

Bone Morphogenetic Protein and Retinoic Acid-Inducible Neural Specific Protein-3 Is Expressed in Gonadotrope Cell Pituitary Adenomas and Induces Proliferation, Migration, and Invasion

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Pituitary tumors are common intracranial neoplasms that often result in endocrine dysfunction due to hormone overproduction or deficiencies from mass effects. Gonadotrope cell or gonadotropinomas are tumors that produce LH and/or FSH and represent 40% of macroadenomas. Little is known about their underlying pathogenic mechanisms. We compared expression profiles of 10 gonadotropinomas with nine normal pituitaries by cDNA array and identified bone morphogenetic protein-3 and retinoic acid-inducible neural-specific protein-3 (BRINP3) as overexpressed in tumors, compared with normals. BRINP3 is a novel, normally brain restricted protein of unknown function. BRINP3 mRNA was expressed selectively in gonadotropinomas. Subcellular localization studies

showed that BRINP3 was targeted to the mitochondria, but BRINP3 overexpression was unable to protect pituitary cells against programmed cell death induced by growth factor withdrawal. However, BRINP3 overexpression in pituitary gonadotrope cells promoted proliferation, migration, and invasion. A BRINP3 antibody was raised that demonstrated clustered expression of BRINP3 protein in gonadotropinomas and not in normal human pituitary samples. Thus, BRINP3 is a mitochondrially localized protein that is selectively up-regulated in human gonadotropinomas. Its actions to increase proliferation, migration, and invasion suggest it may play an important role in pituitary tumorigenesis. (*Endocrinology* 148: 967–975, 2007)

PITUITARY ADENOMAS ARE common, representing at least 10% of intracranial neoplasms (1, 2). These tumors are categorized according to the cells of origin, which include GH, prolactin, ACTH, and the nonfunctional pituitary tumors that have been shown to derive from pituitary gonadotropes and thus are termed gonadotropinomas (3). The gonadotrope tumors arise from 5–10% of the anterior pituitary cells that normally express the α -subunit, LH β , or FSH β . These tumors represent 40–50% of all macroadenomas (tumors > 1 cm) and present clinically in patients with visual field loss, headaches, hypogonadism, and hypopituitarism (4, 5). Currently there are no medical therapies for these tumors and surgery is the standard treatment (1, 2, 6). Recurrence is common and additional surgery or radiation is often required with the risk of subsequent complications.

Little is known about the molecular pathogenesis of pituitary tumors, and in particular, gonadotrope-specific fac-

tors. Initially, investigators assessed whether classic oncogenes or tumor suppressor genes were involved in the development of pituitary tumors. In contrast to other tumors, mutations in classic oncogenes or tumor suppressor genes such as Rb, Ras, and p53 are rarely seen in human pituitary tumors (2, 7).

Recently Evans *et al.* (8, 9) used a cDNA array approach with a chip containing 4500 transcripts and identified that the folate receptor is overexpressed in gonadotropinomas. They suggest this receptor may play a role in vitamin transport and facilitate tumor growth. Additionally, cDNA representational difference analysis was used to identify Gadd45 γ and MEG3A as absent in pituitary tumors but present in normal pituitary (10–12). Reexpression in cell systems diminished cell growth and proliferation, suggesting that these candidates are tumor suppressors. The lack of MEG3 gene expression in pituitary tumors is due to hypermethylation of the promoter region (12). A screen from a rat pituitary tumor library identified pituitary tumor transforming gene, a member of the securin family that plays a role in chromatin separation. Pituitary tumor transforming gene is overexpressed in multiple human pituitary tumor types, correlates with tumor aggressiveness, and its targeted overexpression causes pituitary tumorigenesis (13–16). Growth factors such as the truncated fibroblast growth factor receptor have also been shown to be expressed in pituitary tumors and cause tumorigenesis in a mouse model (17, 18).

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Abbreviations: BMP, Bone morphogenetic protein; BrdU, 5-bromo-2'-deoxyuridine; BRINP, BMP- and RA-inducible neural-specific protein; ER, endoplasmic reticulum; FBS, fetal bovine serum; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; GCOS, GeneChip operating software; GFP, green fluorescent protein; RA, retinoic acid; si, small interfering.

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To identify additional factors involved in gonadotrope cell pituitary tumor development, newer human cDNA chips were used to test the hypothesis that gonadotropinomas would have a unique genetic profile, compared with normal pituitary. Samples from 10 individual human pituitary tumors obtained at the time of surgery and nine pituitaries obtained at autopsy were compared. Bone morphogenetic protein (BMP)- and retinoic acid (RA)-inducible neural-specific protein (BRINP)-3 was identified as a candidate that was overexpressed in gonadotropinomas and was low or undetectable in normal pituitary.

BRINP3 belongs to a recently described family of genes normally restricted to the brain. Semiquantitative RT-PCR confirmed that BRINP3 mRNA was expressed in brain but not in other mouse or human tissues. No or low levels of BRINP3 were detected in adenomas secreting prolactinoma, GH, or ACTH but not human tumor cells of nonpituitary origin. BRINP3 mRNA was detected in immortalized mouse and human gonadotrope cells, α T3, L β T2, and HP75 cells, as well as neuronal cells, NLT and GT-7, but not GH4, and α TSH cells. Cloning of the BRINP3 cDNA from human pituitary tumors showed no evidence of mutations. Overexpression of BRINP3 promoted cell proliferation, migration, and invasion of gonadotrope cells. Subcellular localization studies suggested that BRINP3 is expressed in the mitochondria. Development of a BRINP3 antibody showed that BRINP3 protein was expressed in gonadotropinomas and not in normal pituitary. Thus, we have identified a novel, mitochondrially localized protein in gonadotropinomas whose overexpression leads to proliferation, migration, and invasion. These properties of BRINP3 may provide an advantage for tumor development and growth.

