Identification of Growth Arrest and DNA-Damage-Inducible Gene β (GADD45β) as a Novel Tumor Suppressor in Pituitary Gonadotrope Tumors

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Gonadotrope and null cell pituitary tumors cause significant morbidity, often presenting with signs of hypogonadism together with visual disturbances due to mass effects. Surgery and radiation are the only therapeutic options to date. To identify dysregulated genes and pathways that may play a role in tumorigenesis and/or progression, molecular profiling was performed on 14 gonadotrope tumors, with nine normal human pituitaries obtained at autopsy serving as controls. Bioinformatic analysis identified putative downstream effectors of tumor protein 53 (p53) that were consistently repressed in gonadotrope pituitary tumors, including *RPRM*, *P21*, and *PMAIP1*, with concomitant inhibition of the upstream p53 regulator, *PLAGL1(Zac1)*. Further analysis of the growth arrest and DNA damage-inducible (GADD45) family revealed no change in the p53 target, *GADD45α*, but identified repression of *GADD45β* in pituitary tumors in addition to the previously reported inhibition of *GADD45γ*. Overexpression of GADD45β in LβT2 mouse gonadotrope cells blocked tumor cell proliferation and increased rates of apoptosis in response to growth factor withdrawal. Stable gonadotrope cell transfectants expressing increased GADD45β showed decreased colony formation in soft agar, confirming its normal role as a tumor suppressor. Unlike previous studies of *GADD45γ* in pituitary tumors and α and β in other tumors, bisulfite sequencing showed no evidence of hypermethylation of the *GADD45β* promoter in human pituitary tumor samples to explain the repression of its expression. Thus, GADD45β is a novel pituitary tumor suppressor whose reexpression blocks proliferation, survival, and tumorigenesis. Together these studies identify new targets and mechanisms to explore in pituitary tumor initiation and progression. **(*Endocrinology* 152: 3603–3613, 2011)**

Pituitary tumors are among the most common intracranial neoplasms. Clinically detected pituitary adenomas develop in one per 10,000 persons, but present at an overall prevalence of 16.7% in the population as detected by radiology and autopsy (1, 2). Factors underlying the pathogenesis and progression of these tumors remain poorly understood despite extensive investigation. Pituitary tumors are monoclonal and classified by their cell type of origin (3). These include prolactinomas (35–40% of adenomas), GH adenomas (10–15%), ACTH adenomas (10%), and gonadotrope or null cell (35%) as the most common subtypes.

Gonadotrope tumors derive from gonadotrope cells, which compose 10–20% of the pituitary (1). These tumors express the gonadotrope subunit mRNA and variably secrete and demonstrate immunohistochemical evidence of LH, FSH, and α-subunit (ASU). Gonadotrope tumors represent up to 50% of macroadenomas (>1 cm)

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Abbreviations: ASU, α-Subunit; FBS, fetal bovine serum; IPA, Ingenuity pathway analysis; PARP, poly ADP-ribose polymerase; PCA, principal component analysis; QPCR, quantitative RT-PCR; TBST, Tris-buffered saline/Tween 20.

and are more common in men (4). Clinical presentation can include erectile dysfunction, headache, dizziness, visual field defects resulting from compression of the optic chiasm, and hormonal deficits resulting from normal pituitary gland compression (4, 5). Surgical resection of the tumor can be highly effective but often fails to cure those with invasive or recurrent tumors. These difficulties underscore the need for improved predictive biomarkers of disease progression and the development of mechanism-targeted medical therapies. Oncogenes and tumor suppressors characteristically mutated or dysregulated in the pathogenesis of common cancers typically do not seem to play central roles in pituitary tumorigenesis or in predicting biological behavior of the adenomas (1, 2). In addition, there are no animal models that optimally model gonadotrope tumorigenesis. The advances in genomic microarray technology provide an opportunity to identify and characterize the pathways and genes responsible for the pathogenesis and progression of human pituitary tumors.

We performed gene expression microarray analysis comparing 14 individual gonadotrope tumors with nine individual normal pituitary samples obtained at autopsy. Bioinformatic analysis allowed characterization of differentially expressed transcripts and identification of novel signatures in tumors compared with normal pituitary. Down-regulation was observed in many downstream components in the *TP53* pathway, but with no evidence of alterations in *TP53* transcript itself. We explored the roles of the *GADD45* family, shown previously to mediate extrinsic and intrinsic stress response in cells (6–12). We found no alteration in the expression levels of *GADD45α*, a putative downstream p53 target, and confirmed previous studies showing that *GADD45γ* is suppressed in pituitary tumors (10). In addition, our microarray data showed loss of *GADD45β*, implicating it as a novel tumor suppressor in gonadotrope tumors. Thus, functional analysis of *GADD45β* was performed to examine its potential role in pituitary tumorigenesis, and possible sources of dysregulation.

Materials and Methods

Reagents

Antibodies for caspase 3, p53, phospho-p53, and p14ARF were purchased from Cell Signaling Technology (Beverly, MA). GADD45β antibody was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) antibody was purchased from Millipore (Billerica, MA). β-tubulin antibody was purchased from Abcam (Cambridge, MA). Horseradish peroxidase-conjugated secondary antibodies (goat antirabbit IgG and goat antimouse IgG) were purchased from Bio-Rad (Hercules, CA). Peroxidase AffiniPure donkey antigoat IgG (H+L) was purchased from Jackson ImmunoResearch Laboratories, Inc. (West Grove, PA). RNAlater was from Ambion (Austin, TX).

Tumor and normal pituitary tissue characteristics

With informed consent, pituitary tumor samples were obtained from patients at University of Colorado Hospital at the time of transsphenoidal surgery. Portions of the specimens not used for histology and immunohistochemistry were placed in RNAlater and stored at −80 C. Gonadotrope tumors were defined as demonstrating positive immunostaining for FSH, LH, or ASU in greater than 5–10% of cells. Null cell adenomas were defined by gonadotropic staining for FSH, LH, or ASU in less than 5–10% of cells. Normal pituitary glands used as controls were obtained at autopsy within 2–18 h of death from University of Colorado Denver Pathology Department and stored similarly. A principal component analysis (PCA) was performed on our normalized array data using Partek Genomics Suite software. PCA is a technique for taking very large multivariate datasets, such as those generated from expression arrays, and reducing its dimensionality (for review see Refs. 13 and 14). This widely used visual method accounts for as much of the variability in the data as possible and represents this summation as a point in three-dimensional space. Each axis on the graph represents the best approximation of this variability, termed principal components, and plots the individual samples according to their three calculated principal components. The numbers on the graph correspond to the percentage of the variation accounted for in computing the individual principal components.

