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Maternally Expressed Gene 3, an Imprinted Noncoding RNA Gene, Is Associated with Meningioma Pathogenesis and Progression

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Abstract

Meningiomas are common tumors, representing 15% to 25% of all central nervous system tumors. *NF2* gene inactivation on chromosome 22 has been shown as an early event in tumorigenesis; however, few factors underlying tumor growth and progression have been identified. The chromosomal abnormalities of 14q32 are often associated with meningioma pathogenesis and progression; therefore, it has been proposed that an as yet unidentified tumor suppressor is present at this locus. *Maternally expressed gene 3* (*MEG3*) is an imprinted gene located at 14q32 which encodes a noncoding RNA with an antiproliferative function. We found that MEG3 mRNA is highly expressed in normal arachnoidal cells. However, *MEG3* is not expressed in the majority of human meningiomas or the human meningioma cell lines IOMM-Lee and CH157-MN. There is a strong association between loss of *MEG3* expression and tumor grade. Allelic loss at the *MEG3* locus is also observed in meningiomas, with increasing prevalence in higher grade tumors. In addition, there is an increase in CpG methylation within the promoter and the imprinting control region of *MEG3* gene in meningiomas. Functionally, MEG3 suppresses DNA synthesis in both IOMM-Lee and CH157-MN cells by ~60% in bromodeoxyuridine incorporation assays. Colony-forming efficiency assays show that MEG3 inhibits colony formation in CH157-MN cells by ~80%. Furthermore, MEG3 stimulates p53-mediated transactivation in these cell lines. Therefore, these data are consistent with the hypothesis that *MEG3*, which encodes a noncoding RNA, may be a tumor suppressor gene at chromosome 14q32 involved in meningioma progression via a novel mechanism. *Cancer Res; 70(6); 2350–8. ©2010 AACR.*

Introduction

Meningiomas arise from the arachnoidal cells of the leptomeninges covering the brain and spinal cord, and account for 15% to 25% of all central nervous system tumors (1). Most meningiomas are slow-growing and considered benign (WHO grade 1). However, a subset of grade 1 meningiomas could recur, leading to the compression of critical anatomic structures and clinically significant impairment of neurologic function. Less than 20% of cases are classified as WHO grade 2 (atypical meningioma) or WHO grade 3 (anaplastic/malignant meningioma), and these exhibit more aggressive clinical behavior and have a higher risk of recurrence with increased morbidity and mortality (1).

Cytogenetic studies have revealed several chromosomal abnormalities in meningiomas, with losses of 22q, 1p, and 14q being most common. The inactivation of the *NF2* gene at 22q12 has been identified as an early event in meningioma pathogenesis, but not associated with tumor progression (2). In contrast, abnormalities of chromosome 14, including 14q32, have been reported more frequently in higher-grade (WHO grades 2 and 3) as well as recurrent meningiomas (3–6). Therefore, it has been suggested that gene inactivation in this particular region is associated with progression of meningiomas from lower to higher grade, and may also be associated with tumor recurrence. However, relevant genes of interest in this region have not been discovered.

Maternally expressed gene 3 (*MEG3*) is an imprinted gene with maternal expression which encodes a noncoding RNA. We have shown that MEG3 RNA expression is lost in the majority of clinically nonfunctioning human pituitary tumors and other cancer cell lines, it also suppresses cancer cell growth, stimulates p53-mediated transcriptional activation, and selectively activates p53 target genes (7, 8). *MEG3* is highly expressed in the normal human brain (7). Because *MEG3* is located at 14q32, a region in which chromosomal abnormalities are associated with meningioma progression, we hypothesized that *MEG3* may represent a novel meningioma suppressor gene in this region. In this study, we report the progressive loss of *MEG3* expression in human

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meningiomas and inhibition of meningioma cell proliferation by MEG3.

Materials and Methods

Samples. Human meningioma samples were obtained from surgery and snap-frozen at −80°C. Matched whole blood samples were collected from each patient. Tumors were classified and graded according to the WHO grading system (1). Normal human brain and meningeal samples were obtained from the Harvard Brain Tissue Resource Center (Belmont, MA) and the Pathology Service at Massachusetts General Hospital. This study was approved by the Partners HealthCare Institutional Review Board.

In situ *hybridization.* Samples from normal human arachnoid tissue (including arachnoidal granulations) and human meningiomas were fixed in 4% paraformaldehyde for 3 to 4 h, rinsed with PBS, sectioned (5 μm) with a cryostat, and stored at −80°C. *In situ* hybridization was performed as previously described (7), using MEG3 sense or antisense probes.

