Altered MicroRNA Expression Profile in Human Pituitary GH Adenomas: Down-Regulation of miRNA Targeting HMGA1, HMGA2, and E2F1

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Context: MicroRNA (miRNA) are an important class of regulators of gene expression. Altered miRNA expression has been constantly found in human neoplasias and plays an important role in the process of carcinogenesis.

Objective: The aim of this study was to identify specific miRNA whose expression is altered in GH-secreting pituitary adenomas.

Design: Using a miRNACHIP microarray, we have analyzed the miRNA expression profile of human GH adenomas *vs.* normal pituitary gland.

Results: We report the identification of a set of miRNA, including miR-34b, miR-326, miR-432, miR-548c-3p, miR-570, and miR-603, drastically and constantly down-regulated in GH adenomas. We demonstrate that these miRNA target genes such as high-mobility group A1 (*HMGA1*), *HMGA2*, and *E2F1*, whose overexpression and/or activation plays a critical role in pituitary tumorigenesis. We also show that the enforced expression of the down-regulated miRNA has a negative role on the growth regulation of pituitary adenoma cells. Finally, an inverse correlation is found between the expression of these miRNA and HMGA1 and HMGA2 protein levels in GH adenomas.

Conclusion: Our study identifies a specific subset of miRNA, whose down-regulation might contribute to pituitary tumorigenesis. (***J Clin Endocrinol Metab*** **97: E1128–E1138, 2012)**

Pituitary tumors are in most cases monoclonal adenomas arising from adenohypophyseal cells and represent about 15% of intracranial tumors (1, 2). GH adenomas (somatotrophic adenomas) correspond to about 20% of all pituitary adenomas. In 30–50% of the GH adenomas, they are histologically mixed GH/prolactin (PRL) cell adenomas cosecreting PRL and GH (3). Pituitary adenomas appear to result from a multistep and multicausal process in which hereditary genetic predisposition, endocrine factors, and specific somatic mutations may serve as contributing factors.

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Abbreviations: E2F1, E2 transcription factor 1; HMGA, high-mobility group A; miRNA, microRNA; PRL, prolactin; qRT, quantitative real-time; UTR, untranslated region.

The high-mobility group A (HMGA) protein family consists of four members, HMGA1a, HMGA1b, and HMGA1c (encoded through alternative splicing by the *HMGA1* gene) and HMGA2 (encoded by the *HMGA2* gene) (4, 5). The HMGA proteins interacting with the transcriptional machinery can regulate, positively or negatively, the expression of several genes (6–8). HMGA protein expression is abundant during embryogenesis, whereas it is absent or detected at very low levels in normal adult tissue. Conversely, HMGA overexpression is a constant feature of human malignant neoplasms (9).

We have previously shown that transgenic mice overexpressing *Hmga1* or *Hmga2* genes develop mixed GH/PRL-secreting pituitary adenomas (10, 11) and that the *HMGA2* gene is amplified and overexpressed in several pituitary adenomas (8, 12). The overexpression of the *HMGA* genes would induce pituitary adenomas by enhancing the activity of the E2 transcription factor 1, E2F1, by up-regulating the expression of cyclin B2 and, likely, other genes mainly involved in the regulation of the cell cycle (8, 13).

MicroRNA (miRNA) are a class of small noncoding RNA that regulate gene expression at the posttranscriptional level. They bind to 3′-untranslated regions (3′-UTR) of target mRNA, causing block of translation or mRNA degradation (14). They play important roles in essential processes such as differentiation, cell growth, and cell death (15, 16). Several studies have demonstrated altered expression of specific miRNA in different types of human neoplasias, suggesting that they play a key role in tumorigenesis (17). It has been previously demonstrated that miR-15a and miR16–1 are expressed at low levels in pituitary adenomas. Their expression correlates inversely with tumor diameter and directly with the secretion of the antineoplastic cytokine p43 (18). Other studies have shown alterations of miRNA expression in pituitary adenomas (19–21). In particular, it has been recently reported that 52 miRNA were differentially expressed in GH adenomas and normal pituitaries. Several of these miRNA may be involved in cell proliferation, apoptosis, and cancer development and progression (22).

To identify miRNA potentially involved in the pathogenesis of GH adenomas, we have analyzed the miRNA expression profile of 12 human GH adenomas *vs.* normal pituitary gland. Interestingly, five of the most down-regulated miRNA have as targets *E2F, HMGA1*, and *HMGA2* genes, all of which are already known to play a critical role in pituitary tumorigenesis.

Materials and Methods

Tissue collection and RNA isolation

Pituitary adenomas were obtained from patients operated at E Jouanneau Service de Neurochirurgie U300, Groupement Hospitalier Est Hospices Civils de Lyon. For each tumor, some fragments were frozen in liquid nitrogen and stored at −80 C until RNA extraction. Other fragments were fixed in Bouin-Holland fluid and embedded in paraffin for pathological diagnosis, including immunocytochemistry. Tumors were classified according to the 2004 World Health Organization classification into sparsely or densely granulated adenomas and GH or GH-PRL adenomas. Age, sex, and clinical and hormonal data of the patients and the pathological characteristic of GH adenomas analyzed for miRNACHIP are summarized in Supplemental Table 1 (published on The Endocrine Society's Journals Online web site at http://jcem.endojournals.org). We declare that informed consent for the scientific use of biological material was obtained from all patients.

Total RNA isolation from human pulverized tumors was performed with TRIzol reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. RNA from human normal pituitary glands were used as control. Normal pituitaries were obtained from autopsies of two females and one male, aged from 40–50 yr, and devoid of endocrine diseases.

miRNACHIP microarray

RNA labeling, hybridization on miRNA microarray chip, and microarray analysis were performed as previously described (23). Differentially expressed miRNA in pituitary adenoma tissue samples were analyzed by class comparison using Student's *t* test procedure. Microarray data were analyzed also by significance analysis of microarray test (for more details see Supplemental Information). Each sample was analyzed for miRNA expression profile in triplicate (24).

