MicroRNA miR-107 is overexpressed in pituitary adenomas and inhibits the expression of aryl hydrocarbon receptor-interacting protein in vitro

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Trivellin G, Butz H, Delhove J, Igreja S, Chahal HS, Zivkovic V, McKay T, Patócs A, Grossman AB, Korbonits M. MicroRNA miR-107 is overexpressed in pituitary adenomas and inhibits the expression of aryl hydrocarbon receptor-interacting protein in vitro. Am J Physiol Endocrinol Metab 303: E708–E719, 2012. First published July 17, 2012; doi:10.1152/ajpendo.00546.2011.—Abnormal microRNA (miRNA) expression profiles have recently been associated with sporadic pituitary adenomas, suggesting that miRNAs can contribute to tumor formation; miRNAs are small noncoding RNAs that inhibit posttranscriptional expression of target miRNAs by binding to target sequences usually located in the 3′-UTR. In this study, we investigated the role played by miR-107, a miRNA associated with different human cancers, in sporadic pituitary adenomas and its interaction with the pituitary tumor suppressor gene aryl hydrocarbon receptor-interacting protein (AIP). miR-107 expression was evaluated in pituitary adenoma and normal pituitary samples using microRNA screen TLDA (TaqMan Low-Density Array) and RT-qPCR assays. We show that miR-107 expression was significantly upregulated in GH-secreting and nonfunctioning pituitary adenomas. We found that human AIP 3′-UTR is a target of miR-107 since miR-107 inhibited in vitro AIP expression to 53.9 ± 2% of the miRNA control in a luciferase assay and reduced endogenous AIP mRNA expression to 53 ± 22% of the miRNA control in human cells. However, we did not observe a negative correlation between AIP and miR-107 expression in the human tumor samples. Furthermore, we show that miR-107 overexpression inhibited cell proliferation in human neuroblastoma and rat pituitary adenoma cells. In conclusion, miR-107 is overexpressed in pituitary adenomas and may act as a tumor suppressor. We have identified and confirmed AIP as a miR-107 target gene. Expression data in human samples suggest that the expression of AIP and miR-107 could be influenced by a combination of tumorigenic factors as well as compensatory mechanisms stimulated by the tumorigenic process.

miR-107; pituitary adenoma

PITUITARY ADENOMAS ARE COMMON (accounting for ≤25% of all intracranial tumors) benign neoplasms that often grow invasively but very rarely progress to true carcinomas (3). Several genetic syndromes are known to be associated with the development of pituitary adenomas, but for sporadic adenomas the molecular pathology remains poorly understood. The only mutational changes unequivocally associated with sporadic pituitary adenomas are somatic mutations present in the GNAS1 gene, which occur in around 40% of growth hormone (GH)-secreting adenomas (36).

MicroRNAs (miRNAs) are a class of 20–25 base-pair (bp)-long noncoding RNAs that are found in the genomes of animals, plants, and protozoa (18); miRNA sequences are dispersed throughout the genome and are classified as intergenic (between genes) or intronic (embedded within a gene) (44). Mature miRNAs are incorporated into the miRNA-induced silencing complex, which associates with target messenger RNAs (mRNAs) (32). Depending on the overall degree of sequence complementarity, miRNAs negatively regulate posttranscriptional expression of target genes predominantly by inhibiting protein translation or degrading the target miRNA (18).

Recent studies have shown the abnormal expression of certain miRNAs in various human cancers, demonstrating that some miRNAs may function as oncomiRs or tumor suppressors (21, 64, 65). Pituitary tumors have also recently been shown to be associated with an abnormal miRNA expression profile, suggesting that miRNAs might contribute to pituitary adenoma development (56). However, there is a substantial lack of knowledge regarding the gene targets of deregulated miRNAs, and this limits a full understanding of the cellular mechanisms by which they influence pituitary adenoma pathogenesis.

We and others have previously explored the functions of the putative tumor suppressor gene aryl hydrocarbon receptor-interacting protein (AIP) in pituitary tumors (11, 24, 26, 27, 38, 62). AIP is a 37-kDa cytoplasmic cochaperone protein that has been reported to interact with at least 20 proteins (61). However, it is at the moment uncertain which of the AIP interactions play a role in pituitary tumorigenesis. Mutations affecting AIP were shown to be involved in the predisposition to develop a familial form of pituitary adenoma, although such mutations do not seem to play a role in the pathogenesis of sporadic tumors (12, 13, 38, 63). However, in sporadic somatotroph adenomas, which arise from a cell type normally expressing AIP, lower AIP expression was seen in aggressive tumors (27, 29). In addition, AIP expression in somatotroph adenomas correlates to invasiveness (29) and somatostatin responsiveness (30), and AIP is suggested to be involved in the somatostatin pathway (14). In contrast, expression of AIP was shown to be paradoxically higher in nonfunctioning pituitary adenomas (NFPAs), a tumor type arising from gonadotroph cells that normally do not express AIP (27, 29, 38). In addition, whereas in both normal and adenomatous somatotroph cells AIP is located in the secretory vesicles, the aberrantly ex-
pressed AIP in NFPAs and corticotroph adenomas has a different localization in the cytoplasm (38). The consequences of the aberrant localization of AIP in non-GH-secreting adenomas as well as of its abnormal expression, independent of germline mutations, are at present unknown.