RNA extraction and reverse transcription-PCR. Total RNA was extracted from 46 human meningiomas (16 grade 1, 18 grade 2, and 12 grade 3) and the human meningioma cell lines IOMM-Lee and CH157-NM (obtained from Dr. D. H. Gutmann, Washington University School of Medicine, St. Louis, MO; we did not test these cell lines), using TRIzol Reagent (Invitrogen). Normal meningeal RNA samples were purchased from BioChain and Analytical Biological Services, or extracted from normal meningeal samples (see Samples, above). Reverse transcription-PCR (RT-PCR) was performed as previously described (9), using MEG3-specific primers as well as glyceraldehyde-3-phosphate dehydrogenase–specific primers as a control (sequences available upon request). RT reactions performed in the absence of reverse transcriptase were used as negative controls. Quantitative RT-PCR using TaqMan probes (Applied Biosystems) was performed as previously described (10).

Genomic DNA preparation. Tumor DNA was extracted from 27 snap-frozen meningioma samples using the DNeasy Tissue Kit (Qiagen). In addition, DNA samples from 27 corresponding peripheral blood leukocytes from the same patients were isolated using Puregene DNA extraction kit (Gentra Systems). Following DNA extraction, samples were amplified with the Whole Genome Amplification Kit (Sigma-Aldrich). Normal meningeal genomic DNA samples were either purchased from BioChain and Analytical Biological Services or extracted from normal meningeal samples.

Copy number analysis for chromosomal loss. Quantitative real-time PCR was used to quantify gene copy number. The starting relative copy number DNA at each locus in a tumor sample was given by the formula $2^{-\Delta\Delta CT}$, where $\Delta\Delta CT = C_T^{(\text{tumor} - \text{reference})}$ minus $C_T^{(\text{normal} - \text{reference})}$ (11). *RNase P*, a housekeeping gene, was used as the reference gene. This gene has only one copy per haploid cell and was amplified in parallel with experimental samples for normalizing the results to allow relative quantification analysis. The normal DNA extracted from peripheral blood leukocytes from the same patient was designated as 1.0 by this equation, and all other samples were calculated in relation

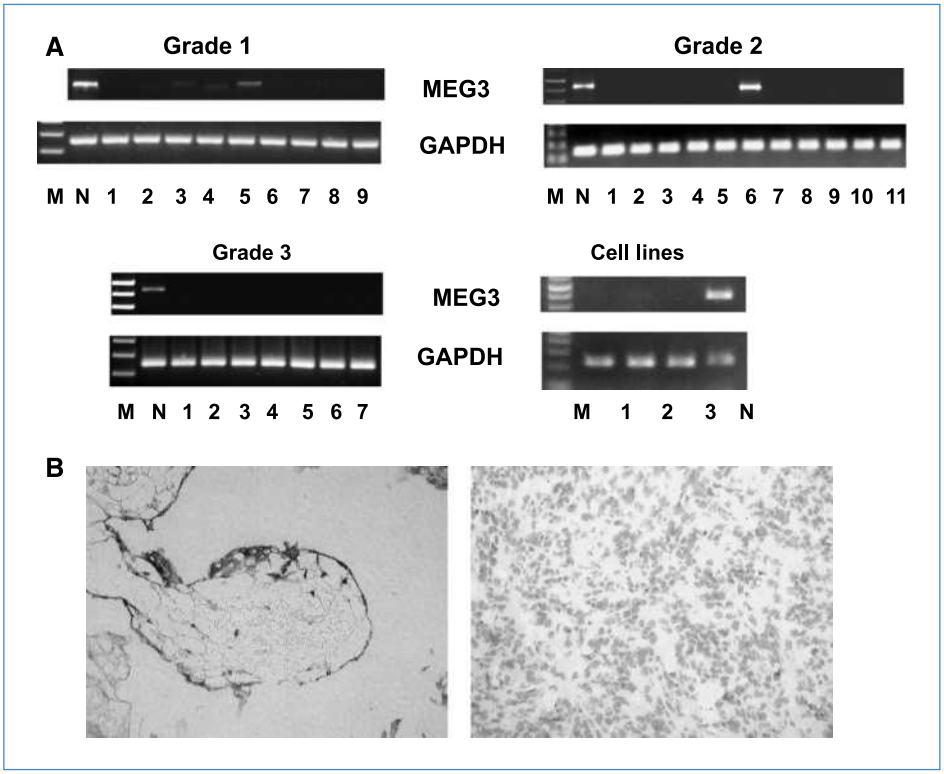

Figure 1. MEG3 RNA is expressed in normal human meninges but not in the majority of human meningiomas. A, RT-PCR readily detected MEG3 RNA in normal human meningeal (lane N), but only in 3 of 9 typical meningiomas (top, left: lanes 3, 4, and 5) and in 1 of 11 atypical meningiomas (top, right: lane 6). None of the seven anaplastic meningiomas (bottom, left) or tumor cell lines (bottom, right: lane 1, IOMM-Lee; lane 2, CH157-MN; lane 3, a pituitary tumor–derived cell line PDFS) expressed MEG3 RNA as examined by RT-PCR. M, molecular weight marker. B, *in situ* hybridization shows that MEG3 RNA is present in the arachnoidal cells of human meningeal samples (left), but no MEG3 RNA was detected in one typical meningioma sample (tumor no. 5 in A). Nuclei were stained by hematoxylin in the tumor slide.