Microarray preparation and data generation

Total RNA was isolated from pituitary tumors or control pituitaries using TRIzol (Invitrogen, Carlsbad, CA), followed by clean-up with a QIAGEN (Valencia, CA) RNeasy minikit. RNA was quantified by spectrophotometry, and RNA integrity was confirmed with the Agilent Bioanalyzer (Agilent Technologies, Santa Clara CA). Microarray targets were prepared and labeled from 300 ng total RNA using the MessageAmp Premier RNA Amplification Kit (Applied Biosystems/Ambion, Austin, TX) following the manufacturer's instructions. Affymetrix HG-U133 plus 2.0 arrays (Santa Clara, CA) were hybridized with 10 μg cRNA and processed and scanned using standard Affymetrix protocols.

Hybridization intensities were quantified and normalized across all arrays using the Robust Multichip Average (RMA) algorithm with an adjustment for guanine/cytosine contest of probesets, available as an array processing tool on Partek Genomics Suite software (St. Louis, MO) (15). Data were filtered to remove all genes considered absent, as determined by the Affymetrix GeneChip Operating Software (GCOS), in greater than 95% of all the samples (22 of 23 total samples). Remaining transcripts (38,932 of 54,675) were used for all subsequent statistical and visual analysis. A mixed-model ANOVA was employed to estimate the batch effect, and the data were adjusted using the Partek Batch Remover tool available on Partek Genomics Suite. The filtered, batch effect-adjusted data are available as a tab-delineated file in an online supplement and has also been deposited, along with the original Affymetrix CEL files used to generate the raw data, in the GEO database (www.ncbi.nlm.nih.gov/geo/).

Biostatistical and bioinformatic analysis

Partek Genomcs Suite software was used to identify differentially expressed transcripts using a one-way ANOVA model with a stringent false discovery rate of less than 0.05 to control for multiple testing. An arbitrary expression change cutoff of more than 2.0 was applied to generate a set of transcripts with differential expression between tumor and normal tissues. Ingenuity pathway analysis (IPA) was then used to identify biological functions and diseases associated with differentially expressed genes. Signaling pathways containing or regulated by differentially expressed genes were identified using the IPA library of canonical pathways. Differentially expressed genes were analyzed for their contribution to specific known pathways in tumor *vs.* normal using the IPA software and a Fisher exact test ($P < 0.05$).

Semiquantitative RT-PCR

Total RNA was extracted from tissues or cells using TRIzol reagent, according to the manufacturer's protocol (Invitrogen), and 0.5 μg RNA was reverse transcribed using Thermo Verso cDNA kit (Fisher Scientific, Pittsburgh, PA). RT-PCR was conducted on tumor and normal pituitary cDNA for the following genes using the indicated primers: p14ARF, *hCDKN2A_ARF*_224-243F 5′-GTTTTCGTGGTTCACATCCC-3′ and *hCDKN2A*_ARF_487–470R 5′-ACCAGCGTGTCCAGGAAG-3′; *MDM2*, *hMdm2*_980-1005F 5′-TGCTGGTGTAAGTGAACATTCAGGTG-3′ and *hMdm2*_1293-1271R 5′-GCCAATTCTCACGAAGGGCCCAA-3′; p53, hp53_var3_62-80F 5′-AGTCTAGAGCCACCGTCCA-3′ and hp53_var3_262-238R 5′-AGGTCTGAAAATGTTTCCTGACTCA-3′; PLAGL1, *hZac1*_F 5′-CCTTTGGGTGTGAGGAGTGT-3′ and *hZac1*_R 5′-GCTTTTGAGGTGACTGAGGC-3′; *RPRM*, *hRPRM*Fwd233 5′-GCAATCTGCTCATCAAGTCCGAG-3′ and *hRPRM*-Rev616 5′-CCCCGCATTCCAAGTAAGTAG-3′; *p21*, *hCDKN1A* (p21)_F 5′-GACACCACTGGAGGGTGACT-3′ and *hCDKN1A* (p21)_R 5′-CCCTAGGCTGTGCTCACTTC-3′; *PMAIP1*, hPMAIP1(NOXA)_F 5′-TCACCGTGTGTAGTTGGCAT-3′ and *hPMAIP1*(NOXA)_R 5′-TTCCATCTTCCGTTTCCAAG-3′; GAPDH, hGAPDH_s 9–32 5′-CGGAGTCAACGGATTTGGTCGTAT-3′ and *hGAPDH*_as 292–315 5′-AGCCTTCTCCATGGTGGTGAAGAC-3′. For assessing endogenous and overexpressed levels of *GADD45β*, semiquantitative PCR was carried out with the primers rmh*GADD45β* 236–257 5′-AAGAGCTGGTGGCGAGCGACA-3′ and rmh*GADD45β* 649–628 5′-AGTAACTGGCCACCTCCACCAA-3′.

Immunoblot analysis

Protein lysates were quantified by bicinchoninic acid assay to ensure even loading (Pierce, Rockford, IL) and electrophoresed through SDS-polyacrylamide gel. Proteins on the gels were electroblotted to polyvinylidene difluoride membranes using the mini transblotter system (Bio-Rad, Hercules, CA). The polyvinylidene difluoride membranes were blocked in 3% BSA in Tris-buffered saline/Tween 20 (TBST) for 1 h. Primary antibodies were diluted 1:1000 in TBST/0.5% BSA/0.1% NaN_3, and the membranes were incubated in primary antibodies at 4 C overnight. Membranes were washed three times in TBST for 10 min each. Horseradish peroxidase-conjugated secondary antibody (Amersham Biosciences, Piscataway, NJ) was diluted 1:3000 in TBST and incubated on the membranes for 1 h at room temperature. The membranes were washed as above and visualized using enhanced chemiluminescence according to the manufacturer's protocol (Pierce).

