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Molecular Pathogenesis of Genetic and Inherited Diseases

The Expression of *AIP*-Related Molecules in Elucidation of Cellular Pathways in Pituitary Adenomas

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Germline mutations in the *aryl hydrocarbon receptor interacting protein* (*AIP*) gene predispose to the development of pituitary adenomas. Here, we characterized *AIP* mutation positive (*AIP*mut+) and *AIP* mutation negative (*AIP*mut−) pituitary adenomas by immunohistochemistry. The expressions of the AIP-related proteins aryl hydrocarbon receptor (AHR), AHR nuclear translocator (ARNT), *cyclin-dependent kinase inhibitor 1B* encoding p27(Kip1), and hypoxia-inducible factor 1-α were examined in 14 *AIP*mut+ and 53 *AIP*mut− pituitary adenomas to detect possible expression differences. In addition, the expression of CD34, an endothelial and hematopoietic stem cell marker, was analyzed. We found ARNT to be less frequently expressed in *AIP*mut+ pituitary adenomas ($P = 0.001$), suggesting that *AIP* regulates the ARNT levels. *AIP* small interfering RNA-treated HeLa, HEK293, or *Aip*-null mouse embryonic fibroblast cells did not show lowered expression of ARNT. Instead, in the pituitary adenoma cell line GH3, *Aip* silencing caused a partial reduction of Arnt and a clear increase in cell proliferation. We also observed a trend for increased expression of nuclear AHR in *AIP*mut+ samples, although the difference was not statistically significant ($P = 0.06$). The expressions of p27(Kip1), hypoxia-inducible factor 1-α, or CD34 did not differ between tumor types. The present study shows that the expression of ARNT protein is significantly reduced in *AIP*mut+ tumors. We suggest that the down-regulation of ARNT may be connected to an imbalance in AHR/ARNT complex formation arising from aberrant cAMP signaling. ***(Am J Pathol 2009, 175:2501–2507; DOI: 10.2353/ajpath.2009.081131)***

Pituitary adenomas are common, benign intracranial neoplasms. They can be nonfunctioning or hypersecrete pituitary hormones, most often prolactin or growth hormone.[1] Although most pituitary adenomas are sporadic, a subset occurs as component tumors in rare familial syndromes.[2,3] Germline mutations in *aryl hydrocarbon receptor interacting protein* (*AIP*) gene have been recently shown to cause pituitary adenoma predisposition.[4] So far, several *AIP* mutations have been identified in different populations in both familial and apparently sporadic cases with pituitary adenomas.[5,6] Typically patients with pituitary adenoma predisposition have a young age at disease onset, but they do not display a strong family history of pituitary adenomas. Occurrence of loss of heterozygosity in the tumors suggests that *AIP* acts as a tumor suppressor gene.[4]

AIP is known to form a complex with the aryl hydrocarbon receptor (AHR) and two 90-kDa heat-shock proteins.[7] AIP has also been reported to interact with at least two phosphodiesterase (PDE) isoforms (PDE2A and PDE4A5), peroxisome proliferation-activated receptor-α,

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survivin, translocase of the outer membrane of mitochondria 20, and thyroid hormone receptor β1.[8–13] In pituitary tumorigenesis, *AHR* appears to be an interesting candidate gene, since AHR is a transcription factor that initiates the dioxin-response of the cell by regulating the expression of several xenobiotic metabolizing enzymes.[14] The binding of dioxins or dioxin-like chemicals, which display high affinity to the AHR complex, leads to shuttling of AHR to the nucleus where it heterodimerizes with aryl hydrocarbon receptor nuclear translocator 1 (ARNT ie, hypoxia-inducible factor [HIF]-1β). ARNT is essential for the normal function of AHR, which encompasses the altered transcription and expression of various target genes of XREs.[14,15] It is postulated that the carcinogenic effect of dioxins is due to their activation of AHR and subsequent regulation of transcription.[16] Several studies are also suggesting that AHR has an endogenous role in normal cell physiology in absence of xenobiotics, eg, in cell proliferation, cell adhesion, and migration.[17]

