doses followed by a 1-week break. Pretherapy prostate biopsies were done to obtain frozen tissue for research studies. Patients underwent a radical prostatectomy procedure within 1 month after completion of chemotherapy and additional prostate tissue samples were frozen (Fig. 1). We selected 31 patients that completed the full courses of chemotherapy and that had sufficient tumor tissue in the pretherapy and posttherapy samples to measure gene expression changes associated with chemotherapy. The clinical characteristics of the patients are shown in Supplementary Table S1. The median age was 67 years (interquartile range, 62-71) and the median Gleason score was 7. Roughly half of the patients (16 of 31) had a clinical stage higher than or equal to T<sub>3</sub>. The median PSA before and after chemotherapy was 9.3 ng/mL (interquartile range, 5.9-17.0) and 5.8 ng/mL (interquartile range, 3.9-10.5), respectively. The majority of patients (81%) had PSA reductions after chemotherapy with a median decline of 42.2% (interquartile range, 19.3-47%). A PSA reduction >50% was observed in 7 of 31 patients. None of the patients showed a complete tumor remission as determined by pathologic analysis of radical prostatectomy specimens.

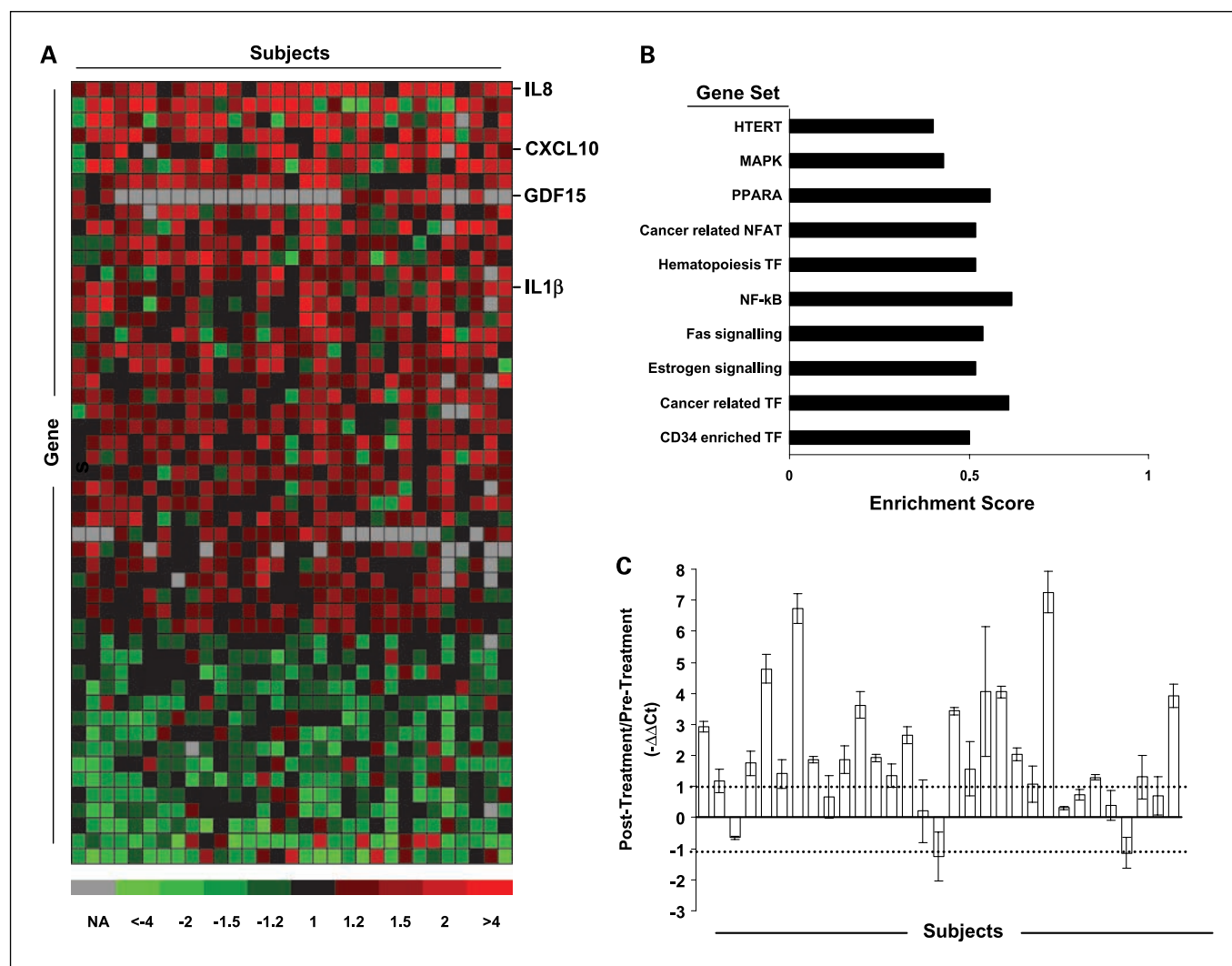
**Chemotherapy-associated alterations in prostate cancer gene expression.** To identify gene expression changes in prostate tumor cells associated with chemotherapy exposure, we used laser capture microdissection to specifically acquire neoplastic epithelium from pretreatment and posttreatment



