

# Somatotroph-Specific *Aip*-Deficient Mice Display Pretumorigenic Alterations in Cell-Cycle Signaling

Mary P. Gillam,<sup>1\*</sup> Cheol Ryong Ku,<sup>3\*</sup> Yang Jong Lee,<sup>3</sup> Jean Kim,<sup>3</sup> Se Hoon Kim,<sup>4</sup>  
Sue Ji Lee,<sup>5</sup> Byungjin Hwang,<sup>6</sup> JaeHyung Koo,<sup>7</sup> Rhonda D. Kineman,<sup>8,9</sup>  
Hiroaki Kiyokawa,<sup>1,2</sup> and Eun Jig Lee<sup>3</sup>

<sup>1</sup>Department of Molecular Pharmacology and Biological Chemistry and

<sup>2</sup>Robert H. Lurie Comprehensive Cancer Center, Northwestern University Feinberg School of Medicine, Chicago, Illinois 60611;

<sup>3</sup>Division of Endocrinology, Department of Internal Medicine and

<sup>4</sup>Departments of Pathology and <sup>5</sup>Radiology, Yonsei University College of Medicine, Seoul, Korea 03722;

<sup>6</sup>Department of Chemistry, Yonsei University, Seoul, Korea 03722;

<sup>7</sup>Department of Brain and Cognitive Science, Daegu Gyeongbuk Institute of Science and Technology, Daegu, Korea 42988; and

<sup>8</sup>Research and Development Division, Jesse Brown Veterans Affairs Medical Center and

<sup>9</sup>Section of Endocrinology, Diabetes, and Metabolism, Department of Medicine, University of Illinois at Chicago, Chicago, Illinois 60612

\*These authors are joint first authors.

Patients with familial isolated pituitary adenoma are predisposed to pituitary adenomas, which in a subset of cases is due to germline inactivating mutations of the aryl hydrocarbon receptor–interacting protein (*AIP*) gene. Using Cre/lox and Flp/Frt technology, a conditional mouse model was generated to examine the loss of the mouse homolog, *Aip*, in pituitary somatotrophs. By 40 weeks of age, >80% of somatotroph specific *Aip* knockout mice develop growth hormone (GH) secreting adenomas. The formation of adenomas results in physiologic effects recapitulating the human syndrome of acromegaly, including increased body size, elevated serum GH and insulin-like growth factor 1 levels, and glucose intolerance. The pretumorigenic *Aip*-deficient somatotrophs secrete excess GH and exhibit pathologic hyperplasia associated with cytosolic compartmentalization of the cyclin-dependent kinase (CDK) inhibitor p27<sup>kip1</sup> and perinuclear accentuation of CDK-4. Following tumor formation, the *Aip*-deficient somatotrophs display reduced expression of somatostatin receptor subtype 5 with impaired response to octreotide. The delayed tumor emergence, even with loss of both copies of *Aip*, implies that additional somatic events are required for adenoma formation. These findings suggest that pituitary hyperplasia precedes adenomatous transformation in somatotroph-specific *Aip*-deficient mice and reveal potential mechanisms involved in the pretumorigenic state that ultimately contribute to transformation.

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**Freeform/Key Words:** aryl hydrocarbon receptor–interacting protein, knockout mice, pituitary adenoma predisposition, pituitary hyperplasia, pituitary tumor

Pituitary adenomas are common monoclonal neoplasms of the anterior pituitary gland, constituting 15% of all intracranial tumors [1, 2]. A number of genetic syndromes such as multiple endocrine neoplasia type 1, the Carney complex, and familial isolated pituitary adenoma predispose individuals to develop pituitary adenomas. Mutations in the gene

Abbreviations: AhR, aryl hydrocarbon receptor; CDK, cyclin-dependent kinase; CDKI, cyclin-dependent kinase inhibitor; CI, confidence interval; ELISA, enzyme-linked immunosorbent assay; ER, estrogen receptor; GH, growth hormone; IGF-1, insulin-like growth factor 1; MRI, magnetic resonance imaging; mRNA, messenger RNA; PAP, pituitary adenoma predisposition; RT-PCR, reverse transcription polymerase chain reaction; sAIPKO, somatotroph-specific *Aip* knockout; SSTR, somatostatin receptor.

encoding the aryl hydrocarbon receptor (AhR)–interacting protein (*AIP*) have been associated with a unique pituitary adenoma predisposition (PAP; Online Mendelian Inheritance in Man #102200) [3]. Somatotropinomas and prolactinomas are the predominant tumor subtypes found among PAP families, although other types have been reported as well. To date, more than 100 different germline mutations or sequence variants in this gene have been identified in families that are predisposed to developing pituitary tumors [4–6]. Altogether 70% of germline mutations are nonsense, deletion, insertion, and/or frameshifts, predicting truncation or absence of the resulting protein. Hence, *AIP* appears to be a classic tumor suppressor gene with tumors in affected patients showing loss of heterozygosity, *i.e.*, somatic loss of the wild-type allele.