Bioinformatic prediction of miRNA target genes

Genes potentially targeted by the selected miRNA were identified by using different tools available online such as TargetScan (www.targetscan.org) and miRanda (www.microrna.org). For more details see Supplemental Information.

Reverse transcription and quantitative real-time (qRT)-PCR

Reverse transcription and qRT-PCR for mature miRNA were performed according to manufacturer's instructions of miScript system kits (QIAGEN, Valencia, CA) (for more details see Supplemental Information). qRT-PCR analyses for *HMGA1*, *HMGA2*, and *E2F1* were performed as previously described (13). To calculate the relative expression levels we used the $2^{-\Delta\Delta CT}$ method (25). Primers for glucose-6-phosphate dehydrogenase (*G6PD*) were used for normalization. The primers used to amplify the above mentioned genes are reported in Supplemental Information.

Cell lines and transfection

GH3, HP75, MEG01, and HEK-293 and transfection methods are described in Supplemental Information.

Western blotting and antibodies

Western blot analysis was performed as previously described (8), and the membranes were incubated with antibodies against E2F1 (C-20; Santa Cruz Biotechnology, Santa Cruz, CA), α-tubulin (SC-8035; Santa Cruz), β-actin (SC-1615; Santa Cruz), and

anti-HMGA1 and anti-HMGA2 antibodies, all previously described (13, 26).

Luciferase target assays

Cells were cotransfected with the modified firefly luciferase vectors described in Supplemental Information, along with the renilla luciferase reporter plasmid and the miRNA oligonucleotides. Firefly and renilla luciferase activities were measured 24 h after transfection with the dual-luciferase reporter assay system (Promega, Madison, WI). Firefly activity was normalized to renilla activity as control of transfection efficiency.

Growth curve assay

Cells were plated in six-well plates and transfected with 50 nmol/ml pre-miR miRNA precursor or scrambled oligonucleotide using siPORT neoFX. Cells were counted in triplicate at daily intervals with a Burker hemocytometer chamber.

Colony-forming assay

Cells were transfected with the pMIRNA expression vectors or the backbone vector, along with the pBABE-puro construct expressing the puromycin-resistance gene. Transfected cells were selected by using 1 mg/ml puromycin diluted in the medium used for culture. After 15 d, cells were fixed and stained with 0.1% crystal violet in 20% methanol, and resulting colonies were counted.

Flow cytometry

After trypsinization, cells were washed in PBS and fixed in 70% ethanol. Staining for DNA content was performed with 2 μg/ml propidium iodide and 20 μg/ml ribonuclease A for 30 min. For each measure, 30,000 events have been analyzed. We used a FACScan flow cytometer (Becton Dickinson, San Jose, CA) that was interfaced with a Hewlett-Packard computer (Palo Alto, CA). Cell cycle data were analyzed with the CELL-FIT program (Becton Dickinson).

Statistical analysis

Student's *t* test was used to determine the significance for all the quantitative experiments. All error bars represent the SEM. Statistical significance for all the tests, assessed by calculating the *P* value, was <0.05.

Results

miRNA expression profile of GH-secreting pituitary adenomas

We used a miRNACHIP microarray (23) to evaluate the miRNA expression profile of 12 GH adenomas *vs.* that of three normal pituitary glands. Eleven patients presented with a GH-secreting adenoma with clinical and hormonal signs of acromegaly, one with a silent GH adenoma. The clinical and hormonal data and the histological features of the tumors are summarized in Supplemental Table 1. By applying biostatistical analysis (see *Materials and Methods*), we obtained a list of miRNA differentially expressed ($P < 0.05$) between normal pituitary and GH adenomas (Table 1). Eighteen miRNA were down-regulated with at least a 2-fold change in the neoplastic tissues compared with the normal gland. Conversely, only one miRNA, the miR-320, was overexpressed (with a 13.3-fold change) in GH adenomas, compared with normal pituitary. To validate the results obtained by microarray Chip, we analyzed the expression of eight down-regulated miRNA (miR-34b, miR-326, miR-374b, miR-432, miR-548c-3p, miR-570, miR-603, and miR-633) and the only one up-regulated (miR-320) by qRT-

TABLE 1. miRNA expression profile of GH-secreting pituitary adenomas *vs.* normal pituitary

	Geometric mean of intensities in pituitary adenomas	Geometric mean of intensities in normal pituitary	Fold change	miRNA unique ID
1	1339.7274209	100.6376509	13.3123876	hsa-miR-320
2	61.0039988	923.4445835	0.0660614	hsa-miR-633
3	554.5981341	4446.917357	0.1247152	hsa-miR-657
4	77.3976401	835.2207126	0.0926673	hsa-miR-663
5	60.6623332	360.2462431	0.1683913	hsa-miR-432
6	93.443452	1320.8216198	0.0707465	hsa-miR-801
7	182.2341266	2893.0851269	0.0629895	hsa-miR-326
8	75.8028902	510.8846675	0.1483757	hsa-miR-585
9	217.0307216	2713.6989391	0.079976	hsa-miR-374b
10	101.5571388	617.4486958	0.1644787	hsa-miR-548b-3p
11	88.1968279	574.4133742	0.1535424	hsa-miR-603
12	9485.5552871	46998.85725	0.2018252	hsa-miR-483-3p
13	74.4222971	331.8440313	0.2242689	hsa-miR-592
14	58.8817238	202.0629337	0.2914029	hsa-miR-561
15	102.7756863	520.9925122	0.197269	hsa-miR-26b
16	230.1809093	1728.076057	0.1332007	hsa-miR-548c-3p
17	4629.3477853	17850.1685191	0.2593448	hsa-miR-636:
18	1259.1293869	4979.3184131	0.2528718	hsa-miR-570
19	71.5553155	228.773205	0.3127784	hsa-miR-34b

The microarray experiment was performed twice using the same RNA samples. The threshold value used to screen up- and down-regulated miRNA was more than 2-fold or less than 0.5-fold change.

