Selective Loss of *MEG3* Expression and Intergenic Differentially Methylated Region Hypermethylation in the *MEG3*/*DLK1* Locus in Human Clinically Nonfunctioning Pituitary Adenomas

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Context: *MEG3* is an imprinted gene encoding a novel noncoding RNA that suppresses tumor cell growth. Although highly expressed in the normal human pituitary, it is unknown which of the normal pituitary cell types and pituitary tumors express *MEG3*.

Objectives: Our objectives were 1) to investigate cell-type- and tumor-type-specific expression of *MEG3* in the human pituitary and 2) to investigate whether methylation in the intergenic differentially methylated region (IG-DMR) at the *DLK1*/*MEG3* locus is involved in the loss of *MEG3* expression in tumors.

Design and Methods: RT-PCR, quantitative RT-PCR, Northern blot, and a combination of *in situ* hybridization and immunofluorescence were used to determine the cell-type- and tumor-type-specific *MEG3* expression. Bisulfite treatment and PCR sequencing of genomic DNA were used to measure the CpG methylation status in the normal and tumor tissues. Five normal human pituitaries and 17 clinically nonfunctioning, 11 GH-secreting, seven prolactin-secreting, and six ACTH-secreting pituitary adenomas were used.

Results: All normal human pituitary cell types express *MEG3*. However, loss of *MEG3* expression occurs only in nonfunctioning pituitary adenomas of a gonadotroph origin. All other pituitary tumor phenotypes examined express MEG3. Hypermethylation of the IG-DMR at the *DLK1*/*MEG3* locus is present in nonfunctioning pituitary adenomas.

Conclusions: *MEG3* is the first human gene identified expressed in multiple normal human pituitary cell types with loss of expression specifically restricted to clinically nonfunctioning pituitary adenomas. The IG-DMR hypermethylation may be an additional mechanism for *MEG3* gene silencing in such tumors. **(*J Clin Endocrinol Metab* 93: 4119–4125, 2008)**

Pituitary adenomas comprise approximately 7% of all intracranial tumors, and clinically nonfunctioning adenomas are among the most common phenotype (1). Although clinically nonfunctioning tumors typically grow slowly, many are locally invasive, causing hypopituitarism and neurological deficits, and recurrences can occur (2, 3). Most importantly, there is no medical therapy available for such tumors. Although it has been known for many years that these tumors are primarily of a gonadotroph cell origin, the underlying pathogenetic factors responsible for their development remain unknown. Clinically

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Abbreviations: IG-DMR, Intergenic differentially methylated region; ORF, open reading frame; PRL, prolactin; qRT-PCR, quantitative (real-time) RT-PCR; Rb, retinoblastoma.

nonfunctioning pituitary adenomas are known to represent monoclonal cell proliferations (4, 5); however, target oncogenes and tumor suppressor genes, such as *ras*, *c-myc*, *MEN-1*, *Rb*, *p53*, *nm23*, and *gsp*, have not been shown to play an important role in their pathogenesis (3, 6, 7). Epigenetic alterations in clinically nonfunctioning tumors, such as hypermethylation of the *p16/CDKN2A/MTS1* gene, have been reported. However, this alteration is not specific to these tumors because it has also been described with high frequency in lactotroph-derived tumors and, rarely, in somatotroph- and corticotroph-derived adenomas (8). Therefore, molecular mechanisms specifically underlying the pathogenesis of clinically nonfunctioning pituitary adenomas remain unknown.

MEG3 is an imprinted gene expressed from the maternal allele (9). Using representational difference analysis, we identified this gene as strongly expressed in the normal human pituitary but not expressed in clinically nonfunctioning tumors derived from gonadotroph cells (10). The link between *MEG3* expression and tumorigenesis has been supported by the finding that expression of *MEG3* cDNA suppresses cell proliferation in a number of human tumor cell lines (10). Of importance, we have recently shown that *MEG3* cDNA is able to stimulate the transactivation function of p53 (11). *MEG3* is the human homolog of *Gtl2* (12), first mapped on mouse distal chromosome 12. *Gtl2/MEG3* gives rise to multiple spliced cDNA isoforms, and each of them has similar functions in suppressing cancer cell growth *in vitro* and stimulating p53-mediated transactivation (11). Interestingly, no strong Kozak consensus sequence for translation has been detected in any of the ATG codons in the potential open reading frames (ORFs) (12, 13), suggesting that this gene might function as a noncoding RNA. We have recently shown that the integrity of each potential ORF in *MEG3* is not required for its ability to suppress tumor cell growth and to activate the tumor suppressor p53, indicating that *MEG3* function is translation-independent. Therefore, the *MEG3* gene encodes a noncoding RNA with growth suppression function (11). We also found no genomic abnormalities in the *MEG3* locus in most clinically nonfunctioning pituitary adenomas (14). However, two 5′-flanking regions, containing a promoter and an enhancer, respectively, were hypermethylated in human tumors lacking *MEG3* expression, and treatment of human cancer cell lines with a methylation inhibitor resulted in *MEG3* expression (14, 15). These data indicate that hypermethylation is a mechanism involved in tumor suppression by *MEG3*.