**Fig. 1.** Clinical trial and analysis plan. Patients were enrolled in a phase I-II neoadjuvant clinical trial with the treatment protocol of four 28-d cycles of 35 mg/m<sup>2</sup> docetaxel and 4 mg/m<sup>2</sup> mitoxantrone administered as 3 weekly doses followed by a 1-wk break. These patients then underwent radical prostatectomy within 1 mo after completion of chemotherapy. All patients underwent pretherapy prostate biopsies to obtain tissue for comparative histopathologic and molecular analyses. Patients continued receiving clinical follow-up after surgery. Direct comparisons of gene expression profiles were done on microdissected cancerous epithelial cells acquired from pretreatment needle biopsy specimens and posttreatment radical prostatectomy specimens.

<sup>8</sup> <http://linus.nci.nih.gov/BRB-ArrayTools.html>





**Fig. 2.** Gene expression analyses. *A*, heatmap of differentially expressed genes in posttreatment specimens compared with pretreatment specimens. Rows, genes; columns, patients. Several genes selected for further study, *IL-8*, *CXCL10*, *GDF15*, and *IL1B*, are indicated. *B*, GSEA pathway analysis. Columns, a gene set. All gene sets have enrichment scores  $>0.4$  and normalized  $P$  values  $<0.05$ . TF, transcription factor; NF- $\kappa$ B, nuclear factor of  $\kappa$  light polypeptide gene enhancer in B-cells; NFAT, nuclear factor of activated T cells; PPARA, peroxisome proliferators regulated genes; HTERT, RNA subunit of telomerase. *C*, GDF15 expression in posttreated radical prostatectomy specimens compared with pretreated needle biopsy specimens as determined by qRT-PCR.  $-\Delta\Delta C_T$  was calculated as the differences of cycle threshold value ( $C_T$ ) of GDF15 in posttreated relative to pretreated specimens.  $C_T$  values of GDF15 were first normalized to the  $C_T$  values obtained during the exponential amplification of GAPDH. Columns, posttreatment/pretreatment; bars, SD. Dashed line, 2-fold differences.

tissue samples. We purposely attempted to collect cells from tumors with the same Gleason pattern from pretreated and posttreated tissues to avoid possible bias introduced by states of cellular differentiation (15). We did cDNA microarray analysis using a head-to-head comparison of specimens from the same subject before and after treatment. After filtering for array artifacts and poor quality cDNAs, 6,600 genes were retained for analysis. We used a random variance  $t$  test to compare transcript abundance measurements between pretreated and posttreated specimens and identified 95 differentially expressed genes ( $P < 0.001$ ; Supplementary Table S3).

We have shown previously that the process of surgical resection with attendant tissue ischemia and other procedural affects can result in a substantial number of gene expression changes in the prostate (20). Because the present study compared needle biopsy tissues with radical prostatectomy tissues, several expression changes hypothesized to be chemo-

therapy influenced may actually have been altered due to the different methods of tissue acquisition. Comparing the cohort of genes in this study with our prior analyses identified 25 (26.3%) genes that we determined previously to be altered in the context of surgical manipulation, such as *DUSP1*, *VMP1*, *JUN*, *CDKN1A*, and *BTG2* (20). Although chemotherapy could induce or augment cellular stress responses similar to those influenced by ischemia, we opted to eliminate these genes from further analyses that were targeted toward defining chemoresistance mechanisms. To this end, we broadened our cohort of potential ischemia-related genes using a less stringent cutoff of statistical significance ( $P < 0.05$ ) than we reported previously resulting in the classification of 441 clones that associated with surgical manipulation. We excluded these 441 potential ischemia-related clones and repeated the analysis, a procedure that resulted in the classification of 70 clones representing 51 unique genes as differentially expressed ( $P < 0.001$ ;

