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Tumorigenesis and Neoplastic Progression

Silencing of the Imprinted *DLK1-MEG3* Locus in Human Clinically Nonfunctioning Pituitary Adenomas

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DLK1-MEG3* is an imprinted locus consisting of multiple maternally expressed noncoding RNA genes and paternally expressed protein-coding genes. The expression of maternally expressed gene 3 (*MEG3*) is selectively lost in clinically nonfunctioning adenomas (NFAs) of gonadotroph origin; however, expression status of other genes at this locus in human pituitary adenomas has not previously been reported. Using quantitative real-time RT-PCR, we evaluated expression of 24 genes from the *DLK1-MEG3* locus in 44 human pituitary adenomas (25 NFAs, 7 ACTH-secreting, 7 GH-secreting, and 5 PRL-secreting adenomas) and 10 normal pituitaries. The effects on cell proliferation of five miRNAs whose expression was lost in NFAs were investigated by flow cytometry analysis. We found that 18 genes, including 13 miRNAs at the *DLK1-MEG3* locus, were significantly down-regulated in human NFAs. In ACTH-secreting and PRL-secreting adenomas, 12 and 7 genes were significantly down-regulated, respectively; no genes were significantly down-regulated in GH-secreting tumors. One of the five miRNAs tested induced cell cycle arrest at the G2/M phase in PDFS cells derived from a human NFA. Our data indicate that the *DLK1-MEG3* locus is silenced in NFAs. The growth suppression by miRNAs in PDFS cells is consistent with the hypothesis that the *DLK1-MEG3* locus plays a tumor suppressor role in human NFAs.** ***(Am J Pathol 2011, 179:2120–2130; DOI: 10.1016/j.ajpath.2011.07.002)

Human pituitary adenomas account for approximately 15% of intracranial neoplasms.[1] They can be classified as clinically nonfunctioning or functioning adenomas, based on clinical phenotype and hormone hypersecretion. Clinically nonfunctioning adenomas (NFAs), mostly derived from gonadotroph cells, are often large and cause symptoms of mass effects such as visual disturbance, cranial nerve palsies, or headache. In contrast, hormone hypersecretion in functioning pituitary adenomas causes specific clinical syndromes. Human pituitary adenomas are monoclonal in origin,[2] indicating that genetic and/or epigenetic events play causal roles in development of these tumors. For example, mutations in the *GNAS1* gene are found in approximately 30% to 40% of GH-secreting tumors.[3] Silencing of *RB1* and *CDKN2A* genes by promoter hypermethylation is found in most human NFAs, although genetic mutations in either gene are rarely found.[4,5] We identified a novel noncoding RNA gene, maternally expressed gene 3 (*MEG3*), which is highly expressed in the normal pituitary. In NFAs of gonadotroph origin, however, its expression is selectively lost.[6,7] Loss of *MEG3* expression has also been found in certain brain tumors and in many human cancer cell lines.[8,9] Furthermore, MEG3 activates p53, selectively stimulates expression of p53 target genes, and inhibits cell proliferation *in vitro*.[10] In mice, deletion of *Gtl2*, the murine ortholog of *MEG3*, causes enhanced embryonic brain angiogenesis and perinatal death.[11,12] These data strongly suggest that *MEG3* functions as a novel noncoding RNA tumor suppressor gene and that it plays a critical role in NFA pathogenesis.

The *MEG3* gene belongs to the imprinted *DLK1-MEG3* locus located on human chromosome band 14q32. The mouse ortholog *DLK1-Gtl2* locus is located on distal chromosome 12. To date, at least 80 imprinted genes have been identified at this locus. Three are paternally expressed genes (PEGs): delta-like homolog 1 (*DLK1*), retrotransposon-like 1 (*RTL1*), and iodothyronine deiodinase type III (*DIO3*). All three are protein-coding genes. The remainder are maternally expressed genes (MEGs), which consist of large noncoding RNAs, including *MEG3*,

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MEG8, and *RTL1* antisense (*RTL1*as), along with many small RNAs. The latter belong to three small-RNA gene families: the C/D small nucleolar RNAs (snoRNAs), the microRNAs (miRNAs), and likely piwi-interacting RNAs.[13]

The microRNA cluster in the *DLK1-MEG3* locus, which is one of the largest miRNA clusters in humans, is organized into two smaller clusters: one is between *MEG3* and *MEG8* and contains approximately 9 miRNAs; the other consists of more than 40 miRNAs mapped downstream to the C/D snoRNA gene cluster.[13,14] These maternally expressed miRNAs are transcribed in the same orientation as *MEG3* and *MEG8*.[15] Imprinting of the *DLK1-MEG3* locus is regulated by an intergenic differentially methylated region (IG-DMR) located 13 kb upstream of the *MEG3* gene. The IG-DMR is hypermethylated on the paternal chromosome.[16,17] Imprinting of this locus plays an important role in growth and development. Loss of imprinting results in a phenotypic spectrum ranging from embryonic lethality to growth retardation and developmental abnormalities.[18,19] In addition, dysregulation of genes at this locus has been found in a subset of human tumors, such as renal cell carcinoma and neuroblastoma,[20,21] suggesting that the *DLK1-MEG3* locus plays a role in the development of a number of human neoplasms.

We have previously shown that methylation in the IG-DMR and the *MEG3* promoter region is increased in human NFAs, suggesting that genes in this locus also play a role in human pituitary tumor pathogenesis.[6,7] This is consistent with the finding that *MEG3* was not expressed in virtually all NFAs examined; however, little is known regarding expression of other imprinted genes in the *DLK1-MEG3* locus in NFAs. We therefore analyzed the expression levels of 21 MEGs and all known PEGs in primary NFAs and other human pituitary tumors types by quantitative real-time PCR. We found that 18 of them were significantly down-regulated in NFAs and 12 and 7 were down-regulated in ACTH-secreting and PRL-secreting tumors, respectively. In contrast, none of the genes examined were significantly down-regulated in GH-secreting tumors. Transfection of the significantly down-regulated miR-134 resulted in cell cycle arrest in PDFS cells, which are derived from a human clinically NFA. Our data are consistent with the hypothesis that silencing of the *DLK1-MEG3* locus plays an important role in human NFA pathogenesis.

Materials and Methods

Tissue and Tumor Samples

Tissues from 44 human pituitary adenomas were obtained during surgery at Massachusetts General Hospital (25 NFA, 7 somatotropin-secreting, 7 adrenocorticotropic hormone-secreting, and 5 prolactin-secreting tumors). Of the total, 40 were macroadenomas and 4 (all ACTH-secreting) were microadenomas. Tumor tissue not used for standard pathological examination was stored in Ambion RNAlater solution (Applied Biosystems, Austin, TX) at −20°C or snap-frozen and stored in liquid nitrogen until use. Immunohistochemical staining for follicle stimulating hormone subunit beta (FSHβ), luteinizing hormone subunit beta (LHβ), thyrotropin subunit beta (TSHβ), prolactin (PRL), somatotropin (GH), adrenocorticotropic hormone (ACTH), and glycoprotein hormone α-subunit was performed on formalin-fixed, paraffin-embedded sections. Immunohistochemical results are given in Table 1, and additional characteristics of nonfunctioning pituitary tumors (ie, patient age at tumor onset, tumor size, and proliferation index) are given in Table 2. The study was approved by the Partners institutional review board.

RNA Extraction and cDNA Synthesis

Total RNA was isolated using TRIzol reagent according to the manufacturer's instructions (Invitrogen, Carlsbad, CA). RNA quality was determined by two methods. The overall integrity of RNAs was determined using the Bioanalyzer platform (Agilent Technologies, Santa Clara, CA). Samples showing significant RNA degradation were excluded from further assays. We also evaluated the transcripts of internal controls, including *GAPDH* for large RNA and *SNORD44* for small RNA, by quantitative RT-PCR. We considered an RNA sample acceptable for further analysis if the cycle threshold counts (Ct values) for these controls were approximately 20 to 22. One microgram of total RNA was subjected to reverse transcription using a ProtoScript M-MuLV first-strand cDNA synthesis kit (New England Biolabs, Ipswich, MA). MicroRNA cDNA synthesis was performed with 1 μg of total RNA using an NCode VILO miRNA cDNA synthesis kit according to the manufacturer's protocol (Invitrogen, Carlsbad, CA).

