0021-972X/03/$15.00/0
Printed in U.S.A.

The Journal of Clinical Endocrinology & Metabolism 88(11):5119–5126

doi: 10.1210/jc.2003-030222

A Pituitary-Derived MEG3 Isoform Functions as a Growth Suppressor in Tumor Cells

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Human pituitary adenomas are the most common intracranial neoplasm. Typically monoclonal in origin, a somatic mutation is a prerequisite event in tumor development. To identify underlying pathogenetic mechanisms in tumor formation, we compared the difference in gene expression between normal human pituitary tissue and clinically nonfunctioning pituitary adenomas by cDNA-representational difference analysis. We cloned a cDNA, the expression of which was absent in these tumors, that represents a novel transcript from the previously described *MEG3*, a maternal imprinting gene with unknown function. It was expressed in normal human gonadotrophs, from which clinically nonfunctioning pituitary adenomas are derived. Additional investigation by Northern blot and RT-PCR demonstrated that this gene was also not expressed in functioning pituitary tumors as well as many human cancer cell lines. Moreover, ectopic expression of this gene inhibits growth in human cancer cells including HeLa, MCF-7, and H4. Genomic analysis revealed that *MEG3* is located on chromosome 14q32.3, a site that has been predicted to contain a tumor suppressor gene involved in the pathogenesis of meningiomas. Taken together, our data suggest that *MEG3* may represent a novel growth suppressor, which may play an important role in the development of human pituitary adenomas. (*J Clin Endocrinol Metab* 88: 5119–5126, 2003)

HUMAN PITUITARY ADENOMAS are the most common intracranial neoplasm, comprising approximately 10% of all diagnosed brain tumors (1, 2). Secretory adenomas produce one or more pituitary hormones such as prolactin, GH, ACTH, and TSH, causing phenotypic clinical syndromes. A significant percentage of macroadenomas is clinically nonfunctioning and does not cause syndromes of hormone overproduction. However, these tumors, typically of gonadotroph cell origin, can cause considerable morbidity because of mass effect, resulting in cranial nerve compression syndromes, other neurological deficits, and the development of hypopituitarism (1–3). Pituitary adenomas are typically monoclonal in origin, and a somatic mutation is a requisite event in tumor formation (4–6). However, mechanisms that underlie selective clonal proliferation remain elusive. Investigation of commonly known oncogenes and tumor suppressor genes, such as *ras*, *MEN-1*, *c-myc*, *Rb*, *p53*, *nm23*, and *gsp*, has revealed that none of these genes are involved in the pathogenesis of the majority of human pituitary tumors (7–9). The recently identified pituitary tumor-transforming gene (*PTTG*) has been shown to have increased expression in pituitary tumors (10–14), and the functions of this gene are still under investigation. In the majority of human pituitary tumors, the identification of specific causal genetic rearrangements, deletions, or mutations remains unknown.

To identify the molecular events responsible for the development of human pituitary tumors, we have compared the difference in gene expression between the normal human pituitary and clinically nonfunctioning pituitary adenomas by cDNA-representational difference analysis (RDA) (15). We identified a novel cDNA that is an alternatively spliced isoform of a previously reported transcript from a maternal imprinting gene, *MEG3*, with unknown function (16). Here, we report the molecular cloning, expression, and antiproliferative function of this *MEG3* cDNA isoform.

Abbreviations: EST, Expressed sequence tag; FSH-β, β-subunit of FSH; ORF, open reading frame; RDA, representational difference analysis; SSC, standard saline-citrate.

Materials and Methods

Molecular cloning of MEG3a

A 200-bp N-terminal fragment of *MEG3* cDNA, isolated by RDA from the normal pituitary (15), was used to screen a human fetal liver library (Clontech, Palo Alto, CA). The cDNAs from the positive clones were subcloned into pBlueScript-SK (Stratagene, La Jolla, CA) via the 5′-EcorRI and 3′-NotI sites, and their sequences were analyzed. The sequence information was used to search GenBank and the Human Genome Sequence Draft to reveal its identity, chromosomal localization, and genomic structure, at the web site National Center for Biotechnology Information (www.ncbi.nlm.nih.gov).

Construction of plasmid for expression

The *MEG3* cDNA isoform MEG3a (see Fig. 1A for sequence), obtained from the library screening, was cloned into pCI-neo (Promega, Madison, WI) via the 5′-EcorRI and 3′-NotI sites to generate a mammalian expression vector, pCI-neo-MEG3a, which was used in the colony formation and growth rate assays.