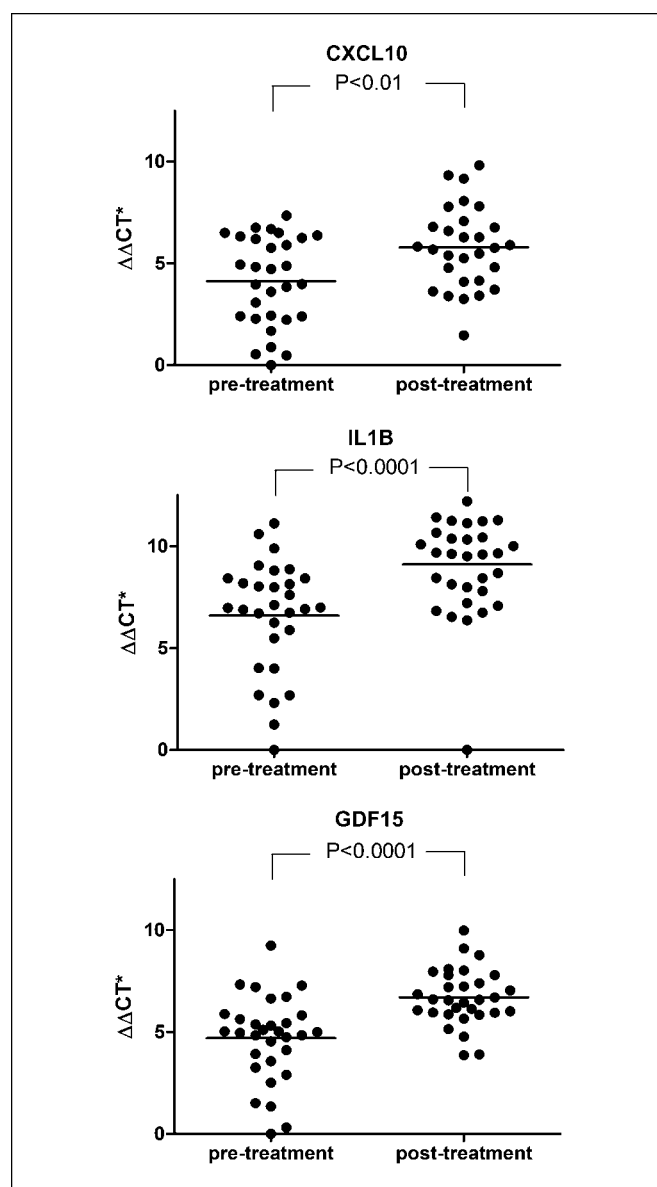
Supplementary Table S2). Of these, the expression of 36 genes increased and 15 decreased following chemotherapy (Fig. 2A). Several altered genes encode cytokines, chemokines, and growth factors, such as IL-6, IL-8, IL1B, CXCL10, and GDF15. Consistent with these *in vivo* findings, a previous *in vitro* study showed that IL-8 and IL-6 expression increased in paclitaxel-resistant ovarian cancer cell sublines (21), and GDF15 has been reported to be up-regulated after neoadjuvant chemotherapy with epirubicin and cyclophosphamide or Taxol in breast cancer (11). We next measured GDF15, CXCL10, and IL1B transcripts by qRT-PCR in the microdissected tumor cells and confirmed that each cytokine was significantly up-regulated ( $P < 0.05$  by paired  $t$  test) in posttreatment tumor cells compared with matched pretreatment biopsy samples (Figs. 2C and 3).