Another heterodimerization partner of ARNT (HIF-1β) is HIF-1α. HIF-1α/ARNT heterodimer regulates several genes involved in tumorigenesis under hypoxia and in response to environmental toxins. In this manner, HIF-1α and AHR share an analogous signal transduction pathway.[18] HIF-1α is present at high levels in many tumors, contributing to angiogenesis, proliferation, metastasis, and resistance to radiation therapy.[19]

One interesting AHR target gene is *cyclin-dependent kinase inhibitor 1B (CDKN1B*) encoding p27(Kip1).[20] *p27*(*Kip1*) is a tumor suppressor that has multiple roles in the cell. p27(Kip1) protein acts as a cell cycle regulator and it shares overlapping pathways with retinoblastoma, a known tumor-suppressor gene linked to pituitary tumors.[21,22] It is reported that *p27*(*Kip1*) is underexpressed or absent in most human pituitary tumors.[23] In addition, the recent studies have shown that germline mutations in *p27*(*Kip1*) cause a multiple endocrine neoplasia type 4 condition that is characterized by pituitary adenomas.[2,24]

The recent functional data has revealed that *AIP* indeed has properties consistent with a tumor suppressor gene.[6] Although *AIP* is the subject of dynamic research, the exact mechanisms by which *AIP* exerts its tumor suppressive action in pituitary tumorigenesis are still obscure. Clearly, more data on the molecular mechanisms by which *AIP* mutations lead to pituitary adenoma predisposition are desired. Especially, the modulating role of *AIP* in the AHR/ARNT signaling raises the question on the outcome of the loss of *AIP* expression in this pathway.[25] Here, we profiled by immunohistochemistry the expression of *AIP* related molecules in an extensive—considering the rarity of the condition—set of *AIP*mut+ and *AIP*mut− pituitary adenomas, to reveal alterations that could contribute to AIP-associated pituitary tumorigenesis. The examined molecules were: ARNT, AHR, p27(Kip1), and HIF-1α. In addition, hematopoietic stem cell marker CD34 was used as a marker for studying vasculature in the tumors.

Materials and Methods

Samples

A total of 67 paraffin blocks from pituitary adenoma tissue were analyzed. Fourteen of the samples were from *AIP*mut+ patients, harboring four different mutations (Q14X, 824insA, IVS3-1G>A and IVS2-1G>C).[4,26] From these *AIP*mut+ adenomas, four secreted growth hormone (GH), three secreted prolactin, and six were mixed adenomas (GH+prolactin). According to the patient records the remaining adenoma secreted at least GH, since the patient had gigantism although the hormonal staining of the adenoma had failed. Of the 53 *AIP*mut− sporadic adenomas, 15 secreted GH, seven prolactin, 14 GH + prolactin, and one adrenocorticotropic hormone.[4,27] One adenoma secreted GH + prolactin + adrenocorticotropic hormone, one GH + prolactin + luteinizing hormone, as well as thyroid-stimulating hormone. Ten adenomas were hormonally silent. The hormonal staining failed in four adenomas, but the diagnosis of acromegaly in these patients proves at least GH oversegregation. Sixty-five patients were from Finland and two *AIP*mut+ individuals were from the United States.