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Table 1. MEG3 RNA expression, gene copy loss, and methylation in promoter (R1), enhancer (R4), and imprinting control region (IG-DMR) in meningiomas

Samples	MEG3 level (%)*	Loss of MEG3 allele	R1 methylation (%)	Mean ± SD	R4 methylation (%)	Mean ± SD	IG-DMR methylation (%)	Mean ± SD
Normal								
NM1†	100	—‡	7	6.0 ± 1.41	26	17.06 ± 12.72	42	50.06.0 ± 1.0
NM2	100	—	5		8		59	
Grade 1								
1-1	UD	No	6	15.4 ± 27.0	71	69.2 ± 17.4	48	56.4 ± 9.9
1-2	0.97	No	90		87		59	
1-3	2.75	No	22		87		74	
1-4	3.17	No	6		43		56	
1-5	7.80	No	3		49		56	
1-6	UD	No	3		85		41	
1-7	0.42	No	1		55		56	
1-8	UD	No	1		89		70	
1-9	UD	No	7		57		48	
1-10	UD	—	—		—		—	
1-11	2.75	—	—		—		—	
1-12	0.23	—	—		—		—	
1-13	4.80	—	—		—		—	
1-14	3.13	—	—		—		—	
1-15	UD	—	—		—		—	
1-16	UD	—	—		—		—	
Grade 2								
2-1	UD	Yes	15	14.4 ± 5.6	57	43.6 ± 13.1		61.0 ± 4.8
2-2	UD	Yes	—		—		62	
2-3	0.33	No	13		48		—	
2-4	UD	No	11		52		71	
2-5	UD	No	—				59	
2-6	161	No	21		42			
2-7	0.39	No	22		34		53	
2-8	0.35	No	11		55		60	
2-9	UD	No	18		47		58	
2-10	UD	No	4		14		64	
2-11	UD	Yes	—		—		61	
2-12	0.13	—	—		—		—	
1-13	UD	—	—		—		—	
2-14	UD	—	—		—		—	
2-15	0.16	—	—		—		—	
2-16	UD	—	—		—		—	
2-17	0.22	—	—		—		—	
2-18	UD	—	—		—		—	
Grade 3								
3-1	UD	Yes	—	27.0 ± 21.26	—	58.3 ± 19.3	—	68.8 ± 5.1
3-2	UD	Yes	—		—		—	
3-3	UD	Yes	58		87		67	
3-4	UD	No	22		45		76	
3-5	UD	No	10		37		73	
3-6	UD	Yes	—		—		—	
3-7	UD	No	18		64		63	
3-8	UD	—	—		—		—	

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Table 1. MEG3 RNA expression, gene copy loss, and methylation in promoter (R1), enhancer (R4), and imprinting control region (IG-DMR) in meningiomas (Cont'd)

Samples	MEG3 level (%)*	Loss of MEG3 allele	R1 methylation (%)	Mean ± SD	R4 methylation (%)	Mean ± SD	IG-DMR methylation (%)	Mean ± SD
3-9	1.09	—	—		—		—	
3-10	UD	—	—		—		—	
3-11	UD	—	—		—		—	
3-12	UD	—	—		—		—	

*Shown as the percentage of the average level in the normal tissue. The average level in the normal tissue, obtained from six normal meningeal samples, is set as 100%. UD, undetectable.
†NM, normal meningeal sample.
‡—, not examined.

to this value. For the ΔΔCT to be valid, the efficiencies of the reference and target should be approximately equal. A calibration curve was constructed using serial dilutions of template DNA (198,000 pg/μL to 19.8 pg/μL) and the plot of log input amount versus ΔCT ($target^{probe}$ – $reference^{RNase\ P}$) had a slope of <0.1 for each primer probe. PCR amplification efficiencies (E) were determined according to the equation: $E = 10^{(-1/slope)}$. The efficiency for each primer probe was: 1.96 for *RNase P*, 2.00 for *DLK1*, 1.97 for *D14S119*; $r = -1.00$. Quantitative real-time PCR was performed using a 25 μL working master mix containing: 50 ng of the template DNA in 1× TaqMan Universal Master Mix (Applied Biosystems), a 200 nmol/L final concentration of the primers, and the probe (FAM labeled; Applied Biosystems). The reaction was run in a SmartCycler II (Cephid, thermal cycler), using the following cycling parameters: 50°C for 2 min, 95°C for 10 min, 40 cycles of 95°C (denature) for 15 s with 60°C for 1 min (annealing extension). The sequence of the genomic probes and primers that mapped to region 14q32.1 to 14q32.3 (*D14S831* and *D14S1006* for *DLK1, WI-16835* for *MEG3*, and *D14S119*) were obtained from the genome databases. Sequences of primers and TaqMan probes are available on request. Single copy loss was considered to be present in tumors in which the highest value of the SD was <1 (12).