Materials and Methods

Gonadotrope tumor, normal pituitary, and tissue characterization

Ten pituitary gonadotrope tumors were obtained from patients at University of Colorado Hospital at the time of transphenoidal surgery after informed consent. Portions of the specimens not used for histology and immunohistochemistry were placed in RNAlater (QIAGEN, Valencia, CA). Gonadotropinomas were characterized by immunostaining for FSH, LH, and/or α -subunit. Nine normal pituitary glands used as controls were obtained at autopsy within 2–18 h of death from University of Colorado at Denver and Health Sciences Center (UCDHSC) Pathology Department. Human smooth muscle was also donated at time of autopsy. All mouse tissues were obtained from normal C57/BL6J mice.

Microarray analysis of gene profiles in gonadotropinomas and human pituitary

Total RNA was isolated from both tumor and normal specimens using TRIzol (Invitrogen, Carlsbad, CA) and purified using RNEasy (QIAGEN). cDNA and cRNA were made using 2–2.5 μ g of total RNA and the One-Cycle eukaryotic target labeling assay (Affymetrix, Valencia, CA); 15–20 μ g cRNA was used for labeling. To ensure intact RNA, both total RNA and cRNA integrity were verified using the Agilent 2100 bioanalyzer in the UCDHSC Cancer Center Array Core. The microarrays were performed using the Affymetrix Human Genome U133 Plus 2.0 chip containing 47,000 transcripts representing approximately 39,500 human genes. Analysis of results were performed using GeneChip operating software (GCOS) (Affymetrix) to compute the cell intensity from the image data, filter the data, and compare individual tumors to each normal pituitary. GeneSpring (Agilent Technologies, Santa Clara, CA)

software was also used for further statistical analysis, eliminating all genes with less than 2-fold difference between tumors and normal pituitaries as well as for pathway classifications. Pairwise comparisons between each tumor and each normal pituitary were performed using GCOS, and genes found to be differentially expressed at least 2-fold in 81 of 90 tumors were determined significant for further study.

Real-time and semiquantitative PCR

For semiquantitative RT-PCR, RNA (1.5 μ g) was reverse transcribed using Superscript II RNase H+RT (Invitrogen). RT-PCR was performed under the following conditions: 94 C for 3 min, 92 C for 45 sec, 55 C for 45 sec, and 72 C for 1 min for 35 cycles and 72 C for 7 min. The primer sequences used to amplify human and mouse BRINP3 were: 5'-CAACTGGAGAACAGCATGAAA-3', 3'-TAAGCCAGTAGGTTGAGGTTTC-5' and 3'-TCCTCAGGTAGATGTGTACTG-5'. PCRs were performed in the GeneAmp PCR system 9700 (Applied Biosystems, Foster City, CA). The quantity of BRINP3 mRNA was normalized to that of GAPDH in the semiquantitative PCR using glyceraldehyde-3-phosphate dehydrogenase (GAPDH) primers 5'-CGGAGTCAACGGATTTGGTCGTAT-3' and 3'-AGCCTTCTCCATGGTGGTGAAGAC-5'. Real-time PCR was performed using BRINP3 primers previously described and human GAPDH primers (Applied Biosystems) under these conditions: 50 C for 2 min, 95 C for 10 min, and 95 C for 15 sec for 40 cycles and 60 C for 1 min. Amplification was performed using an ABI 7700 sequence detector (PerkinElmer Corp./Applied Biosystems) and the TaqMan probe (PerkinElmer) 5-labeled with 6-carboxyfluorescein and 3'-labeled with 6-carboxy-tetramethylrhodamine. After amplification, real-time data acquisition and analysis were performed. The fluorescence data were expressed as Rn (normalized reporter signal), calculated by dividing the amount of reporter signal by the amount of passive reference signal, and the detection threshold was set above the mean baseline fluorescence determined from the first 15 cycles. Amplification reactions in which the fluorescence intensity increased above the threshold were defined as a positive reaction. A standard curve was generated using the fluorescent data from serial dilutions of BRINP3 RNA, and this was used to calculate the relative amounts of BRINP3 in the samples. Quantities of BRINP3 were normalized to the corresponding GAPDH RNA.

BRINP3 constructs, small interfering (si) RNA, and antibody

Human BRINP3 was PCR amplified from several gonadotropinomas into N-terminal flag-tagged pcDNA3 vector (Invitrogen) via 5' *Kpn*I and 3' *Not*I sites and the sequence verified as wild type by comparison with GenBank. BRINP3 siRNA was created using the oligonucleotides, 5'-AAAACCTGCAAGGTTATTTACTCCTGTCTC-3' and 3'-AAAGTA-AATAACCTTGCAAGGTTATTTACTCCTGTCTC-3', and constructed using the Silencer siRNA construction kit (Ambion, Austin, TX) according to the manufacturer's instructions. Human, murine, and rat BRINP3 proteins are highly conserved (>98% identical) at the amino acid level, and a BRINP3 antibody was generated against a sequence that is identical between species (N-EYTFVDRSRQGF-STR-C) (Affinity Bioreagents Golden, CO).

Cell culture and transfections

Pituitary cell lines, α T3, L β T2, and α TSH cells were from P. Mellon (University of California, San Diego, San Diego, CA), HP75 cells were from R. Lloyd (Mayo Clinic, Rochester, MN), and GH4 cells were from A. Gutierrez-Hartmann (UCDHSC). SaOS2 cells were from American Type Culture Collection (Manassas, VA), and HEp3 were from James Quigley (Scripps (La Jolla, CA). Cells were cultured in DMEM supplemented with 10% fetal bovine serum (FBS). Transfections were performed using Lipofectamine (Invitrogen) following the manufacturer's protocol. Selection of stably expressing BRINP3 in α T3 and L β T2 cells was achieved using geneticin (Invitrogen) at 600 μ g/ml. Gene-silencing studies were performed by electroporating 10 nM siRNAs into α T3 vector control cells using a gene pulser (Bio-Rad).

BMP and RA assay

α T3 cells were incubated with BMP2 (10 ng/ml) and/or RA (100 μ M) (Sigma, St. Louis, MO) for 4 d. RNA was extracted and RT-PCR performed using BRINP3 and GAPDH primers as outlined above.