Quantitative Real-Time RT-PCR

Expression of *MEG3* (NR_002766), *MEG8* (NR_024149), *DLK1* (NM_003836), RTL1 (NM_001134888), and *DIO3* (NM_001362) was quantified using TaqMan probes and TaqMan universal PCR master mix (Applied Biosystems, Foster City, CA). GAPDH was used as the internal control. Expression levels of miRNAs and snoRNAs were determined using an EXPRESS SYBR GreenER miRNA qRT-PCR kit (Invitrogen) according to the manufacturer's instructions. PCR reactions were performed using the NCode universal reverse primer (Invitrogen) in conjunction with specific forward primers for each miRNA. The PCR forward primer sequences were obtained using Invitrogen's NCode database (*http://escience.invitrogen.com/ncode*) or were custom-designed based on mature miRNA sequences (*http://www.mirbase.org*), as follows: hsa-miR-127-3p (MIMAT0000446), 5′-GATCCGTCTGAGCTTGGCT-3′; hsa-miR-134 (MIMAT0000447), 5′-TGTGACTGGTTGACCAGAGGGG-3′; hsa-miR-136 (MIMAT0000448), 5′-GCACTCCATTTGTTTTGATGTGGA-3′; hsa-miR-154 (MIMAT0000452), 5′-TAGGTTATCCGTGTTGCCTTCG-3′; hsa-miR-299-5p (MIMAT0002890), 5′-TGGTTTACCGTCCCACATACAT-3′; hsa-miR-323-3p (MIMAT0000755), 5′-CACATTACACGGTCGACCTCT-3′; hsa-miR-329 (MIMAT0001629), 5′-CAACACACCTGGTTAACCTCTTT-3′; hsa-miR-337-5p (MIMAT0004695),

Table 1. Clinical and Immunohistochemical Characteristics of 44 Human Pituitary Adenomas

Clinical diagnosis	Immunohistochemical status*						
	FSH	LH	TSH	α-Subunit	PRL	GH	ACTH
Nonfunctioning adenoma							
1	rare	−	rare	rare	−	−	1+
2	−	−	−	−	−	−	−
3	rare	−	−	rare	−	−	−
4	3+	−	−	1+	rare	−	−
5	3+	4+	3+	1+	−	−	−
6	1+	rare	rare	−	−	−	−
7	rare	−	−	rare	−	−	−
8	−	−	−	−	−	rare	−
9	−	rare	−	−	−	−	−
10	−	−	rare	−	−	−	rare
11	3+	4+	4+	rare	rare	−	−
12	2+	3+	1+	1+	rare	−	−
13	1+	1+	1+	rare	rare	rare	−
14	rare	2+	1+	−	rare	−	−
15	1+	rare	−	−	rare	−	−
16	1+	1+	1+	rare	−	−	−
17	1+	rare	1+	1+	−	−	−
18	4+	4+	−	rare	rare	−	−
19	rare	−	rare	rare	rare	−	rare
20	1+	1+	1+	1+	rare	−	−
21	3+	3+	1+	rare	1+	−	−
22	1+	1+	1+	1+	1+	−	−
23	−	−	−	−	rare	1+	−
24	−	−	−	−	−	−	−
25	−	−	−	−	−	−	−
Acromegaly							
26	ND	ND	ND	−	rare	3+	ND
27	rare	1+	−	−	2+	3+	1+
28	−	−	−	−	3+	4+	−
29	rare	rare	3+	3+	rare	4+	−
30	ND	ND	ND	2+	2+	4+	ND
31	ND	ND	ND	−	ND	4+	ND
32	ND	ND	ND	ND	2+	3+	ND
Prolactinoma							
33	−	−	−	−	4+	2+	−
34	−	−	−	−	4+	2+	−
35	−	−	−	−	4+	−	−
36	−	−	−	−	4+	−	−
37	−	−	−	−	4+	−	−
Cushing's disease							
38	rare	−	−	−	−	−	4+
39	ND	ND	ND	ND	−	ND	4+
40	ND	ND	ND	−	−	ND	4+
41	−	−	−	−	−	−	4+
42	ND	ND	ND	ND	rare	ND	4+
43	−	−	−	1+	rare	−	4+
44	−	−	−	−	1+	−	4+

*Immunostaining was graded by the number of positive cells: −, negative; rare, only a few cells are positive; 1+, 1% to 5% positivity; 2+, 6% to 20% positivity; 3+, 21% to 50% positivity; 4+, >50% positivity; ND, not done (staining was not performed).

ACTH, adrenocorticotropic hormone; FSH, follicle-stimulating hormone; GH, somatotropin (growth hormone); LH, luteinizing hormone; PRL, prolactin; TSH, thyrotropin (thyroid-stimulating hormone); α-subunit of glycoprotein hormones.

5′-GAACGGCTTCATACAGGAGTT-3′; hsa-miR-369-5p (MIMAT0001621, 5′-AGATCGACCGTGTTATATTCGC-3′; hsa-miR-369-3p (MIMAT0000721), 5′-CGGAATAATACATGGTTGATCTTT-3′; hsa-miR-370 (MIMAT0000722), 5′-CTGGGGTGGAACCTGGTAAAA-3′; hsa-miR-376c (MIMAT0000720), 5′-CAACATAGAGGAAATTCCACGT-3′; hsa-miR-377 (MIMAT0000730), 5′-GCATCACACAAAGGCAACTTTTGT-3′; hsa-miR-380 (MIMAT0000735), 5′-CCGTATGTAATATGGTCCACATCTT-3′; hsa-miR-410(MIMAT0002171), 5′-GCGAATATAACACAGATGGCCTGT-3′; hsa-miR-431 (MIMAT0001625), 5′-CAGGCCGTCATGCAAAA-3′; hsa-miR-432 (MIMAT0002814), 5′-TGGAGTAGGTCATTGGGTGG-3′; hsa-miR-433 (MIMAT0001627), 5′-GATGGGCTCCTCGGTGTAAA-3′; and hsa-miR-770-5p (MIMAT0003948), 5′-CCCTGACACGTGGTACTGGAAA-3′; SNORD113-5 (NR_003233.1), 5′-GGATCAATGATGAGTATTGGTGGA-3′. Housekeeping genes *SNORD44* and *SNORD48* were used as internal controls. SNORD44 and SNORD48 are members of a group of snoRNAs that function as guide RNAs in the site-specific ribose methylation of pre-rRNA.[22] Both snoRNAs have been widely used as internal controls for quantitative RT-PCR.[23–27] The forward primers were as follows: SNORD44 (NR_002750.2), 5′-CCTGGATGATGATAGCAAATGCTG-3′; and SNORD48 (NR_002745.1), 5′-AGTGATGATGACCCCAGGTAACT-3′.

Table 2. Clinical Characteristics of 25 Human Clinically Nonfunctioning Pituitary Adenomas

No.	Sex	Age at onset (years)	Tumor size (cm)	Ki-67 (%)
1	M	50	2.2	1.7
2	M	63	3.7	<1
3	M	88	3	<1
4	M	65	2.5	<1
5	F	74	2.1	2.1
6	F	60	2	<1
7	M	60	2.5	2.6
8	F	66	2.5	<1
9	M	52	2.8	1.7
10	M	46	3	3.6
11	M	68	2.3	2.2
12	M	59	1.8	1.5
13	M	53	2	<1
14	F	67	1.6	<1
15	M	50	1.6	<1
16	F	69	1.8	<1
17	M	37	5	3.2
18	M	55	1.5	<1
19	M	53	2	1.7
20	M	48	2.1	2.5
21	F	58	2.7	1.5
22	M	65	3	3.6
23	M	59	3	3.9
24	M	80	1.5	ND
25	M	82	3.1	<1

F, female; M, male; ND, not done (analysis not performed).

PCR efficiency was tested on each primer pair using the 10-fold serial dilution method. The Ct values were plotted against the log of cDNA dilution. The amplification rate was calculated based on a linear regression slope using the equation $m = -(1/\log E)$, where m is the slope and E is the efficiency. In all primer sets, efficiencies ranged between 1.8 and 2.2, which is considered optimal.[28] All PCR reactions were done in triplicate and were repeated once on an Applied Biosystems 7500 fast thermocycler with the following parameters: 50°C for 2 minutes, 95°C for 10 minutes, and 40 cycles of 95°C for 15 seconds and 60°C for 1 minute. Melting curve analysis was performed and a no-template control was included in every qPCR using an Invitrogen SYBR PCR master mix.