In humans, *AIP* gene is located on chromosome 11q13 and consists of seven exons (the first of which is untranslated), spanning 11.4 kb of genomic sequence. It encodes a ubiquitously expressed cytoplasmic protein of 330 amino acids with three typical tetratricopeptide repeat domains at its C-terminus and an extended  $\alpha$ -helix [7]. The protein product forms interactions with a variety of proteins, including AhR, heat shock protein 90, phosphodiesterase 2A, RET, and survivin [8–12], but it remains unclear how the loss of *AIP* leads to the development of tumors.

In mice, the *Aip* gene is located on chromosome 19 and has an exon-intron organization similar to that of the human gene. Murine *Aip* demonstrates 95% identity and 98% similarity to human *AIP* at the amino acid level. As with many tumor suppressor gene knockouts, conventional homozygous *Aip* knockout mice (*Aip*<sup>-/-</sup>) die *in utero* between embryonic days 10 and 14 [13]. These mice display severe cardiovascular defects, including heart deformation; double outlet right ventricle, ventricular-septal defects and pericardial edema [13]. A recent report of heterozygous *Aip* mice created through a gene trapping strategy revealed that *Aip*<sup>+/-</sup> animals are prone to the development of growth hormone (GH) secreting adenomas [14]. However, the embryonic lethality of homozygous *Aip* knockout mice has precluded the ability to precisely study the pretumorigenic process and its effects following loss of both *Aip* alleles—an event that appears to be required to instigate a cascade of tumorigenesis.

To overcome the early lethality of the germline homozygous null state and to study the function of *AIP* during tumorigenesis, we engineered specific deletion of exons 5 to 7 of the *Aip* gene in somatotrophs using a Cre/loxP system. Mice in which exons 5 to 7 of *Aip* were flanked by loxP sites were generated. Exons 5 to 7 were then deleted from somatotrophs by breeding the mice with mice expressing cre recombinase under the control of the rat GH promoter (*rGHP-Cre*) [15]. Mice with conditional *Aip* deletions develop GH excess, which precedes the development of somatotroph pituitary tumors. Findings in this model support the role of the *Aip* gene as an important pituitary tumor suppressor, and specify the cellular and pathological processes associated with the transition to neoplasia.

## 1. Materials and Methods

### A. Animal Handling

All animal care protocols were approved by the Institutional Animal Care and Use committees (Northwestern University Feinberg School of Medicine, Chicago, IL, and Yonsei University College of Medicine, Seoul, South Korea). Mice were housed under standard conditions of light (12-hour light, 12-hour dark cycle; lights on at 7:00 AM) and temperature (22 to 24°C), with free access to standard rodent chow and water. All mice were maintained in accordance with American Association of Laboratory Animal Care guidelines.

### B. Generation of *Aip*<sup>loxP/loxP</sup> Mice

The mouse *Aip* locus was targeted through homologous recombination in embryonic stem cells using the C57BL/6 mouse strain. Details of the strategy used to 464 construct the *Aip*<sup>lox/lox</sup> mice are provided in Supplemental Materials and Methods and illustrated in Supplemental Fig. 1.

### C. Generation of *rGHP-Cre<sup>tg/+</sup>; Aip<sup>lox/lox</sup>* Mice

*rGHP-Cre<sup>tg/+</sup>; Aiplox/+* mice were obtained by crossing *rGHP-Cre<sup>tg/+</sup>* female mice, developed by Luque *et al.* [15], with male *Aip<sup>lox/lox</sup>* mice. Interbreeding of *rGHP-Cre<sup>tg/+</sup>; Aip<sup>lox/+</sup>* mice produced homozygous disruption of the *Aip* gene in somatotrophs *rGHP-Cre<sup>tg/+</sup>; Aip<sup>lox/lox</sup>*. Methods for genotyping of mice are provided in Supplemental Materials and Methods.

### D. Phenotypic and Hormone Analysis

Histomorphometric analyses, including body weight and crown–rump measurements, were taken at the time of euthanization. Pituitary glands were imaged with magnetic resonance imaging (MRI), using two mice per genotype at ages 18 and 80 weeks. Blood for GH and prolactin assays was collected at between 8 and 10 AM after an overnight (12-hour) fast. Additional details regarding the phenotypic, hormonal, and metabolic analyses are available in Supplemental Materials and Methods. In enzyme-linked immunosorbent assay (ELISA) for GH levels, the limit of detection, interassay coefficient of variation, and intra-assay coefficient of variation of GH measurements were 0.14 to ~100.00 ng/mL, 3.2 to ~9.4%, and 2.9% to 4.8%. During serial ELISA for GH levels, at least four samples that had been analyzed in previous experiment were used to validate the in-house assay. ELISAs with less than 10% differences of their values were designated appropriate.