Immunohistochemistry

Immunohistochemical staining was performed according to standard procedures on 4- to 5-μm sections of paraffin-embedded pituitary adenoma specimens. Human subjects provided appropriate informed consent. In some cases permission to use the samples was given by the Head of Section, HUSLAB, Division of Pathology, Meilahti Laboratories of Pathology, Helsinki University Central Hospital, and The National Authority for Medicolegal Affairs. Approval of the study was obtained from the Ethical Board. The samples were anonymized by two of the authors (J.A. and A.P.). Power Vision Poly-HRP IHC Kit (ImmunoVision Technologies, Norwell, MA) or Dako ENVISION Kit (Dako Glostrup, Denmark) was used for detection of ARNT (SC-5580; 1:200; Santa Cruz, CA), AHR (ab2770; 1:2000; Novus Biologicals, Littleton, CO), HIF-1α (610958; 1:100; BD Biosciences, San Jose, CA), p27(Kip1) (610244; 1:500; BD Biosciences), and CD34 (M7165; 1:50; Dako). 3,3′-Diaminobenzidine was used as a chromogen and hematoxylin as a counterstain. AHR staining was scored as negative (score = 0) or positive (1). The staining intensity of ARNT, HIF1α, and p27(Kip1) was scaled as negative (0), weak (1), intermediate (2), or high (3). In addition, the fraction of the positively staining cells was evaluated in the case of HIF1α. Specimens with less than 1% of cells staining were scored as 0, when 1% to 10% of cells positive as 1, 10% to 50% of cells positive as 2, and more than 50% of cells positive as 3. The density of CD34-vessels per square millimeter was recorded. To avoid imprecision, in most samples, the mean of two separate vessel counts was calculated.

Preparation of Mouse Embryonic Fibroblasts

An *Aip* mouse model was created using the gene trapping method.[28] The null allele of *Aip* was generated by inserting a gene trap vector construct between *Aip* exons 2 and 3 (ENSMUST00000117831) into an intronic region of genomic DNA (BayGenomics, University of California, Davis, CA). The inserted vector construct creates a false splice site that impairs the transcription of the gene. Mice heterozygous for *Aip* locus were maintained in C57 Black/6 background. *Aip*-null and wild-type mouse embryonic fibroblast (MEF) lines were established from 12-day–postcoitum embryos and were cultured in 95% air, 5% CO_2 at 37°C. Cells at first or second passage at around 80% confluence were used in the experiments.

Cell Transfection

All cells were cultured in 95% air, 5% CO_2 at 37°C. Human embryonic kidney (HEK293) and HeLa cells were plated on 6-well plates 24 hours before the small interfering (si)RNA treatment. Transfection with 30 nmol/L duplex siRNA strands (*AIP* siRNA or nontargeting control siRNA containing a pool of four different oligos; Dharmacon, Lafayette, CO) was performed to approximately 80% confluent cells using Dharma FECT 1 transfection reagents according to the manufacturer's guidelines (Dharmacon). Rat pituitary adenoma cells (GH3) were electroporated using Amaxa nucleofector (Amaxa Biosystems, Gaithersburg, MD) with 100 nmol/L siRNA oligos (Dharmacon) according to the optimized protocol offered by the manufacturer (Amaxa Biosystems).

Quantitative PCR

RNA was extracted from MEFs, HEK293, HeLa, and GH3 cells with the RNeasy Mini Kit (Qiagen, Hilden, Germany) and cDNA was produced by standard methods. The relative expression levels of *AIP/Aip* in cell experiments were determined using TaqMan chemistry and 7500 Fast Real-Time PCR system (Applied Biosystems, Foster City, CA). *AIP/Aip* TaqMan probes for human, mouse, and rat transcripts were Hs00610222_m1, Mm00479316_m1, and Rn00597273_m1 (Applied Biosystems), respectively. The relative mRNA copy numbers were normalized against β-actin housekeeping gene (4326315E for human, 4352341E for mouse, and 4352340E for rat transcripts; Applied Biosystems).