Expression levels for each tested gene in normal pituitaries and tumors were determined using the formula $2^{-\Delta Ct}$, where $\Delta Ct = Ct_{\text{tested gene}} - Ct_{\text{internal control}}$. The relative tumor expression levels for each gene were calculated by normalizing the value from the tumor against the mean average of values from the 10 normal pituitaries. The statistical significance of gene expression between NFAs, functioning adenomas, and normal pituitary was assessed by a two-tailed Student's *t*-test with unequal variance and multiple range test. $P < 0.05$ was considered significant.

Double Immunofluorescence Staining

Normal anterior pituitaries were fixed in formalin and embedded in paraffin. Sections were cut at 5-μm thickness. Detection of DLK1 and FSHβ in the same sections was performed by double immunofluorescence staining, as described previously,[6] using antibodies against human DLK1 protein (Santa Cruz Biotechnologies, Santa Cruz, CA). The antibody against FSHβ was obtained from Dr. A.F. Parlow, National Hormone and Peptide Program, NIH-National Institute of Diabetes and Digestive and Kidney Diseases.

MicroRNA Transfection and Cell Cycle Analysis

The human pituitary-derived cell line (PDFS) was maintained as described previously[29] and plated into six-well plates in triplicate 24 hours before transfection. The cells were transfected with a miRIDIAN miRNA mimic of hsa-miR-134 (MIMAT0000447), hsa-miR-136 (MIMAT0000448), hsa-miR-154 (MIMAT0000452), hsa-miR-299-5p (MIMAT0002890), and hsa-miR-369-5p (MIMAT0001621) (Thermo Scientific-Dharmacon, Lafayette, CO) using a DharmaFECT 1 transfection reagent according to manufacturer's instructions. Negative control wells were transfected with miRIDIAN microRNA mimic negative control no. 1. FAM-labeled Pre-miR negative control no. 1 (Ambion, Austin, TX) was used to determine transfection efficiency. Final concentrations of all synthetic miRNAs were 25 nmol/L in a 2-mL final transfection volume. At 48 hours after transfection, cells were harvested, fixed in 70% ice-cold ethanol, and stained with propidium iodide. Cell cycle analyses were performed at the Flow Cytometry Core, Massachusetts General Hospital. All experiments were repeated three times.

Statistical Analysis

Expression-level data are reported as means ± SD, except as otherwise specified. Group comparisons were made using a two-sided independent samples *t*-test. Associations between loci were examined by Pearson's correlation coefficient. A P value of <0.05 was interpreted as significant.

Results

Expression of Maternally Expressed Genes in the DLK1-MEG3 *Locus in Human Pituitary Adenomas*

Over 80 genes have been identified in the *DLK1-MEG3* locus (Figure 1).[14,19] Of these, three are paternally expressed protein-coding genes (*DLK1*, *RTL1*, and *DIO3);* all the others are maternally expressed large noncoding RNAs, snoRNAs, and miRNAs. Using quantitative real-time PCR, we investigated the expression of these genes in NFAs and three functioning human pituitary adenoma types. Almost no expression of *MEG3* is detected in clinically NFAs (Figure 2A), in contrast to clinically functioning tumors. *MEG8* is a large noncoding RNA, located is approximately 33 kb downstream of *MEG3*. Its expression is lost in NFAs and significantly reduced in ACTH-secreting tumors; in both PRL-se-

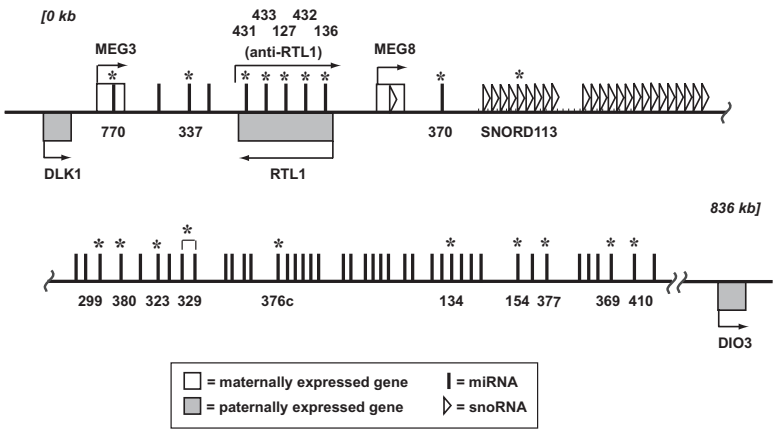

Figure 1. The *DLK1-MEG3* imprinting locus on human chromosome band 14q32. *MEG3*, *MEG8*, snoRNAs, and miRNAs are maternally expressed noncoding RNAs transcribed in the direction from 5′ to 3′. *DLK1*, *RTL1*, and *DIO3* are paternally expressed protein-coding genes transcribed in either direction, as indicated by **arrows**. The SnoRNA and miRNA IDs indicated by names or numbers and marked with **asterisks** were subjected to test by quantitative RT-PCR. The length and distance are not depicted to scale.

creting and GH-secreting tumors, however, its expression is comparable to that in normal pituitary (Figure 2B). Similarly, expression of *SNORD113*, a snoRNA located approximately 80 kb downstream of *MEG3*, is significantly reduced in both NFAs and ACTH-secreting tumors, but not in PRL-secreting and GH-secreting tumors (Figure 2C).

More than 50 miRNAs have been identified in the *DLK1-MEG3* locus. Of these, 18 were examined for their expression (Table 3). These 18 miRNAs were chosen mainly on the basis of their locations, spanning the entire locus (Figure 1). We found that NFAs had 13 miRNAs whose expression was lost or significantly diminished versus normal pituitary (Table 3). ACTH-secreting and PRL-secreting tumors had 9 and 7 significantly down-regulated miRNAs, respectively; no miRNAs were down-regulated in GH-secreting tumors. In contrast, the GH-secreting tumors had 12 significantly up-regulated miRNAs, but NFAs and ACTH-secreting tumors had only 2 and 1 significantly up-regulated miRNAs, respectively, and no miRNA was significantly up-regulated in PRL-secreting tumors. Among the significantly down-regulated miRNAs, the detected expression levels generally remained significantly higher in functioning tumors (ACTH-secreting and PRL-secreting tumors) than in NFAs. For example, levels of, miR-299-5p, miR-377, and miR-433 were significantly higher in ACTH-secreting tumors versus NFA (Table 3). Levels of miR-154, miR-329, and miR-376c in NFAs were significantly higher in PRL-secreting tumors, compared with their levels in NFAs (Table 3). The expression pattern of miRNAs was highly similar between ACTH-secreting tumors and PRL-secreting tumors. In 90% of miRNAs examined, there was no significant difference in expression levels between these two types of tumors (Table 3).

The NFA group consists of 25 tumors, which are derived from patients with specific clinical and pathological characteristics (Table 2). To determine whether gene expression in these tumors is influenced by age of diagnosis, tumor size, or Ki-67 staining, we analyzed the relationship between expression levels of individual genes in NFAs and these characteristics. We found that the expression level of miR-134 is positively correlated with patient age at tumor diagnosis ($r = 0.430$, $P = 0.0318$), and that of miR-337 is positively correlated with Ki-67 index ($r = 0.441$, $P = 0.0311$); however, no significant correlations were found between the rest of the genes and tumor size, proliferation status (as assessed by Ki-67 staining), or age at diagnosis.