Quantitative real-time PCR

Total RNA extracted as above was further purified with the RNeasy minikit (QIAGEN), and 750 ng was reverse transcribed with the iScript cDNA synthesis kit (Bio-Rad). Normal pituitary and gonadotrope tumor cDNA was diluted 1:10, and *GADD45α*, *GADD45β*, and *GADD45γ* expression was determined using FAM-labeled PrimeTime qPCR assays (IDT, Coralville, IA) after normalization to 18s (Hs*99999901*_s1; Applied Biosystems, Foster City, CA) RNA levels. Real-time PCR was performed in duplicate on an ABI7300 system using Gene Expression Master Mix (Applied Biosystems). Sequences for *GADD45* assays are as follows: *GADD45α* (probe 5′-FAM-CCCCTTGGCATCAGTTTCTGTAATCCT-3′ and primers forward 5′-CATTTTCACCTCTTTCCATCTGC-3′ and reverse 5′-CTCAAGCAGTTACTCCCTACAC-3′), *GADD45β* (probe5′-FAM-CGACATCAACATCGTGCGGGTG-3′ and primers forward 5′-CCAGGAGACAATGCAGGTC-3′ and reverse 5′-AATCCACTTCACGCTCATCC-3′), *GADD45γ* (probe5′-FAM-TGAAGGACTTAACCGACTGCCGC-3′ and primers forward 5′-GAAAACGAAGCATTGCCCG-3′ and reverse5′-TTGGTACAGTTTCAGGAGCG-3′).

pcDNA3-flag-GADD45β cloning

The *GADD45β* cDNA was obtained by PCR using normal pituitary cDNA as template and two primers: 5′-GTGCTGGAATTCACCATGGACTACAAGGACGACGATGACAAGATGACGCTGGAAGAGCTC-3′ (forward) and 5′-TGCATGCTCGAGtcagcgttcctgaagagagatgta-3′ (reverse). The PCR product was digested by *Eco*RI and *Xho*I and then inserted to pcDNA3 vector, and the sequence was confirmed.

Cell culture and transfections

LβT2 mouse pituitary cell line was obtained from P. Mellon, University of California, San Diego (San Diego, CA). Cells were cultured in DMEM (Invitrogen) supplemented with 10% fetal bovine serum (FBS) (Atlanta Biologicals, Lawrenceville, GA) at 37 C in humidified 5% CO_2. Transfections were performed using Lipofectamine 2000 (Invitrogen) following the manufacturer's protocol. The mouse *PLAGL1/Zac1* cDNA was transiently and stably expressed in LβT2 cells to assess effects of modulating *PLAGL1/Zac1* on *TP53* downstream targets. Selection of stably overexpressing PLAGL1, GADD45β, and the vector control in LβT2 cells was achieved under Geneticin selection (Invitrogen) at 600 mg/ml, yielding polyclonal stable cell lines.

Apoptosis assays

LβT2-*GADD45β* and LβT2-pcDNA3 stable cells were plated into six-well plates (1.5×10^6 cells per well). After overnight growing in complete medium, cells were serum deprived for 72 h and then harvested for immunoblot to detect the expression of active and total caspase 3.

Proliferation assays

Proliferation was assessed using the CellTiter 96 AQ_{ueous} One Solution cell proliferation assay, which uses the dye [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium] (MTS) (Promega, Madison, WI) to produce a formazon product that can be maximally detected by

spectrophotometer at 490 nm. Five thousand cells were plated in a 96-well plate (triplicate for each condition) in complete medium (DMEM plus 10% FBS) and incubated for 1, 3, 6, or 9 d after plating. CellTiter MTS solution (20 μl) was added to each well, plates were incubated for 1 h at 37 C, and absorbance at 490 nm was measured. Fold change calculations were made in triplicate using mean values from the first observed time point, which were compared with the triplicate mean values of each subsequent time point. A two-way ANOVA test with repeated measures was conducted to determine whether growth curves were significantly different.

Soft agar assay

To assay growth rates of the cells stably overexpressing *GADD45β* or vector control in soft agar, six-well plates were coated with 1.5 ml of a base agar consisting of 0.5% agar, 1× DMEM, and 10% FBS. Cells were trypsinized, counted using a hemocytometer, and then resuspended at low density (10,000 cells/1.5 ml) in 0.35% agar, 1× DMEM, and 10% FBS. After plating on the base agar, cells were grown for 3 wk in a humidified incubator at 37 C with 5% CO_2. Colonies were identified as two to five cells and more than five cells and counted in triplicate plates under ×100 using an Axiovert 200 Zeiss microscope.

GADD45β promoter methylation analysis

DNA from tumor or normal pituitary was extracted with the DNeasy blood and tissue kit (QIAGEN) followed by bisulfite conversion and desulfonation using the EpiTect kit (QIAGEN). The *GADD45β* promoter was amplified from converted DNA with the primers hGADD45B.Bisf.Fwd-700 (5′-GGTTTTTATTATTATT TGGGYGAGA-3′) and hGADD45B.Bisf.Rev-304 (5′-CCTC-CTATTAATAAAAAAACAAAAAC-3′) using the Failsafe PCR System (Epicenter, Madison, WI) and amplimers were purified with GeneJET PCR purification kit (Fermentas, Glen Burnie, MD). The amplified promoters were sequenced directly with the primer *hGADD45β*BisulfPromF1–635 (5′-TATTTTTAGTAGAATTT-GGGAAAGG-3′), allowing the detection of 19 CpG within a 146-bp promoter region. Positive control DNA, CpGenome Universal methylated DNA (Millipore), was also amplified and sequenced.

Results

Clinical characteristics of subjects and samples

Nine normal pituitary glands were obtained at autopsy within 2–18 h of death (clinical characteristics of normal samples are described in Table 1). The mean age of the four males and five females at time of death was 55.9 yr. Cause of death was not related to any known endocrine dysfunction. No significant differences existed in age between tumor and normal controls ($P = 0.39$). Tumor samples from eight males and six females of mean age 61.4 yr with gonadotrope tumors were collected and prepared for microarray analysis as previously described (16). Patient and sample characteristics are described in Table 1. Histological analysis revealed that four samples were classified as null cell adenomas (less than 5% staining for ASU, FSH, or LH), and 10 were classified as gonadotrope tumors. Tumor size ranged from 1.0 × 1.3 cm up to 5.6 × 3.6 × 2.7 cm. Five of the patients had recurrent tumors, defined as regrowth from a previous resection detected by magnetic resonance imaging of the pituitary. Seven patients had tumors that were classified as invasive per radiology, operative, and/or pathology reports (four invasive of the cavernous sinus, one invasive of bone, one invasive of the third ventricle, and one invasive of the dura).