Methylation analysis of genomic DNA. Genomic DNA from six grade 1, eight grade 2, four grade 3 human meningiomas, or from two normal human meningeal samples was treated with sodium bisulfite using the MethylDetector Bisulfite Modification Kit (Active Motif). PCR amplification of treated DNA at *MEG3* promoter (R1) and enhancer (R4), and imprinting control (IG-DMR) region, and the cloning of PCR products were performed as previously described (9, 10). Ten to 20 clones from each PCR product were examined by sequencing. The percentage of methylation at each particular CpG site among these 10 to 20 clones was recorded; then the percentage of methylation at each CpG site within the genomic region was averaged. Therefore, the data represent the overall percentage of methylated CpG sites within a particular genomic region. 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.

Expression vectors. For the bromodeoxyuridine (BrdU) incorporation assay, MEG3 and DLK1 cDNA were cloned into a pCMS-d2EGFP vector, which expresses both destabilized green fluorescent protein (d2EGFP) and MEG3 or DLK1 cDNA. For the colony formation assay and transient transfections and luciferase assays, MEG3, DLK1, and GADD45γ cDNA were cloned into a pCI-neo vector (Promega). Other plasmids used in luciferase assays include p53-Luc (Stratagene) and pCMVβ (BD Clontech).

Cell culture, transfection, and luciferase assay. Human meningioma-derived cell lines IOMM-Lee and CH157-NM were cultured in DMEM supplemented with 10% heat-inactivated fetal bovine serum and penicillin/streptomycin. Cells were transfected with Mirus TransIT-LT1 reagent (Mirus Corp.) as previously described (13). For luciferase assays, cells in 12-well plates were transfected with plasmid DNAs containing 50 ng of p53-Luc, 0.2 μg of pCMVβ, and 50 ng of pCI-neo-MEG3 as indicated. Cells were lysed and luciferase activities were measured as previously described (13). The luciferase activity was normalized against the β-galactosidase activity from the same well. Each experiment was repeated at least four times. Statistical analysis was performed using a t test.

Growth suppression assays. Growth suppression of meningioma cell lines IOMM-Lee and CH157-NM by MEG3 was measured by BrdU incorporation assay and colony formation assay, as previously described (8, 14). Each experiment was repeated at least three times. Statistical analysis was performed using a t test.

5-Aza-2′-deoxycytidine treatment. CH157-MN cells were seeded in 100 mm cell culture dishes and cultured in medium containing 5 μmol/L of 5-aza-2′-deoxycytidine (Sigma-Aldrich) or vehicle for 5 d. The culture medium was changed and fresh agent added daily. RNA extraction and RT-PCR for MEG3 and glyceraldehyde-3-phosphate dehydrogenase RNA was performed as previously described (9).

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Western blot. Cells were lysed with radioimmune precipitation assay buffer to obtain total protein and Western blotting was performed as previously described (13). The blot was probed with antibody DO-1 (Santa Cruz Biotechnology) to detect p53 protein.

Results

MEG3 expression in normal human arachnoidal cells, meningiomas, and meningioma cell lines. We first examined *MEG3* expression in normal human meningeal cells, meningiomas, and meningioma cell lines. MEG3 mRNA was readily detected by RT-PCR in all nine normal human meningeal samples (see Fig. 1A, lane N, for representative samples). However, MEG3 mRNA was present only in 3 of 9 grade 1 (Fig. 1A, top left, lanes 3, 4, and 5) and 1 of 11 grade 2 (Fig. 1A, top right, lane 6) meningiomas. None of the seven grade 3 meningiomas examined expressed MEG3 mRNA (Fig. 1A, bottom left). The difference in *MEG3* expression between normal and combined tumor samples was significant (normal versus all tumors, $P < 0.0001$; normal versus grade 1 tumors, $P = 0.0294$; normal versus grade 2 tumors, $P < 0.0001$; normal versus grade 3 tumors, $P < 0.0001$; grade 1 versus combined grade 2/3, $P = 0.0297$) using Fisher's exact two-tail test.

No MEG3 mRNA was detected in the human meningioma-derived cell lines IOMM-Lee and CH157-MN (Fig. 1A, bottom right, lanes 1 and 2). Using *in situ* hybridization, we observed that MEG3 mRNA was abundantly present in the arachnoidal cells (Fig. 1B, left). In contrast, no MEG3 mRNA was detected by *in situ* hybridization in several grade 1 meningiomas, including tumor no. 5 (Fig. 1B, right); this tumor showed positive MEG3 mRNA expression by RT-PCR (Fig. 1A, top left, lane 5), suggesting that even if MEG3 mRNA is expressed in some tumors, its expression levels are low compared with that in the normal samples.