PCR on a larger set of tumoral samples (18 GH adenomas, 12 of which were already used for the microarray). As shown in Fig. 1A, a drastic down-regulation of miR-34b, miR-326, miR-374b, miR-432, miR-548c-3p, miR-570, miR-603, and miR-633 was observed in GH adenomas in comparison with normal pituitary tissues. As far as the miR-320 expression is concerned, it was highly up-regulated in nine of 18 samples, whereas no

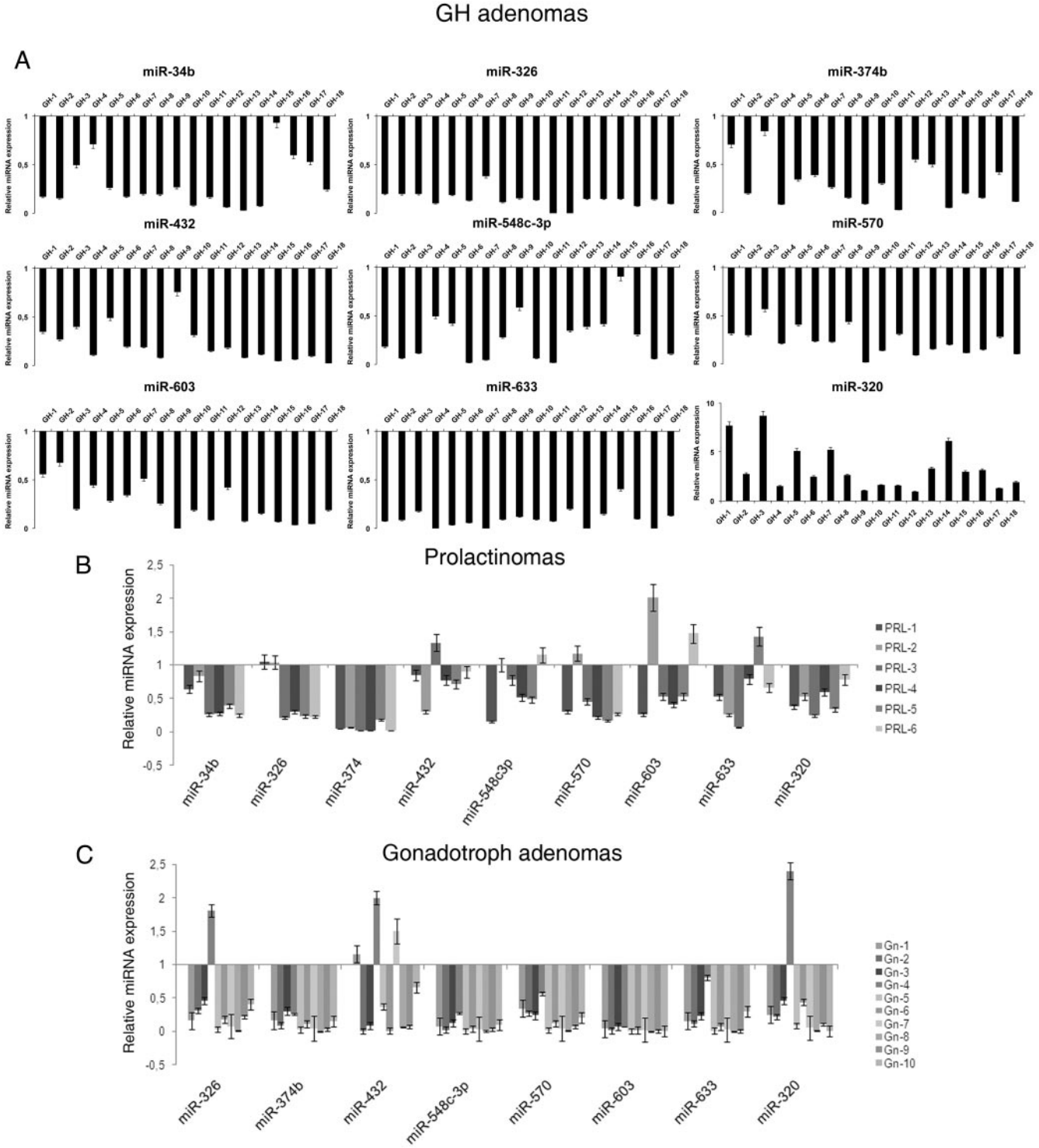

FIG. 1. Analysis of miR-326, miR-374b, miR-432, miR-548c-3p, miR-570, miR-603, miR-633, and miR-320 expression in pituitary adenomas by qRT-PCR. The relative expression values indicate the relative change in the expression levels between GH adenoma (A), PRL adenoma (B), or gonadotroph adenoma (C) samples *vs.* three normal pituitary samples, assuming that the mean value of the normal samples was equal to 1. The range of variability of the expression of these miRNA in normal pituitary tissues is less than 10%. Each *bar* represents the mean value ± SE from three independent experiments performed in triplicate.

or a weak up-regulation was observed in the other adenoma samples.

Next, we investigated whether the altered expression of these miRNA was specific for GH histotype or a more general event in pituitary tumors. Therefore, we examined their expression in six PRL adenoma and in 10 gonadotroph adenoma samples by qRT-PCR. As shown in Fig. 1, B and C, almost all of the analyzed miRNA showed the same expression pattern both in PRL and gonadotroph adenomas with the exception of a few cases. Surprisingly, miR-320 was drastically down-regulated in PRL and gonadotroph adenomas, whereas it was highly up-regulated in GH adenomas.

Identification of the target genes for the miRNA down-regulated in GH adenomas

Using bioinformatic tools (miRanda and TargetScan) to search for potential targets of the above identified miRNA, we identified several genes that could be targeted by them. Interestingly, we found that three of these miRNA (miR-326, miR-432, and miR-570) potentially target the *HMGA2* gene, two miRNA (miR-34b and miR-548c-3p) have both the *HMGA1* and *HMGA2* genes as predicted targets, and the other two (miR-326 and miR-603) are predicted to regulate *E2F1* (Supplemental Table 2). We focused on these targets because it has been previously demonstrated that *HMGA1* and *HMGA 2* are overexpressed in most of the human pituitary adenomas (8, 12) and that transgenic mice overexpressing these genes developed mixed PRL/GH pituitary adenomas (11). It is also well known that increased E2F1 activity or expression can lead to pituitary tumorigenesis (13, 27, 28).