TABLE 1. Normal pituitary and tumor sample characteristics

ID	Age (yr)/sex	Cause of death (normal)/ histology (tumor)
N010	96/M	Old age
N018	56/F	Pulmonary capillary hemangiomatosis
N019	56/F	Hypertension
N022	44/F	Liver cirrhosis
N025	45/M	Pontine myelinolysis, anoxic injury
N027	36/F	Biatrial and biventricular dilatation/hypertrophy
N028	49/M	Cardiac disease
N029	60/M	Pneumonia
N036	61/F	Postoperative complications
T001	57/M	LH (20%)
T002	57/M	ASU, LH (>20%)
T003	63/M	LH (10%), ASU, FSH
T018	66/F	LH (50%)
T023	75/F	Null cell
T028	69/M	LH
T030	70/M	LH (10%)
T024	53/F	LH
T032	78/F	LH
T034	50/M	Null cell
T078	45/M	LH > FSH
T119	60/F	Null cell
T149	58/M	ASU, focal LH, FSH
T176	59/F	Null cell

Shown are age, sex, and cause of death of normal pituitary samples obtained at autopsy and age, sex, tumor size, and histology for each of the tumor samples used in the analysis. F, Female; M, male.

Microarray data analysis reveals a large number of differentially expressed transcripts

A one-way ANOVA model was used to generate *P* values for all present genes (38,932 of 54,675 transcripts probed for on the Affymetrix U133 2.0 array) using the two experimental groups: control pituitaries (n = 9) and gonadotrope tumors (n = 14). Partek Genomics Suite identified differentially expressed transcripts (after elimination of duplicated transcripts) with a false discovery rate of less than 0.05 to control for multiple testing. This analysis revealed 1911 unique genes expressed more than 2.0-fold differentially between tumor *vs.* control. Among these, 1121 genes were down-regulated in gonadotrope

tumors *vs.* normal pituitary, whereas 790 genes were up-regulated. Among the up-regulated genes, the mean fold change was 4.5, whereas the mean fold change among down-regulated genes was −32.2. The relatively large number of differentially expressed transcripts in gonadotrope tumor *vs.* normal pituitary is expected, because the normal pituitary contains multiple cell types (of which gonadotrope cells constitute 10–20%).

Analysis of gonadotrope tumors and normal pituitary genomic profiles

A PCA plot (13, 17) was constructed to examine whether the overall expression profiles differed between tumor and normal samples and whether there were obvious outliers within each subgroup (Fig. 1A). The PCA plot demonstrates clear separation of gene expression profiles between normal pituitary and gonadotrope tumors, with no overlap between the two groups. Tumor and normal pituitary samples demonstrated distinct patterns of gene expression. A clustering analysis demonstrated that tumors and normal pituitary are overall separate in their expression of the 1911 genes. However, after this initial stage of clustering, a single tumor (T176) demonstrated a somewhat greater similarity to normal pituitary than other tumors. This suggests that the tumor sample may have been contaminated with normal pituitary tissue.