In this study, we have investigated the involvement of the microRNA miR-107, known to be involved in tumorigenesis in other tissues, in the pathogenesis of sporadic pituitary adenomas and its interaction with AIP.

METHODS

miRNA Expression in Pituitary Tissues Using TaqMan Low-Density Array and Quantitative Real-Time PCR

Tissue samples and RNA extraction. Six NFPAs, three GH-producing adenomas, five GH- and prolactin (PRL)-producing adenomas (GHP), and five normal pituitary (NP) tissues from autopsy were combined into four pools and studied for global miRNA expression using TaqMan Low-Density Array (TLDA). An additional 49 human tissue samples [10 NP, 11 GH-secreting tumors, 4 GH-secreting adenomas (a typically seen cell type in AIP-positive adenomas), and 24 NFPAs] were subsequently assessed specifically for miR-107 expression using RT-quantitative PCR (qPCR) in two independent experiments. The tumors were collected fresh during surgery, and patients gave written informed consent for the protocol, which was approved by the local institutional review board. Following transsphenoidal surgery, the tumor specimens were immediately frozen in liquid nitrogen and stored at −80°C until use. Autopsy samples were collected within 24 h and stored at −80°C until use. Total RNA was extracted with the RNeasy Mini Kit (Qiagen, Crawley, UK), following the manufacturer’s instructions. RNA concentration was assessed by Nanodrop-1000 (Thermo Scientific, Wilmington, DE), and RNA integrity was measured by Agilent Bioanalyzer 2100 System (Agilent, Stockport, UK). Only samples with an RNA integrity number >7.9 have been analyzed further.

TLDA and quantitative real-time PCR. The miRNA expression profile was analyzed using TLDA Human MicroRNA Panel version 2 (Applied Biosystems, Foster City, CA), following the supplier’s protocol. Briefly, 300 ng/pool of total RNA was reverse transcribed using Megaplex RT Primer Pool A and B (part nos. 4399966 and 4399968; Applied Biosystems), ≤380 stem-looped primers per pool, and TaqMan MicroRNA Reverse Transcription Kit (part no. 4366596). Preamplification was carried out by Megaplex Preampl Primers and TaqMan PreAmp Master Mix. Quantitative real-time PCR (RT-qPCR) amplifications were run in TaqMan Human miRNA Array A and B (part nos. 4398965 and 4398966) using Megaplex Primer Pools, Human Pools Set version 3.0 (part no. 4444750), on the 7900HT Real Time PCR System (Applied Biosystems). RNU6B (part no. 4373381) was chosen as an endogenous control based on the supplier’s application note [Endogenous Controls for Real-Time Quantification of miRNA using TaqMan MicroRNA Assays (Applied Biosystems)], and we have confirmed that this gene’s expression level does not vary among our examined pituitary samples. Expression levels of miR-107 (part no. 4427975) were validated further by RT-qPCR, using the specific TaqMan MicroRNA Assay (Applied Biosystems). Reactions were performed in triplicate in a 384-well plate on a 7900HT Real-Time PCR System (Applied Biosystems), using RNU6B as endogenous control. An initial denaturation step of 10 min at 95°C was followed by 40 cycles of 95°C for 15 s and 60°C for 1 min. Relative gene expression data were analyzed using the ∆∆CT method (10, 40).

Cell Culture

The rat pituitary GH3 cell line was grown in Dulbecco’s modified Eagle’s medium (DMEM; Sigma-Aldrich, Poole, Dorset, UK) supplemented with 10% fetal bovine serum (Biosera, Ringmer, UK), penicillin (100 IU/ml), and streptomycin (100 μg/ml; Sigma-Aldrich) in a humidified atmosphere at 37°C with 5% CO2. This SH-SYSY human neuroblastoma cell line was cultured in a 1:1 mixture of DMEM and Ham’s F-12 medium (Sigma-Aldrich) supplemented and incubated as above.

The protein sequence of human AIP is 93% identical to rat AIP, whereas at the DNA level the similarity is 57%. The mature miR-107 sequence is conserved among different vertebrate and invertebrate species.

Target Prediction

The analysis of miR-107 predicted target genes was done using seven different algorithms: TargetScan version 5.1 (http://www.targetscan.org/) (39), microCosm Targets version 5.0 (http://www.ebi.ac.uk/enright-srv/microcosm/htdocs/targets/v5/) (23), MicroTar (http://tiger.dbs.nus.edu.sg/microtar/) (58), FindTar version 2.0 (http://bio.sz.tsinghua.edu.cn/findtar/) (73), Probability of Interaction by Target Accessibility (PITA; http://genie.weizmann.ac.il/pubs/mir07/mir07_prediction.html) (31), PicTar (http://www.picrar.org/) (33), and miRanda-September 2008 release (http://www.microrna.org/microrna/home.do) (6).