Quantitative RT-PCR was performed to assess the relative *MEG3* expression levels in meningiomas compared with that in the normal human meningeal samples. In addition to the 27 samples used for the regular RT-PCR shown in Fig. 1, 19 additional meningioma samples were included (7 grade 1, 7 grade 2, and 5 grade 3). The relative MEG3 RNA expression level in each tumor was compared with the average level of MEG3 RNA determined from six normal human meningeal samples (Table 1). Among 16 grade 1 tumors, quantitative RT-PCR detected MEG3 RNA in nine tumors, ranging from only 0.23% to 7.8% of the average MEG3 RNA level in normal tissues. In the 18 grade 2 tumors, MEG3 RNA was detectable at low levels in 6 tumor samples, ranging from 0.13% to 0.39% of the average MEG3 RNA level in the normal tissues. Only one grade 2 tumor expressed a level of MEG3 RNA comparable to normal tissue. In the 12 grade 3 tumors, MEG3 RNA was detected in only one sample, at a level of ~1% of that in the normal tissue (Table 1). Overall, *MEG3* is expressed in normal arachnoidal cells but is expressed at low levels in some grade 1 meningiomas and is absent in the majority of grade 2 and almost all grade 3 meningiomas.

Copy number loss at the MEG3 locus in meningiomas. We next performed copy number analysis to determine whether there is a *MEG3* gene loss in meningiomas. Four markers were analyzed: *D14S831* located at 14q32.1; *D14S1006* located at 14q32.2, within the *DLK1* gene; *WI-16835* located at 14q32.2/3, within the *MEG3* gene; and *D14S119* at 14q32.3. As summarized in Table 1, copy number loss between 14q32.1 and 14q32.3, including the *MEG3* gene locus, was found in 3 of 10 grade 2 and 4 of 7 grade 3 meningiomas. No copy number loss at this region was found in any grade 1 meningioma. For those tumors with copy number loss at 14q32, we also analyzed another marker located at 14q12. No copy number loss was detected at 14q12 in any tumors (data not shown). Therefore, there is specific loss at 14q32, containing the *MEG3* gene, in these higher-grade meningiomas. Notably, none of the tumors with *MEG3* gene copy number loss express MEG3 RNA (as determined by RT-PCR).

Genomic DNA methylation in the promoter, enhancer, and imprinting control region of* MEG3 *gene. The status of CpG methylation in the promoter (R1), enhancer (R4), and IG-DMR region of the *MEG3* gene was examined in six grade 1, eight grade 2, four grade 3 human meningiomas, and two normal human meningeal samples. These functional regions have been described in our previous publications (9, 10). In two normal human meningeal samples, the percentage of methylated CpGs in the promoter R1 region was very low (6.0 ± 1.41, mean ± SD). There is an increase in the degree of CpG methylation in this region in tumors (15.4 ± 27 for grade 1, $P = 0.1769$, compared with that in normal tissue; 14.4 ± 5.6 for grade 2, $P = 0.0037$; and 27.0 ± 18.4 for grade 3, $P = 0.0712$; Table 1).

For the R4 region with enhancer activity, ~17% of CpG dinucleotides are methylated in the normal human meningeal samples (17 ± 12.72). The percentage of CpG methylation in the tumors is 69.2 ± 17.4 for grade 1 ($P = 0.0187$, compared with that in normal tissue), 43.63 ± 13.1 for grade 2 ($P = 0.073$), and 58.3 ± 19.3 for grade 3 ($P = 0.02553$; Table 1).

For the imprinting controlling IG-DMR, methylation was found in ~50% of the CpG dinucleotides in the normal meningeal samples (5 0 ± 12%). There was a statistically significant increase in methylation in the tumors (56.4 ± 9.9% for grade 1, 61.0 ± 4.8% for grade 2, and 69.8 ± 5.1% for grade 3). The degree of methylation significantly correlated with tumor grade (one-way ANOVA test, $P = 0.038$; Table 1).

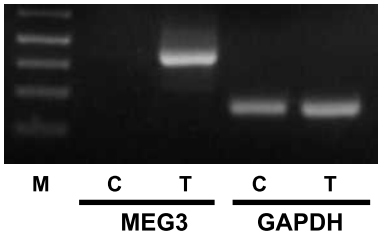

Figure 2. 5-Aza-2′-deoxycytidine treatment results in MEG3 RNA expression in CH157-MN cells as examined by RT-PCR. M, molecular marker; C, vehicle treatment control; T, 5-aza-2′-deoxycytidine treatment.