All maternally expressed genes in the *DLK1-MEG3* locus are transcribed in the same orientation. The *MEG3* gene is upstream of all other MEGs. We have previously shown that deletion of the *Gtl2* gene and a small portion of its upstream promoter in mice resulted in silencing of all downstream maternally expressed genes,[12] suggest-

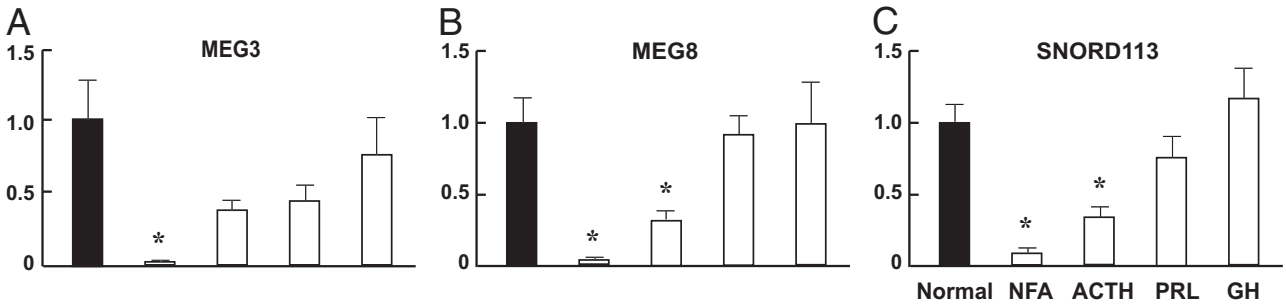

Figure 2. Expression of *MEG3* (**A**), *MEG8* (**B**), and *SNORD113* (**C**) in human pituitary adenomas. Transcripts were detected by quantitative real-time PCR, as described under *Materials and Methods*. Values from normal pituitaries were designated as 1, against which values from pituitary tumors were normalized. Data were obtained using 10 normal pituitaries, 25 NFAs, 7 GH-secreting, 5 PRL-secreting, and 7 ACTH-secreting tumors. Data are reported as means ± SEM. Student's *t*-test was used to compare values between tumors and the normal pituitaries. $^*P < 0.05$.

Table 3. Expression Levels of miRNAs in Human Pituitary Adenomas

	Expression level (mean ± SD) and significance*			
miRNA	NFA	GH	PRL	ACTH
miR-127-3p	0.559 ± 0.569	1.767 ± 1.279	0.482 ± 0.22	0.348 ± 0.143
versus normal	$P = 0.06$	$P = 0.178$	$P = 0.028$	$P = 0.006$
versus NFA		$P = 0.054$	$P = 0.417$	$P = 0.048$
miR-134	0.047 ± 0.190	5.281 ± 2.712	1.44 ± 0.794	0.951 ± 0.926
versus normal	$P < 0.001$	$P = 0.006$	$P = 0.289$	$P = 0.896$
versus NFA		$P = 0.002$	$P = 0.017$	$P = 0.041$
miR-136	0.002 ± 0.01	1.492 ± 0.884	0.932 ± 0.972	0.138 ± 0.26
versus normal	$P < 0.001$	$P = 0.195$	$P = 0.885$	$P < 0.001$
versus NFA		$P = 0.005$	$P = 0.108$	$P = 0.348$
miR-154	0.087 ± 0.140	2.163 ± 0.706	0.512 ± 0.125	0.309 ± 0.217
versus normal	$P < 0.001$	$P = 0.004$	$P < 0.001$	$P < 0.001$
versus NFA		$P < 0.001$	$P < 0.001$	$P = 0.057$
miR-299-5p	0.048 ± 0.127	4.692 ± 2.359	1.003 ± 0.215	0.492 ± 0.295
versus normal	$P < 0.001$	$P = 0.006$	$P = 0.993$	$P = 0.003$
versus NFA		$P = 0.002$	$P < 0.001$	$P = 0.012$
miR-323-3p	0.029 ± 0.111	2.345 ± 1.108	0.737 ± 0.576	0.828 ± 0.591
versus normal	$P < 0.001$	$P = 0.018$	$P = 0.415$	$P = 0.543$
versus NFA		$P = 0.002$	$P = 0.068$	$P = 0.017$
miR-329	0.041 ± 0.090	2.422 ± 0.944	0.57 ± 0.262	0.5 ± 0.296
versus normal	$P < 0.001$	$P = 0.006$	$P = 0.02$	$P = 0.006$
versus NFA		$P = 0.001$	$P = 0.012$	$P = 0.009$
miR-337	1.356 ± 1.234	4.262 ± 4.593	0.26 ± 0.582	0.395 ± 0.68
versus normal	$P = 0.212$	$P = 0.109$	$P = 0.043$	$P = 0.061$
versus NFA		$P = 0.166$	$P = 0.005$	$P = 0.007$
miR-369-5p	0.047 ± 0.071	2.328 ± 0.707	0.254 ± 0.352	0.29 ± 0.32
versus normal	$P < 0.001$	$P = 0.002$	$P = 0.005$	$P < 0.001$
versus NFA		$P < 0.001$	$P = 0.314$	$P = 0.123$
miR-369-3p	0.08 ± 0.188	2.107 ± 1.273	0.747 ± 0.57	0.127 ± 0.17
versus normal	$P < 0.001$	$P = 0.062$	$P = 0.394$	$P < 0.001$
versus NFA		$P = 0.006$	$P = 0.061$	$P = 0.635$
miR-370	0.290 ± 0.270	4.004 ± 2.353	0.707 ± 0.182	0.676 ± 0.278
versus normal	$P < 0.001$	$P = 0.015$	$P = 0.074$	$P = 0.068$
versus NFA		$P = 0.006$	$P = 0.004$	$P = 0.016$
miR-376c	0.039 ± 0.102	2.382 ± 0.931	0.442 ± 0.27	0.271 ± 0.245
versus normal	$P < 0.001$	$P = 0.007$	$P = 0.013$	$P = 0.001$
versus NFA		$P = 0.001$	$P = 0.033$	$P = 0.064$
miR-377	0.067 ± 0.132	4.058 ± 1.403	1.072 ± 0.334	0.619 ± 0.291
versus normal	$P < 0.001$	$P = 0.001$	$P = 0.699$	$P = 0.023$
versus NFA		$P < 0.001$	$P = 0.002$	$P = 0.002$
miR-380	1.496 ± 1.174	3.095 ± 2.5	1.101 ± .556	0.806 ± 0.315
versus normal	$P = 0.119$	$P = 0.069$	$P = 0.754$	$P = 0.399$
versus NFA		$P = 0.187$	$P = 0.148$	$P = 0.007$
miR-410	0.036 ± 0.174	4.285 ± 1.936	1.122 ± 1.208	1.104 ± 0.835
versus normal	$P < 0.001$	$P = 0.004$	$P = 0.835$	$P = 0.767$
versus NFA		$P = 0.001$	$P = 0.134$	$P = 0.019$
miR-431	2.441 ± 2.240	6.157 ± 3.569	1.585 ± 1.055	1.876 ± 0.833
versus normal	$P = 0.007$	$P = 0.009$	$P = 0.291$	$P = 0.033$
versus NFA		$P = 0.042$	$P = 0.135$	$P = 0.186$
miR-432	0.067 ± 0.198	4.247 ± 1.582	1.253 ± 0.734	1.213 ± 0.671
versus normal	$P < 0.001$	$P = 0.001$	$P = 0.511$	$P = 0.486$
versus NFA		$P < 0.001$	$P = 0.029$	$P = 0.006$
miR-433	0.065 ± 0.098	0.979 ± 0.985	0.402 ± 0.331	0.539 ± 0.309
versus normal	$P < 0.001$	$P = 0.958$	$P = 0.011$	$P = 0.007$
versus NFA		$P = 0.061$	$P = 0.131$	$P = 0.011$
miR-770-5p	3 ± 3.704	2.95 ± 2.301	0.984 ± 0.67	1.248 ± 1.027
versus normal	$P = 0.018$	$P = 0.067$	$P = 0.963$	$P = 0.566$
versus NFA		$P = 0.928$	$P = 0.013$	$P = 0.035$

*Values from normal pituitaries were designated as 1, against which values from pituitary tumors were normalized. Data were obtained using 10 normal pituitaries, 25 NFAs, and 7 GH-secreting, 5 PRL-secreting, and 7 ACTH-secreting tumors. *P* values were obtained from two-tailed Student's *t*-test with unequal variance.

ing a possible dependence of downstream MEG expression on *MEG3*. We therefore analyzed the relationship between *MEG3* expression and its downstream MEGs in pituitary tumors and normal pituitary tissue using their expression data obtained by quantitative real-time PCR. We found that expression of 14/20 MEGs significantly correlated with *MEG3* expression (Table 4). The two genes whose expression was highly correlated with *MEG3* expression were *MEG8* ($r = 0.863$) and *SNORD113* ($r = 0.642$).