### E. Histologic Analysis

Pituitaries were fixed in 10% neutral buffered formalin, embedded in paraffin, and sectioned at 5  $\mu$ m. Paraffin-embedded sections were stained with hematoxylin and eosin or silver stain for reticulin matrix. Immunohistochemical staining was performed using the streptavidin–biotin peroxidase method, or the Gordon–Sweet silver staining method, as described in Supplemental Materials and Methods. Staining conditions and primary antibody concentrations are available in Supplemental Materials and Methods.

### F. Octreotide Acetate Suppression Test

Control (30 weeks), and hyperplastic (18 weeks) or adenomatous somatotroph-specific *Aip* knockout (sAIPKO; 30 weeks) mice were used for octreotide acetate suppression test. Octreotide acetate was diluted with 0.9% sterile saline to the final concentration using two different doses, as previously described [16]. Octreotide acetate (30  $\mu$ g/kg or 500  $\mu$ g/kg) was injected subcutaneously after overnight fasting. Each group consisted of five mice. For blood sampling, the animals were anesthetized by inhalation of isoflurane. Blood samples for GH were collected by periorbital sampling prior to injection and 60 and 120 minutes postinjection.

### G. Real-Time Reverse Transcription Polymerase Chain Reaction (RT-PCR)

Extraction of total RNA and real-time RT-PCR was conducted as described previously [17]. The primers for amplification are described in Supplemental Table 1. The results for relative expression in mice with pituitary gland of control (30 weeks), hyperplasia (18 weeks), and tumor (30 weeks) were normalized to GAPDH messenger RNA (mRNA) levels in each sample. Results are expressed as means  $\pm$  standard errors from five mice for each group.

### H. Statistical Analysis

Statistical analysis was performed using Microsoft Excel software and SPSS software package for Windows (Version 18.0; IBM Corp., Armonk, NY). Tumor incidence data were evaluated with two-tailed Student's *t* test. Differences were assessed by Mann–Whitney *U* test when comparing three groups and Wilcoxon signed rank test when comparing within

each group. Results are expressed as mean  $\pm$  standard errors of the mean. A  $P$  value  $< 0.05$  was considered statistically significant.