In Fig. 2, A and B, we report the miRNA-targeting sites on *HMGA1* and *HMGA2* 3′-UTR, respectively. To validate the influence of the identified miRNA on the expression of *HMGA* genes, we transfected them into MEG-01 and HEK-293 cells, which express significant levels of both HMGA1 and HMGA2, searching for changes in HMGA1/2 protein levels. The expression levels of the transfected miRNA are reported in Supplemental Fig. 1. Transfection of miR-34b and miR-548c-3p decreased both HMGA1 and HMGA2 protein levels, whereas miR-326, miR-432, and miR-570 overexpression resulted in the decrease of HMGA2 protein levels only (Fig. 2C and Supplemental Fig. 2A) compared with the scrambled-transfected cells. Interestingly, changes in *HMGA1* and/or *HMGA2* mRNA levels, even though quite moderate, were observed in cells transfected with miR-548c-3p, miR-432, and miR-570 (Fig. 2D and Supplemental Fig. 2B), suggesting that these miRNA act, at least in part, through *HMGA1/2* mRNA degradation. Conversely, no changes in *HMGA1* and *HMGA2* mRNA levels were detected after transfection with miR-34b and miR-326, thus excluding their role in *HMGA1/2* mRNA degradation.

To demonstrate that the direct interaction between the analyzed miRNA and the *HMGA1* and *HMGA2* mRNA was responsible for protein level decrease, we inserted the 3′-UTR of *HMGA1* and *HMGA2* mRNA downstream of the luciferase open reading frame, either in sense (Luc-HMGA1-3′-UTR and Luc-HMGA2-3′-UTR) or in antisense (Luc-HMGA1rev-3′-UTR and Luc-HMGA2rev-3′-UTR) orientation. These reporter vectors were transfected in MEG-01 cells together with miRNA oligonucleotide precursors or a scrambled oligonucleotide. As shown in Fig. 2E, the luciferase activity of the Luc-sense-3′-UTR constructs was markedly diminished after transfection of each analyzed miRNA compared with the scrambled, whereas the luciferase activity of Luc-antisense-3′-UTR constructs did not vary, indicating that these miRNA interfere with *HMGA1* and *HMGA2* translation through direct interaction with their 3′-UTR. Moreover, we also performed luciferase assays using 3′-UTR constructs of *HMGA1* and *HMGA2* in which we deleted the seed sequence of miR-548c-3p and miR-326, respectively. Our results show that both *HMGA1* and *HMGA2* 3′-UTR deletion mutants were not affected by miR-548c-3p or miR-326 transfection (Supplemental Fig. 2C).

As described above, *E2F1* was one of the predicted targets for miR-326 and mir-603. The miRNA-targeting sites on *E2F1* 3′-UTR are represented in Fig. 3A. To validate *E2F1* as target for miR-326 and miR-603, we transfected these miRNA into MEG-01 cells, and quantification of E2F1 protein and mRNA levels was performed by Western blot and qRT-PCR, respectively. As shown in Fig. 3B, transfection of miR-326 and miR-603 decreased E2F1 protein levels with respect to the scrambled-transfected cells. A reduction of mRNA levels was also observed indicating that miR-326 and miR-603 regulate E2F1 protein levels also through *E2F1* mRNA degradation (Fig. 3C). Similar results were obtained by transfecting HEK-293 cells (Supplemental Fig. 3, A and B). To validate the direct inhibition of E2F1 protein by these miRNA, we inserted 3′-UTR of the *E2F1* mRNA either in sense or in antisense orientation (Luc-E2F1-3′-UTR and Luc-E2F1rev-3′-UTR) downstream of the luciferase open reading frame, and reporter vectors were transfected into MEG-01 cells along with oligonucleotide precursors of miR-326 and miR-603. As shown in Fig. 3D, overexpression of the predicted E2F1-targeting miRNA significantly reduced luciferase activity of the Luc sense construct, but not Luc antisense