Construction of 3’-Untranslated Region Reporter Plasmid

A 931-bp segment of human AIP-3’-untranslated region (UTR) containing the putative miR-107 target sites was PCR amplified from the genomic DNA of a healthy control donor and cloned into the PGEM-T Easy Vector (Promega, Southampton, UK) using primers containing an XbaI binding site (forward: 5′-GGGTCTAGACGT-CAAGGCTACTTCAAGGC-3′; reverse: 5′-GGGTCTAGACGT- TCAAAGGGGACACT-3′). The XbaI fragment was then subcloned into the pGL3-Control Vector (Promega) using the XbaI site immediately downstream from the stop codon of the luciferase reporter gene. To examine whether the observed repression of a reporter gene by miR-107 was due specifically to the presence of predicted binding sites in the AIP 3’-UTR fragment, we also disrupted these sites by site-directed mutagenesis. For interrupting a perfect “seed” pairing, four nucleotides were deleted from each site of perfect complementarity using the QuickChange XL-site-directed mutagenesis kit (Agilent) and the following primers: site 1: forward 5′-GCAAGAGCCACTCAGCTGCAGAC-3′ and reverse 5′-GGCTGAGCACGTCAGTGGTGCGAGG-3′; site 2: forward 5′-GGCTGAGCACGTCAGTGGTGCGAGG-3′ and reverse 5′-AGGCTGAGCACGTCAGTGGTGCGAGG-3′. Because there are two predicted binding sites for miR-107 in the AIP 3’-UTR, three variants were generated, with one of each mutated and both mutated. Wild-type (WT) and mutant inserts were confirmed by sequencing.

Luciferase Gene Reporter Assay

GH3 cells were seeded in six-well plates at a density of 6 × 10⁴ cells/well. After 24 h, cells were cotransfected with Lipofectamine 2000 (Invitrogen, Paisley, UK) according to the manufacturer’s protocol, using 2 μg of the pGL3-control vector and 0.2 μg of the Renilla vector [pRL-cytomegalovirus (CMV)]. For each plate, the pre-miR-107 precursor or the corresponding scrambled miRNA was cotransfected at a final concentration of 50 nM.

Firefly and Renilla luciferase activities were measured consecutively 24 h posttransfection using the Dual-Luciferase Reporter Assay System (Promega). Ratios of Firefly vs. Renilla luminescence signals served as a measure for reporter activity normalized for transfection efficiency.

Endogenous AIP Expression After miR-107 Overexpression/Inhibition

GH3 and SH-SYSY cells were seeded in 12-well plates at a density of 3 × 10⁵ cells/well on the day before transfection. Cells were transfected with the pre-miR-107 precursor (PM10056; Applied Biosystems), the anti-miR-107 inhibitor (AM10056; Applied Biosys-
miR-107 INHIBITS AIP

miR-107 overexpression/inhibition by RT-qPCR for miR-107 using the specific TaqMan MicroRNA Assay (Applied Biosystems). Cell growth was measured at 48 h after transfection by [3H]thymidine incorporation, as described previously (45). Lentiviral-transduced GH cells were seeded in 24-well plates at 1 × 10^5 cells/well, and cell growth was measured after 48 h.

**Colony Formation Assay**

Lentiviral-transduced GH3 cells were seeded in six-well plates at a density of 1,500 cells/well to form colonies and were cultured at 37°C for 7 days. Cells were fixed in 4% paraformaldehyde, and colony formation was detected by staining the cells with a 0.1% crystal violet solution. Colony number and average colony size were determined using Image J (http://imagej.nih.gov/ij/).

**Statistical Analysis**

Statistical analysis was performed with StatsDirect software (Addison-Wesley-Longman, Cambridge, UK). Data are presented as the mean ± SE of two to six independent experiments, each performed in triplicate. Comparisons were calculated using the unpaired, two-tailed Student t-test and the Kruskal-Wallis test followed by the Conover-Inman test as appropriate. P < 0.05 was considered significant.

**RESULTS**

miR-107 is Overexpressed in GH-Secreting and NFPA Adenomas Compared with Normal Pituitaries

The miRNA expression profiles of pooled pituitary adenoma samples were determined using TLDA. Out of 738 miRNA genes available on the TLDA cards, we found 174 miRNAs expressed in all four pituitary tissue pools, whereas we could not detect the expression of 488 miRNAs. Examination of the expression profiles revealed that nine miRNAs were expressed in normal tissues but not in the adenomas, and seven miRNAs were expressed in the adenomas but not in normal tissue (Table 1), whereas 66 miRNAs were differentially expressed in adenomas and normal tissue, with ≥2.5-fold difference. One of these miRNAs, miR-107, is known to be associated with human cancer (20). The TLDA experiment showed a 3.1-fold increase in the expression of miR-107 in the NFPA pool and no change in GH/GHP pools compared with NP. In validation studies using RT-qPCR in a first set of samples (4 GH, 4 GHP, 14 NFPA, and 1 NP), miR-107 upregulation was confirmed for NFPA (3.6 ± 1.6-fold), and we also observed miR-107 overexpression in both GH and GHP adenomas (1.7 ± 2.9-fold and 1.6 ± 0.25-fold, respectively). The analysis was also extended to a second larger series of independent samples (10