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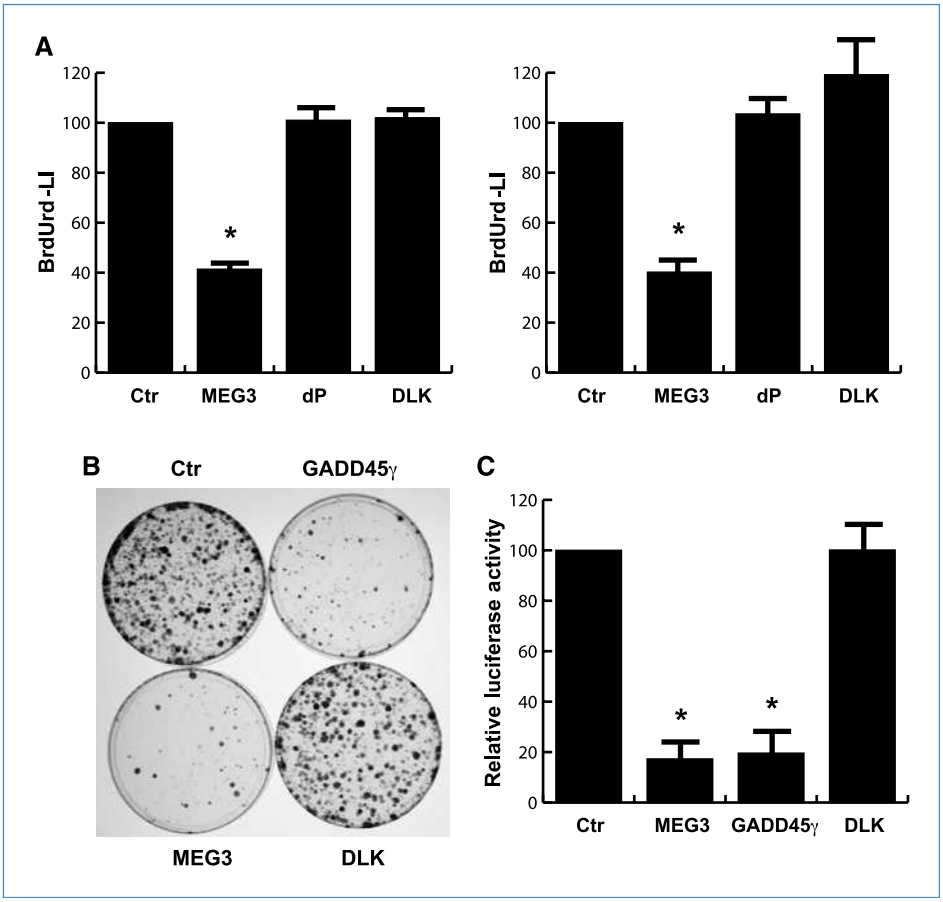

Figure 3. MEG3 suppresses human meningioma cell proliferation. A, left, suppression of DNA synthesis by MEG3 expression as measured by BrdU incorporation assay in IOMM-Lee cells; right, suppression of DNA synthesis by MEG3 expression as measured by BrdU incorporation assay in CH157-MN cells. Ctr, transfection with an empty expression vector as a control; MEG3, transfection with MEG3-expressing vector; dP, transfection with the same MEG3 expression vector but without CMV promoter; DLK, transfection with a DLK1-expressing vector. No inhibition of DNA synthesis was observed when cells were transfected with a MEG3 containing vector with the CMV promoter deleted (dP), or a DLK-expressing vector (DLK). Data are represented as mean ± SD for BrdU-labeling index from at least three independent experiments. B, in colony formation assays, transfection of MEG3- or GADD45γ-expressing vector into CH157-MN cells resulted in a significant decrease in colony number compared with the empty expression vector (Ctr), whereas transfection of DLK-expressing vector does not affect colony formation. C, the percentage of reduction in colony numbers in each transfected cell culture plate. Data are represented as mean ± SD for BrdU-labeling index from at least three independent experiments (*, $P < 0.001$).

There is no statistically significant correlation between the extent of CpG methylation in each individual region and MEG3 RNA expression. Clearly, mechanisms other than DNA hypermethylation also contribute to MEG3 gene silencing in meningiomas. However, in samples without MEG3 RNA expression, the percentages of methylation are significantly higher at CpG positions 1, 10, and 17 in the enhancer region (R4), and at positions 2, 3, and 5 in the IG-DMR region compared with those in the samples with MEG3 RNA expression. Therefore, these are potential hotspots of methylation which may be linked to transcriptional silencing of MEG3.

To explore the functional role of DNA methylation in the silencing of MEG3 transcription in meningioma cells, we treated the human meningioma cell line CH157-MN cells with 5-aza-2′-deoxycytidine, a demethylating agent. As shown in Fig. 2, treatment of 5-aza-2′-deoxycytidine resulted in MEG3 RNA expression.

Suppression of meningioma cell growth by MEG3 cDNA. To investigate the functional relevance of *MEG3* in human meningiomas, we tested its ability to suppress *in vitro* cell growth of meningioma cell lines IOMM-Lee and CH157-MN. Transfection of a MEG3 expression vector into IOMM-Lee and CH157-MN cells resulted in suppression of BrdU incorporation by ~60% (Fig. 3A). However, when the transfection was performed with a similar expression vector in which the CMV promoter sequence controlling MEG3 expression was deleted, no suppression of BrdU incorporation was observed, indicating that expression of MEG3 RNA in the transfected cells is required for suppression of DNA synthesis. Transfection of a DLK1 expression vector showed no suppression of BrdU incorporation (Fig. 3A). In colony-forming efficiency assays, MEG3 suppressed colony formation in CH157-MN cells by ~80%, similar to GADD45γ, a known growth suppressor (15). Again, DLK1 failed to suppress colony formation in CH157-MN cells (Fig. 3B and C).