The ultimate objective of these studies is the identification of chemotherapy resistance mechanisms and pathways that could be exploited as therapeutic targets to improve treatment responses. To this end, we further analyzed the functional categories of the 51 chemotherapy-altered genes based on Genome Ontology biological processes using EASE methods (22). We found a significant enrichment of genes involved in cellular responses operating through the mitogen-activated protein kinase and nuclear factor- $\kappa$ B pathways that include the chemokines and cytokines described above (Fig. 2B). We also found a significant enrichment of genes expressed in CD34<sup>+</sup> cells, genes involved in telomere function, estrogen signaling, and apoptosis. These findings suggest that chemokines may play important roles in mediating chemotherapy resistance and response. Although chemokine activation after chemotherapy could be explained by generalized inflammatory reactions induced by cell death after toxin exposure, growing evidence indicates that chemokines are important survival factors for cancer cells under chemotherapy treatment (21, 23). Importantly, chemokines and their receptors have the therapeutic advantage of relatively straightforward modulation by agonists or antagonist such as small molecules or antibodies.

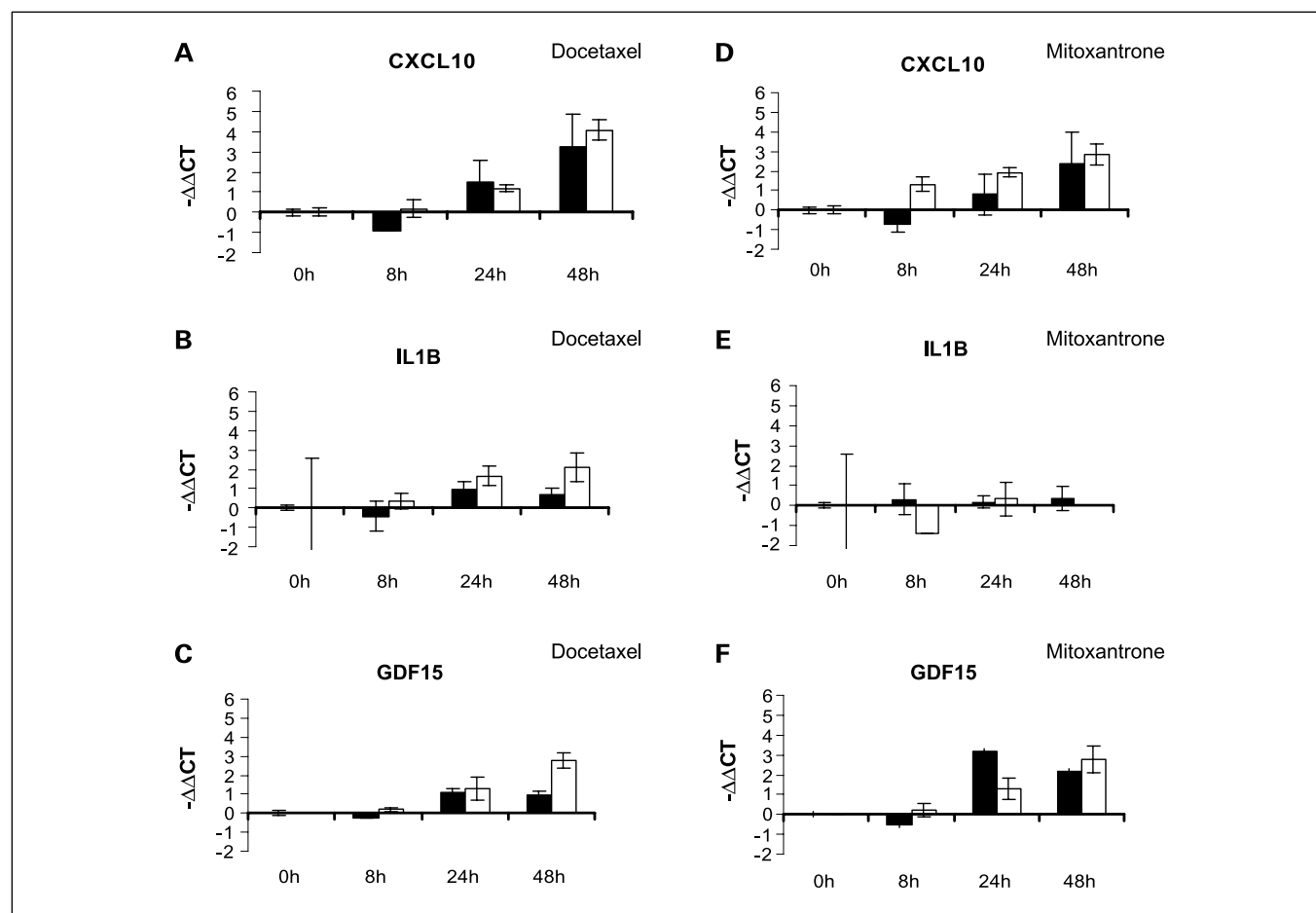
**GDF15 influences chemotherapy resistance.** Of the cytokine-encoding transcripts exhibiting chemotherapy-associated differential expression, GDF15 (alias macrophage-inhibitory cytokine 1), a transforming growth factor- $\beta$  superfamily member, has been reported to be associated with cancer progression and metastasis (24). The role of GDF15 in chemotherapy resistance has yet to be determined. We first confirmed the expression results derived from the microarray analyses. GDF15 mRNA abundance in pretreated biopsy and posttreated prostatectomy tissue samples was measured by quantitative PCR in aRNA obtained from the microdissected cancer cells. Of 31 samples, 28 had measurable increases in GDF15 expression (90%) with 22 of 31 showing a 2-fold or greater change (Fig. 2C). The consistency of these findings suggested that GDF15 induction could be a generic response to chemotherapy stress or could represent a tumor response gene because the majority of subjects did have PSA reductions in the context of chemotherapy. Alternatively, GDF15 changes, measured here in cells surviving treatment, could represent an important modulator of resistance because there were no complete tumor responses in any of the subjects treated in this study.