**Table 1. Differentially expressed miRNAs in normal and tumor pituitary samples**

<table>
<thead>
<tr>
<th>miRNAs</th>
<th>hsa-miR-509-3-5p, hsa-miR-508-5p, hsa-miR-452, hsa-miR-330-5p, hsa-miR-200a, hsa-miR-503, hsa-miR-424, hsa-miR-499a, hsa-miR-199-5p</th>
</tr>
</thead>
<tbody>
<tr>
<td>miRNAs expressed in all types of adenomas but not in normal pituitary</td>
<td>hsa-miR-378, hsa-miR-516-3p, hsa-miR-151-3p, hsa-miR-224, hsa-miR-618, hsa-miR-455-3p, hsa-miR-29b</td>
</tr>
<tr>
<td>miRNAs detected only in the NFPA group</td>
<td>hsa-miR-217, hsa-miR-216a, hsa-miR-215, hsa-miR-502, hsa-miR-338, hsa-mir-10b, hsa-miR-96, hsa-miR-202, hsa-miR-501, hsa-miR-18a, hsa-miR-450a, hsa-miR-329</td>
</tr>
<tr>
<td>miRNAs detected only in the GH group</td>
<td>hsa-miR-1, hsa-miR-760, hsa-miR-196b, hsa-miR-188-5p, hsa-miR-146b-3p</td>
</tr>
<tr>
<td>miRNAs detected only in the GHP group</td>
<td>hsa-miR-205, hsa-miR-132*, hsa-miR-523</td>
</tr>
</tbody>
</table>

miR, microRNA; NFPA, nonfunctioning pituitary adenoma; GH, growth hormone; GHP, GH- and prolactin-producing adenomas.
miR-107 INHIBITS AIP

Fig. 1. miR-107 is expressed abnormally in pituitary tumors. The relative expression levels of miR-107 in normal pituitary (NP), nonfunctioning pituitary adenomas (NFPA), and growth hormone (GH)-secreting adenomas were assessed by RT-quantitative PCR (qPCR) in NP (n = 9), NFPA (n = 10), and GH (n = 7) samples. Data are expressed as mir-107/RNU6B ratio to NP. *P < 0.05.

GH, 7 NFPA, and 9 NP samples). In this second set of samples, miR-107 was found to be overexpressed 3.5 ± 0.97-fold in NFPA and 2.5 ± 0.39-fold in GH-secreting adenomas (P < 0.05 for both; Fig. 1), thus confirming miR-107 overexpression in both tumor types.

**miR-107 is Predicted to Bind the Human AIP 3'-UTR at Two Sites**

To identify a miR-107 putative target gene that might be involved in pituitary adenoma development, we conducted an in silico analysis. Five out of seven target prediction algorithms used in this study predicted miR-107 as potentially targeting the 3'-UTR of human AIP. A 7mer-A1 site (for the definition of the different types of target sites, see Ref. 5) located 38–44 bp downstream of the stop codon of AIP (site 1) was predicted by the TargetScan, FindTar, and PITA programs, whereas a 6mer site at position 65–70 bp downstream of the stop codon of AIP (site 2) was predicted by the MicroCosm, MicroTar, FindTar, and PITA programs (Fig. 2). Site 2 is predicted to have better accessibility than site 1 based on PITA and FindTar outputs. In the rat AIP sequence, no miR-107 binding sites were identified.

**miR-107 Represses AIP Through Binding at Site 2**

To experimentally verify the in silico predicted interaction between miR-107 and AIP, we cloned the human WT AIP 3'-UTR into a pGL3 reporter vector downstream to the coding sequence of Firefly luciferase. In this way, the total luciferase output of the AIP 3'-UTR-Firefly luciferase hybrid transcript depends on the effect of the AIP 3'-UTR. To reduce the background noise due to possible endogenous miR-107 interaction with the AIP-3'-UTR, we chose GH3 cells as reporter cells because they express relatively low levels of endogenous miR-107 (Fig. 3A). As shown in Fig. 3B, the construct containing the human WT AIP 3'-UTR reduced luciferase expression to 58 ± 0.9% of the empty vector (P < 0.05). We next assessed the effect of miR-107 on the activity of the hybrid transcript. When the pre-miR-107 precursor was transfected into GH3 cells along with the WT AIP 3'-UTR, a 50% reduction of luciferase activity was observed compared with the control scrambled miRNA (Fig. 3C). We then investigated whether one or both predicted miR-107 target sites in AIP 3'-UTR (site 1 and site 2) were functional. For this end, two deletion mutants targeting the two putative binding sites were generated. We refer to them as MUT1 for the mutated binding site 1, MUT2 for site 2, and MUT1 + 2 for the sequence carrying both mutations. The luciferase assay demonstrated that miR-107 still inhibits luciferase activity at comparable levels after mutation of site 1, whereas it loses the inhibitory effect on luciferase activity with both MUT2 and the combined MUT1 + 2 (Fig. 3C).