Western Blot Analyses

Proteins from MEFs, HEK293, HeLa, and GH3 cells were extracted with M-PER Mammalian Protein Extraction reagent (Pierce, Rockford, IL) or radioimmunoprecipitation assay buffer (Sigma-Aldrich, St. Louis, MO) supplemented with proteinase inhibitor (Roche, Mannheim, Germany). Twenty-five μg of protein was loaded into a 10% Tris-HCL gel (Bio-Rad, Hercules, CA). Primary antibodies against AIP/Aip (NB100–127; 1:1000 in MEF experiments and 1:500 in siRNA experiments; Novus Biologicals) and ARNT/Arnt (ab14829; 1:200; Abcam, Cambridge, UK) were used. α-Tubulin (T5168; 1:5000; Sigma-Aldrich) was used as a loading control protein. Proteins were visualized by Amersham ECL Plus Western Blotting Detection System (GE Health Care, Buckinghamshire, UK). Western blot band intensities were calculated with FluorChem 8800 using the Spot Denso analysis tool (α Innotech Corporation, San Leandro, CA). The program calculates integrated density values for the bands based on band areas and intensities. Arnt band values were normalized with band values from α-Tubulin of the same sample. Band intensity was reported as percentages relative to the siRNA control band set at 100%.

Table 1. ARNT Immunohistochemistry in *AIP* Mutation Positive and Negative Pituitary Adenomas

ARNT intensity in tumor	Samples with *AIP* mutation	Samples without *AIP* mutation	*P* value*
0 (negative)	5	1	0.001
1 (weak)	2	3	
2 (intermediate)	3	7	
3 (high)	3	33	

*Two-sided *P* value with Fisher's exact test, 0,1 vs. 2,3 staining intensities.

Proliferation Assays

The proliferative status of *AIP/Aip* siRNA-transfected and nontreated HEK293, HeLa, and GH3 cells was determined by the 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2*H*-tetrazolium, inner salt (MTS) assay (G3580; Promega, Madison, WI). HEK293 (3×10^3 per well), HeLa (3×10^3 per well), and GH3 (2×10^4 per well) were transfected as previously described and plated in five parallel wells to a 96-well plate. Conversion of MTS into formazan was detected at the absorbance of 490 nm according to manufacturer's instructions (Promega). Measurement time points of HEK293 and HeLa cells were from 20 hours until 72 hours and for GH3 the timescale was from 6 hours until 72 hours.

Results

Immunohistochemistry

To study the impact of *AIP* mutations on the expression of a set of AIP-related proteins, immunohistochemical analysis was performed on *AIP*mut+ and *AIP*mut− pituitary adenomas. When negative (score 0) and weak (1) intensities were considered as negative and intermediate (2) and high (3) as positive stainings ARNT was found to be significantly ($P = 0.001$, two-sided *P* value with Fisher exact) more frequently expressed in *AIP*mut− tumors (40/44, 90.9%) compared with *AIP*mut+ tumors (6/13, 46.2%) (Table 1, Table 2, Figure 1A). This association remained significant when weak (1) intensity was also considered positive staining ($P = 0.0016$). No correlation between the expression of ARNT and the hormone se-

Table 2. Immunohistochemical Staining of ARNT, AHR, HIF-1α, and p27(Kip1) in *AIP* Mutation Positive and Negative Pituitary Adenomas; Positive Staining/Total Number of Samples

Protein analyzed	Number of samples with *AIP* mutation	Number of samples without *AIP* mutation	*P* value*
Normal tissue ARNT	13/13	44/45	ND
Tumor tissue ARNT	6/13	40/44	0.001
Cytoplasmic AHR	8/13	38/53	0.51
Nuclear AHR	6/13	10/53	0.067
HIF-1α	9/13	36/40	0.09
HIF-1α staining cells†	11/13	38/40	0.25
p27(Kip1)	7/7	31/41	0.32

*Two-sided *P* value with Fisher's exact test; ND, not determined.
†Fraction of cells staining with HIF-1 α: 0, less than 1% of cells staining; 1, 1% to 10% of cells staining; 2, 10% to 50% of cells staining; 3, >50% of cells staining. Scoring from 2 to 3 is considered as significant positive staining.