To explore these possibilities, we evaluated whether expression of GDF15 could confer cellular chemoresistance using an

*in vitro* cell culture system. We first determined if the chemotherapy agents used in the *in vivo* studies, mitoxantrone and docetaxel, also induced the expression of GDF15 and other cytokines in prostate cancer cells *in vitro*. PC3 and DU145 prostate cancer cells were each treated with docetaxel and mitoxantrone separately at concentrations near their respective IC<sub>50</sub> levels. We measured cytokine mRNA abundance by qRT-PCR in cDNA obtained from cells at 0, 8, 24, and 48 h after drug exposure. Transcripts encoding CXCL10, IL1B, and GDF15 increased 2- to 8-fold over the 48-h time course after exposure to docetaxel in both PC3 and DU145 cells (Fig. 4A-C). Exposure to mitoxantrone induced CXCL10 and GDF15 expression but not IL1B in both PC3 and DU145 cells (Fig. 4D-F). We further evaluated if these findings could be



**Fig. 3.** Confirmation of chemotherapy-influenced gene expression alterations *in vivo*. qRT-PCR measurements of transcripts encoding CXCL10, GDF15, and IL1B in posttreated radical prostatectomy specimens and pretreated needle biopsy specimens.  $C_T$ , cycle threshold value.  $\Delta C_T = C_T$  of cytokines -  $C_T$  of GAPDH.  $\Delta\Delta C_T = \Delta C_T$  - lowest  $\Delta C_T$  in pretreatment group.



**Fig. 4.** Chemotherapy-induced gene expression changes *in vitro*. Time course of transcript levels of cytokines in PC3 (black columns) and DU145 (white columns) prostate cancer cells after treatment with docetaxel or mitoxantrone.  $-\Delta\Delta C_T$  was calculated as differences of cycle threshold value ( $C_T$ ) of cytokines in 8, 24, and 48 h relative to 0 h.  $C_T$  values of cytokines were first normalized to the  $C_T$  values obtained during the exponential amplification of GAPDH. Columns,  $-\Delta\Delta C_T$ ; bars, SD.

generalized to metastatic prostate cancer. Cytokine-encoding transcripts were quantitated in human LuCaP 35V xenografts treated with docetaxel, 20 mg/kg/wk, or placebo, by qRT-PCR. In these tumors, propagated in immune-deficient mouse hosts, expression of CXCL10, IL1B, and GDF15 increased following chemotherapy.

To assess the functional consequences of GDF15 expression and cellular responses to chemotherapy, we increased GDF15 levels by transfecting the DU145 and PC3 cells with plasmids designed to express GDF15 (pIRES2-EGFP-GDF15; gift from Dr. S. Breit; ref. 25). Cells overexpressing GDF15 and control cells expressing vector only (pIRES2-EGFP) were treated with increasing concentrations of docetaxel or mitoxantrone for 3 days. The percentage of viable cells was determined using MTS assays. We found that DU145 cells expressing GDF15 exhibited significantly greater resistance to both docetaxel- and mitoxantrone-induced cytotoxicity, and differences in cell survival were enhanced with increasing drug concentrations (Fig. 5). PC3 cells expressing GDF15 also showed resistance to docetaxel and mitoxantrone, although the attenuation of mitoxantrone-induced cell death was not as substantial.