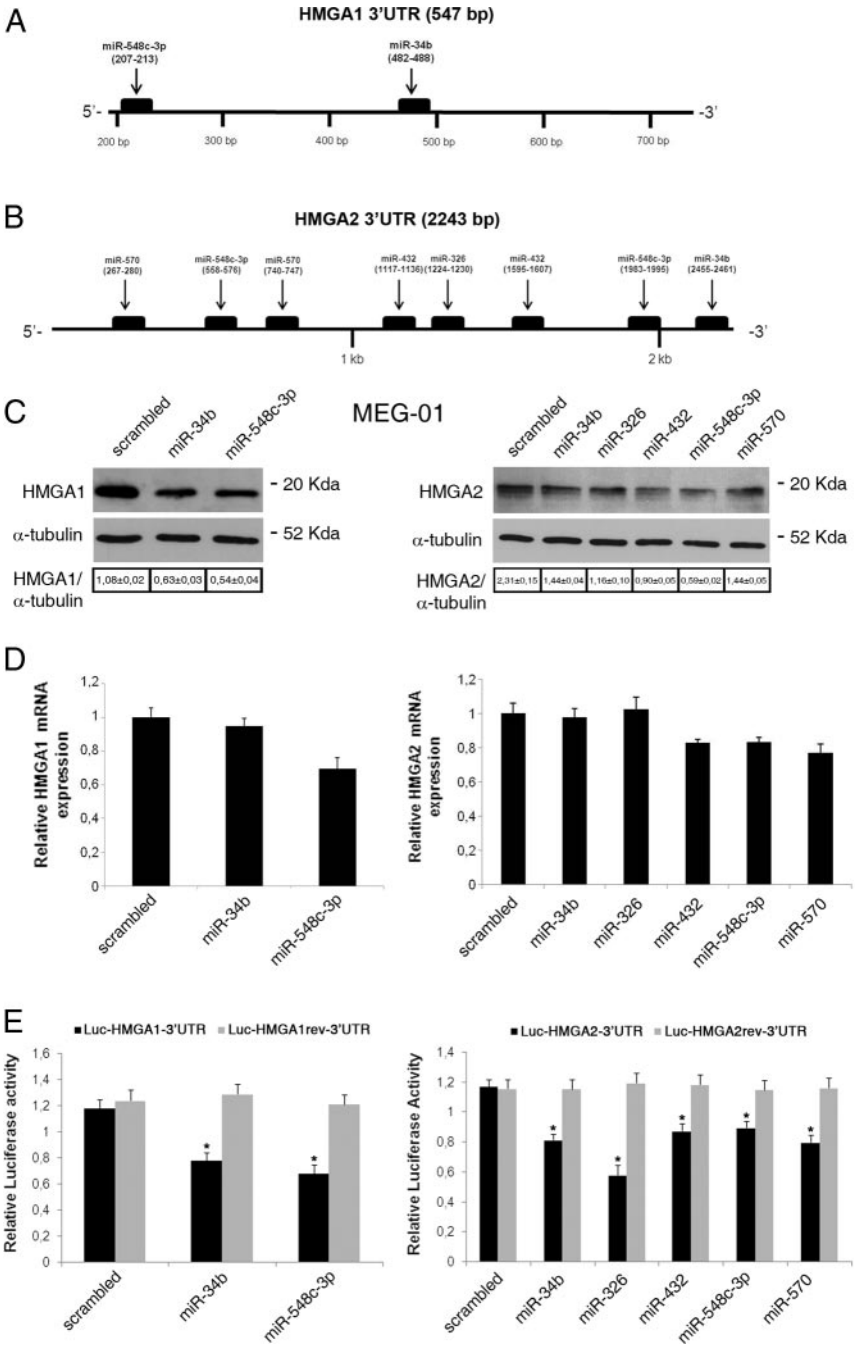

FIG. 2. miR-34b, miR-326, miR-432, miR-548c-3p, and miR-570 target HMGA1 and HMGA2. A and B, Schematic representation of human *HMGA1* and *HMGA2* 3′-UTR and the relative position of the predicted miRNA-binding sites. C, Western blot analysis of HMGA1 (*left panel*) and HMGA2 (*right panel*) protein levels in MEG-01 cells transfected with indicated miRNA precursors or scramble oligonucleotide. α-Tubulin was analyzed as loading control. A representative experiment is shown. The mean ± SE of densitometric analysis performed on three different experiments, using ImageJ software and normalizing to the α-tubulin, is reported on the *bottom*. D, qRT-PCR analysis of *HMGA1* (*left panel*) and *HMGA2* (*right panel*) mRNA in the same samples shown in C. Relative expression values indicate the relative change in *HMGA1* and *HMGA2* mRNA expression levels between miR-treated and scrambled oligonucleotide-treated cells, normalized with *G6PD*. The *error bars* represent the mean ± SE of three independent experiments performed in triplicate. E, Relative luciferase activity in MEG-01 cells transiently transfected with Luc-HMGA1-3′-UTR and Luc-HMGA1-rev-3′-UTR (*left panel*) or Luc-HMGA2-3′-UTR and Luc-HMGA2rev-3′-UTR (*right panel*) along with the indicated miRNA oligonucleotides or with a no-targeting scrambled oligonucleotide. The relative activity of firefly luciferase expression was standardized to a transfection control, using renilla luciferase. The *scale bars* represent the mean ± SE of three independent experiments performed in triplicate. *, $P < 0.05$ compared with scrambled transfected cells.

construct, indicating that the inhibition of E2F1 protein expression by these miRNA was dependent on their direct binding to its 3′-UTR. These results were further supported by similar experiments, in which we used the Luc-E2F1-3′-UTR-carrying deletion in the seed sequences of miR-326 and miR-603. These reporter vectors were not affected by miR-326 and miR-603 transfection, demonstrating that the inhibitory activity of these miRNA on E2F1 expression is dependent on their direct interaction with their binding sites on E2F1 3′-UTR (Supplemental Fig. 3, C and D).

The miRNA down-regulated in GH adenomas inhibit cell proliferation

To understand the role of miRNA down-regulation in pituitary tumorigenesis, we analyzed their effects on cell proliferation, performing cell growth curves using HP75 pituitary adenoma cells transiently transfected with miR-34b, miR-326, miR-432, miR-548c-3p, miR-570, miR-603, or scrambled oligonucleotide. As shown in Fig. 4A, a significant reduction of cell number was observed 96 h after transfection with the selected miRNA compared with scrambled-transfected cells. Similar results were also obtained when rat GH3 pituitary tumor cells were transfected with miR-326, miR-432, miR-548c-3p, miR-570, or scrambled oligonucleotide (Fig. 4B). Because miR-34b and miR-603 targeting sites on *HMGA* and *E2F1* 3′-UTR are not conserved in rat, their effects were not analyzed in these cells. Moreover, the inhibitory role of the analyzed miRNA on cell growth was also demonstrated by a growth curve performed on human HEK-293 cells (Supplemental Fig. 4A).

Next, we performed a colony-forming assay on GH3 cells (Fig. 4C) after transfection with pMIRNA expression vector carrying miR-326 precursor and on MEG-01 cells (Fig. 4D) after transfection with pMIRNA expression vectors carrying miR-34b and miR-326 precursors along with