cretion status of the adenoma was found. By contrast, the expression of ARNT in adjacent normal tissue was consistent in *AIP*mut+ (13/13) and *AIP*mut− samples (44/45, 97.8%) (Table 2). Nuclear *AHR* was more frequently expressed in *AIP*mut+ samples (6/13, 46.2%) compared with *AIP*mut− samples (10/53, 18.9%), although the result was not statistically significant ($P = 0.067$). The cytoplasmic expression of AHR was even between tumor types: *AIP*mut+ (8/13, 61.5%), *AIP*mut− (38/53, 71.7%) ($P = 0.51$) (Figure 1B, Table 2).

HIF-1α was expressed clearly in most of the pituitary adenoma samples. Staining intensity considered as positive (scores from 2 to 3) was observed in 9/13 (69.2%) of *AIP*mut+ and in 36/40 (90%) of *AIP*mut− samples ($P = 0.09$). A significant fraction of positive tumor cells (scoring from 2 to 3) was seen in 11/13 (84.6%) *AIP*mut+ and in 38/40 (95%) *AIP*mut− samples ($P = 0.25$) (Table 2). For reference, some samples contained adjacent normal pituitary tissue, which did not display HIF-1α staining (data not shown). All *AIP*mut+ tumors (7/7) showed a prominent p27(Kip1) expression (intensity score 3). There was more staining variation in *AIP*mut− tumors (intensity scores 0 to 3), but when staining intensities 2 and 3 were considered as positive staining the expression of p27(Kip1) was found to be even between tumor types ($P = 0.32$; Table 2).

CD34 was present at high levels both in *AIP*mut+ (13/13), as well as in *AIP*mut− adenomas (43/43). The density of CD34-vessels was slightly elevated in *AIP*mut+ samples (mean 319.5/mm^2) compared with *AIP*mut− samples (mean 253.1/mm^2), although not statistically significantly ($P = 0.11$). However, the standard deviations were high (± 140.0 in *AIP*mut+ samples and ± 120.8 in *AIP*mut− samples), presumably due to the small size of adenoma pieces, which impedes tissue cutting in surgery and in preparation of paraffin sections.

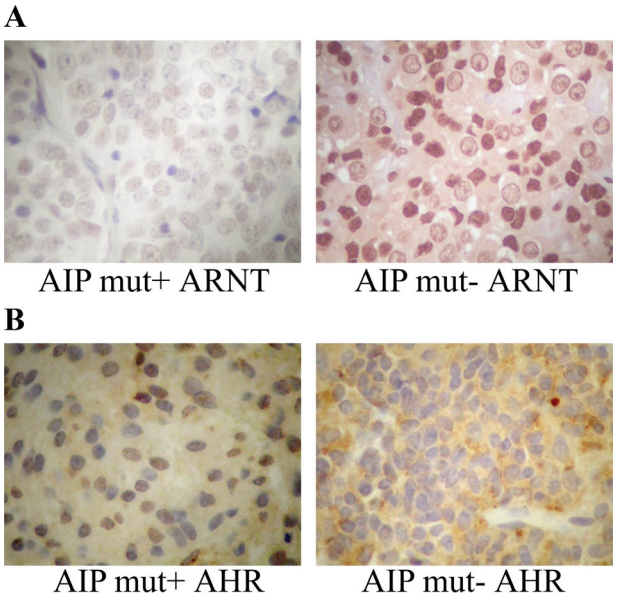

Figure 1. ARNT and AHR immunohistochemistry in *AIP*mut+ and *AIP*mut− pituitary tumors at an original magnification of ×356. **A:** Negative ARNT staining in a patient with *AIP* Q14X mutation (**left**); positive ARNT staining in *AIP*mut− adenoma (**right**). **B:** Positive nuclear AHR staining in a patient with *AIP* Q14X mutation (**left**); negative nuclear AHR staining in *AIP*mut− adenoma (**right**).