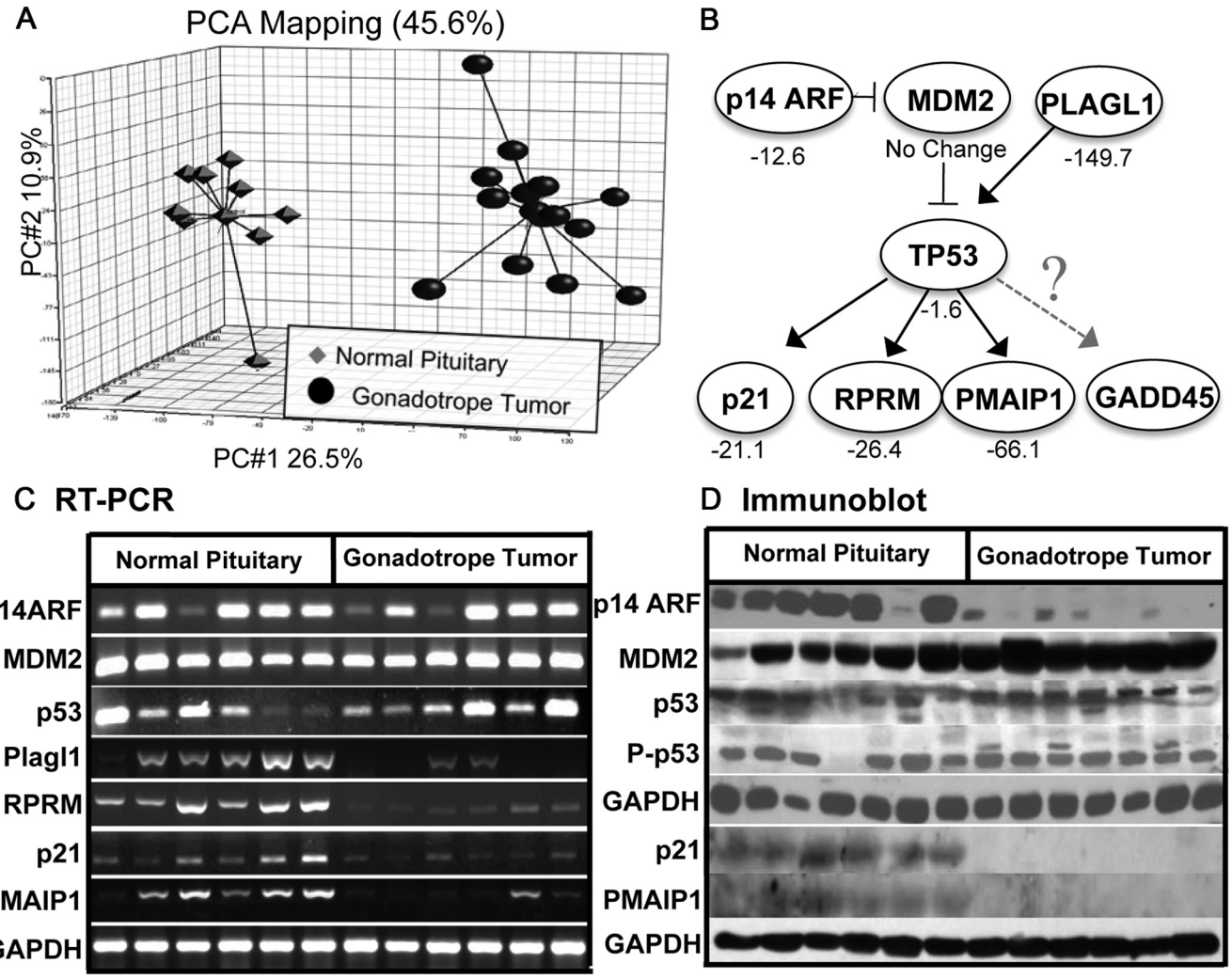

FIG. 1. *TP53* effectors that are significantly altered in gonadotrope pituitary tumors compared with normal pituitary. A, PCA of tumor compared with normal pituitaries. Control samples are shown in *diamonds*, and tumors are displayed in *spheres* (see *Materials and Methods* for details of analysis). B, IPA of TP53 signaling demonstrates the network cyclin-dependent kinase inhibitor 2A (P14 ARF), pleiomorphic adenoma gene-like 1 (PLAGL1/Zac1), murine double minute 2 (MDM2), p53 (tumor suppressor protein 53), cyclin-dependent kinase inhibitor 1A (P21), growth arrest and DNA-damage-inducible 45 (GADD45), Reprimo (RPRM), and phorbol-12-myristate-13-acetate-induced protein 1 (PMAIP1). C, mRNA protein levels of components of TP53 pathway in tumors compared with normal samples. Six normal pituitary and six gonadotrope tumors were compared for mRNA levels of P14/ARF, MDM2, p53, PLAGL1(Zac1), RPRM, p21, and PMAIP1 by semiquantitative RT-PCR with GAPDH used as an internal standard. D, Immunoblots of seven normal pituitary and seven gonadotrope tumors compared for protein levels of active phospho-p53 (53 kDa), total p53 (53 kDa), p14ARF (14 kDa), MDM2 (90 kDa), p21 (21 kDa), and PMAIP1 (15 kDa) with GAPDH (38 kDa) used as an internal control.

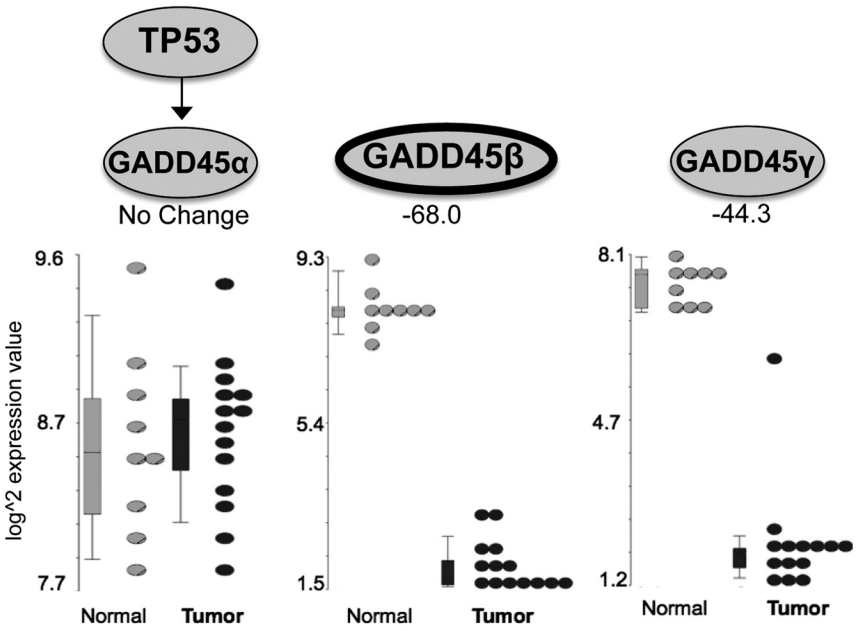

FIG. 2. GADD45 family members are differentially regulated in human gonadotrope pituitary tumors. IPA of GADD45 signaling shows that GADD45α, a downstream target of p53, is not altered in gonadotrope tumors in contrast to suppression of GADD45β and -γ. Transcript profiles of GADD45 family members in individual pituitary tumors compared with normal pituitary samples.

Downstream targets of p53 are suppressed in gonadotrope tumors

Previous studies have demonstrated that *TP53* is not typically mutated or abnormally expressed in human gonadotrope pituitary tumors (18). However, analysis of our microarray data using IPA showed that *TP53* was one of the most down-regulated pathways ($P = 0.03$) with widespread repression of putative *TP53* targets as well as dysregulation of proteins known to modify p53 activity (Fig. 1B). Upstream of *TP53*, *MDM2*'s activity as a inhibitor is potentially increased due to the 12.6-fold loss of its inhibitor *p14ARF/CDKN2A*. Furthermore, losses were apparent in *TP53 trans*-activator *PLAGL1(Zac1)* (−149.7-fold). Downstream of *TP53*, we observed a uniform down-regulation signature of targets related to cell cycle arrest and apoptosis: *Reprimo* (RPRM), −26.4-fold; *p21/CDKN1A*, −21.1-fold; and *PMAIP1*, −66.1-fold. Suppression of selected candidates in tumors was confirmed with semiquantitative RT-PCR (Fig. 1C). *P14ARF* expression by semiquantitative PCR did not concur with the down-regulation observed by array, but protein by immunoblot was consistent. *TP53* and *MDM2* mRNA levels were unchanged between normal pituitary and gonadotrope tumor samples as predicted. Consistent suppression of *p21*, *RPRM*, and *PMAIP mRNA* was confirmed. Immunoblots for each of the genes demonstrated protein loss in p14ARF, PLAGL1(Zac1), p21, RPRM, and PMAIP1, although showing no differences in mdm2 and p53 (Fig. 1D), demonstrating that the expression differences detected by our gene expression microarray were similar to the protein expression levels.

To test whether the altered *TP53* signature was related to down-regulation of the upstream modulator, *PLAGL1 (Zac1)*, a vector expressing the mouse *PLAGL1(Zac1)* cDNA was overexpressed in LβT2 gonadotrope cells, and the mRNA levels of *TP53*, *p14ARF*, *PMAIP*, and *p21* were measured. No alteration in mRNA levels were observed in the presence of reexpressed *PLAGL1(Zac1)* (data not shown), suggesting the putative downstream targets of *TP53* are modulated by other upstream regulators.

GADD45 family members are divergently expressed in human pituitary tumors

Previous studies identified three GADD45 isoforms that play cell-specific roles as stress responders modulating cell cycle and survival (6, 8, 9, 11, 12, 19). GADD45α has been identified as a downstream effector of p53 (11), and work by Klibanski and co-workers (10) has reported the loss of GADD45γ in pituitary tumors. Thus, we examined the potential role of GADD45β in pituitary tumors. We documented no significant changes in *GADD45α* transcript levels between tumor and normal samples (1.04, $P = 0.75$, Fig. 2) and confirmed the previous studies showing repression of *GADD45γ* in our analysis (−44.3-fold, $P = 8.8 \times 10^{-12}$, Fig. 2). *GADD45β* transcript was significantly and consistently repressed (−68-fold, $P = 1.10 \times 10^{-16}$, Fig. 2). Changes in mRNA and protein of GADD45 family members were assessed by quantitative RT-PCR (QPCR) and immunoblot, respectively (Fig. 3). QPCR analysis confirmed the lack of changes in *GADD45α* (1.5-fold, $P = 0.34$), suppression of *GADD45β* (−10.8, $P = 0.0016$), and the previously reported inhibition of *GADD45γ* (−138-fold, $P = 0.002$) (Fig. 3A). Immunoblot analysis of GADD45β demonstrated that its protein is normally present in the normal pituitary but is uniformly absent in gonadotrope tumors (Fig. 3B).