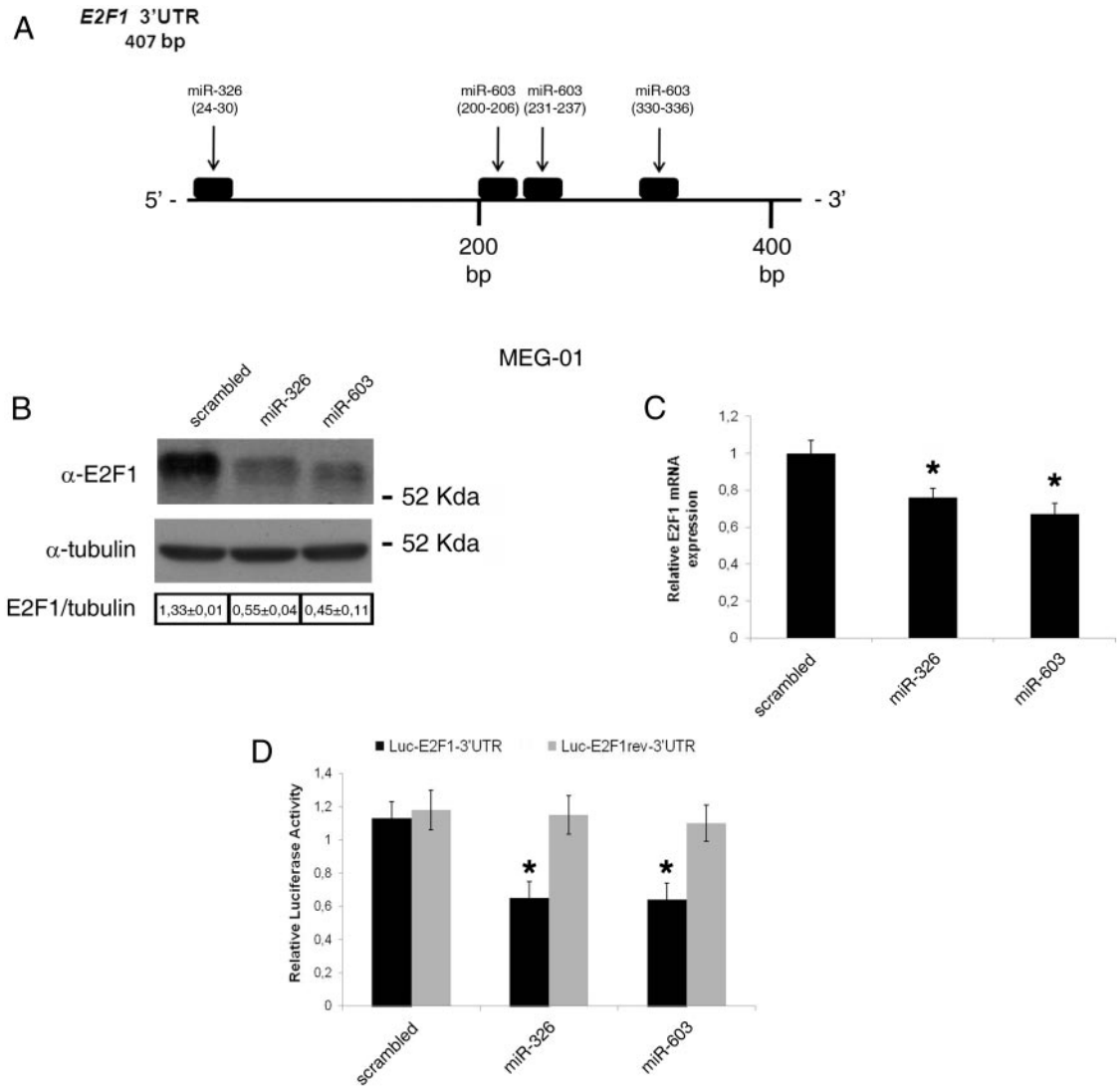

FIG. 3. miR-326 and miR-603 target E2F1. A, Schematic representation of the 3′-UTR sites of the *E2F1* gene targeted by miR-326 and miR-603. B, Western blots of the E2F1 protein expression in MEG-01 cells transfected with miR-326, miR-603, and a scrambled oligonucleotide. α-Tubulin was analyzed as loading control. A representative experiment is shown. The mean ± SE of densitometric analysis performed on three different experiments, using ImageJ software and normalizing to the α-tubulin, is reported on the *bottom*. C, qRT-PCR analysis of *E2F1* mRNA in the same samples shown in B. Relative expression values indicate the relative change in *E2F1* mRNA expression levels between miRNA-treated and scrambled oligonucleotide-treated cells, normalized with *G6PD*. The *error bars* represent the mean ± SE. *, $P < 0.05$ compared with scrambled transfected cells. D, Relative luciferase activity in MEG-01 cells transiently transfected with Luc-E2F1-3′-UTR and Luc-E2F1rev-3′-UTR along with miR-326 and miR-603 oligonucleotide. A no-targeting scrambled oligonucleotide was used as a control. The relative activity of firefly luciferase expression was standardized to a transfection control, using renilla luciferase. The *scale bars* represent the mean ± SE of three independent experiments performed in triplicate. *, $P < 0.05$ compared with scrambled transfected cells.

pBABE-puro-expressing gene for the resistance to puromycin. We focused our attention on these miRNA because miR-34b was predicted to down-regulate both *HMGA1* and *HMGA2* mRNA, and miR-326 was predicted to target both *HMGA2* and *E2F1* mRNA. As control of the pBABE-puro transfection efficiency in the presence of pMIRNA or backbone vectors, we also transfected cells with pMIRNA expression vector carrying miR-887 that does not have matching sequences in the 3′-UTR of *HMGA1*, *HMGA2*, and *E2F1* and does not have as predicted target genes coding for protein involved in cell growth regulation. As shown in Fig. 4, C and D, cells transfected with miR-34b and miR-326 generated a lower number of colonies in comparison with the same cells transfected with the backbone vector or the pMIRNA-887, thus confirming the negative role of these miRNA in the regulation of cell growth.

Finally, to better characterize the effects of the analyzed miRNA on cell cycle progression, miRNA precursors or scrambled oligonucleotide were transfected on GH3 (Fig. 4E), MEG-01 (Fig. 4F), and HEK-293 cells (Supplemental Fig. 4B) and analyzed by flow cytometry.