To determine if GDF15 could exert a cytoprotective function via a paracrine mechanism, we treated DU145 and PC3 cells with various concentrations of recombinant GDF15 protein

and the chemotherapy drugs at around  $LD_{50}$  concentrations. The concentration of GDF15 protein in these experiments ranged from 0.1 to 100 ng/mL; this range covered the physiologic serum concentration in patients with prostate cancer ( $0.7 \pm 0.5$  ng/mL; ref. 26), although local concentrations within tumors may be substantially higher. We found that GDF15 exposure slightly inhibited proliferation of DU145 cells at concentrations above 50 ng/mL, consistent with previous findings that GDF15 may suppress *in vitro* tumor cell growth (27). However, GDF15 did not affect PC3 cell proliferation. We found that concentrations of GDF15  $\geq 1$  ng/mL resulted in measurable increases in both PC3 and DU145 cell viability with docetaxel exposure (Fig. 6A and B). Following 72 h of exposure to docetaxel, 1 ng/mL GDF15 increased DU145 cell viability by 15%, and PC3 cell viability 33% relative to controls. A significant GDF15-mediated cytoprotective effect was also observed in mitoxantrone-treated DU145 cells, but not PC3 cells, indicating that drug- and cell type-specific resistance mechanisms are operative.

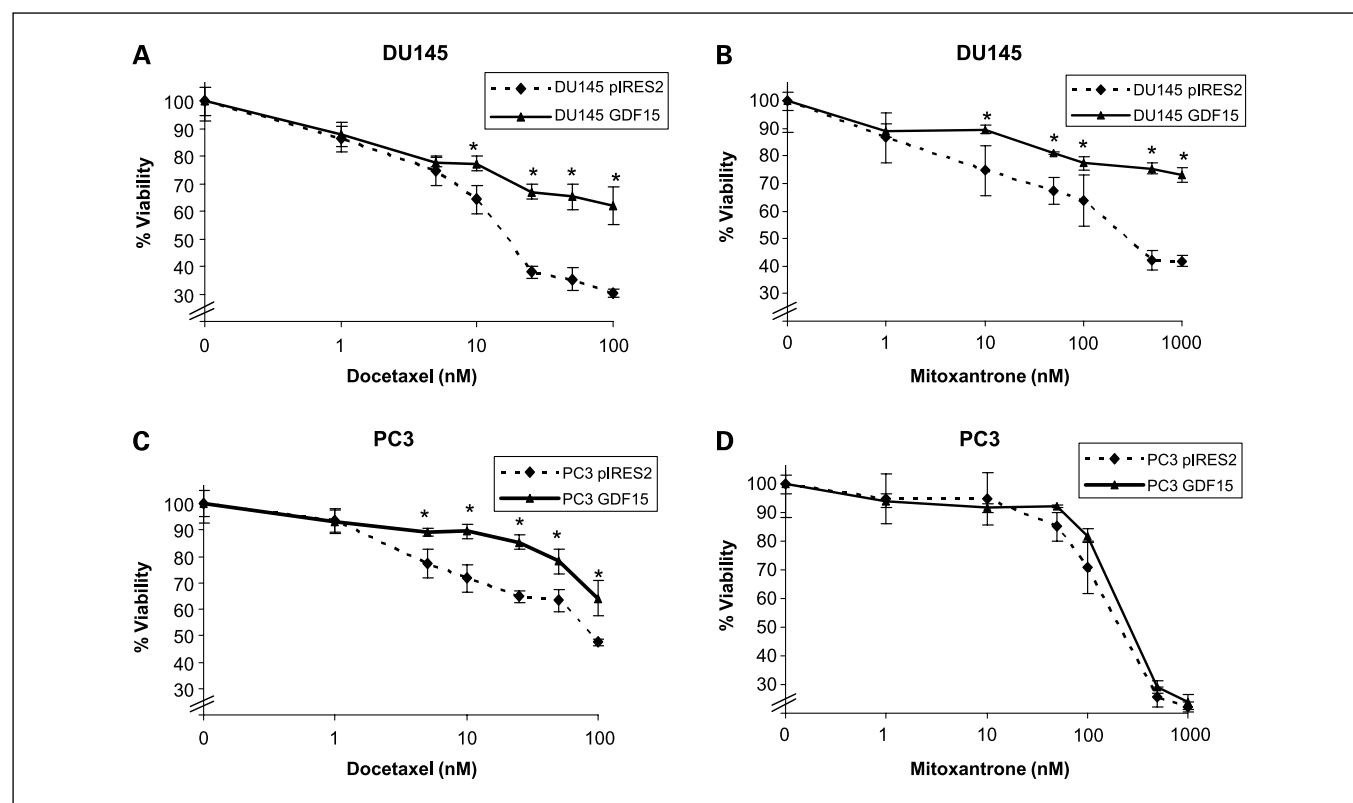
## Discussion

Identifying the biological mechanisms that underlie intrinsic or acquired resistance to chemotherapies designed to eradicate

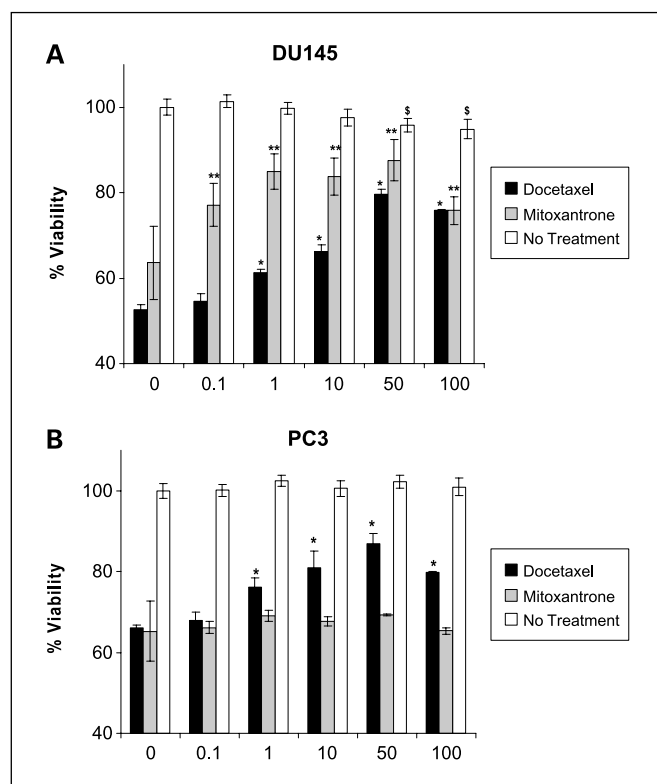
cancer cells is of critical importance for improving the morbidity and mortality associated with human malignancies. For reasons that have yet to be determined, prostate cancers seem to be particularly resistant to the cytotoxic effects of commonly used antineoplastic drugs. Most studies evaluating drug resistance use cell culture models and little data are available translating the relevance of these findings to the complexity and variability of the clinical environment. In this study, we have used a clinical trial system designed specifically to assess molecular features of tumor cells in response to drug interventions. Several features were used to reduce noise and artifacts and optimize our ability to identify relevant therapy-related tumor effects. First, tumor tissue was acquired *in situ* before and after drug exposure from the same individual to control for genotypic differences that may influence gene expression measurements between individuals. Second, we used a cell type-specific analysis approach that focused specifically on tumor epithelium to minimize issues consequent to heterogeneity in sample composition. Third, we filtered the experimental results to specifically exclude a cohort of genes whose expression is influenced by the process of surgical resection. The resulting analyses determined that a group of 51 genes is significantly associated with chemotherapy exposure to docetaxel and mitoxantrone in neoplastic prostate epithelium. Further, many of these genes are components of complex cellular pathways of interacting proteins that were also statistically associated with chemotherapy exposure, supporting

the concept that a network of responses contribute to cell survival and therapy resistance (28).