RNA preparation, dot blot and Northern blot hybridization, and RT-PCR

Total RNA from the tumor samples or cultured cells was isolated using TRIzol Reagent (Invitrogen, Carlsbad, CA) according to manufacturer's protocol. This study was approved by the Massachusetts General Hospital Subcommittee for Human Studies. For Northern blot, 20 μg of each RNA sample were analyzed by electrophoresis in a 1% agarose-formaldehyde gel, blotted onto Hybond-N transfer membrane (Amersham, Piscataway, NJ), cross-linked by UV, and baked at 80 C in a vacuum oven. The membrane was probed with ^{32}P-labeled, full-length MEG3a cDNA using Ultrahyb solution (Ambion, Austin, TX). mRNA levels were standardized with β-actin. For the tissue distribution study, a quantitative human tissue blot membrane was purchased from Clontech. The RNA amount from each tissue blotted on this membrane was

A

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GCAGAGAGGG AGCGCGCCTT GGCTCGCTGG CCTTGGCGGC GGCTCCTCAG GAGAGCTGGG 60
GCGCCCACGA GAGGATCCCT CACCCGGGTC TCTCCTCAGG GATGACATCA TCCGTCCACC 130
TCCTTGTCTT CAAGGACCAC CTCCTCTCCA TGCTGAGCTG CTGCCAAGGG GCCTGCTGCC 180
CATCTACACC TCACGAGGGC ACTAGGAGCA CGGTTTCCTG GATCCCACCA ACATACAAAG 240
CAGCCACTCA CTGACCCCCA GGACCAGGAT GGCAAAGGAT GAAGAGGACC GGAACTGACC 300
AGCCAGCTGT CCCTCTTACC TAAAGACTTA AACCAATGCC CTAGTGAGGG GGCATTGGGC 360
ATTAAGCCCT GACCTTTGCT ATGCTCATAC TTTGACTCTA TGAGTACTTT CCTATAAGTC 420
TTTGCTTGTG TTCACCTGCT AGCAAACTGG AGTGTTTCCC TCCCCAAGGG GGTGTCAGTC 480
TTTGTCGACT GACTCTGTCA TCACCCTTAT GATGTCCTGA ATGGAAGGAT CCCTTTGGGA 540
AATTCTCAGG AGGGGGACCT GGGCCAAGGG CTTGGCCAGC ATCCTGCTGG CAACTCCAAG 600
GCCCTGGGTG GGCTTCTGGA ATGAGCATGC TACTGAATCA CCAAAGGCAC GCCCGACCTC 660
TCTGAAGATC TTCCTATCCT TTTCTGGGGG AATGGGGTCG ATGAGAGCAA CCTCCTAGGG 720
TTGTTGTGAG AATTAAATGA GATAAAAGAG GCCTCAGGCA GGATCTGGCA TAGAGGAGGT 780
GATCAGCAAA TGTTTGTTGA AAAGGTTTGA CAGGTCAGTC CCTTCCCACC CCTCTTGCTT 840
GTCTTACTTG TCTTATTTAT TCTCCAACAG CACTCCAGGC AGCCCTTGTC CACGGGCTCT 900
CCTTGCATCA GCCAAGCTTC TTGAAAGGCC TGTCTACACT TGCTGTCTTC CTTCCTCACC 960
TCCAATTTCC TCTTCAACCC ACTGCTTCCT GACTCGCTCT ACTCCGTGGA AGCACGCTCA 1020
CAAAGGTTCC CAGTGCCCCC GACAAGCCCC TGCTGGTGTC TCCATCTCCT GCCAAGCATC 1080
CTCCAGTGCC TCCTCCTGTG GGCCTGGCCT CAGGGCTATG GACAGACTCC TGTCCCATCC 1140
CAGAGACCCC TCGTGATCGT TCCCTGGCAC GTGGGCCGTG GCCCGGCTGG GTCGGCTGAA 1200
GAACTGCGGA TGGAAGCTGC GGAAGAGGCC CTGATGGGGC CCAACATCCC GGACCCAAGT 1260
CTTCTTCCTG GCGGGCCTCT CGTCTCCTTC CTGGTTTGGG CGGAAGCCAT CACCTGGATG 1320
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GACCCGGGGC CTCCCCTTGA GTAGAGACCC GCCCTCTGAC TGATGGACTC CGCTGACCTG 1500
GGGTCAGACC CGTGGGCTGG ACCCCTGCCC ACCCCGCAGG AACCCTGAGG CCTAGGGGAG 1560
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B

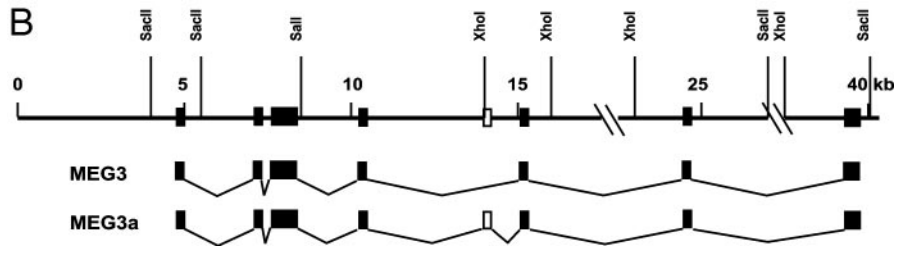


FIG. 1. A, The nucleotide sequence of MEG3a, isolated from human cDNA library screening. The two putative ORFs are *underlined*, with the start and stop codens in *bold*, and the sequence missing in the MEG3 cDNA published previously is *bold* and *italic*. The two *boxed* sequences represent the primers used in RT-PCR. B, Genomic structure of the human *MEG3* gene. The *solid boxes* represent the common exons for both MEG3 and MEG3a. The additional exon 5 used in MEG3a, but not in the MEG3 reported previously, is shown as an *open box*, which represents the *bold* and *italic* sequence in A.