To confirm that the decreased luciferase activity was really caused by miR-107 interaction with the cloned fragment and not by nonspecific binding with the backbone sequence of the pGL3 reporter vector [an effect seen before (10)], we compared the effect exerted by the pre-miR-107 precursor and the scrambled miRNA on the empty pGL3 vector. As shown in Fig. 3D, miR-107 did not change the luciferase activity of the empty vector compared with the scrambled miRNA control. To verify the reliability of our assay, we included in each experiment a reporter vector containing the 3'-UTR of the NFI-A gene (Fig. 3E), which had previously been demonstrated to be targeted by miR-107 using the same technique (22).

**Regulation of Endogenous AIP Expression by miR-107 in Vitro and in Human Pituitary Samples**

It is well known that miRNAs are able to downregulate mRNA and/or the protein levels of their target genes (18). Therefore, to further characterize the interaction between miR-107 and AIP, we measured mRNA and protein levels of endogenous AIP after miR-107 overexpression in a human cell line. Because no human pituitary cell lines are presently available, we evaluated the effect of miR-107 overexpression in AIP 3'-UTR-expressing transfected human cell lines because they express relatively low levels of endogenous AIP 3'-UTR.

**Fig. 2. Graphic representation of the 2 predicted miR-107 target sites in human aryl hydrocarbon receptor-interacting protein (AIP)-3'-untranslated region (UTR).** The localization of the seed regions is reported. The underlined sequences represent the 4 deleted nucleotides in MUT1 (for mutated binding site 1) and MUT2 (for mutated binding site 2) AIP-3'-UTR plasmids.
available, we chose a neuroblastoma cell line (SH-SY5Y) because of its human origin and its relatively high expression levels of AIP (62).

As shown in Fig. 4A, transient miR-107 overexpression, but not the scrambled miRNA, caused endogenous human AIP expression to decrease at both the mRNA and protein levels. At the mRNA level, miR-107 decreased AIP expression to 53 ± 22% of the scrambled miRNA control at 48 h (P < 0.05). The decrease in AIP protein expression was already detectable at 24 h posttransfection and became more pronounced at 48 and 72 h, reaching statistical significance (P < 0.001). Similar AIP mRNA and protein knockdown levels were obtained in SH-SY5Y cells stably overexpressing miR-107 (Fig. 4B).

We have shown that overexpression of miR-107 inhibits AIP expression. We tried to shift the balance of these two factors in the opposite direction. Via reducing endogenous miR-107 using transient transfection of anti-miR-107, one could expect an increase in AIP expression if the regulation of AIP via miR-107 is working in both directions, i.e., inhibition of AIP when miR-107 levels are high and stimulation of AIP if miR-107 levels are low. We observed no change in AIP levels when miR-107 was inhibited (Fig. 4C), suggesting that miR-107-induced regulation works in one direction but not the other, a phenomenon previously observed for other miRNAs (54). To confirm that our miR-107 overexpression has an effect on other miR-107 targets, ARNT was studied as a positive control. We observed that miR-107 overexpression results in downregulation of ARNT, as expected (data not shown).

Because we used GAPDH as an endogenous control housekeeping gene in our experiments, it is important to show that GAPDH expression is not influenced by miR-107. In silico analysis does not predict miR-107 target sites on GAPDH, and our transfection/transduction experiments showed no changes...
in GAPDH expression, in agreement with previous studies (Fig. 4) (43, 72).

To see whether miR-107 levels correlate with AIP expression in human pituitary adenoma samples, we studied AIP mRNA and protein levels in normal pituitary, NFPAs, and GH-secreting adenomas. At the mRNA level, we did not see any significant difference in AIP expression among the NP, NFPA, and GH groups (Fig. 5A), whereas we found higher AIP mRNA and protein levels in normal pituitary, NFPAs, and GH-secreting adenomas. At the mRNA level, we did not see any significant difference in AIP expression among the NP, NFPA, and GH groups (Fig. 5A), whereas we found higher AIP expression levels in the 3 groups was measured by RT-qPCR. No statistically significantly different mRNA levels were observed among the groups. B: AIP protein expression in NP (n = 7), NFPA (n = 5), and GH-secreting tumors (n = 4) assessed by Western blot. Significantly higher miR-107 levels were found in GH-secreting and NFPA tumors compared with NP. Western blot images coming from different parts of the same gel were separated. *P < 0.05; **P < 0.01.