The expression of *MEG3* in normal gonadotrophs and loss of *MEG3* expression in pituitary adenomas of gonadotroph origin have been reported in our previous studies (10, 14). However, an important unanswered question is whether loss of *MEG3* expression is specific for gonadotroph-derived tumors or also occurs in other pituitary tumor types. It is also unknown whether *MEG3* is expressed in other normal human pituitary cell types. We therefore investigated the specificity of *MEG3* expression in normal human pituitary cell types and examined *MEG3* expression in different human pituitary tumor phenotypes. We also determined whether hypermethylation of the imprinting control region for the *MEG3* locus serves as another mechanism for the loss of MEG3 expression in gonadotroph-derived pituitary tumors.

Materials and Methods

Tissue and tumor samples

Normal human pituitary glands were obtained 2–16 h postmortem from autopsies performed at the Massachusetts General Hospital. Fresh samples from 17 clinically nonfunctioning, 11 GH-, seven prolactin (PRL)-, and six ACTH-secreting adenomas with immunohistochemical confirmation were obtained from surgical specimens at the Massachusetts General Hospital and the National Institutes of Child Health and Human Development (Bethesda, MD). The median age was 43 (range, 12–83 yr old). The patients included 16 females and 25 males. All pituitary tumors were macroadenomas except the six ACTH-secreting adenomas, which were microadenomas. The study was approved by the Institutional Review Boards of Partners HealthCare and The National Institute of Child Health and Human Development/National Institutes of Health.

In situ hybridization

Digoxigenin-labeled RNA probes (sense and antisense) of human *MEG3a* were generated by *in vitro* transcription from the cloned cDNA in pBlueScript-SK vector using the DIG-RNA labeling kit (Roche, Indianapolis, IN) according to the manufacturer's instructions. This probe recognizes all *MEG3* RNA isoforms. Normal pituitaries and tumor samples were fixed with 4% paraformaldehyde for 3–4 h, rinsed with PBS, and washed with 30% sucrose. Four-micrometer frozen sections were obtained using a cryostat and stored at −80 C. *In situ* hybridization was performed as previously described (10). The *in situ* hybridization slides with tumor tissue were stained with nuclear fast red (Sigma Chemical Co., St. Louis, MO).

Immunofluorescence

After *in situ* hybridization, immunofluorescence was performed on the same slides using primary antibodies against GH, FSHβ, PRL, ACTH, and TSHβ (obtained from Dr A. F. Parlow, National Institutes of Health National Hormone and Pituitary Program, Torrance, CA). The sections were rinsed briefly with PBS, incubated with 1% BSA in PBS at room temperature for 30 min, and then incubated overnight at 4 C with primary antibodies diluted in 1% BSA (1:400 dilution). After washes with PBS, the sections were incubated with a secondary immunofluorescent antibody in PBS (antirabbit goat IgG conjugated with rhodamine; Jackson ImmunoResearch, West Grove, PA) at room temperature for 2 h, washed with PBS, and mounted with Vectashield (mounting medium with 4′,6-diamidino-2-phenylindole; Vector Laboratories, Burlingame, CA).

Colocalization of *MEG3* and pituitary hormones

The images for the *in situ* hybridization and immunostaining signals were photographed using a Nikon dual-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). The *in situ* hybridization images were converted to dark field with a green color and superimposed on the immunofluorescence images.