normalized with eight different housekeeping gene products to ensure the proper quantitation. This blot was hybridized with ^{32}P-labeled, full-length MEG3a cDNA using Express-Hyb solution (Clontech). RT-PCR was performed as described previously (17). The primers used to amplify *MEG3* cDNA were 5′-CAGGATCTGGCATAGAGGAGG-3′ and 5′-GAGGATGCTTGGCAGGAGATGG-3′. [This primer recognizes a sequence specific to the MEG3a isoform obtained from our library screening. This sequence is not present in the previously reported expressed sequence tag (EST) clone of *MEG3*.] The primers for cyclophilin A as a control were 5′-CATGGTCAACCCCACCGTGTTCTT-3′ and 5′-TAGATGGACTTGCCACCAGTGCCAT-3′.

In situ hybridization and immunohistochemical staining

Digoxingenin-labeled RNA probes (sense and antisense) of human MEG3a as well as GH and the β-subunit of FSH (FSH-β) (for controls) were generated by *in vitro* transcription from the cloned cDNA in pBlue-Script-SK using the DIG RNA labeling kit (Roche, Indianapolis, IN), according to the manufacturer's instructions. Antibody against FSH-β was obtained from the National Hormone and Pituitary Program (sponsored by the National Institutes of Health; Torrance, CA). Other secondary antibodies were purchased from Roche. Normal human pituitary glands (containing both anterior and posterior) were obtained 2–16 h postmortem from the Harvard Brain Tissue Resource Center (Belmont, MA) and fixed with 4% paraformaldehyde for 3–4 h, rinsed with PBS, washed with 30% sucrose, sectioned (4 μm) by a cryostat, and stored at −80 C. For *in situ* hybridization, sections warmed to room temperature were rinsed briefly in DEPC-H_2O and PBS, then treated with 0.1 M triethanolamine for 2 min and 0.25% acetic anhydride in 0.1 M triethanolamine for 10 min. After rinsing with 2× standard saline-citrate (SSC), prehybridization was performed at room temperature for 1 h, with 50% deionized formamide, 4× SSC, 10% dextran sulfate, 1× Denhardt's solution, and 0.25 mg/ml yeast RNA. The sections were then rinsed briefly with 2× SSC and hybridized in the same solution containing 1 μg/ml RNA probe at 42 C overnight. After hybridization, the sections were washed serially in 2× SSC at room temperature for 1 h, 1× SSC at room temperature for 1 h, 0.5× SSC at 37 C for 30 min, and 0.5× SSC at room temperature for 30 min. To detect the hybridization signals, the sections were incubated in 0.1 N HCl for 2 min to block nonspecific staining; rinsed with 100 mM Tris-HCl (pH 7.5) and 150 mM NaCl; and incubated first with 100 mM Tris-HCl (pH 7.5), 150 mM NaCl, 0.3% Triton X-100, and 2% BSA for 30 min at room temperature and then with 100 mM Tris-HCl (pH 7.5), 150 mM NaCl, 0.3% Triton X-100, and 1% BSA containing an antidigoxigenin antibody conjugated with alkaline phosphatase for 3 h. Hybridization signals were detected using colorimetric reaction by incubation with 5-bromo-4-chloro-3-indolylphosphate/nitro-blue tetrazolium solution. Cellular nuclei were visualized by staining with Mayer's hematoxylin solution (Sigma, St. Louis, MO).

To observe the colocalization of MEG3a and FSH-β, the tissue slides were first used for *in situ* hybridization with the MEG3a probe as described above. After colorization, the same sections were used to perform immunohistochemical staining with the anti-FSH-β antibody. The sections were rinsed briefly with PBS, incubated with 1% BSA in PBS at room temperature for 30 min, and then incubated with the anti-FSH-β antibody in PBS (1:500 dilution) for 1 h. After washes with PBS, the sections were incubated with a secondary antibody in PBS (antirabbit

IgG conjugated with rhodamine) at room temperature for 1 h and washed with PBS. After the sections were mounted with Gel/Mount (Biomeda Corp., Foster City, CA), the images for the hybridization and immunostaining signals were photographed using a Nikon duel-field microscope (bright field for the *in situ* hybridization and fluorescence for the immunostaining) and analyzed with Adobe Photoshop software (Adobe Systems Inc., San Jose CA).

Cell culture and colony formation assays

Human cervical carcinoma cell line HeLa, breast adenocarcinoma MCF-7, and neuroglioma H4 were obtained from American Type Culture Collection (Manassas, VA) and cultured in DMEM with 10% fetal bovine serum (Invitrogen), incubated at 37 C with a humidified atmosphere of 5% CO_2. The colony formation assays were performed as described previously (15).