Fig. 4. Effect of miR-107 on endogenous AIP mRNA and protein expression in SH-SY5Y cells. Both AIP mRNA and protein levels were downregulated significantly in SH-SY5Y cells transiently (n = 3; A) or stably overexpressing miR-107 (n = 3; B), as measured by RT-qPCR and Western Blot. C: no increase in AIP protein expression was observed in SH-SY5Y cells transiently transfected with anti-miR-107 (n = 3). In miR-107-transduced cells, RT-qPCR and Western blot experiments were performed 72 h posttransduction. RT-qPCR data are expressed as AIP/GAPDH ratio to scrambled control-transfected/green fluorescent protein (GFP) control-transduced cells. Western blot data are expressed as AIP/GAPDH ratio to scrambled control-transfected/GFP control-transduced cells. Western blot images coming from different parts of the same gel were separated. *P < 0.05; **P < 0.01; ***P < 0.001.

Fig. 5. AIP expression levels in NP, NFPA, and GH-secreting adenomas. A: AIP mRNA expression in the 3 groups was measured by RT-qPCR. No statistically significantly different mRNA levels were observed among the groups. B: AIP protein expression in NP (n = 7), NFPA (n = 5), and GH-secreting tumors (n = 4) assessed by Western blot. Significantly higher miR-107 levels were found in GH-secreting and NFPA tumors compared with NP. Western blot images coming from different parts of the same gel were separated. *P < 0.05; **P < 0.01.
protein expression in both the NFPA and GH groups compared with normal pituitaries (Fig. 5B). No significant correlation was seen between AIP mRNA/protein levels and miR-107 expression in the samples as a whole or in the individual tissue types.

miR-107 Suppresses Cell Proliferation in SH-SY5Y Cells

To determine the biological functions of miR-107, we investigated its effect on cell proliferation in SH-SY5Y cells. We overexpressed/inhibited miR-107 by transient transfection of pre-miR-107 and anti-miR-107, respectively, and we confirmed successful upregulation/knockdown by RT-qPCR (Fig. 6A). We observed a significant decrease in cell proliferation in pre-miR-107-transfected cells compared with controls (\( P < 0.05 \); Fig. 6B). Anti-miR-107 did not increase cell proliferation, suggesting that baseline proliferation rate is not affected by miR-107 under normal conditions (Fig. 6B), similar to AIP levels (Fig. 4C).

We also generated cell lines stably overexpressing the mature miR-107 using lentiviral transduction (>90% GFP-positive cells; Fig. 6C). The levels of miR-107 increased 92-fold compared with cells transduced with LV-GFP only (\( P < 0.01 \); Fig. 6D). A few days after lentiviral transduction of miR-107, SH-SY5Y cells started to exhibit a different morphology compared with GFP control-transduced cells; cells became rounder in shape and started to die (Fig. 6E), suggesting that high miR-107 levels have a detrimental effect on cell viability in the long term.

miR-107 Effects on GH3 Cells

miR-107 is not predicted in silico to bind to the 3′-UTR of rat Aip. We have proven this experimentally in rat GH3 cells,
which were shown previously to express WT Aip at appropriate levels (14, 38), since no reduction in Aip protein expression was seen after transient or stable expression of miR-107 (Fig. 7, A and B). However, cell proliferation was reduced after transient (P < 0.05) and stable miR-107 expression (P < 0.001) (Fig. 7, C and D), whereas no effect was seen on colony size (P = 0.08) or number (P = 0.71).

**DISCUSSION**

In this study, we investigated the role of miR-107 in pituitary tumorigenesis and its interaction with the pituitary tumor suppressor gene *AIP*. miR-107 is a member of the miR-15/107 superfamily and has been shown to be associated with human cancer (20), with overexpression in several tumor types (41, 53, 64) but underexpression in others (19, 53, 57, 72). Members of this miR family have previously been found to be altered in pituitary adenomas (2, 8, 9), suggesting that they may play a role in the pathogenesis of pituitary tumors. The expression of miR-107 has been observed in many mammalian tissues, with the highest concentration found in the brain (51, 57), and in this study we show, using TLDA and RT-qPCR on normal human pituitaries and pituitary adenomas, that miR-107 is significantly upregulated in both sporadic GH-secreting pituitary adenomas and NFPAs. Our TLDA findings are in agreement with recently published miRNA array data showing miR-107 overexpression in GH-secreting tumors compared with normal pituitary (48) and are compatible with previous data by Bottoni et al. (9) showing increased expression of miR-107 in NFPAs. Moreover, our validation with additional RT-qPCR strengthens the results.