RT-PCR and Northern blot

Total RNA from the normal anterior pituitaries and the pituitary adenomas was extracted using TRIzol reagent (Invitrogen Life Technologies, Inc., Carlsbad, CA) and reverse transcribed using the RT System from Promega Corp. (Madison, WI) according to the manufacturer's protocol. PCR was performed under the following condition for both *MEG3* and glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*): 94 C for 2 min, 94 C for 30 sec, 60 C for 30 sec, and 72 C for 1 min for 30 cycles and 72 C for 10 min. The PCR primer sequences used

were as follows: *MEG3* forward, 5′-ATCATCCGTCCACCTCCTT-GTCTTC-3′, and *MEG3* reverse, 5′-GTATGAGCATAGCAAAGGT-CAGGGC-3′; *GAPDH* forward, 5′-AATGCCTCCTGCACCACCAAC-3′, and *GAPDH* reverse, 5′-AAGGCCATGCCAGTG-AGCTTC-3′. The *MEG3* primers used here amplify all *MEG3* cDNA isoforms. Positive controls with three different normal pituitaries and negative controls without cDNA template were used in each reaction. Northern blot was performed as previously described (10).

Quantitative (real-time) RT-PCR (qRT-PCR)

qRT-PCR for *MEG3* expression was performed in a subset of the pituitary adenomas, including 11 clinically nonfunctioning, seven GH-secreting, four PRL-secreting, and six ACTH-secreting tumors. Four normal human pituitaries were used for control. The PCR was performed using a 20-μl working mix containing 1.0 μl of the cDNA template in 1× TaqMan universal Master Mix (Applied Biosystems, Foster City, CA) and 200 nM final concentration of the primers and the probe for *MEG3* (Hs01087966_m1, FAM labeled; Applied Biosystems). The TaqMan primers and the probe for *MEG3* amplify all *MEG3* cDNA isoforms. As an endogenous control, *GAPDH* (Hs99999905_m1, FAM labeled; Applied Biosystems) was amplified in parallel and used to normalize the results to allow relative quantitative analysis of *MEG3* expression. The reaction was run in an Applied Biosystems 7500 Fast Real-Time PCR Sequence Detection System using the following parameters: 50 C for 2 min, 95 C for 10 min, and 40 cycles of 95 C (denature) for 15 sec, with 60 C for 1 min (annealing and extension). Each qRT-PCR was performed in duplicate experiments. Data were expressed as CT values (the cycle number at which logarithmic PCR plots cross a calculated threshold line). Relative RNA expression was given by the formula $2^{-\Delta\Delta CT}$, where $\Delta\Delta CT = CT^{(\text{MEG3 tumor} - \text{GAPDH tumor})} - CT.^{(\text{MEG3 normal pituitary gland} - \text{GAPDH normal pituitary gland})}$

Genomic DNA extraction

Genomic DNA from normal human pituitaries and from pituitary tumors was extracted using a DNeasy Tissue Kit (QIAGEN, Valencia, CA) according to the manufacturer's protocol.

Sodium bisulfite treatment of genomic DNA

A total of 1.5 μg of genomic DNA from the 11 nonfunctioning pituitary adenomas, three GH-secreting adenomas, three PRL-secreting adenomas, and four ACTH-secreting adenomas as well as from the five normal human pituitaries were treated with sodium bisulfite using the MethylDetector Bisulfite Modification Kit (Active Motif, Carlsbad, CA) according to the manufacturer's protocol. A semi-nested PCR was performed to amplify the intergenic differentially methylated region (IG-DMR) at the *MEG3* locus (position 51021–51180; GenBank accession no. AL117190) (15). For the first round, the primer sequences were as follows: IG forward, 5′-TTTTGAGGAGATTGATATTTTTAG-TTT-TATT-3′, and IG reverse, 5′-ATAAACTACACTACTAAAAACTA-CATTTAAA-3′. The second round was performed using the same IG reverse primer and a new primer IG-Fnes (nested forward primer), 5′-TTAGGTTGGAATTGTTAAGAGTTTGTGGATT-3′. PCR was performed using Hotstart Plus DNA polymerase (QIAGEN) under the following conditions: 95 C for 5 min, 94C for 30 sec, 53 C for 30 sec, and 72 C for 1 min for 35 cycles and 72 C for 10 min. PCR products were purified using a QIAquick purification kit (QIAGEN) according to the manufacturer's protocol. Purified PCR products were subcloned into a TOPO TA cloning vector (Invitrogen). Twenty clones from each PCR product were examined by sequence analysis. All data are expressed as the mean ± SD for descriptive statistics and ± SEM for comparing groups. Repeated measures of ANOVA were used to analyze data where appropriate. $P < 0.05$ was considered significant.