Growth rate assay of the transfected cells

Using the Lipofectamine Plus Reagent (Invitrogen) according to the manufacturer's protocol, we cotransfected HeLa cells in 100-mm culture dishes with expression construct pCI-neo (blank vector; Promega), pCI-neo-LacZ (negative control), pCI-neo-MEG3a, or pCI-neo-GADD45γ (positive control) (15), along with the plasmid pMACS K^k.II (Miltenyi Biotec, Auburn, CA), which expresses a truncated mouse H-$2K^k$ molecule as a cell surface marker. The ratio between the expression construct and pMACS K^k.II was 20:1. Forty-eight hours after the transfection, cells were collected and resuspended in 320 μl PBS with 5 mM EDTA and mixed with 80 μl MACSelect K^k Microbeads (Miltenyi Biotec), which bind to the surface marker H-$2K^k$ expressed on the cell surface of the transfected cells. After incubation on ice for 15 min, the transfected cells were isolated by MS Separation Columns (Miltenyi Biotec) via magnetic separation according to the manufacturer's protocol. The purified cells were seeded on 6-well plates with 5×10^4 cells per well and cultured in the CO_2 incubator at 37 C. The growth rate of these transfected cells was determined by counting the cell numbers in each well at 48 and 72 h after plating.

Results

Molecular cloning of MEG3a

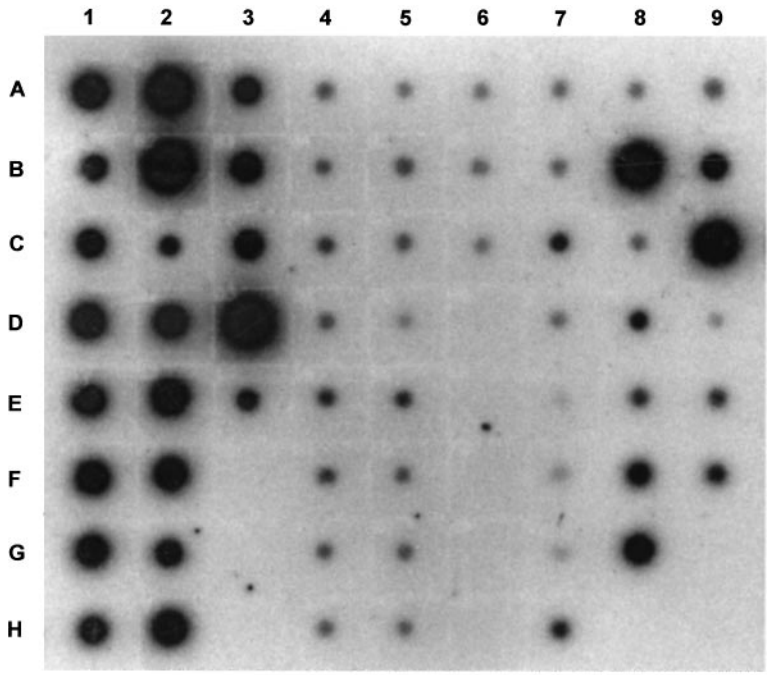
Using cDNA-RDA, we identified individual cDNA fragments expressed highly in normal pituitary but lower or undetectable in clinically nonfunctioning pituitary tumors (15). Of a total of 309 fragments analyzed, 49 represented one cDNA fragment (16%). This cDNA fragment was used to screen a human fetal liver library. Positive clones from the library were identified and subcloned, and the DNA sequences were analyzed. All the positive cDNA clones contained DNA sequences very similar to the previously reported MEG3, a cDNA identified from the Human EST Database based on the homology to the mouse imprinting gene *Gtl2*, with an unknown function (16). However, these cDNA sequences from the cDNA library screening were not identical to MEG3, with an additional 141-bp DNA sequence inserted in the middle of the cDNAs (Fig. 1A). Therefore, we designated this particular cDNA as MEG3a. Two single-nucleotide insertions were also found in our cDNA clones but not in the MEG3 sequence published previously, presumably as a result of the previous sequencing error, because our cDNA sequences completely matched the Human Genome Sequence at the National Center for Biotechnology Information web site. The cDNA sequence of MEG3a has been submitted to GenBank (accession no. AY314975). No cDNA containing the exact sequence of the previously reported MEG3 was found among the positive cDNA clones obtained from our library screening. There were two putative open reading frames (ORFs) within the cDNAs of MEG3a as well as MEG3 (Fig. 1A), however, no consensus Kozak sequence was identified for either ORF.

To understand the difference between MEG3, the previously reported cDNA obtained from the EST Database, and MEG3a, the cDNA that we cloned from human cDNA library screening, we analyzed the *MEG3* gene structure by comparing these cDNA sequences with the human genome sequence working draft (http://www.ncbi.nlm.nih.gov/genome/seq/). The Blast search of the human genome sequence showed that the *MEG3* gene was located on chromosome 14q32.3. Comparison of *MEG3* cDNA isoform sequences and the human genomic sequence in this region revealed the *MEG3* genomic structure (Fig. 1B). The difference of the insertion of a 141-bp DNA fragment between the MEG3a and the MEG3 sequence published previously was caused by the use of an extra exon 5 in the MEG3a cDNA, which is absent in MEG3, probably because of alternative splicing (Fig. 1B, open box). All the introns started with nucleotide sequence GT and ended with AG, standard donor and acceptor sites for RNA splicing in eukaryotes. Only one matched genomic sequence was detected in the human genome, suggesting that *MEG3* is a single-copy gene.