Three previous studies have evaluated the use of docetaxel before radical prostatectomy. Dreicer et al. (5) administered six doses of docetaxel on a weekly schedule before surgical resection of the prostate to 29 men with features of high-risk adenocarcinoma. Despite most subjects achieving some reduction in serum PSA (24% achieved more than a 50% decrease), there were no pathologic complete tumor responses. Febbo et al. (6) treated 19 patients with high-risk localized prostate cancer for 6 months on a weekly regimen of docetaxel followed by prostatectomy. PSA declines >50% were observed in 11 of 19 patients and magnetic resonance imaging documented tumor volume reductions of at least 25% in 13 of 19 subjects. However, of the 16 patients that completed chemotherapy and had surgery, there were no pathologic complete responses. Microarray-based gene expression studies were done on residual tumors from the radical prostatectomy samples and compared with samples from a separate group of untreated patients. Unsupervised clustering of the treated versus untreated samples could not partition subjects according to therapy group, indicating substantial interindividual variability in baseline gene expression across prostate tissues or variables in sample composition that precluded the robust partitioning of samples. However, the study did identify significant changes in genes involved in androgen metabolism that were associated with cells surviving after docetaxel exposure; these genes were



**Fig. 5.** Influence of GDF15 expression on cell growth and chemotherapy resistance. DU145 (A and B) and PC3 (C and D) prostate cancer cells transfected with GDF15 expression vectors (solid line) versus empty (pIRES2) vector (dashed line) were exposed to docetaxel (A and C) or mitoxantrone (B and D) chemotherapy or vehicle control and quantitated by MTS assays. Y-axis, cell numbers relative to vehicle control after 72 h of treatment; X-axis, concentration of docetaxel and mitoxantrone treatment. \*,  $P < 0.05$  by Student's *t* test compared with cells with empty vector.



**Fig. 6.** Influence of exogenous GDF15 on cell growth and chemotherapy resistance. MTS cell viability assay for prostate cancer cells treated with recombinant GDF15 protein. *A*, DU145 cells. *B*, PC3 cells. Drug concentrations were docetaxel (50 nmol/L) and mitoxantrone (100 nmol/L). Y-axis, percentage of cells surviving after 72 h of chemotherapy exposure relative to vehicle-treated controls; X-axis, concentrations of GDF15 in growth medium. \*,  $P < 0.05$  by Student's *t* test compared with no GDF15 treatment in docetaxel treatment group (black columns); \*\*,  $P < 0.05$  by Student's *t* test compared with no GDF15 treatment in mitoxantrone treatment group (shaded columns); \$,  $P < 0.05$  by Student's *t* test compared with no GDF15 treatment in control (vehicle) group (white columns).

hypothesized to account for declines in PSA concentrations and contribute to chemotherapy resistance. The present study, using an arguably more intensive chemotherapy intervention with two drugs targeting different cellular pathways, also failed to produce any complete tumor remissions. Together, these studies suggest either a failure in delivering adequate drug concentrations to prostate tissues or an intrinsic difference in

the phenotypes of prostate cancer cells that confers widespread, rather than rare acquired resistance to cytotoxic drugs.

In this study, gene expression analyses determined that stress response pathways, particularly those involving inflammation-associated cytokines, might be involved in the mechanism of chemotherapy resistance of prostate cancer. Several, including IL-8 and IL-6, are up-regulated in taxane-resistant tumor cell lines, and IL-6 can specifically modulate resistance (21, 23). Further experimental studies of chemoresistant prostate cancer may clarify whether targeting these cytokines or their receptors could reduce tumor cell viability following cytotoxic therapies. The cytokine GDF15, also known as macrophage-inhibitory cytokine 1, is a member of the human transforming growth factor- $\beta$  superfamily. Similar to other transforming growth factor- $\beta$  family genes, the effect of GDF15 in tumorigenesis may be context specific and differ depending on tissue type, tumor stage, and/or type of treatment. Although prior studies have found that GDF15 can exert proapoptotic (25) and growth-inhibitory effects on tumor cells (27), gene expression studies of GDF15 in colon, prostate, and pancreas have measured increased expression levels of GDF15 in tumor compared with benign tissue (24). In the context of therapy, Modlich et al. (11) compared gene expression changes in the setting of neoadjuvant chemotherapy for patients with primary breast cancer. Expression profiles of paired tumor samples obtained before and 24 h after chemotherapy found increased expression of GDF15 and several other genes following treatment. Shimizu et al. (29) reported an analysis of gene expression differences between 5-fluorouracil-chemoresistant and 5-fluorouracil-chemosensitive colon cancer cell lines and determined *GDF15* to be one of the most significantly up-regulated genes in resistant cells. The results of our experiments with GDF15 are consistent with these reports. Furthermore, we found that overexpression of GDF15 or exposure to GDF15 protein may confer a component of chemoresistance to prostate cancer cells, a finding that supports further studies designed to manipulate GDF15 for therapeutic benefit.

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