Apoptosis assays

3–5 $\times 10^6$ α T3 cells (stably overexpressing vector or BRINP3) were serum deprived for 24 or 48 h and then treated with dual-sensor Mitocasp (Cell Technology, Inc., Mountain View, CA) using the manufacturer's protocol. Cells were analyzed by flow cytometry at the University of Colorado Cancer Center Flow Cytometry Core on a FACSCalibur (Becton Dickinson, San Jose, CA) or an FC500 cytometer (Beckman Coulter, Hialeah, FL) using CXP analysis software (Beckman).

Proliferation assays

Proliferation was assessed using both direct cell counts and 5-bromo-2'-deoxyuridine (BrdU) incorporation. Briefly, 10,000 cells (stably overexpressing vector control or BRINP3) were plated in a 96-well plate in complete medium (DMEM + 10% FBS). Wells were trypsinized (in duplicate) on d 1, 3, 5, and 7 after plating and viable cells counted by trypan blue exclusion. BrdU incorporation was assessed using the BrdU cell proliferation ELISA (Roche Applied Science, Indianapolis, IN) using the manufacturer's protocol.

Migration and invasion assays

Migration was assessed in a modified transwell chamber as previously described (20). α T3 cells stably overexpressing BRINP3, vector control, with or without BRINP3 siRNA, were incubated in serum reduced (0.2%) medium for 6 h before the experiment. Cells (5×10^4) per 0.1 ml were added to the upper chamber, and medium (1 ml) containing 0.2% or 10% FBS served as a stimulus. Dishes were incubated 16–18 h at 37 C. Inserts were removed, rinsed with PBS, and stained with Difquick (Dade Behring, Newark, DE) staining solution. Adherent but nonmigrated cells were removed from the insert and migrated cells were directly counted under $\times 20$ on an inverted microscope (Zeiss, New York, NY). Invasion assays were performed similarly, using inserts coated in Matrigel (CLONTECH, Mountain View, CA) following the manufacturer's protocol.

Immunocytochemistry and Immunohistochemistry

α T3 cells ($1.5\text{--}2 \times 10^5$) expressing endogenous BRINP3 were incubated with an anti-BRINP3 antibody and fluorescein isothiocyanate antirabbit IgG antibody (Jackson ImmunoResearch, West Grove, PA) for visualization, or transiently transfected with a construct containing the rat BRINP3 cDNA fused to green fluorescent protein (BRINP3-GFP) (21). These cells were stained alone or with pDsRed1-Mito Vector (CLONTECH) to label the mitochondria. Cells transfected with rBRINP3-GFP were stained with ER tracker (Molecular Probes, Eugene, OR) to label the endoplasmic reticulum (ER). BRINP3 and cell organelles were visualized by fluorescence deconvolution microscopy using an Axioplan 2 EPI fluorescence microscope (Carl Zeiss), and images digitally captured using Slidebook software (Olympus, Center Valley, PA). Cell fractionations were done using NE PER and mitochondrial isolation kits (Pierce, Rockford, IL). Cox IV and lamin A/C (Cell Signaling, Beverly, MA) were used in Western blots to confirm subcellular fractionation. For immunohistochemistry, 4- μ m tissue sections on Superfrost slides were deparaffinized in xylene and rehydrated with four changes of absolute ethanol (2 min each), followed by four changes of 95% ethanol (2 min each), and deionized water for 2 min. Slides were processed on a Ventana automated system, using antigen retrieval by pronase digestion, and blocking before the addition of primary (BRINP3) antibody. The BRINP3 antibody was added manually at a 1:200 dilution. Slides were counterstained with hematoxylin and eosin.

Statistics

Data were analyzed by unpaired Student's *t* test and ANOVA with Bonferroni's multiple comparison test using GraphPad Prism software

(GraphPad, San Diego, CA). Values are expressed as means \pm SE. Values of *P* < 0.05 were considered statistically significant.

Results

BRINP3 is overexpressed in human gonadotropinomas

Microarray analysis was used to identify genes differentially expressed in 10 human gonadotrope tumors *vs.* nine normal pituitaries. Tumors were selected from six males and four females, classified immunohistochemically and stained variably for α -subunit, FSH, or LH. Normal pituitaries used as controls were from four males and five females aged 44–84 yr. Analysis using GeneSpring software to detect fold changes and GCOS software to evaluate differences in individual tumors *vs.* normal by pairwise comparison (81 of 90 to 90 of 90) was performed. Genes with significant 2-fold changes in at least 81 of 90 pairwise comparisons were considered relevant genes to study. Using these standards, 120 genes were detected as up-regulated and 206 genes down-regulated in the tumors, compared with normal pituitary (Fig. 1A), and their expression profiles are currently being verified by RT-PCR. Genes differentially expressed between gonadotropinomas and normal pituitaries fell within all gene categories listed in the Affymetrix program (Fig. 1B). Although many genes were up- or down-regulated, BRINP3 was unique in being low or undetectable in normal pituitaries but highly overexpressed (584-fold) in nine of 10 gonadotrope tumors

To ask whether the array data were robust, the expression profiles of genes previously shown to be abnormally expressed in gonadotrope cell pituitary tumors were determined. As previously reported (9–11), the folate receptor was up-regulated in gonadotropinomas (25-fold). In contrast,