Tissue distribution

We used a 200-bp N-terminal fragment of *MEG3* cDNA as a probe to investigate its expression in different tissues. Hybridization with a quantitative human tissue RNA blot (Clonetech), which was normalized with eight different housekeeping genes, revealed that *MEG3* was highly expressed in the pituitary and cerebellum, as well as other areas of the brain. It was also expressed in placenta, adrenal gland, pancreas, and ovary, suggesting an endocrine-related function (Fig. 2).

Because *MEG3* was identified in the normal human pituitary by comparing gene expression between the normal pituitary, which contains different cell types, and clinically nonfunctioning tumors, which were primarily gonadotroph derived, we performed *in situ* hybridization to determine whether *MEG3* is expressed in normal human pituitary gonadotrophs. Using nonradioactive-labeled MEG3a cDNA as a probe (digoxigenin labeling system; Roche Molecular Biochemicals, Indianapolis, IN), we observed that scattered cells expressing *MEG3* mRNA were widely distributed throughout the human anterior pituitary (Fig. 3A), but none were detected in the posterior pituitary (Fig. 3B). Next, immunohistochemical staining was performed on the same pituitary tissue section slide, using an antibody against FSH-β (obtained from Dr. A. F. Parlow, National Hormone and Peptide Program, National Institute of Diabetes and Digestive and Kidney Diseases), a gonadotroph-specific hormone. We were able to colocalize the expression of *MEG3* mRNA and FSH-β protein in the same cells, indicating that *MEG3* is expressed in normal gonadotrophs in the human pituitary (Fig. 3, C–E). However, other cells expressing *MEG3* mRNA but not FSH-β were also observed (data not shown), indicating that *MEG3* mRNA expression is not restricted to gonadotrophs.



	1	2	3	4	5	6	7	8	9
A	whole brain	cerebellum, left	substantia nigra	heart	esophagus	colon, transverse	kidney	lung	liver
B	cerebral cortex	cerebellum, right	nucleus accumbens	aorta	stomach	colon, desending	skeletal muscle	placenta	pancreas
C	frontal lobe	corpus callosum	thalamus	atrium, left	duodenum	rectum	spleen	bladder	adrenal gland
D	parietal lobe	amygdala	pituitary gland	atrium, right	jejunum		thymus	uterus	thyroid gland
E	occipital lobe	caudate nucleus	spinal cord	ventricle, left	ileum		peripheral blood leukocyte	prostate	salivary gland
F	temporal lobe	hippo-campus		ventricle, right	ileocecum		lymph node	testis	mammary gland
G	p.g.* of cerebral cortex	medulla oblongata		inter-ventricular septum	appendix		bone marrow	ovary	
H	pons	putamen		apex of the heart	colon, ascending		trachea		

FIG. 2. Tissue distribution of *MEG3* mRNA. p.g.*, Paracentral gyrus.

Lack of MEG3 expression in human tumors

We next examined whether there were true differences in *MEG3* expression between normal and neoplastic pituitary cells, as well as in human carcinoma cell lines. Northern blot showed a *MEG3* mRNA of approximately 1700 nucleotides (1.7 K) in normal fibroblasts HS27 and WI38, but not in any human carcinoma cell lines examined (Fig. 4A). Because of sample size limitation, we could not examine most pituitary tumors by Northern blot. Therefore, RT-PCR was used to detect specific MEG3a isoform expression in different human pituitary tumor phenotypes. Figure 4B shows that there was no MEG3a expression in three GH-secreting tumors and eight clinically nonfunctioning pituitary tumors, in contrast to the five normal human pituitaries, using a primer set that will only amplify the MEG3a isoform but not the MEG3 EST sequence reported previously. MEG3a was also undetectable in a human pituitary tumor-derived cell line, PDFS (18). As a positive control, cyclophilin A cDNA was detected in all samples by RT-PCR (data not shown). RT-PCR also confirmed MEG3a expression in the normal fibroblast cells HS27 and WI38 (data not shown). The identities of all PCR products have been verified by DNA sequence analysis. Therefore, the loss of *MEG3* expression is not restricted to gonadotroph tumors but also to tumors of other pituitary cell types as well as other human cancer cell lines.