ARNT/Arnt Protein Levels in Aip-Null MEFs and AIP siRNA-Treated Cell Lines

Expressions of AIP/Aip and ARNT/Arnt were studied in model cell lines including *Aip*$^{-/-}$ MEFs and *AIP* siRNA-treated cell lines. Quantitative PCR showed lack of *Aip* transcription in *Aip*$^{-/-}$ MEFs when compared with *Aip*$^{+/-}$ and *Aip*$^{+/+}$ MEF cell lines (Figure 2). The Western blot analysis in an *Aip*-null MEF line confirmed the lack of Aip

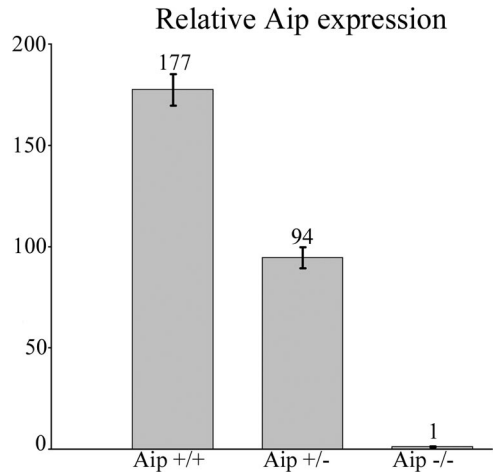

Figure 2. Relative expressions of *Aip* in Aip$^{+/+}$, Aip$^{+/-}$, and Aip$^{-/-}$ MEF cell lines. SD is shown by error bars.

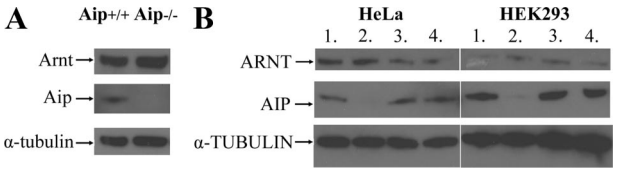

Figure 3. Western blot results from MEF, HeLa, and HEK293 cell lines. (**Arrows** indicate the bands corresponding to the proteins detected.) **A:** Arnt, Aip and α-Tubulin expression in Aip wild-type ($Aip^{+/+}$) and Aip-null ($Aip^{-/-}$) MEF cell lines. **B:** ARNT, AIP, and α-tubulin expression in HeLa and HEK293 cell lines: **1)** untreated; **2)** AIP siRNA-transfected; **3)** control siRNA-transfected; and **4)** transfection reagent-treated (DF1) cells.

protein. To assess the putative effect of *Aip* knockdown the differences in the amount of Arnt protein were studied by western blotting. $Aip^{-/-}$ and $Aip^{+/+}$ MEF cells showed no differences in the amounts of Arnt among the cell lines evaluated (Figure 3A). The *AIP* siRNAs reduced the expression of *AIP/Aip* in HEK293, HeLa, and GH3 cell lines approximately 85%, 90%, and 25% to 87%, respectively (data not shown). Western blot detection of *AIP* siRNA-treated HEK293 and HeLa cells at 48 hours showed notable reduction of AIP protein in both cell lines, but equal presence of ARNT protein, when compared with control siRNA-treated cells (Figure 3B). Thus, a total lack or considerable reduction of AIP/Aip protein did not alter ARNT/Arnt protein levels in *Aip*-null MEFs or siRNA-treated human cell lines. Instead, in GH3 pituitary adenoma cell line the reduction of Arnt was notable as early as 6 hours after *Aip* siRNA transfection by electroporation (60% transfection efficiency). The Arnt band intensity of *Aip* siRNA-treated cells was 51.6% in contrast to the Arnt intensity of control siRNA-transfected cells (Figure 4A). At the 48-hour time point with 59% transfection efficiency, no reduction of Arnt was observed (data not shown).