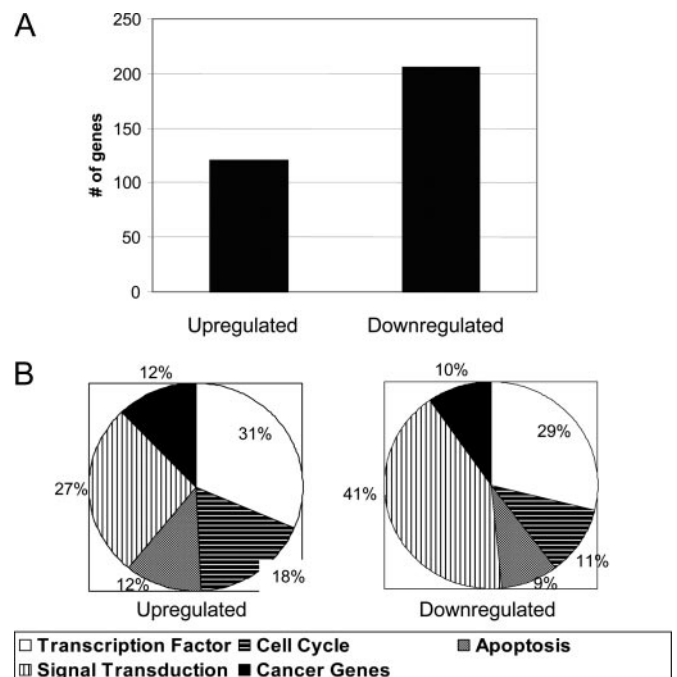


FIG. 1. A, Bar graph displaying the number of genes up- or down-regulated in tumor *vs.* normal pituitary. B, Chart illustrating the classification of genes differentially regulated in tumor versus normal pituitary.

MEG3A and GADD45 γ were down-regulated 10- and 35-fold, respectively.

The differential expression of BRINP3 by cDNA array was confirmed by semiquantitative RT-PCR on nine of the tumors and six normal pituitaries used in the array (Fig. 2A). All but one of the gonadotropinomas expressed high levels of BRINP3, compared with the low or undetectable levels in control pituitaries. In addition, real-time RT-PCR on five of the tumors and six normal pituitaries confirmed this differential expression pattern (Fig. 2B). An additional 11 human gonadotropinomas were analyzed by RT-PCR, and nine of the 11 were positive for BRINP3 mRNA expression (data not shown).

BRINP3 is not mutated in gonadotropinomas

Because prior work in fibroblast cells suggested the BRINP proteins would inhibit rather than stimulate cell proliferation (22), we cloned the BRINP3 cDNA from several human gonadotropinomas to ensure that there were no mutations that would alter its functional role. The BRINP3 cDNA was not mutated in the human tumors (data not shown).

BRINP3 expression is higher in gonadotropinomas compared with other pituitary tumors

To investigate whether increased BRINP3 expression was characteristic of all pituitary tumors, RNA was isolated from a prolactinoma, a GH and an ACTH tumor. Semiquantitative PCR was performed as above (Fig. 3A). The gonadotropinoma expressed a strong BRINP3 mRNA signal, and a low-level signal was detected in the ACTH but not the GH tumor. Further studies are needed to further define the selectivity of BRINP3 expression in different pituitary tumor cell types. To begin to explore BRINP3 expression in other tumor settings, BRINP3 was analyzed in two nonpituitary cancer lines. HEP3, a human epidermoid carcinoma, and SaOS2, a human

osteosarcoma line, were both negative for BRINP3 mRNA, again suggesting that BRINP3 may be a pituitary-specific tumor marker,

BRINP3 mRNA expression is brain specific

The expression profile of BRINP3 mRNA in various normal tissues was then examined (Fig. 3B). Consistent with the initial report by Matsuoka and colleagues (21), high levels of BRINP3 mRNA were detected in mouse total brain. However, no detectable BRINP3 was observed in mouse liver or lung or human smooth muscle. These data confirm that BRINP3 is normally restricted to the brain.

BRINP3 is expressed in immortalized gonadotrope cell lines

BRINP3 mRNA expression levels were analyzed in immortalized cell lines of pituitary origin (Fig. 3C). BRINP3 was detected in HP75 cells derived from a SV₄₀ immortalized human pituitary adenoma (23, 24), as well as in the mouse gonadotrope cells, α T3-1 and L β T2 cells (25), but not the thyrotrope-derived [α TSH (26)] or GH (GH4) cells. It was detected at a low level in two mouse neuronal cell lines, NLT and GT, consistent with its previous detection in mouse total brain. To investigate the functional role of BRINP3, we used α T3 and L β T2 cells as our model system, contrasting cells expressing BRINP3 at low endogenous levels with those stably overexpressing BRINP3.

BRINP3 is down-regulated by BMP and RA in α T3 cells

BRINP3 belongs to a recently described brain restricted family (BRINP1, BRINP2, and BRINP3) (21). In contrast to BRINP3, BRINP1, the first family member to be characterized, was expressed at very low levels, higher in normal pituitary, compared with tumor (raw numbers = 107 and 56,

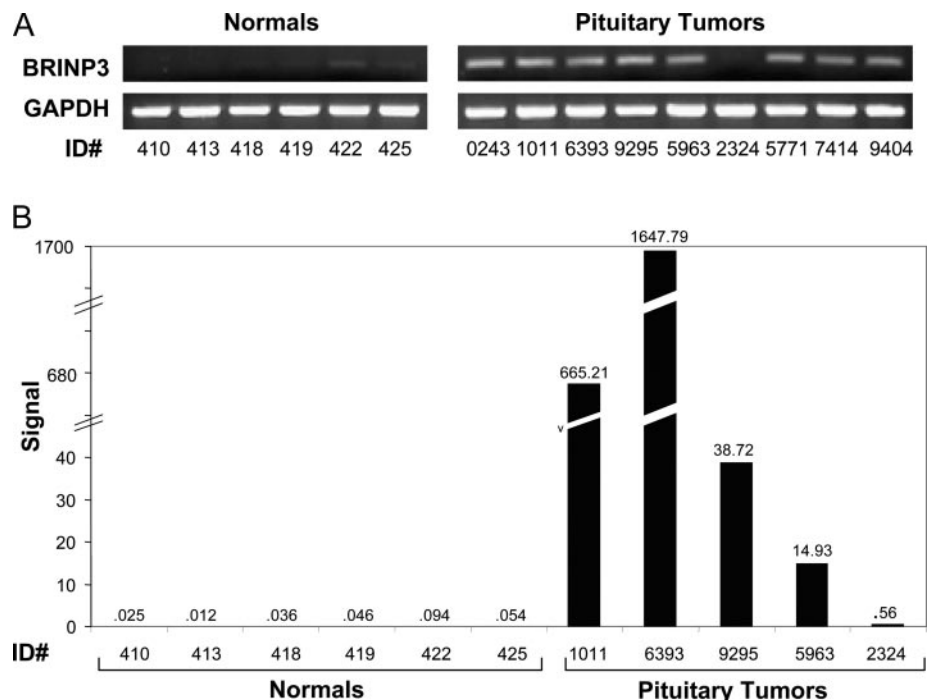


FIG. 2. RT-PCR analysis of BRINP3 expression in normal human pituitaries and gonadotropinomas. A, RT-PCR of six normal and nine tumors showing BRINP3 and GAPDH products. Gel is representative of two independent experiments. B, Signal intensity of real-time RT-PCR products for six normal pituitaries and five tumors, standardized to GAPDH levels. Normalized signal values are shown.