MEG3 inhibits cell proliferation of human carcinoma cell lines

Because MEG3a is highly expressed in normal pituitary cells and fibroblasts but undetectable in pituitary tumors and most human cancer cells, we hypothesized that the biological functions of *MEG3* may involve control of cell proliferation. To examine this possibility, the cDNA for the MEG3a isoform was cloned into a mammalian expression vector pCI-neo and used to transfect human cancer cell lines HeLa, MCF-7, and H4, which showed no *MEG3* mRNA expression in Northern analysis, to perform colony formation assays. Blank vector and similar expression vectors for β-galactosidase gene

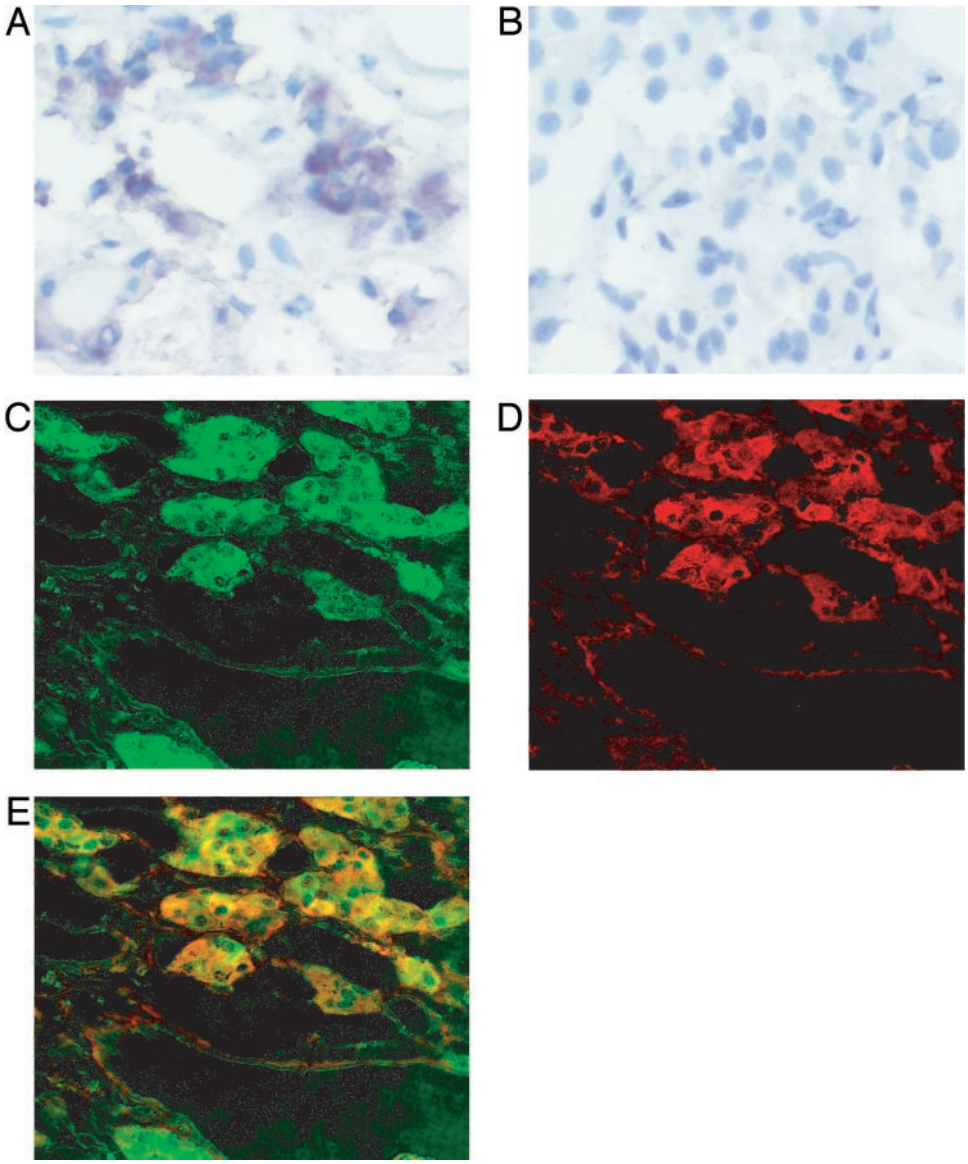


FIG. 3. Colocalization of *MEG3* mRNA with FSH protein. A, *In situ* hybridization with MEG3a cDNA shows *MEG3* expression in the normal human anterior pituitary. The cells containing *MEG3* mRNA are shown in *dark brown*, and cellular nuclei were stained with Mayer's hematoxylin, shown in *blue*. B, No *MEG3* mRNA was detected in the normal human posterior pituitary. C, An area in the human anterior pituitary rich in *MEG3*-positive cells (*green*). D, The same area with immunohistochemical staining using anti-FSH-β antibody on the same slide. The cells containing FSH protein are shown in *red*. E, Overlapping images of C and D reveals coexpression of MEG3 and FSH within the same cells (*yellow*).

(*LacZ*) and a known growth suppressor gene, *GADD45-γ* (19), were used as controls. Transfection with the pCI-neo-MEG3a expression vector caused a 70% decrease in cell clone numbers in all of these cancer cell lines (Fig. 5, A and B). For controls, the *LacZ* expression vector produced similar colony numbers as the blank vector, whereas the expression vector for a known growth suppressor gene, *GADD45-γ*, caused an 85% decrease in colony numbers, similar to results reported previously (19, 20).