Results

Expression of *MEG3* in the normal pituitary

In situ hybridization detected strong positive *MEG3* RNA expression in most pituitary cells with stronger expression in the lateral wings of anterior pituitary (data not shown). Using a combination of *in situ* hybridization and immunofluorescence, *MEG3* RNA expression was detected in most of the pituitary cell types (90–95%), including gonadotrophs, somatotrophs, lactotrophs, thyrotrophs, and corticotrophs (Fig. 1). The GH-producing cells had the strongest and most diffuse reaction for MEG3.

Selective loss of MEG3 expression in clinically nonfunctioning pituitary adenomas

RT-PCR showed *MEG3* RNA expression in all 11 GH-secreting, all seven PRL-secreting, and all four ACTH-secreting tumors (Fig. 2, A–C). RT-PCR also detected *MEG3* RNA in three normal human pituitaries (Fig. 2D). *MEG3* expression in the PRL-secreting tumors was more variable than that in the other functioning tumors. Interestingly, the two prolactinomas with lower *MEG3* expression (Fig. 2B, lanes 5 and 6) came from patients with poorly functioning prolactinomas with sparse PRL immunostaining and PRL levels of 68 and 46 ng/ml, respectively, much lower than that in other patients (ranging from 289-8150 ng/ml). In contrast, none of the 17 clinically nonfunctioning tumors expressed *MEG3* RNA as determined by RT-PCR (Fig. 2E). Northern blot also showed that *MEG3* RNA is detectable in one normal human pituitary, two GH-secreting tumors and one PRL-secreting tumor, but undetectable in five clinically nonfunctioning tumors (Fig. 2F).

Furthermore, qRT-PCR demonstrated detectable and variable levels of *MEG3* RNA in GH-secreting, PRL-secreting, and

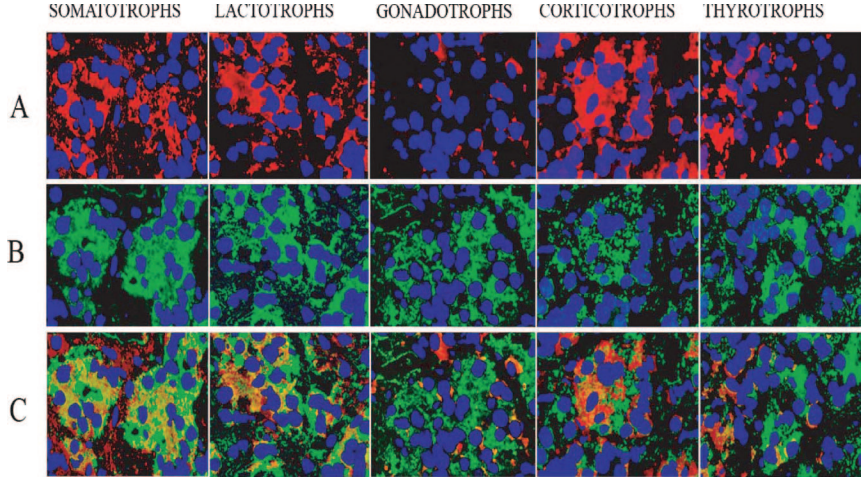

FIG. 1. A, Immunofluorescence to detect each pituitary hormone (GH, PRL, FSH, ACTH, and TSH) as indicated, thus identifying different types of pituitary cells; B, *in situ* hybridization with an antisense probe for *MEG3* RNA; C, overlapping of A and B. The areas with *yellow* represent the cells with coexpression of *MEG3* RNA (*green*) and the pituitary hormone (*red*), thus showing the expression of *MEG3* in each type of pituitary cell.