Table 4. Correlation between Expression of *MEG3* and Other Maternally Expressed Genes in the *DLK1-MEG3* Locus in Normal Pituitary and in Pituitary Adenomas

Gene symbol*	Pearson's *r*	*P* value
MEG8	0.863	<0.001
SNORD113-5	0.642	<0.001
MIR369-5p	0.533	<0.001
MIR376c	0.522	<0.001
MIR323-3p	0.463	<0.001
MIR329	0.463	<0.001
MIR154	0.453	<0.001
MIR369-3p	0.439	<0.001
MIR377	0.436	0.001
MIR433	0.424	0.001
MIR136	0.410	0.002
MIR370	0.378	0.005
MIR432	0.377	0.005
MIR410	0.332	0.014
MIR299-5p	0.28	0.040
MIR134	0.259	0.058
MIR127-3p	0.175	0.206
MIR337	0.053	0.701
MIR431	−0.050	0.722
MIR380	−0.113	0.418
MIR770-5p	−0.189	0.170

Associations between *MEG3* expression levels and the rest of the maternally expressed genes were examined by Pearson's correlation coefficient. A *P* value of <0.05 was interpreted as significant. (n = 54).

*For miRNA genes, -3p and -5p denote miRNAs derived from the 3′ arm and the 5′ arm of the precursor miRNA, respectively.

Expression of Paternally Expressed Genes in the DLK1-MEG3 *Locus In Human Pituitary Adenomas*

The expression of three known paternally expressed genes in the *DLK1-MEG3* locus (ie, *DLK1*, *RTL1*, and *DIO3*) was examined by quantitative real-time PCR. We found no significant change in *DIO3* expression in either NFAs or functioning tumors. *RTL1* expression was significantly down-regulated in NFAs, but not in any functioning tumors. *DLK1* expression was reduced 2500-fold in NFAs, compared with normal pituitary (Figure 3A). *DLK1* expression was also significantly reduced in ACTH-secreting tumors, but not in GH-secreting or PRL-secreting tumors (Figure 3A). The finding that *DLK1* was virtually undetectable in all NFAs examined raised the possibility that *DLK1* is not expressed in normal gonadotroph cells, from which most NFAs were derived. We therefore performed double immunofluorescence staining on normal human anterior pituitaries using antibodies against DLK1 and FSHβ to determine the *DLK1* expression status in normal gonadotroph cells. *DLK1* was readily detected in FSH-expressing cells, indicating that gonadotroph cells normally express *DLK1* (Figure 3B).

Ectopic Expression of miR-134 Caused Cell Cycle Arrest in Pituitary Tumor Cells

Of the miRNAs examined, 70% were significantly down-regulated in NFAs, suggesting that loss of expression of these miRNAs in this locus plays a role in development of these tumors. We therefore selected five miRNAs (miR-134, miR-136, miR-154, miR-299-5p, and miR-369-5p) to test this possibility. These five miRNAs were selected because all five were significantly down-regulated and detected at very low expression levels among all of the miRNAs tested in NFAs and because these miRNAs were not detected in PDFS cells (data not shown), which are derived from a human NFA.[29] Individual synthetic miR-

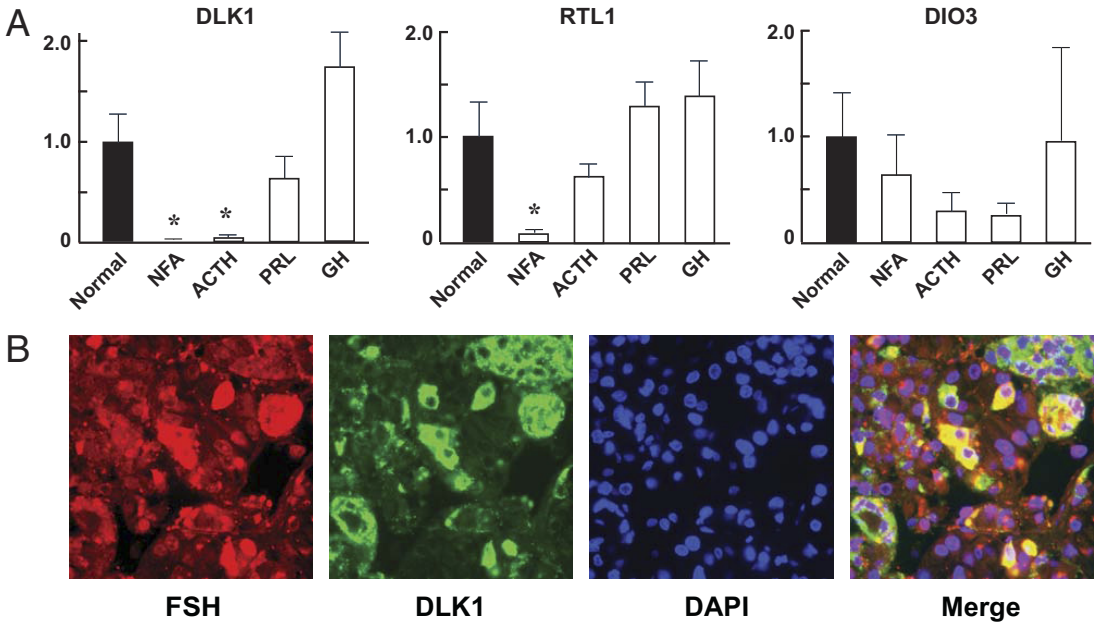

Figure 3. Paternally expressed genes. **A:** Expression of *DLK1*, *RTL1*, and *DIO3* in clinically NFA, ACTH-secreting, PRL-secreting, and GH-secreting pituitary adenomas was detected by quantitative real-time PCR as described under *Materials and Methods*. Values from normal pituitaries were designated as 1, against which values from pituitary tumors were normalized. Data are reported as means ± SEM. Student's *t*-test was used to compare values between tumors and the normal pituitaries. *$P < 0.05$. **B:** Coexpression of DLK1 and FSHβ in normal human anterior pituitary. Sections of normal pituitaries were immunostained with antibodies against DLK1 and FSHβ, and nuclei were stained by DAPI. The merged image demonstrates the co-localization (yellow) of DLK1 and FSHβ in normal pituitary cells. Original magnification, 400×.

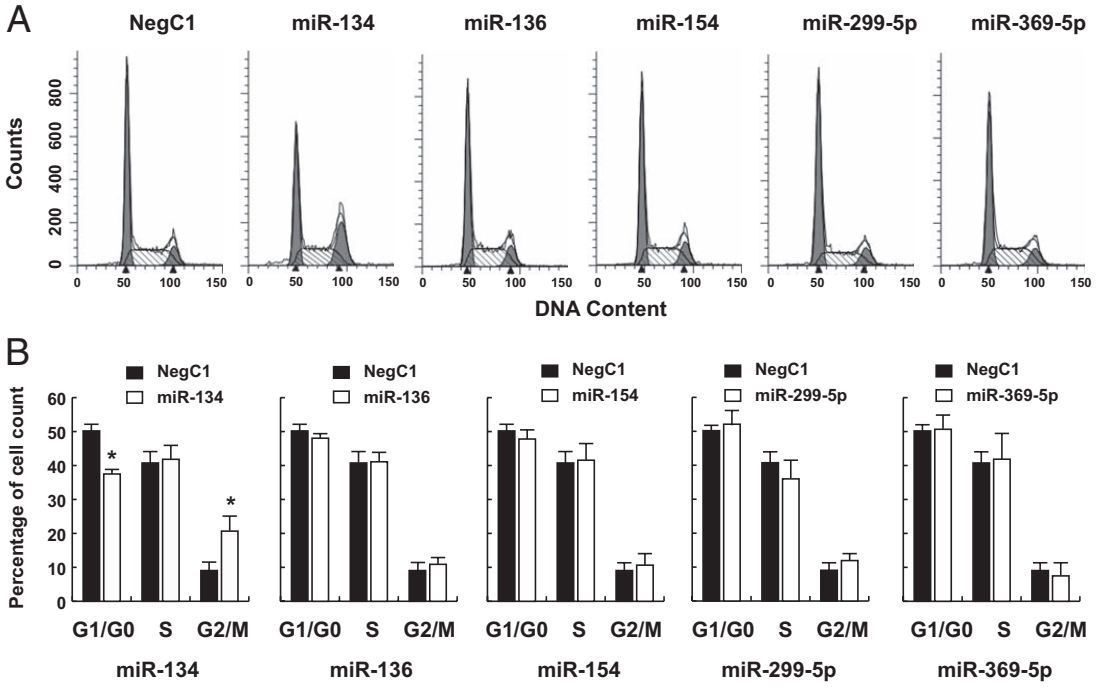

Figure 4. Cell cycle arrest induced by miRNA. PDFS cells were transfected with synthetic miRIDIAN microRNA mimic negative control no. 1 (NegC1), miR-134, miR-136, miR-154, miR-299-5p, and miR-369-5p. At 48 hours after transfection, cells were harvested, fixed, stained with propidium iodide, and subjected to cell cycle analysis. **A:** Representative histograms of flow cytometry analysis for PDFS cells transfected with synthetic miRNAs. **B:** Percentages of cells in each phase of the cell cycle were calculated using data from at least three independent experiments (mean ± SD). Student's *t*-test was used to compare values between cells transfected with miRNAs and the negative control. $^*P < 0.05$.