To confirm the growth suppression function of *MEG3*, we also examined the growth rate of HeLa cells transfected with the pCI-neo-MEG3a expression vector. After cotransfection with the selection plasmid pMACS K^k.II, the transfected cells were purified by a magnetic separation column and the growth rate was determined by direct counting of cell number. As shown in Figure 5C, transfection of the pCI-neo-MEG3a expression vector resulted in a decrease in cell growth rate by 70% compared with the blank vector or a *LacZ* expression vector. Taken together, these data indicate that expression of *MEG3* in tumor cells leads to an inhibition of cell proliferation, consistent with the observation that all human tumor cells examined do not express this gene.

Discussion

We have identified a cDNA isoform from *MEG3*, a previously reported maternal imprinting gene with unknown function (16), in the normal human pituitary, which strongly suppresses cancer cell growth. Blot hybridization and RT-PCR revealed that *MEG3* mRNA is expressed in the normal pituitary but is not present in pituitary tumors and human cancer cell lines.

MEG3 was first discovered as the human homolog of a mouse gene, *Gtl2*. *Gtl2* was identified as an imprinting gene by gene trapping (21). Transgenic mice $Gtl2^{lacZ}$ bearing insertion mutations close to this gene locus are considerably smaller than the normal mice (with ~60% of body weight of nontransgenic mice), starting at d 16.5 of embryonic devel-

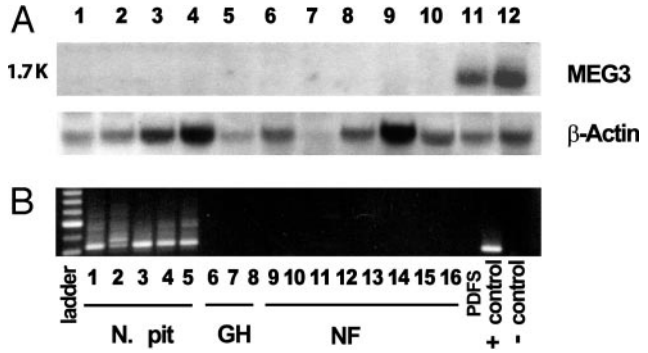


FIG. 4. A, *MEG3* mRNA is expressed in the normal human fibroblast cells but is undetectable in human cancer cell lines by Northern blot. Lanes 1–10, Human carcinoma cells HeLa, MCF-7, T47-D, T24, 5637, Du145, K562, HT29, H1299, H4; lanes 11 and 12, human normal fibroblasts HS-27 and WI38. B, RT-PCR shows that MEG3a is expressed in the normal human pituitary but not in pituitary tumors. N. pit., RNA from the normal human pituitary; GH, RNA from GH-secreting pituitary tumors; NF, RNA from clinically nonfunctioning pituitary tumors; PDFS, RNA from a human pituitary tumor-derived folliculostellate cell line (18); + control, PCR performed with cloned MEG3 cDNA as a template; – control, PCR performed in the absence of the template.

opment and throughout the postnatal growth period (21). Thus, it was originally proposed that the function of *Gtl2* may be involved in growth control during early development. Several groups subsequently reported that *Gtl2/Meg3* is closely linked to another imprinting gene, *Dlk1* (22–25). *Dlk1* encodes a transmembrane protein containing six epidermal growth factor repeat motifs closely related to those present in the δ/notch/serrate family of signaling molecules (26, 27), and it has been shown to modulate proliferation and differentiation of preadipocytes, stromal cells, and hematopoietic progenitor cells (28–32). Interestingly, Dlk1 is induced by GH in the fetal and maternal pancreas, leading to increased β-cell proliferation and insulin production (33). Based on the most recent evidence, it has been proposed that the previously observed dwarfism in $Gtl2^{lacZ}$ mice is likely caused by the destruction of a regulatory element for *Dlk1* function positioned at the 3′ end of the *Dlk1* gene, because the *lacZ* insertion is located between the *Dlk1* and *Gtl2* genes and at the 3′ end of the *Dlk1* gene (23). However, the function of *Glt2/Meg3* remained undetermined until now, when we found that MEG3a, a cDNA isoform from human *MEG3*, functions to inhibit cell growth.

The MEG3a cDNA that we have identified is an isoform of the previously reported MEG3. Among the multiple positive clones obtained from our screening of a human fetal liver library, none of them contains the exact sequence as the MEG3 cDNA sequence reported previously, suggesting that MEG3 mRNA does not represent a major form of transcript, at least in the fetal liver. The difference in the sequences of MEG3 and MEG3a cDNA was generated by the use of an additional exon in the middle of the cDNA of the MEG3a. It has been reported that the *Gtl2* gene, the mouse homolog of human *MEG3*, also encodes multiple alternatively spliced transcripts (34). The physiological significance of the multiple alternatively spliced isoforms is currently unknown. Because of the lack of a Kozak sequence, it is unknown whether the two putative ORFs within the *MEG3* cDNA encode for