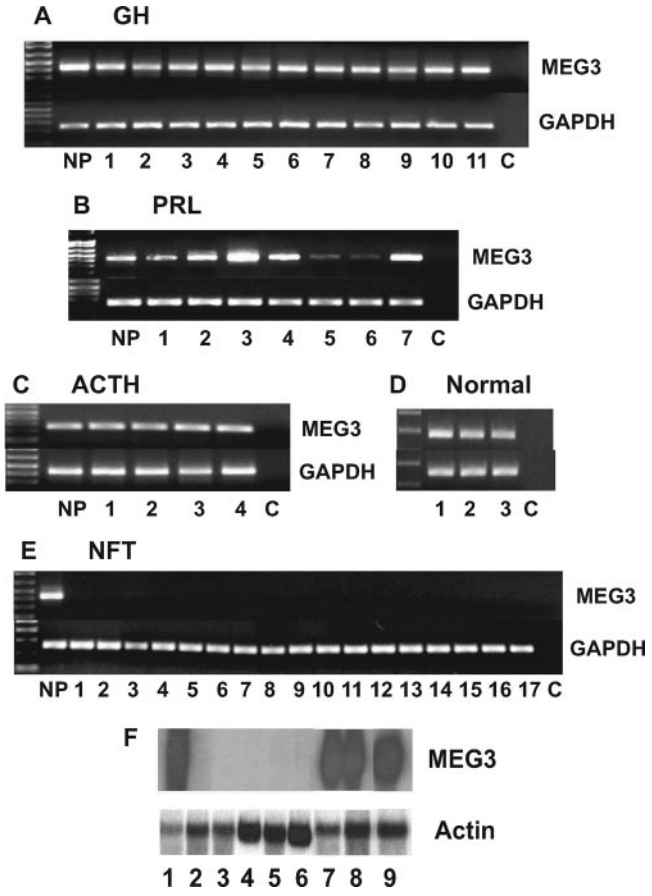

FIG. 2. A–D, RT-PCR showing *MEG3* RNA expression in all cases of functioning pituitary adenomas (A, GH-secreting tumors; B, PRL-secreting tumors; and C, ACTH-secreting tumors) and normal human pituitaries (D). Some prolactinomas have a weaker expression (in two cases) (B). E, No *MEG3* RNA is detected in any of the 17 clinically nonfunctioning tumors (NFT). C, Negative control without cDNA template; NP, positive control with normal pituitary cDNA. F, Northern blot showing *MEG3* RNA expression in a normal human pituitary (lane 1), 2 GH-secreting tumors (lanes 7 and 8), and a PRL-secreting tumor (lane 9), but not in five clinically nonfunctioning tumors.

ACTH-secreting adenomas (Table 1). GH-secreting adenomas had a 38-fold (38.0 ± 31.0, mean ± SD) increase and PRL-secreting adenomas had a 12-fold increase (12.0 ± 15.0) in the levels of *MEG3* RNA compared with that seen in the normal pituitaries (1.00 ± 0.0). Most ACTH-secreting adenomas also had increased *MEG3* RNA expression (2.85 ± 2.72) compared with that in the normal pituitaries. In contrast, the clinically nonfunctioning pituitary adenomas had a −100-fold (0.01 ± 0.01) decrease ($P < 0.001$) in the levels of *MEG3* RNA compared with that in normal pituitaries (Table 1). Tissue slides from four GH-secreting tumors (tumors number 1–4, as shown in Fig. 2) and four clinically nonfunctioning tumors (tumors number 1–4, as shown in Fig. 2) were used for *in situ* hybridization. Strong expression of *MEG3* RNA was observed in all cells of the GH-secreting tumors, but no *MEG3* RNA expression was detected in the clinically nonfunctioning tumors, consistent with the RT-PCR results (Fig. 3).

TABLE 1. *MEG3* RNA levels in human pituitary tumor samples

Nonfunctioning adenomas	GH-secreting adenomas	PRL-secreting adenomas	ACTH-secreting adenomas
0.001	90.0	0.50	1.10
0.022	32.0	0.16	3.10
0.001	64.0	15.0	0.62
0.002	5.00	32.0	1.30
0.004	4.00		3.00
0.021	42.0		8.00
0.010	32.0		
0.013			
0.000			
0.033			
0.003			

Each number indicates the relative *MEG3* RNA level in each tumor compared with the average *MEG3* RNA level in the normal human pituitaries (n = 4), which is designated as 1, as determined by qRT-PCR.

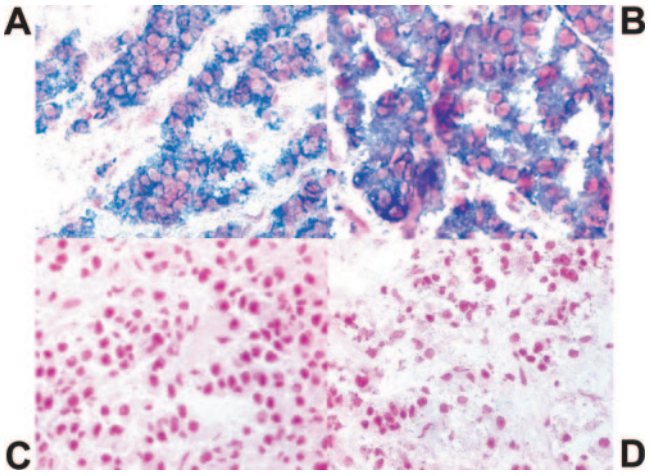

FIG. 3. Representative results of *in situ* hybridization for *MEG3* RNA in frozen tissues. A, Normal human pituitary gland; B, GH-secreting pituitary tumor; C, clinically nonfunctioning pituitary tumor; D, negative control, the normal pituitary section hybridized with a *MEG3* sense probe. The *blue* indicates *MEG3* RNA-positive signal.