Proliferation Assays

To evaluate the possible effect of *AIP/Aip* silencing to the proliferation, siRNA experiments of HEK293, HeLa, and GH3 were analyzed in the MTS assay. As compared with the control siRNA, *AIP* siRNA-treated HEK293 cells showed slightly increased proliferation at 72 hours after transfection (data not shown). No difference on proliferation was detected in siRNA-treated HeLa cells (data not shown). Transfection of rat pituitary GH3 cells with *Aip* siRNA resulted in a clearly increased rate of proliferation already at 48 hours, as compared with the control siRNA (Figure 4B).

Discussion

Germline mutations in *AIP* have been reported to cause pituitary adenoma predisposition, but little is known of the molecular mechanisms of the subsequent tumorigenesis.[4] Reported *AIP* mutations may have an impact on the expression and function of several proteins, and therefore have potential to interfere with multiple cellular and environmental signals. In the present study, we have scrutinized immunohistochemically *AIP*-related molecules by comparing their expression in an extensive collection of *AIP*mut+ and *AIP*mut− pituitary adenomas. The main finding of our study was the reduced expression of ARNT in *AIP*mut+ pituitary adenomas, as compared with *AIP*mut− samples ($P = 0.001$) (Table 1, Figure 1A). Also a trend for increased expression of nuclear AHR in *AIP*mut+ adenomas was detected, although the difference was not statistically significant ($P = 0.06$) (Table 2, Figure 1B).

Protein expressions of HIF-1α and p27(Kip1) were uniform between tumor types when staining intensities 2 and 3 were considered as positive staining (Table 2). In p27(Kip1), all seven successfully stained *AIP*mut+ tumors showed a prominent p27(Kip1) staining (intensity 3). In the *AIP*mut− tumors, intensities varied between 0 and 3. This is in accordance with the earlier literature where it has been reported that p27(Kip1) can be underexpressed or even absent in human pituitary tumors.[23] However, additional *AIP* mut+ adenomas are needed to study the role of p27(Kip1) in these tumors.

CD34 marker was used to detect possible changes in vasculature resulting from HIF-1α/ARNT complex imbalance. The expression and density of CD34-vessels were high in both tumor types. The high vessel density detected in ARNT deficient *AIP*mut+ tumors indicates that the hypoxia response seems to be functional despite reduced ARNT levels. It is reported that only 15% of the total ARNT pool is sequestered by HIF-1α when the signaling pathway is saturated and that certain HIF-1α target genes require only very small amounts of HIF-1α/ARNT heterodimer.[29,30] Thus, it is possible that the expression of ARNT protein is not totally abolished and the required amount of protein still exists to induce the expression of hypoxia-regulated genes, eg, *VEGF*. On the other hand, for instance ARNT2 has shown to be capable to compensate the lack of ARNT through binding with HIF-1α in response to hypoxia.[31,32]

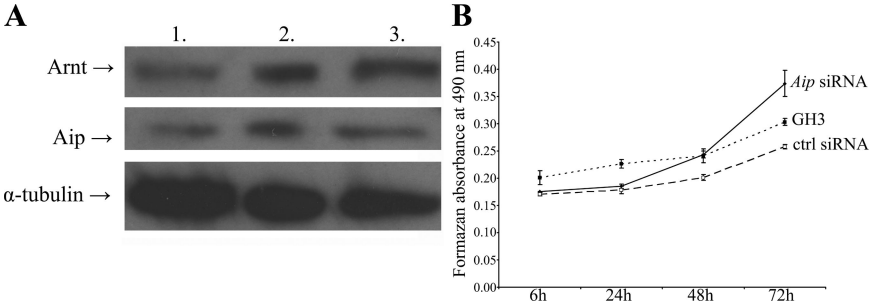

Figure 4. Western blot and proliferation assay results from GH3 cell line. **A:** Arnt, Aip, and α-tubulin expression in **1)** Aip siRNA transfected; **2)** untreated; and **3)** control siRNA-transfected cells. In each lane the intensity of Arnt protein has been normalized against α-tubulin. **B:** Cell proliferation of *Aip* siRNA, control siRNA-transfected, and untreated cells performed by MTS assay at different time points.