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Stimulation of p53-mediated transactivation by MEG3 in meningioma cells. To begin to understand the molecular mechanism by which MEG3 suppresses meningioma cell growth, we examined whether MEG3 could affect the function of p53, one of the most important tumor suppressors which functions as a sequence-specific transcription factor. Both meningioma cell lines IOMM-Lee and CH157-MN express p53 protein (Fig. 4A). When a p53-responsive reporter plasmid was transfected into these cells, luciferase activity was detected in the cell lysate. When a MEG3 expression vector was cotransfected with this p53-responsive reporter, reporter activity was increased by ~4-fold (Fig. 4B and C). Therefore, MEG3 is able to stimulate p53-mediated transactivation in IOMM-Lee and CH157-MN cells.

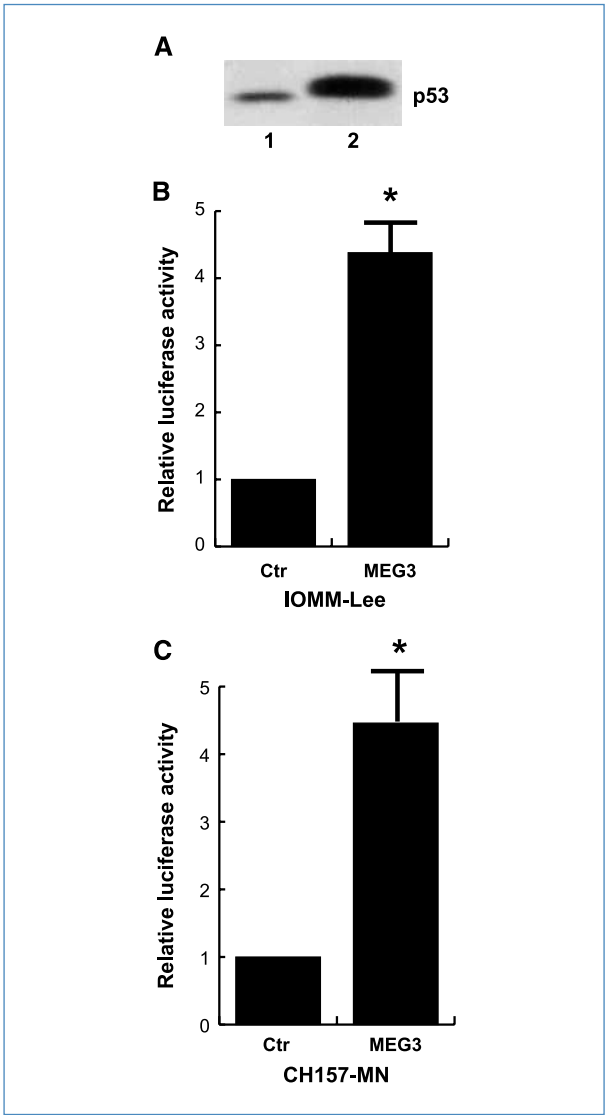

Figure 4. A, both CH157-NM (lane 1) and IOMM-Lee (lane 2) cell lines express p53 protein as examined by Western blot. B and C, MEG3 stimulates p53-mediated transactivation in IOMM-Lee (B) and CH157-NM (C) cells. The relative luciferase activity from cells without MEG3-expressing vector cotransfection is designated as 1. Data are represented as mean ± SD from at least three independent experiments (*, $P < 0.001$).

Discussion

It has long been suggested that chromosome 14q32 contains a tumor suppressor gene involved in meningioma pathogenesis and progression (3–6, 16–18). However, the potential 14q32 tumor suppressor has not yet been discovered. Our data indicate that *MEG3* might be an excellent candidate for this tumor suppressor because (*a*) the MEG3 gene is located at chromosome 14q32; (*b*) MEG3 RNA is highly expressed in normal arachnoidal cells, the likely cell of origin for meningiomas, but not expressed in the majority of meningiomas; (*c*) loss of MEG3 RNA expression as well as loss of *MEG3* gene copy number is more common in higher grade meningiomas and there is an overall increase in CpG methylation in tumors associated with tumor grade; and (*d*) MEG3 RNA expression in human meningioma cell lines strongly suppresses tumor cell growth and activates p53-mediated transactivation.