Overexpression of GADD45β alters gonadotrope cell proliferation and survival

Based upon these data, we focused on potential functional effects of diminished GADD45β levels in gonadotrope tumors. Because GADD45β is inherently low in go-

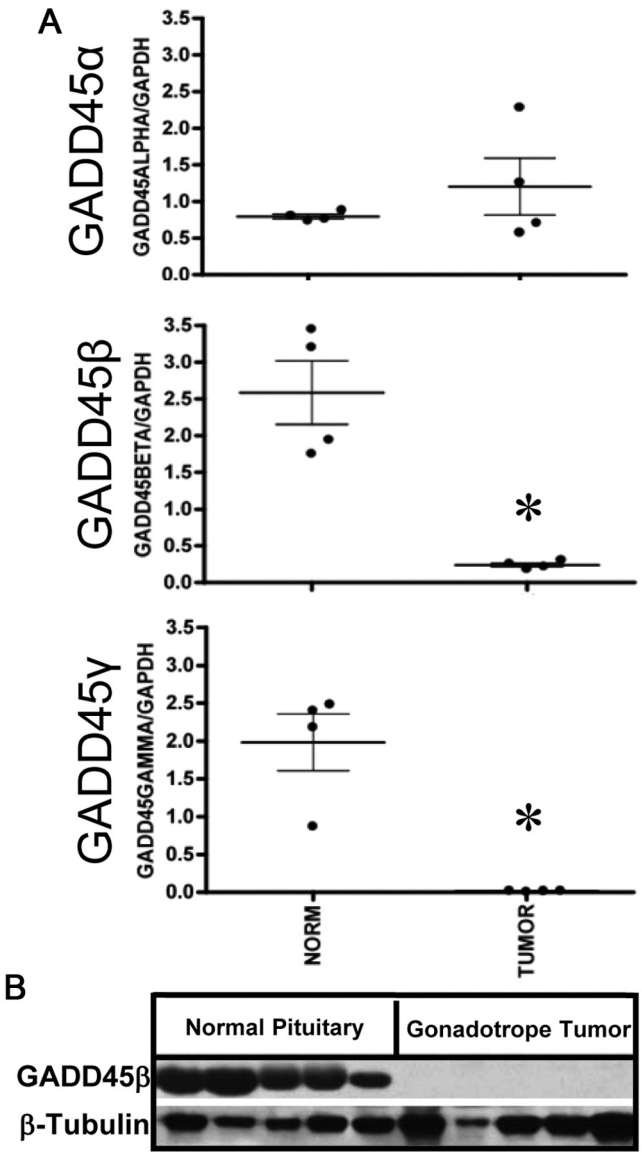

FIG. 3. GADD45β mRNA and protein are repressed in gonadotrope pituitary tumors. A, QPCR measurement of mRNA levels of *GADD45* family members in gonadotrope tumors and normal pituitary samples (*, $P = 0.002$); B, immunoblot of GADD45β (18 kDa) levels in normal pituitary and pituitary tumor samples with β-tubulin (50 kDa) used as an internal control. Repression of both GADD45β and GADD45γ was confirmed, with tumors expressing −10.8 and −138-fold less transcript than normal pituitary respectively; *, $P = 0.002$.

nadotrope tumors and immortalized mouse gonadotrope cell lines, it was stably overexpressed in mouse LβT2 gonadotrope cells (resulting in a 3.2-fold increase in mRNA and 14.4-fold increase in protein levels, Fig. 4A, *left and right panels*, respectively), and functional assays were performed.

To assess the effects of GADD45β on gonadotrope cell growth, vector control and GADD45β experimental cells were plated (n = 9 per group) at a density of 5000 cells per well and observed for rates of proliferation (Fig. 4B). Cells overexpressing GADD45β slowed their rate of growth significantly over a 9-d time course ($P = 0.02$), with a 20 and 30% inhibition of growth compared with vector controls at d 6 and 9, respectively.

To ask whether changes in GADD45β expression altered cell survival, LβT2 gonadotrope cells were stably transfected with vector control or the GADD45β construct and grown in serum-free media for 3 d. The levels of total and activated caspase 3 and poly ADP-ribose polymerase (PARP) as indices of increased programmed cell death were measured by immunoblot (Fig. 4C). Overexpression of GADD45β resulted in a 10.4-fold increase in cleaved caspase 3 and a 2.9-fold increase in cleaved PARP after growth factor withdrawal, which were 2.3-fold and 1.6-fold higher than vector controls, respectively. These studies suggest that whereas increased GADD45β decreases proliferation and increases programmed cell death, the repression of GADD45β in human pituitary tumors would allow for increased survival and cell growth during progression of tumorigenesis.

Induction of GADD45β represses colony formation in soft agar

To determine whether the low GADD45β levels in human gonadotrope tumors modulated tumorigenicity, LβT2 cells stably overexpressing GADD45β or vector controls were plated on soft agar for 22 d, and colonies were counted. Compared with vector controls, increased GADD45β resulted in a decrease in colony formation (two to five cell colonies, 189 ± 9 *vs.* 86 ± 2; more than five cell colonies, 401 ± 25 *vs.* 68 ± 9) or overall (590 ± 30 *vs.* 153 ± 7, a decrease of 74%) (Fig. 5). These data suggest that similar to GADD45γ, GADD45β acts as a tumor suppressor whose function is lost in pituitary tumorigenesis.