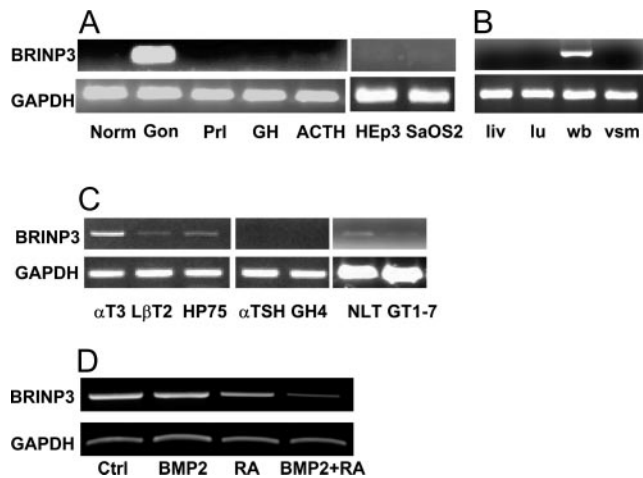


FIG. 3. Semiquantitative RT-PCR analysis of BRINP3 in pituitary tumors, tissues, and cell lines. A, BRINP3 expression patterns in representative normal pituitary (norm), gonadotropinoma (Gon), prolactinoma (Pr1), GH, and ACTH tumor. B, BRINP3 expression in murine liver (liv), lung (lu), whole brain (wb), and human smooth vascular muscle (vsm). C, BRINP3 expression in α T3, L β T2, HP75 gonadotrope cell lines; α TSH thyrotrope-derived and GH4 GH pituitary cell lines; Hep3 and SaOS cancer cell lines; and NLT, GT1–7 neuronal cell lines. D, Effects of BMP2 and RA on BRINP3 mRNA levels. Cells were treated for 4 d with BMP2 (10 ng/ml), RA (100 μ M), or the combination. Total RNA was isolated and RT-PCR performed using BRINP3 primers, with GAPDH as the control (Ctrl). Gel is representative of three independent experiments.

respectively). Probe sets corresponding to BRINP2 are not found on the human Affymetrix Chip. Because BRINP1 was initially identified as a gene up-regulated by BMP2 and RA, we tested the effects of these growth factors on BRINP3 regulation in gonadotrope cells. α T3 cells were incubated with BMP2, RA, or both for 4 d. In contrast to the up-regulation of BRINP1 mRNA by BMP and RA previously observed in sympathetic ganglion cells (21), this combination repressed BRINP3 mRNA levels in gonadotrope cells (Fig. 3D). There was little effect of BMP2 alone and RA alone repressed BRINP3 to a lesser degree than the combination. These data support that BRINP3 is not activated by BMP/RA in pituitary cells.

BRINP3 localizes to the mitochondria

BRINP3 is a novel protein without homology to other known proteins (21). To gain insight into its function in gonadotropinomas, the subcellular localization of BRINP3 was assessed. A BRINP3-GFP fusion construct was expressed in α T3 cells, alone or with reagents that target specific cellular compartments. pDsRed1-Mito vector labeled the mitochondria and ER tracker identified the ER. Immunofluorescence revealed that in pituitary gonadotrope cells, BRINP3-GFP colocalized with the mitochondrial marker (Fig. 4A, panel 3) and not the ER marker (panel 6). Mitochondrial expression of endogenous BRINP3 was also detected using a newly developed BRINP3 antibody (Fig. 4B).

Because prior work had suggested BRINP1 was localized to the ER in fibroblast cells, we also tested the localization of BRINP3 when overexpressed in GH4 pituitary cells to determine whether the localization pattern was gonadotrope

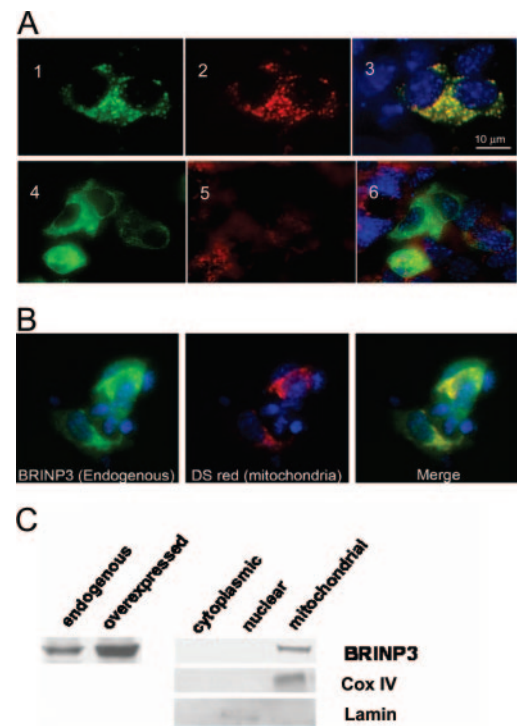


FIG. 4. BRINP3 protein expression and subcellular localization in α T3 cells. A, α T3 cells transiently transfected with rat BRINP3-GFP (panels 1 and 4), pDs-Red1-Mito to detect mitochondria (panel 2), or stained with ER tracker to detect endoplasmic reticulum (panel 5). The BRINP3-GFP image merged with pDs-Red1-Mito shows mitochondrial colocalization (panel 3) in which BRINP3 image merged with ER tracker does not show colocalization (panel 6). B, Localization of endogenous BRINP3 detected in α T3 cells stained with anti-BRINP3, pDs-Red1-Mito, and colocalization shown by the merged panels. C, Western blot representing endogenous and stably overexpressed BRINP3 in α T3 cells. Subcellular fractionation evaluating endogenous BRINP3 protein localization in soluble (cytoplasmic), nuclear, and mitochondrial fraction. Lamin A/C used as a control for the nuclear fraction and Cox IV used as a control for the mitochondrial fraction. Experiments were performed at least three times.

specific. Similar to effects in gonadotrope cells, BRINP3 was targeted to the mitochondria in GH4 cells (data not shown), suggesting that this localization is characteristic of pituitary cells.