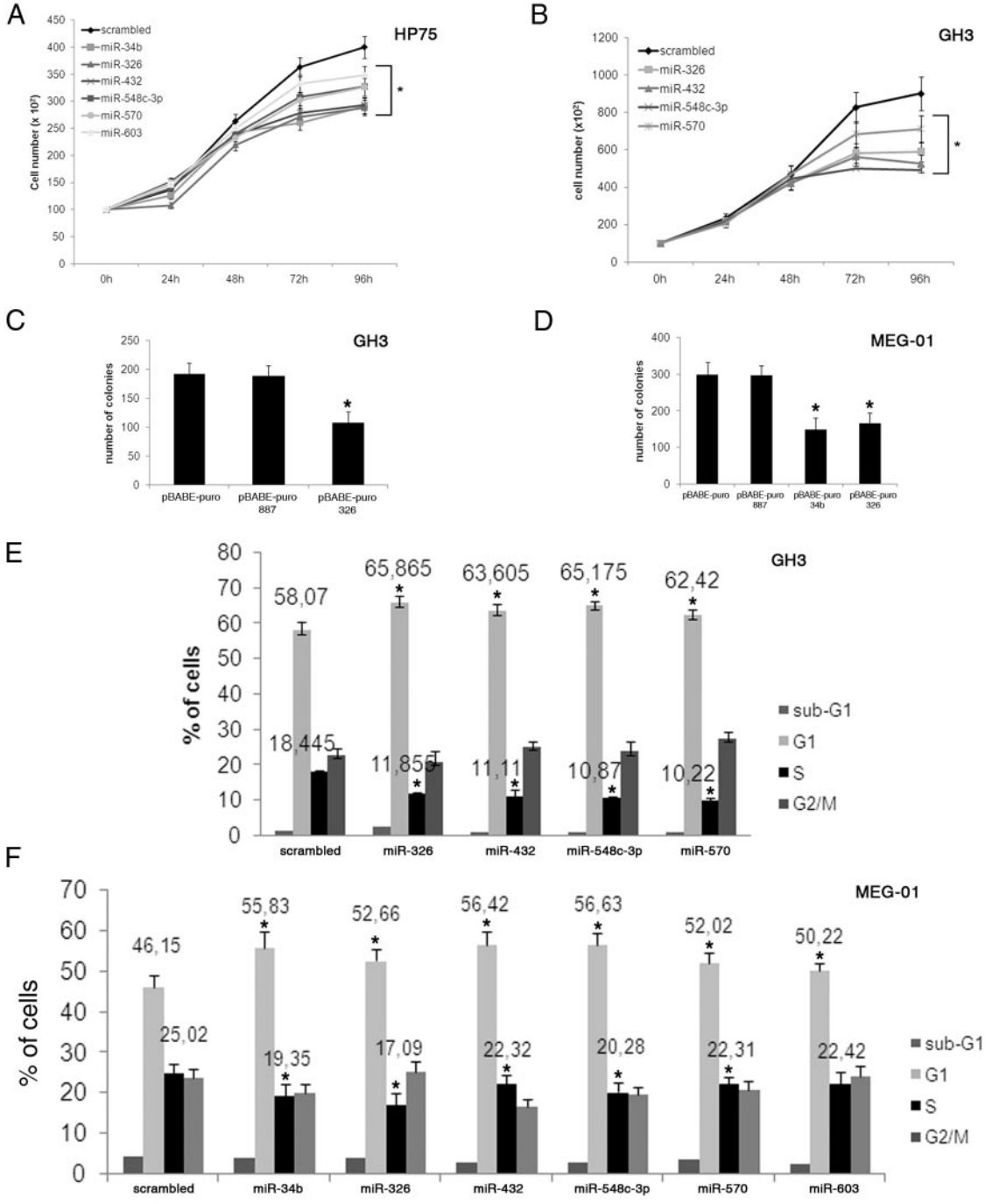

FIG. 4. miR-34b, miR-326, miR-432, miR-548c-3p, miR-570, and miR-603 inhibit cell proliferation. A and B, Cell growth curves of HP75 (A) and GH3 (B) cells transfected with miR-34b, miR-326, miR-432, miR-548c-3p, miR-570, and miR-603 and counted each 24 h for 96 h after plating. The y-axis represents absolute viable cell count. The mean ± SE of three independent experiments is reported. *, $P < 0.05$ at 96 h compared with scrambled transfected cells. C and D, Colony-forming assay performed on GH3 (C) and MEG-01 (D) cells transfected with a vector expressing miR-34b, miR-326, or miR-887 under the transcriptional control of the CMV promoter. The empty vector was used as a control. Reported results are the mean of two independent experiments performed in triplicate, and *error bars* show SE. *, $P < 0.05$ compared with empty vector-transfected cells. E and F, Flow cytometric analysis of GH3 (E) and MEG-01 (F) cells transfected with the indicated miRNA precursors or scrambled oligonucleotide. After transfection, the DNA of the transfected cells was analyzed 72 h later by flow cytometry after propidium iodide staining. The percentage of cells in G1 and S phase is also reported. Each *bar* represents the mean ± SE from three independent experiments performed in triplicate. *, $P < 0.05$ compared with scrambled transfected cells.

Interestingly, miRNA-transfected cells displayed an increase in the G1-phase population and a decrease in the S-phase compared with scrambled-transfected cells. These results indicate that the overexpression of these miRNA affects the G1-S transition of the cell cycle progression, as expected by their targets (*HMGA1*, *HMGA2*, and *E2F1*), known to be involved in the regulation of this phase of the cell cycle.

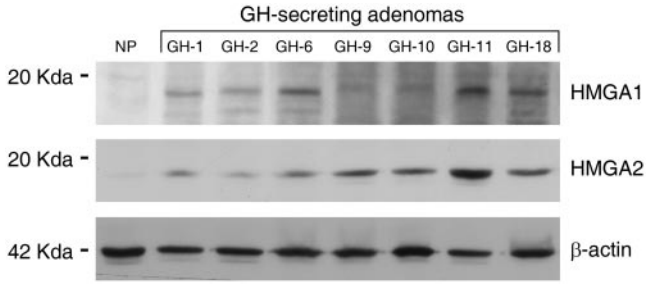

FIG. 5. HMGA1 and HMGA2 protein expression is inversely correlated with miRNA expression in GH adenomas compared with normal pituitary (NP), as shown by Western blot analysis of HMGA1 and HMGA2 protein expression. The level of β-actin was used as loading control.