Inhibition of GADD45β in pituitary tumors is not due to increased promoter methylation

Because previous work suggested that *GADD45*γ is turned off in pituitary tumors due to increased CpG island methylation, we asked whether *GADD45*β was similarly regulated. Bisulfite sequencing of 19 CpG islands in a 146-bp region of the *GADD45*β promoter from 12 gonadotrope tumors and eight normal pituitaries demonstrated that no CpG were hypermethylated and the majority were unmethylated (Fig. 6). Partially methylated CpG were observed at one site in five normal samples and three tumors, two sites in one normal and two tumors, three sites in four tumors, and four sites in one tumor. Two tumors and two normal samples showed no methylated CpG. Control methylated DNA showed complete methylation at all sites. Consistent with these results, treatment of gonadotrope cells with 5-aza-2′-deoxycytidine resulted

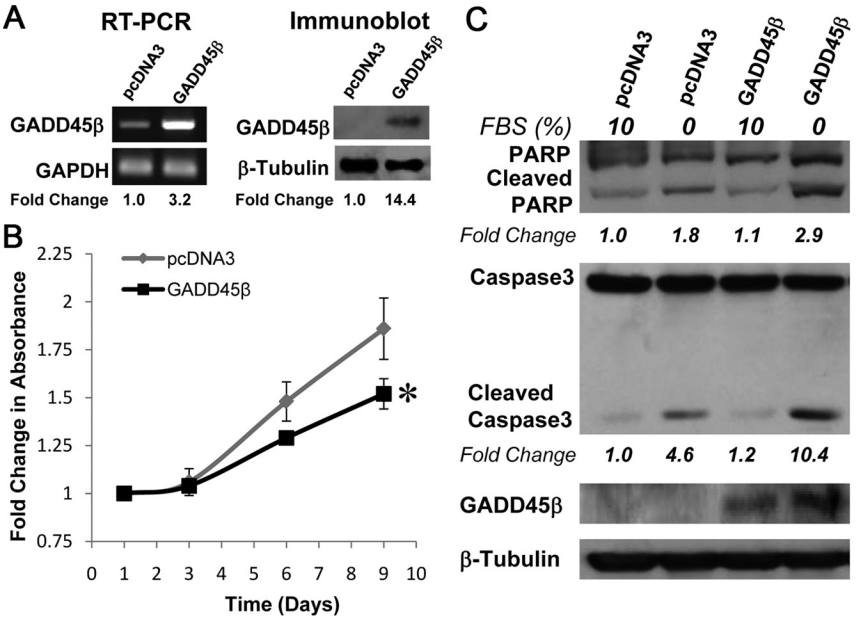

FIG. 4. Analysis of the functional effects of overexpression of GADD45β in gonadotrope cells. A, Semiquantitative RT-PCR and immunoblot confirm overexpression of GADD45β. B, GADD45β stable overexpression leads to decreased proliferation in LβT2 gonadotrope cells. GADD45β and vector control cells were grown in medium supplemented with 10% FBS and cell counts performed at 1, 3, 6, and 9 d. *, P = 0.02. C, Increased apoptosis as assessed by PARP activation and caspase 3 cleavage in gonadotrope cells overexpressing GADD45β. Transfectants were plated in serum-replete or serum-free conditions for 72 h, lysates were harvested, and immunoblots were performed for total (116 kDa) and active PARP (89 kDa) and cleaved (35 kDa) and total (19 kDa) caspase 3 as an index of cell death by apoptosis. β-Tubulin was used as an internal control.

in no overexpression of *GADD45β* mRNA (data not shown). Thus, in contrast to *GADD45γ*, the mechanism of suppression of *GADD45β* in gonadotrope pituitary tumors is not by promoter CpG island methylation.

Discussion

Using gene expression microarrays comparing 14 human gonadotrope tumor samples with nine normal pituitary gland samples, 1911 transcripts were identified that differentiate tumor and normal tissue with a clinically useful 2-fold cutoff. Semiquantitative PCR and immunoblot analysis confirmed down-regulation of multiple targets and pathways that modulate *TP53*. Acute reactivation of *PLAGL1 (Zac1)* in gonadotrope cells did not modulate expression of targets downstream of *p53*. Based upon previous studies of GADD45γ in pituitary tumors (10), and the absence of changes in the *TP53* target *GADD45α*, we chose to further investigate the functional significance of repression of *GADD45β* in pituitary tumors. Our data demonstrate that modulation of its expression alters pituitary cell growth, survival, and tumorigenesis. Unlike inhibition of *GADD45γ* in pituitary tumors, however, promoter CpG island methylation is not a mechanism of *GADD45β* repression.

Other investigators have performed gene expression microarray analysis of gonadotrope tumors. Some studies have compared carcinomas and adenomas (20), other subtypes of pituitary tumors (21–25), gonadotrope tumors and immortalized HP75 pituitary cells (26), and invasive gonadotrope tumors *vs.* noninvasive gonadotrope tumors (26, 27). Several groups (22, 23, 25) compared tumor and normal pituitary using the same DNA array chip as the present study (Affymetrix GeneChip HG U133 Plus 2.0), whereas Evans *et al.* (21) used the UniGEM v.1 microarray, and Moreno and co-workers (28) used HG-U95Av2 GeneChips for their analyses.

A metaanalysis of the four previous microarray studies of gonadotrope tumors and normal pituitary identified 67 genes related to cell cycle, proliferation, and cell growth that were significantly altered in three of the four studies (29). Bioinformatic analysis of our data concurs with 64 of these genes; however, NOTCH3, GPC4, and CITED1 were not differentially expressed between tumor and normal in our analysis. This metaanalysis precludes identification of transcripts not included on the UniGEM v.1 or the HG-U95Av2 microarrays, both of which survey only a fraction of the genes included on current chips. We identified a greater number of transcripts significantly altered in the same three functional networks of proliferation of cells, growth of cells, and cell division process of eukaryotic cells totaling 455 unique genes (see Supplemental Table 1, published on The Endocrine Society's Journals Online web site at http://endo.endojournals.org).

The microarray approach is particularly well suited for the study of pituitary tumorigenesis for a variety of reasons. Pituitary tumors are monoclonal in their nature (4), providing a relatively homogeneous population of cells for analysis. Furthermore, the microarray approach provides a robust exploratory method to examine the entire pituitary tumor transcriptome, which is known to differ from that of classic malignancies. Important to consider, however, is the lack of a pure population of normal gonadotropes for comparison. Our study compares a single monoclonal tumor type to the mixed population of five cell types in the normal pituitary of which gonadotropes only constitute 10–20%. Thus, the differential expression of many genes identified is related to cellular subtype