To confirm that endogenous BRINP3 was localized to the mitochondria, α T3 cells were fractionated into nuclear, cytosolic, and mitochondrial subcellular fractions, and BRINP3 was detected by immunoblot using an anti-BRINP3-specific antibody to detect endogenous protein (Fig. 4C). The fractions were also immunoblotted using anti-cox IV (mitochondrial) and anti-lamin A/C (nuclear) antibodies to detect the adequacy of subcellular fractionation. This biochemical analysis confirmed that the endogenous BRINP3 protein is localized to the mitochondria in gonadotrope cells.

BRINP3 expression does not protect cells against programmed cell death

Because of its mitochondrial localization, we investigated the role of BRINP3 overexpression in mediating sensitivity to apoptosis triggered by growth factor withdrawal. Cells stably expressing control vector or BRINP3 were incubated in

the presence or absence of serum for 24 h (Fig. 5A) or 48 h (Fig. 5B). Activated caspases (specifically caspase-1, -3, -7, -8, -9, and -10) were detected by flow cytometry using MitoCasp detection reagent (Cell Technology), which detects these multiple activated caspases with similar affinity. Apoptosis was triggered at 24 and 48 h of serum deprivation in both cell types, shown by an increase in active caspases. At 24 h there was a slight but nonsignificant increase in the amount of active caspases seen from BRINP3 overexpressing cells, compared with control cells (Fig. 5A), with no differences observed at 48 h (Fig. 5B). Thus, BRINP3 expression in pituitary cells does not protect from programmed cell death caused by growth factor withdrawal.

BRINP3 overexpression increases proliferation of α T3 cells

Although BRINP3 had no protective effect on susceptibility to apoptosis, its overexpression promoted increased proliferation of gonadotrope cells. When α T3 cells stably overexpressing BRINP3 were compared with vector controls in proliferation assays, both groups grew similarly over the first 3 d in culture. However, by d 5, the growth rate plateaued in the controls, whereas the BRINP3 overexpressors contin-

ued to proliferate, increasing 2-fold, compared with controls by 7 d (Fig. 6). This pattern was observed by direct cell count as well as by BrdU incorporation (2.2-fold, data not shown). These data suggest a role for BRINP3 in proliferation under growth-restricted conditions.

BRINP3 overexpression increases migration of gonadotrope cells during trophic factor withdrawal

We next asked whether BRINP3 modulated the migratory ability of pituitary gonadotrope cells using a modified transwell chamber. α T3 cells stably overexpressing BRINP3 migrated similarly toward serum replete medium, compared with vector controls. However, in contrast to control cells that did not migrate toward low (0.2%) serum, BRINP3-expressing α T3 cells migrated robustly (7.8-fold) (Fig. 7A). Similar results were seen with L β T2 cells stably overexpressing BRINP3 (data not shown). These data suggest that BRINP3 allows migration in a growth-restricted environment. Additional studies were then performed to knock down endogenous BRINP3 and assess its functional effect. Silencing of endogenous BRINP3 using BRINP3-specific siRNA significantly blocked vector control cell migration under stimulatory conditions (10% serum containing medium), confirming that BRINP3 is necessary and sufficient for pituitary cell migration (Fig. 7B).

BRINP3 overexpression promotes invasion of gonadotrope cells

To further explore potential actions of BRINP3, invasion assays were performed. BRINP3-overexpressing α T3 cells invaded through matrigel more efficiently than vector controls (Fig. 7C). This invasive phenotype was observed in both growth factor replete (6.7-fold) and deficient (9-fold) conditions. Together these data suggest that BRINP3 allows for proliferation, migration and invasion, especially in trophic factor restricted environments, all features that would allow for increased growth of a pituitary tumor *in vivo*.

BRINP3 protein is expressed in gonadotropinomas but not in normal pituitary

To confirm that BRINP3 protein was differentially expressed *in vivo*, a BRINP3 antibody was raised to sequences homologous between the mouse and human protein and