NAs of miR-134, miR-136, miR-154, miR-299-5p, and miR-369-5p were transfected into PDFS cells and cell cycle analysis was performed using these transfected cells. Transfection of miR-134 significantly decreased the G0/G1 cell population and increased the G2/M population in PDFS cells by 33% ($P = 0.002$) and 128% ($P = 0.014$), respectively, compared with transfection of the negative control (Figure 4). In contrast, transfection of miR-136, miR-154, miR-299-5p, and miR-369-5p did not affect the cell cycle profiles in PDFS cells, compared with transfection of a nonspecific negative control miRNA (Figure 4).

Discussion

The *DLK1-MEG3* locus consists of multiple maternally expressed and paternally expressed genes whose imbalanced expression has been implicated in a number of human malignancies. *MEG3*, the first noncoding RNA identified at this locus, is selectively lost in human clinically NFA,[6] and its ectopic expression activates p53[10] and inhibits cell proliferation *in vitro*.[9,10] These data indicate that loss of MEG3 noncoding RNA plays an important role in the pathogenesis of human NFAs. We found that, in clinically NFAs, expression of 75% of maternally expressed and paternally expressed genes examined was virtually abolished. Although a significant percentage of down-regulated genes was also found in ACTH-secreting and PRL-secreting tumors, their expression levels were significantly higher than their expression in NFAs. In contrast, none of the genes examined were significantly down-regulated in GH tumors, but 50% of the genes were significantly up-regulated. These data indicate that the *DLK1-MEG3* locus is silenced exclusively in human NFAs and suggest that the silencing of this locus contributes to NFA development.

Emerging evidence indicates that noncoding RNAs play an important role in the pathogenesis of many human neoplasms. Noncoding RNAs, such as miRNAs, may function as tumor suppressors. For example, loss of miR-15a and miR-16-1 expression contributes to the development of chronic lymphocytic leukemia.[30,31] Various miRNAs may also function as oncogenes. Up-regulation of miR-21 occurs in several types of solid tumors and leukemia[32–34] and may promote tumor growth by targeting tumor suppressive genes, including *PTEN*, *PDCD4*, and *TPM1*.[35–37] The possible involvement of miRNAs in human pituitary tumors has also been reported. Stilling et al[38] profiled miRNA expression using a microarray technique and found that 188 miRNAs are up-regulated and 160 miRNAs are down-regulated in corticotroph adenomas, compared with normal pituitaries. Bottoni et al[39] reported that expression of miR-15a and of miR-16-1 is significantly reduced in both corticotroph adenomas and prolactinomas. Amaral et al[25] also reported that several miRNAs, including let-7a, miR-15a, miR-16, miR-21, miR-141, miR-143, miR-145, and miR-150, are significantly down-regulated in corticotropinomas compared with normal pituitary.

We examined expression of 18 miRNAs within the *DLK1-MEG3* locus in normal pituitaries and pituitary tumors. Expression of miR-134, miR-323, miR-370, miR-

410, and miR-432 were significantly down-regulated in NFAs but not in functioning tumors, suggesting that these miRNAs play a role in the pathogenesis of clinically NFAs. The mechanism whereby loss of expression of these miRNAs contributes to the development of NFAs is unknown. One possibility we investigated is the involvement of these miRNAs in cell cycle regulation. Guo et al[40] reported that miR-134 induced cell cycle arrest in the lung cancer cell line H68AR. In accord, we found that miR-134 arrested the cell cycle at the G2/M phase in PDFS cells. The PDFS line has characteristics of folliculostellate cells. Normal folliculostellate cells account for up to 10% of cell population of the anterior pituitary and are known to be part of pituitary tumors. The PDFS cell line was established because of spontaneous transformation of human NFA tumor cells, which suggests that these cells have acquired additional genetic or epigenetic mutations during cell culture, making the cell line phenotypically aggressive. Given that miR-134 is capable of inhibiting proliferation of PDFS in culture, it is highly likely that miR-134 can suppress proliferation of other cell types in the pituitary.

Other miRNAs at the *DLK1-MEG3* locus whose expression was also significantly down-regulated in NFAs may also play a role in suppression of NFA development. For example, miR-377 expression was found to be significantly elevated in senescent human fibroblast WI-38 cells,[41] and miR-369 was elevated in senescing mesenchymal stem cells,[42] suggesting that these miRNAs are involved in regulating cellular senescence, which is one of the most important tumor suppression mechanisms. Luo et al[43] reported that miR-433 was down-regulated in human gastric cancers. It is thought that miR-433 may suppress tumor growth by regulating its target, GRB2,[43] which is a crucial adaptor protein mediating growth signaling to the cell.[44] We also observed that two miRNAs (miR-431 and miR-770) were significantly up-regulated in NFAs. Little is known about their functions, however, and whether their high expression in NFAs plays a role in promoting tumor growth remains unknown.

The imprinting of the *DLK1-MEG3* locus is controlled by the IG-DMR. Deletion of this region in mice caused complete silencing of all maternally expressed genes and activation of paternally expressed genes.[16] The *MEG3* gene also plays a pivotal role in imprinting regulation. We previously reported that deletion of the *Gtl2* gene and a small portion of its upstream promoter in mice silences MEGs and activates PEGs, such as *DLK1*.[12] Expression of *DLK1* was increased twofold in mice with deletion of the IG-DMR or the *Gtl2* gene. In human NFAs, expression of *MEG3* is virtually completely abolished. Surprisingly, *DLK1* expression was completely lost in all NFAs examined.

DLK1 (synonym: Pref-1) functions as an inhibitor of adipocyte differentiation.[45] Its role in tumor development is not clear; the function of *DLK1* is cell-type dependent, acting either as an oncogene or as a tumor suppressor. Expression of *DLK1* is elevated in gliomas, and ectopic expression of *DLK1* stimulates glioblastoma cell line proliferation.[46] *DLK1* can also be activated by PTTG and is thought to mediate inhibition of differentiation by PTTG.[47] Kim et al[48] reported that high levels of *DLK1* expression inhibits differentiation and enhances tumorigenic potential in tumor cells. In contrast, Li et al[49] reported that expression of *DLK1* resulted in inhibition of proliferation in hematopoietic cells. Furthermore, Kawakami et al[21] demonstrated that *DLK1* expression is lost in human renal cell carcinomas and that its re-expression increases anchorage-independent cell death and suppresses tumor growth in nude mice. The role of *DLK1* in human pituitary tumor development requires further investigation.