Several direct miR-107 target genes have been identified and experimentally confirmed in various studies (4, 7, 15, 22, 25, 37, 41, 43, 47, 48, 57, 60, 66–68, 72). We decided to identify miR-107 target genes that could be involved in pituitary adenoma pathogenesis by using various in silico software programs (5, 6, 23, 31, 33, 39, 58, 73). Combining all the outputs, the best miR-107 functional candidate target was the pituitary tumor suppressor gene *AIP*. In particular, two predicted target sites were repeatedly detected in the 3′-UTR of human *AIP*. In agreement with a previous report (72), we also confirmed in this study that ARNT (also known as hypoxia-inducible factor-1β) is downregulated when miR-107 is overexpressed. Since ARNT is involved in one of AIP-mediated pathways, it is interesting that both of these proteins are targeted by the same miRNA, suggesting a potential role for miR-107 in the regulation of this pathway. To determine whether miR-107 binds in vitro to AIP, we fused the entire human *AIP* 3′-UTR containing the predicted miR-107 target sites to a luciferase reporter plasmid. As shown in Fig. 3B, the luciferase activity of the hybrid transcript was lower compared with the luciferase vector without the insert. The 3′-UTR of *AIP* could thus be considered a negative regulator of gene expression. We then observed that high levels of miR-107 exert a negative effect on the luciferase expression of the vector containing the WT AIP 3′-UTR, suggesting that miR-107 can bind to the AIP 3′-UTR. The luciferase assay was then performed with different AIP 3′-UTR mutants for the two putative miR-107 binding sites. Our data suggest that only site 2 is responsible for miR-107-mediated *AIP* repression. This result indicates that binding to site 1 has a lower miR-binding ability under experimental conditions, probably because of poor site accessibility that impedes the miRNA-mRNA duplex formation. Furthermore, if we applied the most stringent criteria for miR-binding predictions (73), only one program out of seven would predict site 1.

According to the classification made by Ye et al. (73), the miR-107-AIP duplex forming at site 2 possesses a type I decentered loop, which was shown to decrease the repressive efficiency of miRNAs. It is thus likely that miR-107 acts cooperatively with other negative and positive regulators in the fine-tuning of global AIP expression. Whatever is the precise mechanism, we demonstrate here that in our experimental conditions miR-107 overexpression alone is sufficient to significantly inhibit endogenous AIP mRNA and protein levels in a human cell line (Fig. 4). Interestingly, our experimental results are in agreement with a microarray analysis showing

![Fig. 7. Effect of miR-107 on endogenous AIP protein expression and cell proliferation in GH3 cells. AIP protein levels do not differ in GH3 cells transiently (A) or stably overexpressing miR-107 (B) compared with their relative controls, as measured by Western blot (n = 3). Western blot data are expressed as AIP/GAPDH ratio to scrambled control-transfected/GFP control-transduced cells. In miR-107-transduced cells, Western blot experiments were performed 72 h posttransduction. GH3 cells were either transfected with pre-miR-107 and anti-miR-107 (C) or transduced with LV-miR-107 (D), and proliferation was measured by [3H]thymidine incorporation 48 h after transfection/transduction. Data are expressed as ratio to scrambled control-transfected cells or LV-GFP-transduced cells (n = 3). *P < 0.05; ***P < 0.001.](http://ajpendo.physiology.org/)

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miR-107 INHIBITS AIP

AIP downregulation in miR-107-overexpressing human lung cancer cells (57).

On the other hand, when we transiently silenced the endogenous miR-107 in human SH-SYSY cells we did not observe an increase in AIP expression, and we also did not see a change in cell proliferation, suggesting that regulation of AIP is primarily inhibitory by elevated miR-107 but not influenced by low miR-107 levels. However, we cannot fully exclude either that anti-miR-107 has off-target effects (59), for instance, inhibition of the closely related miR-103, which has high sequence homology with miR-107 [only 1-bp difference (20)], or, alternatively, that miR-103 might compensate for the absence of miR-107, since miRNAs are often redundant (1).

Taken together, these results suggest that in vitro miR-107 can bind to the 3′-UTR of the human AIP mRNA and inhibit protein synthesis by RNA degradation.

Since miR-107 was found to be upregulated in sporadic pituitary adenomas and shown to negatively regulate AIP expression, we expected to find low AIP levels in such tumors. However, we found no significant differences in AIP mRNA levels between the normal and adenomatous tissues. In NFPAs, which arise from gonadotroph cells that normally do not express AIP (38), high AIP protein expression was observed, in agreement with other studies (27, 29, 38). Here, we report an overall higher expression of AIP in GH-secreting tumors compared with normal pituitaries. Previously, overall normal (27, 38), high, or low (29) AIP levels were found in somatotropinomas (27, 29). Actually, lower AIP expression is now suggested to predict aggressive behavior and somatostatin resistance of somatotroph adenomas (14, 29, 30). Thus, miR-107 inhibition of AIP expression could be responsible for the relatively low levels of AIP observed in aggressive somatotroph adenomas, a cell type where AIP is physiologically expressed and therefore sensitive to normal regulators, whereas miR-107-induced gene expression regulation may not be able to act on the aberrantly expressed AIP in NFPAs. In normal pituitaries, we observed a negative correlation trend ($r^2 = 0.49$, $P = 0.09$) between miR-107 and AIP protein expression levels. Therefore, these findings suggest that in tumor cells miR-107 and AIP expression could be influenced by a combination of tumorigenic factors as well as compensatory mechanisms stimulated by the tumorigenic process.