Inverse correlation between miRNA expression and HMGA1/2 expression

To verify whether miRNA down-regulation might have a role also *in vivo* in the process of pituitary tumorigenesis, we analyzed HMGA1 and HMGA2 expression in some GH adenomas by Western blot. As shown in Fig. 5, increased HMGA1 and HMGA2 protein levels, in comparison with normal pituitary, were observed in all GH adenomas analyzed, all of which showed a decreased miRNA expression (Fig. 1A) in comparison with normal pituitary tissue. These data suggest that there may be an inverse correlation between miRNA down-regulation and HMGA1/2 expression.

Discussion

To investigate the possible role of alterations of miRNA expression in GH adenomas, we analyzed the miRNA expression profile of 12 GH adenomas in comparison with the normal pituitary gland by microarray analysis. Nineteen miRNA were identified as differentially expressed between pituitary adenomas and normal pituitary with a significant (>2-fold) change. We focused our attention on eight of the down-regulated miRNA because they were predicted to target *HMGA1*, *HMGA2*, and/or *E2F1* genes. Interestingly, these miRNA were also down-regulated in another six GH adenoma samples and in a large majority of PRL and gonadotroph adenomas tested, suggesting that their down-regulation might represent a general event in pituitary tumorigenesis. However, the validation of this hypothesis requires the analysis of a higher number of PRL and gonadotroph adenoma samples, because only a limited number of these tumors were analyzed in our study. Subsequently, we focused on the miRNA that had as target genes *HMGA1*, *HMGA2*, and *E2F1*, whose role in pituitary tumorigenesis has been already demonstrated. Interestingly, three of these miRNA (miR-326, miR-432, and miR-570) have *HMGA2* as a target, two miRNA (miR-34b and miR-548c-3p) have both the *HMGA1* and *HMGA2* genes as targets, and the other two miRNA (miR-326 and miR-603) have *E2F1* as a target. Therefore, the down-regulation of these miRNA may account for the increased HMGA and E2F1 protein levels observed in pituitary adenomas. This appears extremely interesting because *HMGA2* overexpression, after amplification and/or rearrangement of the *HMGA2* gene associated with trisomy of chromosome 12, where the *HMGA2* gene is located, has been frequently observed in PRL adenomas (12). Moreover, high protein levels of HMGA2 have been correlated with invasion, tumor size, and higher Ki-67 index in PRL, silent ACTH, and gonadotropin adenomas (29). Consequently, the down-regulation of the miRNA targeting *HMGA2* might synergize with *HMGA2* gene alterations in the induction of high expression levels of HMGA2 protein in pituitary adenomas. Accordingly, we report an inverse correlation between HMGA1/2 protein levels and the expression of the analyzed miRNA in GH adenomas. This down-regulation could, at least partially, account for the HMGA2 overexpression in pituitary adenomas where no genomic *HMGA2* alterations have been observed, because it occurs for most of the human nonfunctioning pituitary adenomas, which rarely harbor trisomy of chromosome 12 (30). Certainly, the down-regulation of miR-34b and miR-548c-3p, both targeting *HMGA1*, could account for the overexpression of HMGA1 protein detected in most human pituitary adenomas (13) in the absence of any alteration of the *HMGA1* gene locus.

Among the miRNA down-regulated in GH adenomas, two target *E2F1*. Also in this case, the increased E2F1 levels could have a role in pituitary tumorigenesis. Indeed, mice carrying a germline mutation of one Rb allele, causing an increase in the E2F1 activity, are highly predisposed to develop pituitary tumors (31), and this occurs also in mice with impaired function of p27 or p18, which both converge on pRB (32, 33). Moreover, the induction of the E2F1 activity plays a critical role also in the pituitary adenomas induced in transgenic mice overexpressing *Hmga1* or *Hmga2* (13).

Functional studies validated a role for the down-regulation of these miRNA in tumorigenesis. Indeed, the overexpression of the selected miRNA inhibited cell growth. Moreover, fluorescence-activated cell sorting analysis demonstrated that, consistent with the identified targets, these miRNA retain the cells in the G1 phase of the cell cycle. The results reported here are consistent with a recent study of our group showing that five miRNA targeting the *HMGA1* and *HMGA2* genes are down-regulated in human pituitary adenomas (34).

As mentioned in the introductory section, the miRNA expression profile of GH adenoma has been already re-

ported (19, 22). These studies reveal different down-regulated miRNA but do not show conflicting results. The differences between these studies may depend on 1) a different miRNA platform used for miRNA screening and 2) pharmacological treatment preceding the surgical intervention. Our microarray data were analyzed also by significance analysis of microarray test, a more stringent statistical technique for determining whether changes in gene or miRNA expression are statistically significant (for more details see Supplemental Information). Therefore, it is likely that, given the selectivity of our statistic analysis, our system has excluded some miRNA that have been previously identified as altered in GH adenomas. Of interest is the finding, in the study of Mao *et al.* (22) that miR-126 and miR-381 are down-regulated in GH adenomas. The target of these miRNA is the *PTTG* gene, which is overexpressed in most pituitary adenomas and is involved in multiple cellular pathways including proliferation, transformation, and angiogenesis (35, 36). Nevertheless, there are some common findings between our data and the ones previously published; miR-801 appears down-regulated in GH adenomas also in the work by Mao *et al.* (22), whereas miR-26 is reported as down-regulated in the study of Bottoni *et al.* (19).

It is worth noting that our study, as other ones analyzing RNA and miRNA expression profile in pituitary adenomas, do not use, for technical reasons, the appropriate control that should be represented by normal somatotrophs. However, the validity of our findings is supported by the demonstration that our selected down-regulated miRNA seem to have a role in pituitary tumorigenesis because they are able to regulate the growth of pituitary adenoma cells.

In conclusion, we report the identification of miRNA drastically and constantly down-regulated in GH adenomas. Because these miRNA target genes, such as *HMGA1*, *HMGA2*, and *E2F1*, whose overexpression and/or activation plays a critical role in pituitary tumorigenesis, it is reasonable to conclude that their down-regulation might contribute to this process, suggesting an approach to the therapy of pituitary adenomas based on the restoration of the down-regulated miRNA.

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