Methylation status of the IG-DMR in clinically nonfunctioning pituitary tumors

Genomic DNA from 11 nonfunctioning, three GH-secreting, three PRL-secreting, and four ACTH-secreting pituitary adenomas as well as five normal pituitaries was examined for methylation in CpG dinucleotides in *DLK1/MEG3* IG-DMR. There are a total of eight CpG dinucleotides in this region. As shown in Table 2, there is an approximately 50% methylation in these CpGs in the normal pituitaries, consistent with the fact that *MEG3* is an imprinted gene; therefore, the paternal allele is hypermethylated for the silencing of gene expression from this allele. In contrast, the percentages of CpG methylation in clinically nonfunctioning tumors are significantly higher at each CpG position ($P < 0.05$), indicating that this region is hypermethylated in such tumors. In the pituitary tumors with hormone secretion, we observed an increased percentage of methylation at the CpG positions 1–6. However, in secretory tumors, the percentages of methylation at CpG position 7 and 8 are similar to those in the normal pituitaries, much lower than the percentages of methylation at these two positions observed in the clinically nonfunctioning tumors (Table 2). As shown in Fig. 4, there is a uniform increase of methylation in each individual CpG in the clinically nonfunctioning tumors compared with the normal pituitaries,

TABLE 2. Percentages of CpG methylation within the IG-DMR in normal pituitaries and pituitary tumors

	CpG1	CpG2	CpG3	CpG4	CpG5	CpG6	CpG7	CpG8
Secretory tumors								
GH-secreting								
1	65	95	85	60	60	90	45	70
2	65	55	70	75	90	70	85	70
3	35	85	90	55	35	75	65	50
PRL-secreting								
1	100	45	100	100	100	40	35	35
2	95	50	100	100	100	100	45	45
3	40	60	65	55	40	60	55	45
ACTH-secreting								
1	60	70	70	70	80	25	60	30
2	80	100	95	85	95	90	55	55
3	75	60	75	70	90	45	40	35
4	70	90	95	75	85	70	70	80
Mean	69%	71%	85%	75%	78%	67%	56%	52%
NFT								
1	50	100	100	66	66	66	75	50
2	40	50	86	95	60	66	95	95
3	57	87	74	57	87	57	61	74
4	33	86	95	57	48	81	33	24
5	33	50	83	67	50	58	100	83
6	100	68	95	77	59	65	100	100
7	57	48	57	57	95	43	100	48
8	90	60	100	40	90	40	40	40
9	60	90	70	70	70	70	80	50
10	94	65	100	82	76	76	65	59
11	70	76	100	88	82	47	88	82
Mean	62%	71%	87%	69%	71%	61%	76%	64%
Normal								
1	43	56	75	50	56	68	55	50
2	44	44	75	56	37	44	37.5	62.5
3	31	56	75	56	62	25	60	37
4	44	44	75	56	62.5	50	62.5	68
5	31	56	75	56	68	25	50	37.5
Mean	39%	51%	75%	55%	57%	42%	53%	51%

Each number indicates the percentage of methylation at each CpG dinucleotide position with the IG-DMR among 20 clones analyzed. Normal indicates normal human pituitaries. NFT, Clinically nonfunctioning pituitary adenomas.

and in particular, the percentage of methylation at the CpG positions 7 and 8 in the clinically nonfunctioning tumors is much higher that that in the hormone-secreting tumors (76 *vs.* 56% for CpG 7, $P = 0.031$; 64 *vs.* 52% for CpG 8, $P = 0.189$).