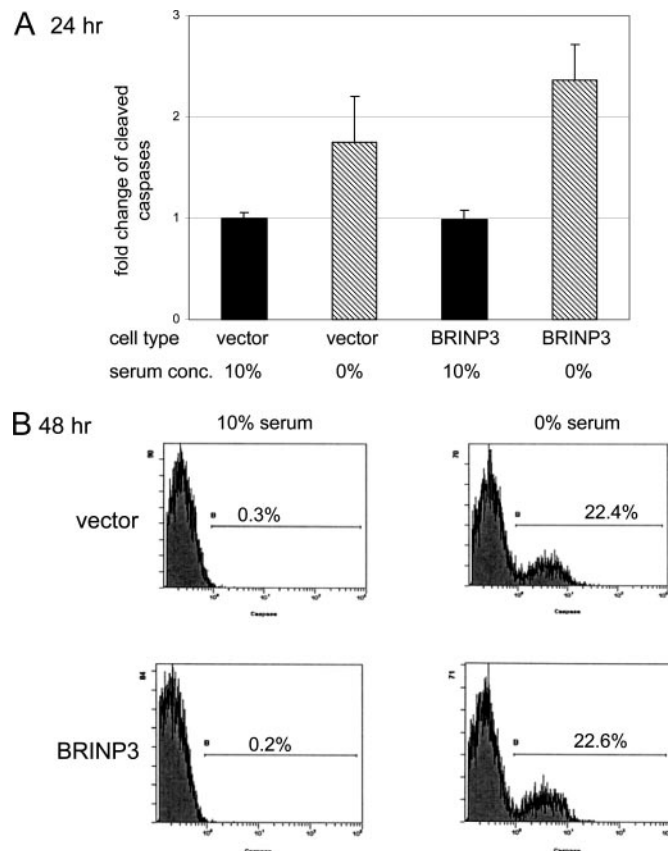


FIG. 5. BRINP3 does not protect against programmed cell death. A, α T3 cells were serum deprived 24 h (A) or 48 h (B). Cells were stained with MemCasp and activated caspase levels were analyzed by flow cytometry. Fold increases were calculated based on vector control cells in the presence of serum replete medium, standardized to 1. Data in A are the mean of four experiments, with fold changes calculated based on vector cells in 10% FBS. Histogram in B represents one of two experiments.

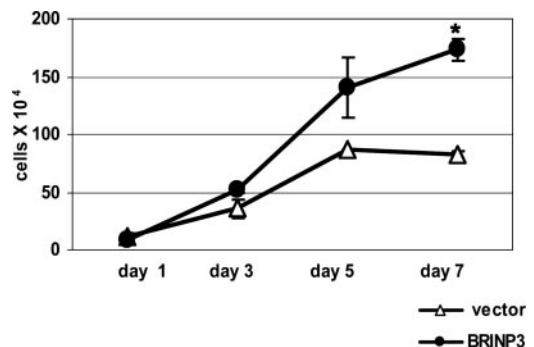
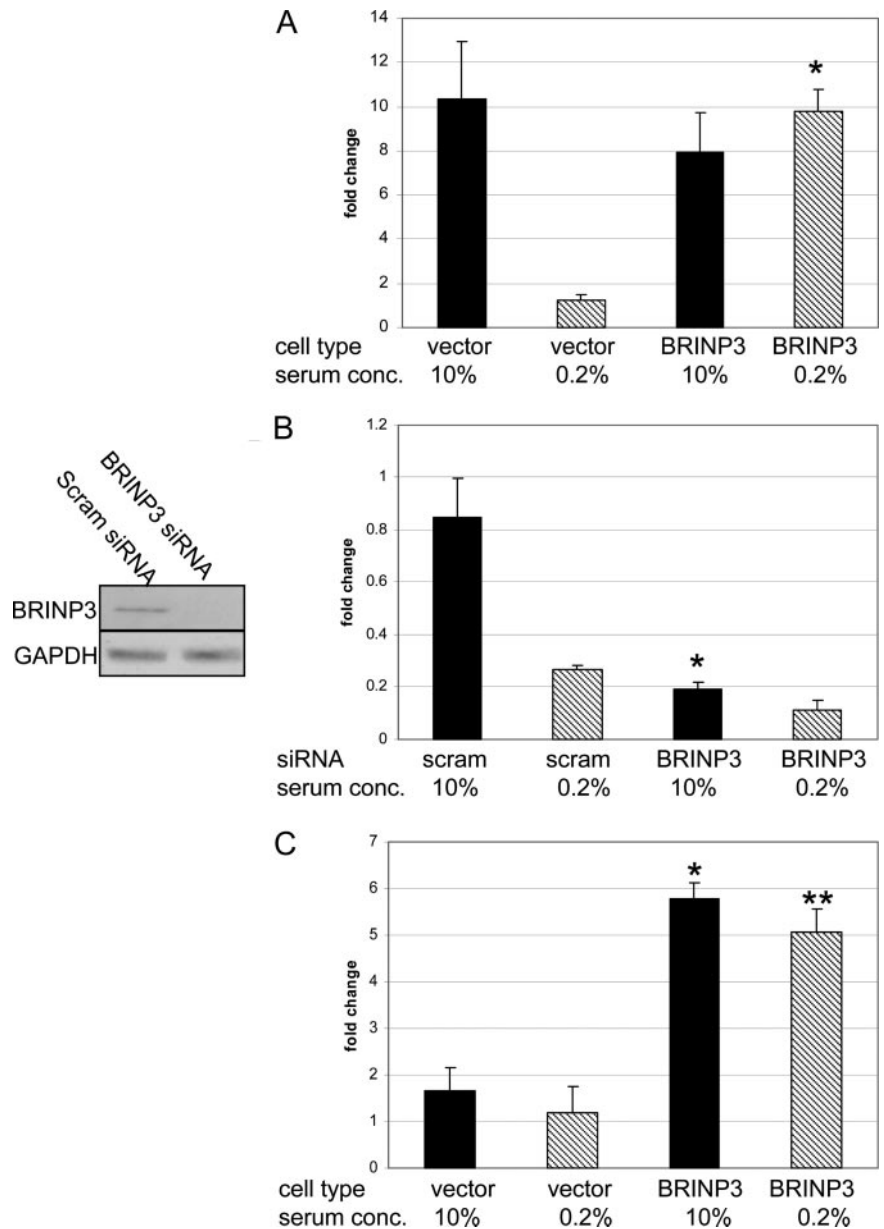


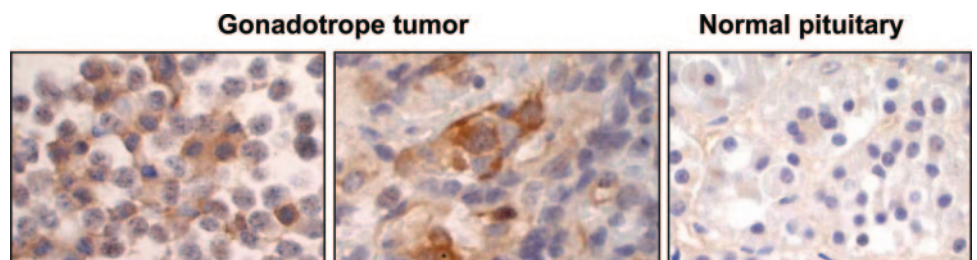
FIG. 6. Cell proliferation was analyzed for 7 d by direct cell count using trypan blue. *, $P = 0.0061$. Data represent one of four experiments.