The IG-DMR and the *MEG3* promoter may be the positive control elements regulating expression of *MEG3* and all its downstream MEGs.[16] It has been hypothesized that the maternally expressed genes are derived from one giant transcript initiated from the *MEG3* gene. We have previously reported that methylation in both the IG-DMR and the *MEG3* promoter was significantly increased in human clinically NFAs.[6,7] This increased methylation may, at least in part, contribute to the silencing of *MEG3* in these tumors. In agreement with this hypothesis, 12 downstream MEGs (including the large noncoding RNA *MEG8*, snoRNA *SNORD113*, and 10 miRNAs) were expressed at similar levels as the *MEG3* gene in NFAs. Not all MEGs are expressed in parallel with the *MEG3* gene. The MEG miR-770, for example, which is located in an intron of the *MEG3* gene, was up-regulated by threefold in NFAs, compared with normal pituitaries, although *MEG3* expression was virtually silenced in these tumors. In GH-secreting tumors, 12 miRNAs were up-regulated by more than twofold, compared with normal pituitaries, even though *MEG3* expression in these tumors was unchanged. These data suggest that these MEGs are also controlled by gene-specific regulatory elements, independent of the *MEG3* gene. For example, Song and Wang[50,51] showed that expression of miR-127, miR-134, miR-154, miR-329, miR-337, and miR-433 were increased by 40-, 12-, 38-, 9-, 15-, and 4-fold, respectively, in the orphan nuclear receptor short heterodimer partner (SHP)-null mice, compared with wild-type controls, and have cloned promoters controlling miR-127 and miR-433. We have shown that the IG-DMR and the *MEG3* promoter regions were not fully methylated in NFAs, although overall methylation was significantly increased,[6,7] suggesting the possibility that the IG-DMR and the *MEG3* promoter remain partially active in these tumors. Thus, another possible cause for the varied levels of MEG expression detected in pituitary tumors is post-transcriptional regulation. This may be the case, especially in functioning tumors, in which *MEG3* expression was not significantly changed; however, the expression levels for individual MEGs vary significantly compared with normal pituitaries. We note that these two possible mechanisms are not mutually exclusive.

Expression of the *DLK1-MEG3* locus was mostly silenced in human NFAs, but not in functioning adenomas. The silenced genes include *MEG3* and many of its downstream miRNAs. Our previous studies indicated that *MEG3* itself may function as a tumor suppressor. Because *MEG3* is required for expression of its downstream maternally expressed genes, it is possible that loss of *MEG3* expression is an initiation event in NFA develop-

ment. However, data from the present study suggest that the maternally expressed miRNAs are capable of tumor suppression. This raises the question of whether loss of *MEG3* alone is sufficient to cause NFAs, or whether loss of other MEGs including miRNAs is also required for NFA development. Future studies, particularly using mouse models, are needed to determine the specific role of *MEG3* and its downstream genes in pituitary tumor development. Our data are consistent with the hypothesis that silencing of the *DLK1-MEG3* locus plays an important role in the development of human NFAs. The tumor suppressor function of this locus in NFAs is at least in part attributed to the anti-proliferation function of several genes within the locus, including *MEG3* and *MIR134* (miR-134).

References

1. Lloyd RV, Kovacs K, Young WF Jr, Farrell WE, Asa SL, Trouillas J, Kontogeorgos G, Sano T, Scheithauer BW, Horvath E: Tumours of the pituitary. Pathology and Genetics of Tumours of Endocrine Organs, ed 3. WHO Classification of Tumours 8. Edited by RA DeLellis, RV Lloyd, PU Heitz, C Eng. Lyon, IARC Press, 2004, pp 9–39
2. Alexander JM, Biller BM, Bikkal H, Zervas NT, Arnold A, Klibanski A: Clinically nonfunctioning pituitary tumors are monoclonal in origin. J Clin Invest 1990, 86:336–340
3. Pertuit M, Barlier A, Enjalbert A, Gérard C: Signalling pathway alterations in pituitary adenomas: involvement of Gsalpha, cAMP and mitogen-activated protein kinases. J Neuroendocrinol 2009, 21:869–877
4. Simpson DJ, Bicknell JE, McNicol AM, Clayton RN, Farrell WE: Hypermethylation of the p16/CDKN2A/MTSI gene and loss of protein expression is associated with nonfunctional pituitary adenomas but not somatotrophinomas. Genes Chromosomes Cancer 1999, 24:328–336
5. Simpson DJ, Hibberts NA, McNicol AM, Clayton RN, Farrell WE: Loss of pRb expression in pituitary adenomas is associated with methylation of the RB1 CpG island. Cancer Res 2000, 60:1211–1216
6. Gejman R, Batista DL, Zhong Y, Zhou Y, Zhang X, Swearingen B, Stratakis CA, Hedley-Whyte ET, Klibanski A: Selective loss of MEG3 expression and intergenic differentially methylated region hypermethylation in the MEG3/DLK1 locus in human clinically nonfunctioning pituitary adenomas. J Clin Endocrinol Metab 2008, 93:4119–4125
7. Zhao J, Dahle D, Zhou Y, Zhang X, Klibanski A: Hypermethylation of the promoter region is associated with the loss of MEG3 gene expression in human pituitary tumors. J Clin Endocrinol Metab 2005, 90:2179–2186
8. Zhang X, Gejman R, Mahta A, Zhong Y, Rice KA, Zhou Y, Cheunsuchon P, Louis DN, Klibanski A: Maternally expressed gene 3, an imprinted noncoding RNA gene, is associated with meningioma pathogenesis and progression. Cancer Res 2010, 70:2350–2358
9. Zhang X, Zhou Y, Mehta KR, Danila DC, Scolavino S, Johnson SR, Klibanski A: A pituitary-derived MEG3 isoform functions as a growth suppressor in tumor cells. J Clin Endocrinol Metab 2003, 88:5119–5126
10. Zhou Y, Zhong Y, Wang Y, Zhang X, Batista DL, Gejman R, Ansell PJ, Zhao J, Weng C, Klibanski A: Activation of p53 by MEG3 non-coding RNA. J Biol Chem 2007, 282:24731–24742
11. Gordon FE, Nutt CL, Cheunsuchon P, Nakayama Y, Provencher KA, Rice KA, Zhou Y, Zhang X, Klibanski A: Increased expression of angiogenic genes in the brains of mouse Meg3-null embryos. Endocrinology 2010, 151:2443–2452
12. Zhou Y, Cheunsuchon P, Nakayama Y, Lawlor MW, Zhong Y, Rice KA, Zhang L, Zhang X, Gordon FE, Lidov HG, Bronson RT, Klibanski A: Activation of paternally expressed genes and perinatal death caused by deletion of the Gtl2 gene. Development 2010, 137:2643–2652
13. Royo H, Cavaillé J: Non-coding RNAs in imprinted gene clusters. Biol Cell 2008, 100:149–166
14. Kircher M, Bock C, Paulsen M: Structural conservation versus functional divergence of maternally expressed microRNAs in the Dlk1/Gtl2 imprinting region. BMC Genomics 2008, 9:346
15. Seitz H, Royo H, Bortolin ML, Lin SP, Ferguson-Smith AC, Cavaille J: A large imprinted microRNA gene cluster at the mouse DLK1-Gtl2 domain. Genome Res 2004, 14:1741–1748
16. Lin SP, Youngson N, Takada S, Seitz H, Reik W, Paulsen M, Cavaille J, Ferguson-Smith AC: Asymmetric regulation of imprinting on the maternal and paternal chromosomes at the DLK1-Gtl2 imprinted cluster on mouse chromosome 12. Nat Genet 2003, 35:97–102
17. Murphy SK, Wylie AA, Coveler KJ, Cotter PD, Papenhausen PR, Sutton VR, Shaffer LG, Jirtle RL: Epigenetic detection of human chromosome 14 uniparental disomy. Hum Mutat 2003, 22:92–97
18. Ogata T, Kagami M, Ferguson-Smith AC: Molecular mechanisms regulating phenotypic outcome in paternal and maternal uniparental disomy for chromosome 14. Epigenetics 2008, 3:181–187
19. da Rocha ST, Edwards CA, Ito M, Ogata T, Ferguson-Smith AC: Genomic imprinting at the mammalian Dlk1-Dio3 domain. Trends Genet 2008, 24:306–316
20. Astuti D, Latif F, Wagner K, Gentle D, Cooper WN, Catchpoole D, Grundy R, Ferguson-Smith AC, Maher ER: Epigenetic alteration at the DLK1-GTL2 imprinted domain in human neoplasia: analysis of neuroblastoma, phaeochromocytoma and Wilms' tumour. Br J Cancer 2005, 92:1574–1580
21. Kawakami T, Chano T, Minami K, Okabe H, Okada Y, Okamoto K: Imprinted DLK1 is a putative tumor suppressor gene and inactivated by epimutation at the region upstream of GTL2 in human renal cell carcinoma. Hum Mol Genet 2006, 15:821–830
22. Kiss-László Z, Henry Y, Bachellerie JP, Caizergues-Ferrer M, Kiss T: Site-specific ribose methylation of preribosomal RNA: a novel function for small nucleolar RNAs. Cell 1996, 85:1077–1088
23. Poliseno L, Salmena L, Zhang J, Carver B, Haveman WJ, Pandolfi PP: A coding-independent function of gene and pseudogene mRNAs regulates tumour biology. Nature 2010, 465:1033–1038
24. Mees ST, Mardin WA, Wendel C, Baeumer N, Willscher E, Senninger N, Schleicher C, Colombo-Benkmann M, Haier J: EP300–A miRNA-regulated metastasis suppressor gene in ductal adenocarcinomas of the pancreas. Int J Cancer 2010, 126:114–124
25. Amaral FC, Torres N, Saggioro F, Neder L, Machado HR, Silva WA Jr, Moreira AC, Castro M: MicroRNAs differentially expressed in ACTH-secreting pituitary tumors. J Clin Endocrinol Metab 2009, 94:320–323
26. Schuster C, Budczies J, Faber C, Kirchner T, Hlubek F: MicroRNA expression profiling of specific cells in complex archival tissue stained by immunohistochemistry. Lab Invest 2011, 91:157–165
27. Pallasch CP, Patz M, Park YJ, Hagist S, Eggle D, Claus R, Debey-Pascher S, Schulz A, Frenzel LP, Claasen J, Kutsch N, Krause G, Mayr C, Rosenwald A, Plass C, Schultze JL, Hallek M, Wendtner CM: miRNA deregulation by epigenetic silencing disrupts suppression of the oncogene PLAG1 in chronic lymphocytic leukemia. Blood 2009, 114:3255–3264
28. Schmittgen TD, Livak KJ: Analyzing real-time PCR data by the comparative C(T) method. Nat Protoc 2008, 3:1101–1108
29. Danila DC, Zhang X, Zhou Y, Dickersin GR, Fletcher JA, Hedley-Whyte ET, Selig MK, Johnson SR, Klibanski A: A human pituitary tumor-derived folliculostellate cell line. J Clin Endocrinol Metab 2000, 85:1180–1187
30. Calin GA, Cimmino A, Fabbri M, Ferracin M, Wojcik SE, Shimizu M, Taccioli C, Zanesi N, Garzon R, Aqeilan RI, Alder H, Volinia S, Rassenti L, Liu X, Liu CG, Kipps TJ, Negrini M, Croce CM: MiR-15a and miR-16-1 cluster functions in human leukemia. Proc Natl Acad Sci USA 2008, 105:5166–5171
31. Cimmino A, Calin GA, Fabbri M, Iorio MV, Ferracin M, Shimizu M, Wojcik SE, Aqeilan RI, Zupo S, Dono M, Rassenti L, Alder H, Volinia S, Liu CG, Kipps TJ, Negrini M, Croce CM: miR-15 and miR-16 induce apoptosis by targeting BCL2 [Erratum appeared in Proc Natl Acad Sci U S A 2006, 103:2464]. Proc Natl Acad Sci USA 2005, 102:13944–13949
32. Volinia S, Calin GA, Liu CG, Ambs S, Cimmino A, Petrocca F, Visone R, Iorio M, Roldo C, Ferracin M, Prueitt RL, Yanaihara N, Lanza G, Scarpa A, Vecchione A, Negrini M, Harris CC, Croce CM: A microRNA expression signature of human solid tumors defines cancer gene targets. Proc Natl Acad Sci USA 2006, 103:2257–2261
33. Calin GA, Ferracin M, Cimmino A, Di Leva G, Shimizu M, Wojcik SE, Iorio MV, Visone R, Sever NI, Fabbri M, Iuliano R, Palumbo T, Pichiorri