Early cytogenetic studies revealed monosomy of chromosome 22 in up to 70% of meningiomas, and subsequent studies have identified loss of heterozygosity at polymorphic markers on 22q in 40% to 70% of meningiomas (19–22). At 22q12.2, a key gene of interest, *NF2* has been identified to be associated with meningioma pathogenesis (23, 24), which encodes a tumor suppressor known as merlin or schwannomin, a member of the protein 4.1 superfamily, functioning to link cell surface signaling to intracellular pathways (25). Because loss of merlin expression is observed in meningiomas regardless of tumor grade, *NF2* inactivation is an early event in meningioma pathogenesis and is not associated with tumor progression (2). In contrast, loss of *MEG3* expression and loss of *MEG3* gene copy is more common in higher grade meningiomas, suggesting that loss of MEG3 function may not only be associated with tumor pathogenesis but also with progression. Of the two human meningioma cell lines used in our functional studies, IOMM-Lee is merlin-positive, but CH157-NM is merlin-negative. The fact that MEG3 suppresses *in vitro* proliferation of both cell lines indicates that the function of *MEG3* is independent of merlin. Consistent with our data, previous studies with large tumor numbers have shown that there is no correlation or minimal correlation of 14q and 22q loss in human meningiomas (16, 26–28).

MEG3 was identified as the human counterpart of a mouse-imprinted gene *Gtl2* (29), identified by gene trapping in an attempt to isolate genes involved in early development (30). *Gtl2* is closely linked to another imprinted gene, *Dlk1* (31, 32), a paternally expressed gene, the function of which may be involved in the control of growth and differentiation (33–37). Studies have intensively focused on the genomic characterization and imprinting control of the *Dlk1* and *Gtl2/Meg3* locus (38–41). However, the physiologic function of *MEG3* remained unknown until we reported its antiproliferative activity in human cancer cells (7), and showed loss of *MEG3* expression and promoter hypermethylation in

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pituitary adenomas (9). Subsequently, a number of reports have shown loss of *MEG3* expression and promoter hypermethylation in several types of human tumors, including pituitary adenomas, neuroblastomas, pheochromocytomas, Wilms tumors, and other carcinomas, underscoring its potential tumor-suppressive function (9, 42, 43).

In our study, *DLK1* served as an important control. *DLK1*, also located at 14q32 and closely linked to *MEG3*, is an imprinted gene but with paternal expression. *DLK1* encodes a protein that contains an extracellular domain with six epidermal growth factor–like repeats, a transmembrane domain and a short cytoplasmic tail. *DLK1* regulates the differentiation of different cell lineages, including preadipocytes, skeletal stem cells, thymocytes, and adrenal gland cells (34–37). Upregulation of *DLK1* has recently been reported in some tumors (44, 45). However, our data show that only MEG3 suppresses meningioma cell growth, whereas DLK1 has no such effect on these cells. These data are consistent with our hypothesis that *MEG3* is a specific candidate tumor suppressor at 14q32.

Mutations in the *TP53* tumor suppressor gene have been identified in >50% of human tumors, and >90% of cancers contain defects in the p53 pathway (46). However, the involvement of p53 in meningiomas remains elusive. In general, high levels of p53 protein expression (2, 47) and occasional *TP53* mutations have been found in high-grade meningiomas (48). The p53 protein is regulated by MDM2, which inhibits p53 function and promotes its degradation. This p53/MDM2 interaction is inhibited by $p14^{ARF}$. In the absence of a *TP53* gene mutation, loss of MDM2 protein expression and a high percentage of $p14^{ARF}$ gene methylation have been reported in high-grade meningiomas (49), consistent with our previous observations that *MEG3* expression leads to p53 protein accumulation and MDM2 downregulation (8). Here, we report that MEG3 enhances p53-mediated transcription in meningiomas. Therefore, it is possible that in normal arachnoidal cells, p53 and MEG3 function together to keep cell proliferation under control. In this conceptual schema, loss of *MEG3* expression would lead to the impairment of p53 function, resulting in uncontrolled cell proliferation and subsequent tumor development, even though the meningioma cells could respond by expressing more p53 protein to reverse this impairment. Future studies to investigate MEG3 and p53 expression in these tumors would be important to support this potential mechanism.

It has yet to be determined how MEG3 interacts with p53. Lacking a solid open reading frame and lacking a Kozak consensus sequence in any of its short open reading frames, it was suggested that MEG3 functions as a noncoding RNA (50). Recently, using untranslatable MEG3 cDNA mutants, we have provided the first experimental evidence for its noncoding RNA nature (8). As shown in this study, a MEG3 expression vector without a promoter fails to suppress DNA synthesis in both IOMM-Lee and CH157 cells, indicating that transcription of MEG3 RNA is necessary for its growth-suppressive function. Further investigation of the molecular interaction between MEG3 and p53 may reveal a novel mechanism for control of cell proliferation and meningioma pathogenesis involving noncoding RNAs.

In conclusion, our data strongly suggest *MEG3* as a candidate tumor suppressor gene at 14q32 associated with the pathogenesis and progression of human meningiomas. As an imprinted gene encoding a noncoding RNA, it seems to suppress tumor development via entirely novel mechanisms. Further investigation of *MEG3* could therefore provide important information regarding the pathogenesis of human meningiomas, reveal novel mechanisms to broaden our knowledge of the involvement of noncoding RNAs in human tumor biology, and eventually, point to new therapeutic strategies for these tumors.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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