FIG. 7. BRINP3 overexpression promotes migration and invasion of α T3 cells. A, Cell migration was analyzed in a modified Boyden chamber. Cells were incubated in DMEM + 0.2% FBS for 6 h and stimulated with 0.2% FBS or 10% FBS overnight. Fold changes are calculated based on migration of vector cells toward 0.2% serum in one experiment, which is set at 1. (*, $P < 0.05$ between vector and BRINP3 groups exposed to 0.2% serum, $n = 3$ experiments). B, Vector control α T3 cells were transfected with a nonspecific (scram) siRNA- or BRINP3-specific siRNA before performing the migration assay. Fold changes are based on vector cells transfected with scram siRNA migrating toward 10% serum (*, $P < 0.01$ between scram and BRINP3 siRNA exposed to 10% serum, $n = 3$ experiments). C, Invasion was evaluated in matrigel-coated chambers in response to 10 or 0.2% FBS. Fold changes are based on invasion of vector cells toward 0.2% serum (*, $P < 0.05$ between vector and BRINP3 groups in serum replete medium; **, $P < 0.01$ between vector and BRINP3 groups in low serum, $n = 3$ experiments).



used to stain sections from human gonadotropinomas and normal pituitary (Fig. 8). Gonadotropinomas demonstrated variable clusters of intense and weak staining of BRINP3 that was not observed in normal pituitary. Preliminary analysis did not suggest that BRINP3 protein is selectively expressed in areas that stain with the specific gonadotropin antisera (data not shown).

FIG. 8. BRINP3 protein is expressed in gonadotrope tumors but not normal pituitary. Immunohistochemistry was performed on a representative gonadotrope tumor (left and middle) and a normal pituitary (right) using the BRINP3 antibody. Staining was detected in intense clusters in some areas and weaker staining in others but not in the normal pituitary tissue.



Discussion

We used cDNA array technology to compare the gene profiles in individual human gonadotrope tumors and normal pituitaries. A limitation to the experimental design was the inability to compare gonadotropinomas to isolated gonadotrope cells from human pituitaries because of the lim-

ited availability of human samples. Recognizing this, we used a large number of individual tumors and normal pituitary samples.

BRINP3 was identified as a candidate that was overexpressed in gonadotropinomas and was low in normal pituitary. BRINP3 belongs to a recently described family of genes normally restricted to the brain consisting of BRINP1, -2, and -3 (21, 22). They were named for the first member of the family that was cloned from a screen of genes up-regulated in superior cervical ganglion cells after 4 d exposure to BMP2 and RA. Subsequently, two related members were identified. BRINP2 and -3 are homologous (70%) with less homology to BRINP1 (50%). Homology across species, however, is high (mouse, rat, and human > 98%). Whereas BRINP1 was activated by BMP and RA in neuronal and fibroblast cells (21), BRINP3 was repressed in pituitary gonadotrope cells. These data suggest tissue-specific effects of the BRINP family members. Further studies will be necessary to determine whether BRINP3 is expressed at any time during pituitary development during which BMPs are known to play major roles in the ontogeny of the organ and cell specification (27, 28).

BRINP1 was also cloned by others as deleted in bowel and bladder cancer (DBCCR1), in a chromosomal region that is missing in some cancers (19). Because Matsuoka and co-workers have shown that the BRINP1 promoter has a non-neuronal silencing element [*i.e.* is repressed in nonbrain tissues (22)], the functional relevance of the BRINP1 deletion in these peripheral tumors is unclear. Because BRINP1 (DBCCR1) was putatively repressed in these cancers (19), but BRINP3 was conversely overexpressed in pituitary tumors, we cloned and sequenced BRINP3 from human gonadotropinomas and found the intact wild-type sequence, suggesting that BRINP3 in human gonadotrope tumors is not a mutant protein.

Kawano *et al.* (21) showed that BRINPs are divergently restricted in the brain by *in situ* hybridization, similar to our data on BRINP3. In their study, BRINP3 was highly expressed in the anterior olfactory nuclei, hypothalamus, and dentate gyrus, the cerebellum in the Purkinje, and lower levels in granule cells (21). No analysis of the BRINP members in the pituitary or across development was performed. Future studies on the normal role of BRINP3 in brain may shed light into its mechanism of action in pituitary tumors.

Because BRINP3 has no homology to any known protein, several approaches were used to explore its functional role in pituitary tumors. Cellular subfractionation studies and testing of tagged overexpressed protein suggested BRINP3 was localized to the mitochondria in pituitary cells. These observations were then confirmed using a new BRINP3 antibody. Considering a mitochondrial localization, our initial hypothesis was that BRINP3 would protect pituitary tumor cells from programmed cell death. In the absence of available primary human gonadotrope cells, the immortalized mouse gonadotrope cells were used as a model. In contrast to our hypothesis, there was no protective effect of BRINP3 overexpression on trophic factor withdrawal-induced apoptosis.

BRINP3 overexpression, however, was shown to have potent effects on proliferation, migration, and invasion. The proliferative effects were observed with increased time in culture. Whereas the growth rate of control cells plateaued

over time, the BRINP3 overexpressing gonadotropes continued to proliferate. This effect of BRINP3 could play a role in the pervasive growth of gonadotropinomas to ultimately present clinically as macroadenomas. The migration assays demonstrated an ability of BRINP3 to confer to pituitary cells the capacity to migrate in growth restricted environment. RNA interference silencing of endogenous BRINP3 blocked the ability of the gonadotrope cells to migrate, confirming the functional importance of the protein in gonadotrope movement. The invasion assays confirmed that BRINP3 directs an invasive phenotype in growth replete or restricted conditions. Together these studies support a potential role for BRINP3 in the process of tumorigenesis in human gonadotropinomas.

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