ARNT has been considered to be a constitutively stable protein and ubiquitously expressed in nearly all cell types.[33] Recent findings have, however, suggested that the levels of ARNT expression are not constitutive, but are altered in diseases such as diabetes, breast cancer, and lung cancer, thereby supporting our assumption that ARNT may be a factor also in pituitary tumorigenesis.[34–36] The down-regulation of ARNT in *AIP*mut+ tumors may be connected to imbalance in the AHR/ARNT complex formation arising from aberrant cAMP signaling often detected in pituitary tumorigenesis.[37] deOliveira et al[13] introduced that PDE2A is targeted by AIP to the cytoplasmic AHR complex. Consequently, PDE2A inhibits the nuclear translocation of AHR by lowering the local cAMP levels.[8,13] Hence, it is conceivable that the lack of functional AIP results in the nuclear abundance of AHR in the presence of elevated cAMP levels. cAMP-mediated AHR has also been shown to adopt a unique structure, and to prevent the formation of the AHR/ARNT complex in nucleus.[38] One might envisage that *AIP* gene defects causing such disturbances in the formation of AHR/ARNT and possibly HIF-1α/ARNT complexes may unbalance transcription of a multitude of target genes of these complexes leading to pituitary tumorigenesis. In the study of Jaffrain-Rea et al,[39] no nuclear immunostaining of AHR was detected in their *AIP* mutation–positive adenomas. Such a discrepancy may arise from IHC assay differences between laboratories and the type of mutations studied. Instead, in the study of Nakata et al the weak nuclear accumulation of AHR was observed after *AIP* siRNA treatment in Arnt-deficient mouse hepatoma Hepa1c1c7 cell line supporting hereby our observation.[40]

Our *AIP/Aip* siRNA experiments showed that partial reduction of Arnt occurred only in the GH3 cell line, the most relevant cell model for acromegaly (Figure 4A). Reduction of Arnt was already detected, 6 hours after siRNA silencing, probably due the fast delivery of the *Aip* siRNA strands directly into the nucleus by electroporation. However, Arnt protein levels were restored 48 hours after knockdown of *AIP*. This suggests that ARNT might have a tissue-specific role in the pituitary tumorigenesis and that the stable underexpression of ARNT associated with *AIP* germline mutations possibly requires some additional cellular events to occur. GH3 was also the only cell line where we were able to show that knockdown of *Aip* led to a clear increase in cell proliferation rate (Figure 4B), hereby further supporting a tumor suppressor role of *AIP*. This finding is in agreement with the work of Leontiou et al,[6] where they noticed that overexpression of wild-type AIP in HEK293, GH3, and TIG 3 cells led to a reduction of cell proliferation.

In summary, we find that the expression of ARNT protein is reduced and the localization of AHR in the nucleus is somewhat increased in *AIP*mut+ pituitary adenoma samples. These findings suggest an interactive role between AIP and these molecules in pituitary tumorigenesis. The increased expression of nuclear *AHR* in *AIP*mut+ samples may involve loss of PDE2A (and possibly PDE4A5) signaling leading to an increase of cAMP, instability of ARNT, and subsequent adenoma formation.[6,8,37] However, we cannot fully exclude the possibility that the observed ARNT reduction is a bystander effect of AIP reduction. Therefore more information on the contribution of AIP in the protein levels of AHR and ARNT is needed. Experiments with an *AIP* deficient pituitary adenoma cell line and additional AIPmut+ pituitary tumors to test these hypotheses would be useful. This would provide insight into the mechanisms of tumor suppression by *AIP* not only in the tissue of interest, but also in an environment with the relevant molecular background.

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