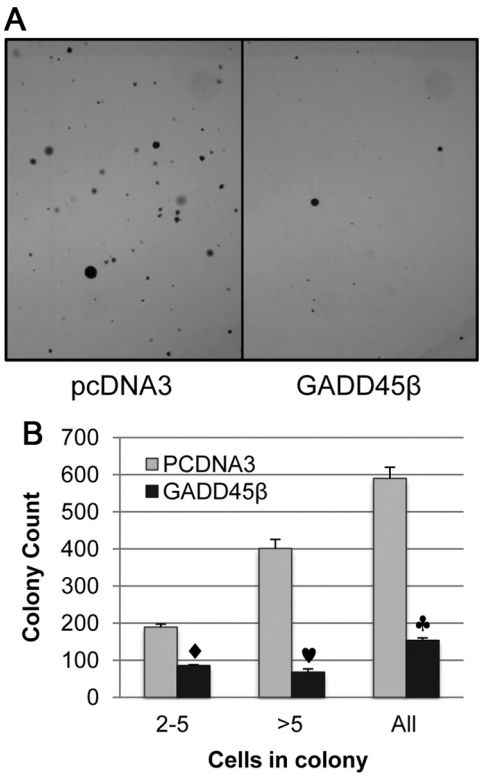

FIG. 5. Increased GADD45β represses colony formation in soft agar. Vector control and GADD45β-overexpressing LβT2 gonadotrope cells were plated in soft agar in media with supplemented 10% FBS. Colonies of two to five cells (♦, $P < 0.001$) and more than five cells (♥, $P < 0.0004$) or all (♣, $P < 0.007$) were counted at 22 d.

rather than tumor state. As described for *GADD45β* below, any candidate identified must be studied for differential expression in other tumor types and normal pituitary to confirm or refute its functional significance.

Bioinformatic analysis showed that among classical cancer pathways, the TP53 pathway was among the most differentially expressed in gonadotrope tumors compared with normal pituitary ($P = 0.03$). Although *TP53* mutations are the most frequently identified genetic aberration

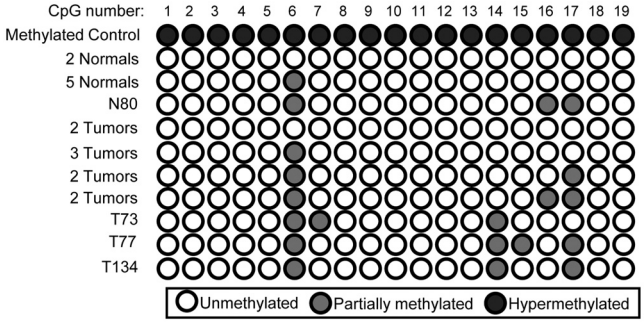

FIG. 6. Hypermethylation of the *GADD45 β* promoter is not observed in pituitary tumors. Bisulfite sequencing of the *GADD45β* CpG island region from 12 tumor and eight normal samples reveals that most CpG are unmethylated in the majority of samples. CpG are portrayed as *circles* and the methylation state is indicated by shading intensity: unmethylated CpG (*no shading*), partially methylated CpG (*light shading*), and complete methylation (*dark shading*).

in cancer, extensive research demonstrates that *TP53* mutations are not typically present in pituitary tumors (18). Immunohistochemical studies have yielded markedly varied results regarding the extent to which p53 protein is present in pituitary tumors and whether p53 levels significantly correlate to adenoma subtype or aggressiveness (30–36). No significant differences in *TP53* mRNA or protein levels were detected in our analysis of gonadotrope tumors. In addition, subcellular fractionation studies showed no alterations in compartmental localization of p53 (data not shown) to account for the consistent down-regulation of components downstream of p53.

Spengler and co-workers (37, 38) showed that *PLAGL1 (Zac1)* is repressed in pituitary tumors, consistent with our finding of a 150-fold decrease in gonadotrope tumors. These authors demonstrated that its loss leads to decreased apoptosis and increased cell proliferation (39). In our studies, reexpression of PLAGL1 in gonadotrope cells did not alter expression of p53 or the downstream effectors, suggesting the coincident suppression of these effectors is not via PLAGL1 modification of p53. The overall molecular signature, however, suggests that uniform loss of these downstream targets crucial to inducing apoptosis, cell cycle arrest, and limiting proliferation would allow for tumor initiation and/or progression.

GADD45β belongs to a family of stress sensor proteins that play roles in cell survival and proliferation but whose cell-specific effects have only recently been appreciated (12, 19). Our functional analysis of *GADD45β* demonstrates that its normal role in the pituitary includes acting as a brake to cell proliferation and survival. Previous work had shown that *GADD45γ* was lost in pituitary tumors and reexpression was tumorigenic, but further functional studies had not been explored (10). We showed no change in *GADD45α*, confirming isoform-specific actions in pituitary tumors. Loss of *GADD45β* was recently predicted in a metaanalysis of previous expression microarray datasets (40). Similarly, reanalysis of a published GEO dataset of single pooled cDNA arrays of different pituitary tumor types (22) predicts that losses in *GADD45β* is not specific to gonadotrope tumors but may be also observed to a lesser extent in prolactinomas and GH tumors. In contrast, ACTH tumors were noted to demonstrate a slight up-regulation of *GADD45β*, a consistent trend common among *TP53* targets in ACTH compared with gonadotrope tumors in our analyses (unpublished observations). We observed a 68.0-fold loss in *GADD45β* transcript that was confirmed at the RNA level with resultant low or absent protein expression. Stable overexpression of GADD45β led to a decreased proliferative capacity of gonadotrope cells, increased apoptosis,

and reduced tumorigenicity in a soft agar assay. As such, it can be inferred that the loss of *GADD45β* in human gonadotrope tumors may contribute to the initiation and/or progression of tumorigenesis.

GADD45 family members are variably repressed in human cancers. *GADD45α* is methylated in most tumors (6, 9). *GADD45β* but not -α or -γ is methylated by hepatitis virus,tk;1 in hepatocellular carcinomas (7), whereas *GADD45α* and -β are silenced by methylation in prostate cancer (41). Loss of *GADD45γ* expression in pituitary tumors was shown to correlate with methylation of its promoter, with 82% of samples with loss of ***GADD45γ*** demonstrating methylation of the gene's CpG island (42). In contrast, we observed low levels of methylation of the promoter in tumors and normal samples, discounting CpG island methylation as a mechanism for loss of *GADD45β* in gonadotrope tumors. In other systems, constitutive activation of NF-κB represses *GADD45α* and -γ but not -β as an alternative mechanism of suppression of GADD45 (8). In our dataset, however, NF-κB expression is not up-regulated in pituitary tumors (unpublished observations). Thus, other factors may modulate the pituitary tumor-specific repression of *GADD45β*.

In summary, our data implicate inhibition of many putative components upstream and downstream of p53 in human gonadotrope tumors but with no alteration in p53 mRNA or protein, suggesting an independent pathway. GADD45β is identified as an important tumor suppressor in pituitary tumorigenesis where its repression modulates cell proliferation, survival, and tumorigenicity. Additional studies are needed to understand the precise mechanisms by which it is down-regulated in human pituitary tumors and whether it may be a useful target for future medical therapies.

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