F, Roldo C, Garzon R, Sevignani C, Rassenti L, Alder H, Volinia S, Liu CG, Kipps TJ, Negrini M, Croce CM: A MicroRNA signature associated with prognosis and progression in chronic lymphocytic leukemia [Erratum appeared in N Engl J Med 2006, 355:533]. N Engl J Med 2005, 353:1793–1801
34. Garzon R, Volinia S, Liu CG, Fernandez-Cymering C, Palumbo T, Pichiorri F, Fabbri M, Coombes K, Alder H, Nakamura T, Flomenberg N, Marcucci G, Calin GA, Kornblau SM, Kantarjian H, Bloomfield CD, Andreeff M, Croce CM: MicroRNA signatures associated with cytogenetics and prognosis in acute myeloid leukemia. Blood 2008, 111:3183–3189
35. Frankel LB, Christoffersen NR, Jacobsen A, Lindow M, Krogh A, Lund AH: Programmed cell death 4 (PDCD4) is an important functional target of the microRNA miR-21 in breast cancer cells. J Biol Chem 2008, 283:1026–1033
36. Meng F, Henson R, Wehbe-Janek H, Ghoshal K, Jacob ST, Patel T: MicroRNA-21 regulates expression of the PTEN tumor suppressor gene in human hepatocellular cancer. Gastroenterology 2007, 133: 647–658
37. Zhu S, Si ML, Wu H, Mo YY: MicroRNA-21 targets the tumor suppressor gene tropomyosin 1 (TPM1). J Biol Chem 2007, 282:14328–14336
38. Stilling G, Sun Z, Zhang S, Jin L, Righi A, Kovacs G, Korbonits M, Scheithauer BW, Kovacs K, Lloyd RV: MicroRNA expression in ACTH-producing pituitary tumors: up-regulation of microRNA-122 and -493 in pituitary carcinomas. Endocrine 2010, 38:67–75
39. Bottoni A, Piccin D, Tagliati F, Luchin A, Zatelli MC, degli Uberti EC: miR-15a and miR-16-1 down-regulation in pituitary adenomas. J Cell Physiol 2005, 204:280–285
40. Guo L, Liu Y, Bai Y, Sun Y, Xiao F, Guo Y: Gene expression profiling of drug-resistant small cell lung cancer cells by combining microRNA and cDNA expression analysis. Eur J Cancer 2010, 46:1692–1702
41. Maes OC, Sarojini H, Wang E: Stepwise up-regulation of microRNA expression levels from replicating to reversible and irreversible growth arrest states in WI-38 human fibroblasts. J Cell Physiol 2009, 221:109–119
42. Wagner W, Horn P, Castoldi M, Diehlmann A, Bork S, Saffrich R, Benes V, Blake J, Pfister S, Eckstein V, Ho AD: Replicative senescence of mesenchymal stem cells: a continuous and organized process. PLoS One 2008, 3:e2213
43. Luo H, Zhang H, Zhang Z, Zhang X, Ning B, Guo J, Nie N, Liu B, Wu X: Down-regulated miR-9 and miR-433 in human gastric carcinoma. J Exp Clin Cancer Res 2009, 28:82
44. Tari AM, Lopez-Berestein G: GRB2: a pivotal protein in signal transduction. Semin Oncol 2001, 28:142–147
45. Smas CM, Sul HS: Pref-1, a protein containing EGF-like repeats, inhibits adipocyte differentiation. Cell 1993, 73:725–734
46. Yin D, Xie D, Sakajiri S, Miller CW, Zhu H, Popoviciu ML, Said JW, Black KL, Koeffler HP: DLK1: increased expression in gliomas and associated with oncogenic activities. Oncogene 2006, 25:1852–1861
47. Espina AG, Méndez-Vidal C, Moreno-Mateos MA, Sáez C, Romero-Franco A, Japón MA, Pintor-Toro JA: Induction of Dlk1 by PTTG1 inhibits adipocyte differentiation and correlates with malignant transformation. Mol Biol Cell 2009, 20:3353–3362
48. Kim Y, Lin Q, Zelterman D, Yun Z: Hypoxia-regulated delta-like 1 homologue enhances cancer cell stemness and tumorigenicity. Cancer Res 2009, 69:9271–9280
49. Li L, Forman SJ, Bhatia R: Expression of DLK1 in hematopoietic cells results in inhibition of differentiation and proliferation. Oncogene 2005, 24:4472–4476
50. Song G, Wang L: MiR-433 and miR-127 arise from independent overlapping primary transcripts encoded by the miR-433-127 locus. PLoS One 2008, 3:e3574
51. Song G, Wang L: Transcriptional mechanism for the paired miR-433 and miR-127 genes by nuclear receptors SHP and ERRgamma. Nucleic Acids Res 2008, 36:5727–5735