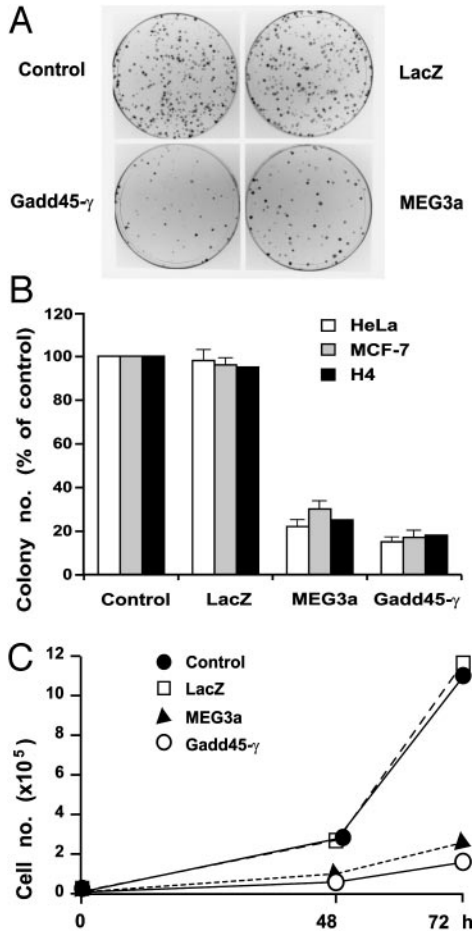


FIG. 5. MEG3a suppresses tumor cell growth. A, H4 cells were transfected with the blank vector pCI-neo (control) or expression vector for *LacZ*, *MEG3*, or *GADD45-γ*. After 2 wk of neomycin selection, the plates were fixed and stained with crystal violet solution. B, Viable colonies of HeLa, MCF-7, and H4 cells in similar experiments were counted and normalized to control. The data are represented as mean ± SD for counts from at least three independent experiments. C, HeLa cells were cotransfected with expression construct pCI-neo, pCI-neo-*LacZ*, pCI-neo-MEG3a, or pCI-neo-GADD45-γ, along with the plasmid pMACS K^k.II for selection. After purification with a magnetic column, the growth rate for the transfected cells was determined by direct counting.

functional proteins. There is no evidence that *MEG3* gene products are expressed at the protein level. It has been suggested that the transcripts from this gene may act as RNAs based solely on the lack of a Kozak sequence (34). In the current study, we have only focused on the newly identified MEG3a isoform, based on its functional activities to suppress tumor growth and its discovery from the original RDA comparing normal human pituitary tissue and pituitary tumors. It will be of interest in future studies to compare MEG3 and MEG3a.

In colony formation and growth rate assays, MEG3a exhibited a strong ability to inhibit cell growth and proliferation even in several extremely malignant and fast-growing human cancer cell lines, such as cervical carcinoma HeLa, breast adenocarcinoma MCF-7, and neuroglioma H4, indicating its powerful function to suppress cell growth. It is unknown whether this growth-suppressive function is me-

diated by the RNA transcript or an unknown translated protein. At the present time, there is no direct evidence that MEG3a can regulate pituitary tumor cell growth or the loss of MEG3a expression can result in pituitary tumor formation. However, its strong activity to cause growth suppression and its expression in the normal gonadotrophs but absence in the gonadotroph-derived tumors suggest that it may play an important role in the maintenance of normal gonadotroph cell growth. Additional investigations using an animal model will be critical to address whether the loss of *MEG3* gene function is directly associated with tumor development. The *MEG3* gene is located at chromosome 14q32.3. Interestingly, abnormalities at this locus are observed frequently in meningiomas (35–40). For examples, genomic alterations of chromosome 14q32.2 have been observed in approximately 50% of aggressive meningiomas (40, 41). Therefore, it has been suggested that there is a potential tumor suppressor gene at this chromosomal location associated with the pathogenesis and progression of meningiomas (41). As shown here for the first time, the expression of the *MEG3* gene at this exact locus is lost in the majority of human pituitary adenomas. Functionally, we have shown that MEG3a has a strong ability to inhibit proliferation of several malignant human carcinoma cell lines. Taken together, these exciting new findings suggest *MEG3* as a candidate tumor suppressor gene at chromosome 14q32.3, the deletion or mutation of which may be involved in the pathogenesis of human pituitary adenomas and other tumors.

Acknowledgments

We thank Drs. Y. Huang and D. Brown (Renal Unit, Massachusetts General Hospital) and Dr. M. E. Shomali (Endocrine Unit, Massachusetts General Hospital) for kind help in *in situ* hybridization and immunohistochemistry; Dr. A. F. Parlow (Harbor-UCLA Medical Center, Torrance, CA) and the National Hormone and Peptide Program of the National Institute of Diabetes and Digestive and Kidney Diseases for providing antibody against human FSH-β; and the Harvard Brain Tissue Resource Center (supported by United States Public Health Service Grant MH/NS 31862) for providing the normal human pituitary tissue.

Received February 27, 2003. Accepted July 28, 2003.

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This work was supported in part by National Institutes of Health Grant R01-DK-40947, American Cancer Society Grant IRG-87-007-13, the Massachusetts General Hospital/Giovanni Armenise Neuro-Oncology and Related Disorder Program, and the Jarislowsky Foundation.

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