Discussion

We found that *MEG3* is strongly expressed in all cell types of the normal human pituitary gland as determined by *in situ* hybridization and immunohistochemistry, consistent with the strong *MEG3* expression found in the normal human pituitary using both regular RT-PCR and qRT-PCR. *MEG3* RNA expression was observed in tumor cells of all functioning pituitary adenomas examined, including lactotroph, somatotroph and corticotroph tumors. In striking contrast, no expression of *MEG3* was found in clinically nonfunctioning tumors. This specific loss of expression in gonadotroph-derived tumors is consistently demonstrated by regular RT-PCR and qRT-PCR as well as *in situ* hybridization. Therefore, *MEG3* represents the first human gene identified whose expression is selectively lost only in clinically nonfunctioning pituitary tumors. These data suggest that *MEG3* may play an important role in a specific pathway controlling clinically nonfunctioning pituitary tumor pathogenesis. qRT-PCR revealed the highest *MEG3* expression in somatotroph-derived pituitary adenomas. This is likely due to the almost pure population of somatotroph cells in these tumors, because we have observed the highest *MEG3* expression in the lateral wings of the anterior pituitary where there are more somatotroph and lactotroph cells. We would like to point out that because *MEG3* expression levels in different cell types in the normal pituitary are different, we cannot conclude that *MEG3* is overexpressed in the functioning tumors.

It had been hypothesized that the *MEG3* gene may be unable to generate protein products based on the lack of Kozak sequences near the potential ORFs within its RNA (12). We recently reported that *MEG3* is capable of stimulating p53-mediated transcription activation, thus providing a strong functional link between *MEG3* and p53, one of the most important tumor suppressors. *MEG3* expression leads to cellular accumulation of p53 protein and selective activation of p53 downstream target genes. Using p53-mediated transactivation as a functional assay,

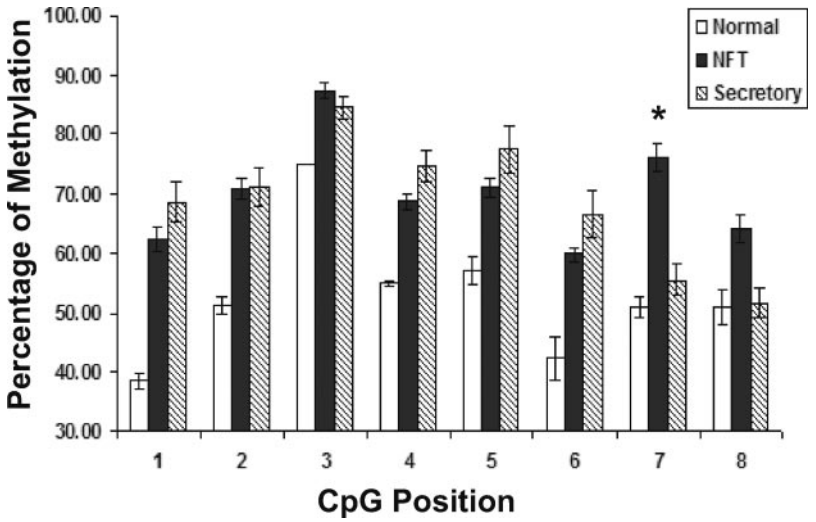

FIG. 4. Comparison of methylation at each CpG position (from position 1 to position 8) within the IG-DMR in the normal human pituitaries (*white bars*), clinically nonfunctioning pituitary adenomas (NFT, *black bars*), and the secretory pituitary adenomas (*striped bars*). The methylation status of each CpG in this region is quantified by the percentage of methylated CpGs among all PCR products analyzed. Values are the mean ± SEM. Difference with statistical significance is observed at each CpG position between the normal human pituitaries and the clinically nonfunctioning pituitary adenomas. Between the clinically nonfunctioning adenomas and the hormone-secreting adenomas, the percentage of methylation at the CpG positions 7 and 8 in the clinically nonfunctioning tumors is much higher that that in the hormone-secreting tumors (76 *vs.* 56% for CpG 7, $P = 0.031$; 64 *vs.* 52% for CpG 8, $P = 0.189$).

we have provided experimental evidence demonstrating that *MEG3* functions as a noncoding RNA (11). Furthermore, we have reported that suppression of tumor cell growth by *MEG3* is retinoblastoma (Rb) dependent (11). The tumor suppressor Rb is well known for its involvement in preventing pituitary tumorigenesis. Loss of expression of Rb or its activator $p16^{Ink4a}$ in human pituitary tumors has been reported by several groups (8, 16–19). Inactivation of Rb by simian virus 40 T antigen in mice results in the development of pituitary tumors derived from gonadotrophs (20), which resemble human clinically nonfunctioning pituitary tumors. Therefore, *MEG3* may play an important tumor suppressor role in Rb-related pathways to prevent the development of clinically nonfunctioning pituitary tumors.