The discrepancy observed for AIP mRNA and protein levels in our samples could be due to the high stability of the AIP protein [in vitro half-life of 20.9 h (42)] vs. AIP mRNA. AIP phosphorylation (17) might be a posttranslational modification that positively influences AIP stability and expression.

An explanation for the elevated concomitant miR-107 and AIP levels could lie in a compensatory mechanism trying to oppose the tumorigenic process induced by reduced AIP levels, including other positive regulators of AIP that provide a mechanism to restore AIP levels. Similar compensatory up-regulation of tumor suppressor genes has been described previously in pituitary adenomas (35, 50, 52) and could explain the generally benign nature of these tumors. Interestingly, the lack of correlation between miR-107 and AIP expression in human tumor sample levels recapitulates the relationship between miR-107 and its host gene PANK1. PANK1 encodes an enzyme involved in the regulation of cellular coenzyme A (CoA) levels (20), but although miR-107 was predicted to act synergistically with PANK1 in pathways involving the acetyl-CoA and lipid levels (70), the expression of the mature miRNA is not synchronized with that of the host gene (34, 51).

Our results reflect the complex nature of gene regulation and expression during pituitary tumorigenesis and could open up new investigations. The fact that this and other studies (27, 29, 38) found increased AIP expression in pituitary adenomas (unexpected for a pituitary-related tumor suppressor gene) supports the complexity of the situation.

Experimental evidence supports a role for miR-107 in cell cycle arrest and growth suppression in lung and pancreatic cancers (53, 57). In sharp contrast, in breast cancer cells, high levels of miR-107 have been shown to promote invasiveness and metastatic dissemination (41). Thus, it is apparent that miR-107 can act as a tumor suppressor or as an oncomiR, depending on the cell type, similarly to other miRNAs (46, 49, 55, 69), suggesting that the net outcome depends on the relative expression levels of its targets. In the present study, we show that miR-107 decreases cell proliferation in human SH-SYSY and rat GH3 cells. Because human pituitary cell lines are not available, data gained using the neuroblastoma cell line need to be interpreted with caution regarding the behavior of human pituitary adenomas. These results demonstrate that miR-107 behaves as a tumor suppressor gene in neuroblastoma and pituitary adenoma cell lines. In particular, the tumor-suppressive effect detected in neuroblastoma cells complements previous findings in these cells showing reduced cellular migration after miR-107 overexpression (43). Moreover, results gained in the GH3 cells indicate that miR-107 explicates its tumor-suppressive effects through binding to a combination of target genes other than AIP.

Interestingly, we noted that stable miR-107 overexpression in both pituitary adenoma and neuroblastoma cells induces cell death. This effect was quite rapid in neuroblastoma cells, occurring just a few days after lentiviral transduction, whereas in pituitary adenoma cells was a bit more delayed, suggesting that there is a threshold after which the cells are not able to sustain any more continuously elevated miR-107 levels. Interestingly, it has been reported that miR-129 overexpression leads to G1 cell cycle arrest and eventually to cell death (71). Since miR-107 has also been found to arrest the cell cycle in the G1 phase (19, 25, 37, 57), we could speculate that this mechanism underlies our observations in GH3 and SH-SYSY cells. However, further studies are necessary to confirm this hypothesis.

Taking all these data together, we believe that AIP is differentially regulated in various pituitary cell types, as supported by 1) the different expression in normal pituitary cells (38), 2) the clinical data from AIP mutant patients primarily having somatotrop adenomas (12, 16), 3) the aberrant emergence of AIP expression in sporadic non-GH/PRL adenomas (38), and 4) the aberrant subcellular localization of AIP in sporadic non-GH-secreting adenomas (27, 38). Our data on miR-107 inhibiting AIP expression could contribute to the aggressiveness of somatotroph adenomas, a cell type where AIP is physiologically expressed and therefore sensitive to normal regulators, whereas miR-107-induced gene expression regulation may not be able to act on the aberrantly expressed AIP in NFPAs.

In conclusion, miR-107 is overexpressed in pituitary adenomas and may act as a tumor suppressor. We have identified and experimentally validated AIP as a miR-107 target gene and suggest that both AIP and miR-107 interact and may play roles in pituitary adenoma tumorigenesis.
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DISCLOSURES

The authors have nothing to disclose.

AUTHOR CONTRIBUTIONS

G.T., S.I., A.P., and M.K. were involved with the conception and design of the research; G.T., H.B., J.D., S.I., H.S.C., and V.Z. performed the experiments; G.T. analyzed the data; G.T., T.M., and M.K. interpreted the results of the experiments; G.T. prepared the figures; G.T. drafted the manuscript; G.T., T.M., A.B.G., and M.K. edited and revised the manuscript; M.K. approved the final version of the manuscript.

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