We previously reported that *MEG3* expression was stimulated by cAMP (21). cAMP is one of the most important second messengers. It is involved in mediating functions or regulating production of all major pituitary hormones (22, 23). We have now observed that *MEG3* is expressed in all cell types of the normal human pituitary. Interestingly, loss of *MEG3* expression is restricted only to gonadotroph-derived clinically nonfunctioning tumors, which coincidently lack significant hormone secretion. In addition, the two prolactinomas with low levels of *MEG3* expression (nos. 5 and 6; see Fig. 2B) are poorly functioning PRL-secreting tumors. Taken together, these data may also suggest that *MEG3* is physiologically involved in the control of pituitary hormone production and function.

We have previously shown that the mechanism for the loss of *MEG3* expression in human pituitary tumors is not related to genomic DNA abnormalities such as gene deletion or mutation. Rather, *MEG3* gene promoter and enhancer hypermethylation has been demonstrated as an epigenetic mechanism for the loss of *MEG3* expression in nonfunctioning pituitary adenomas (14). The expression of *MEG3* in human cancer cell lines can be induced by a demethylation reagent (14). In this study, we observed additional hypermethylation in the IG-DMR in 14q32, between *DLK1* and the *MEG3* gene and known to control imprinting status, in clinically nonfunctioning tumors. In mice, the IG-DMR of the *Dlk1/Gtl2* domain is a control element for the whole imprinting gene cluster in chromosome 14q. Paulsen *et al.* (24) first identified this CpG-rich tandem repeat in the intergenic region of *Dlk1/Gtl2* that was conserved between mouse, sheep, and humans. Takada *et al.* (25) identified this CpG island located 15 kb upstream of the *Gtl2* and 70 kb downstream of the *Dlk1* promoter. Deletion in the IG-DMR from the maternal chromosome causes loss of imprinting of all imprinting genes in the maternal allele. However, deletion of the paternal allele does not affect the imprinted gene expression, indicating that it is the IG-DMR in the maternal allele that controls genetic imprinting (26, 27). The presence of hypermethylation in this IG-DMR in clinically nonfunctioning pituitary tumors compared with the normal pituitaries reported here may be an additional mechanism and likely works together with the promoter and enhancer hypermethylation to contribute to the loss of *MEG3* expression in human pituitary tumors. Hypermethylation has been found in relation to the loss of expression of various genes in pituitary adenomas such as *GADD45γ*, *p16*, *Rb*, and *death-associated protein kinase* genes (28, 29). Considering that pituitary adenomas are slowly growing benign tumors, it is likely that the epigenetic mechanisms play a more important role in the pathogenesis of these tumors than genomic defects, which often lead to more malignant features.

Interestingly, when we examined the methylation patterns of this IG-DMR in the hormone-secreting pituitary tumors, which all express *MEG3*, we observed a methylation increase at the CpG positions 1–6. However, the percentages of methylation at CpG positions 7 and 8 in hormone-secreting tumors are similar to those in the normal pituitaries, much lower than the percentages of methylation at these two positions observed in nonsecreting tumors. Recent studies with detailed analysis of particular CpG methylation patterns have shown that methylation at very few specific CpG positions can dramatically affect gene expression. For example, Demura and Bulun (30) reported that in the *CYP19 I.3/II* promoter fragment of 571 bp, differential methylation at only three CpG dinucleotide positions determined cAMP responsiveness of aromatase expression. Weaver *et al.* (31, 32) showed that methylation of a single CpG was capable of affecting glucocorticoid receptor gene expression. In human adrenocortical adenomas, methylation at one single CpG position in the *H19* promoter region is correlated with decreased H19 expression and increased IGF-II expression in adenomas compared with that in the normal adrenal glands (33). Taking

together, these data clearly indicated that differences in methylation status even at one or very few CpG sites, such as what we reported here between secretory and nonsecretory pituitary tumors, is sufficient to cause gene expression or silencing.

In summary, we have found specific loss of *MEG3* expression in clinically nonfunctioning pituitary adenomas. Thus, *MEG3* is the first gene identified whose loss of expression is restricted to clinically nonfunctioning pituitary adenomas, suggesting that it may play a critical role in control of tumor formation from this cell type. The increased CpG methylation within the IG-DMR in the *DLK1/MEG3* locus, especially at CpG positions 7 and 8, along with that in *MEG3* promoter and enhancer region as we have reported before, demonstrate an overall hypermethylation in the important regulatory regions of *MEG3* gene in human clinically nonfunctioning tumors. This increased methylation contributes to loss of expression and antiproliferative function of this noncoding RNA gene specifically in such tumors.

Acknowledgments

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