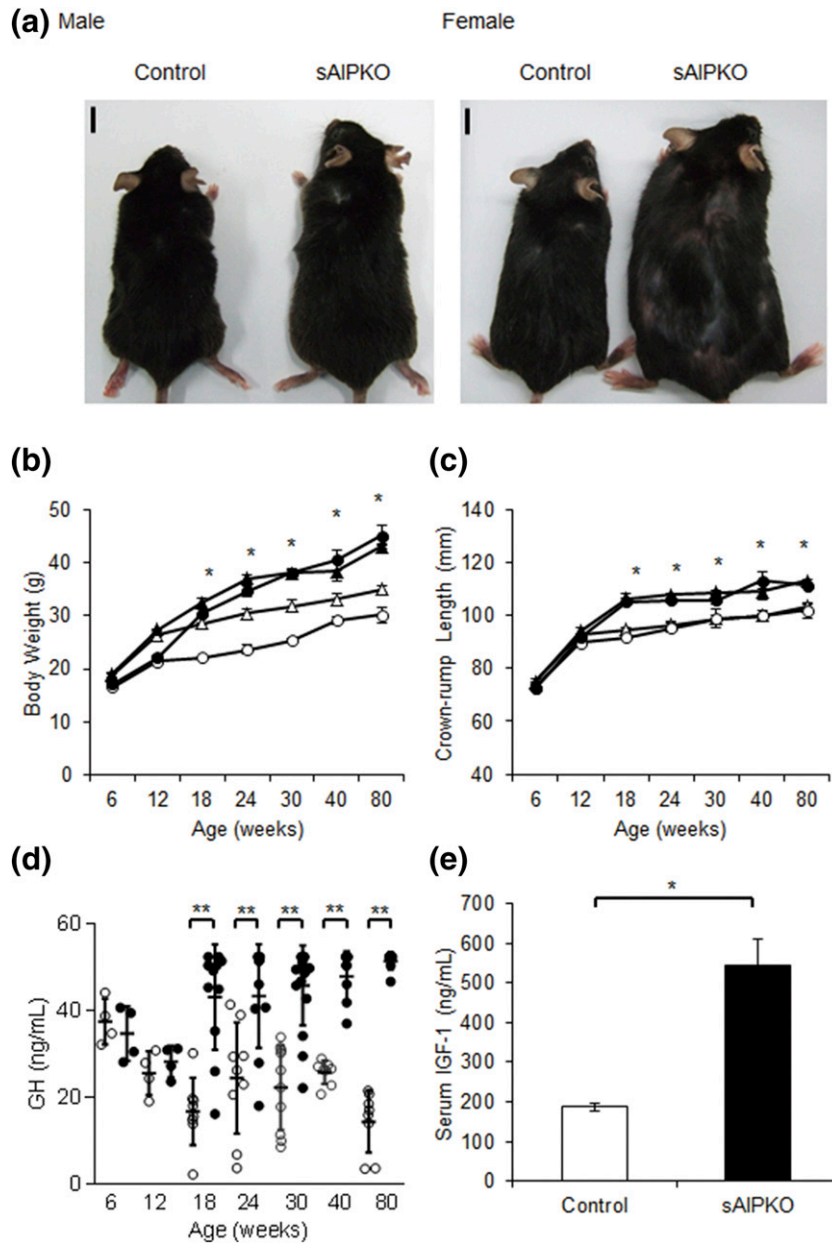
## 2. Results

### *A. Conditional Disruption of Aip in Pituitary Somatotrophs Alters Adult GH Secretion, Resulting in Enhanced Somatic Growth*

The design of the *Aip* targeting vector was constructed with a goal of ascertaining the pathogenetic relevance of C-terminal sequence alterations or truncations that have been reported in humans (Supplemental Fig. 1). Thus, exons 5 through 7 of the *Aip* gene, encompassing the tetratricopeptide repeat domain, were floxed to permit Cre-mediated excision. The *Aip*<sup>lox/lox</sup> mice did not display any gross physical or behavioral abnormalities. Interbreeding of *Aip*<sup>lox/lox</sup> mice with mice expressing Cre recombinase under control of the rat GH promoter (*rGHP-Cre*<sup>tg+</sup>) followed by additional intercrossing of progeny, led to the generation of homozygous sAIPKO (*rGHP-Cre*<sup>tg+</sup>;*Aip*<sup>lox/lox</sup>) mice. The *rGHP-Cre*<sup>tg+</sup> mice were previously shown to express the Cre recombinase almost exclusively in somatotrophs, with expression observed in less than 10% of lactotroph [15]. Mice with monoallelic somatotroph inactivation of *Aip* were followed during the course of the creation and development of mice with biallelic inactivation of *Aip*. Approximately 30 such mice were generated. None of these animals developed visible pituitary tumors at necropsy. The *rGHP-Cre*<sup>tg+/+</sup> mice used in this study are on a C57BL/6 background. The *rGHP-Cre*<sup>tg+</sup>;*Aip*<sup>lox/lox</sup> mice served as controls for all subsequent experiments in this study.

Control and sAIPKO animals were evaluated for up to 80 weeks for physiologic characteristics, hormone secretion patterns, and pituitary tumor development. At 6 weeks of age, the body weights and crown–rump lengths of male and female sAIPKO mice were similar to those of control mice, but beginning at 12 weeks of age, the growth rates for these parameters began to diverge, with a period of accelerated linear growth and weight gain observed between 12 and 18 weeks. Beyond 18 weeks, linear growth leveled off and rate of weight gain tapered for both sAIPKO and control mice, though both parameters remained significantly different throughout the remainder of their lifespan [Fig. 1 (a–c)]. Mean body weight of sAIPKO females ranged from 38.3% to 51.6% greater than controls and for sAIPKO males, ranged from 13.9% to 23.0% greater than controls. The mean difference in body weight was significantly greater for sAIPKO females [15.2 g at 80 weeks old, 95% confidence interval (CI), 9.5 to 19.8] compared with males (9.1 g at 80 weeks old, 95% CI, 4.6 to 16.2), such that the sAIPKO females achieved body weights similar to their male genotypic counterparts, whereas the typical sex differential in weights was observed in controls (4.2 g at 80 weeks old, 95% CI, 0.8 to 8.6). Along with increased body size, sizes of visceral organs were also increased in sAIPKO mice, showing significantly bigger hearts, livers, and kidneys than controls ( $3.4 \pm 0.6$  g vs  $2.2 \pm 0.4$  g,  $37.7 \pm 4.4$  mg vs  $24.1 \pm 5.8$  mg, and  $0.6 \pm 0.1$  g vs  $0.4 \pm 0.1$  g at 80 weeks old, respectively, all  $P$  values  $< 0.05$ ).

To determine the somatotroph hormonal response to the functional loss of *Aip*, we obtained sera from sAIPKO and control mice for measurement of GH levels. Peripheral GH levels of overnight-fasted control and sAIPKO mice were  $37.5 \pm 5.2$  ng/ml and  $34.8 \pm 6.3$  ng/ml at 6 weeks of age, respectively [Fig. 1(d)]. In accordance with the onset of accelerated somatic growth and increase in body weight, mean GH hormone levels of sAIPKO mice were significantly elevated as compared with control mice by 18 weeks. The mean GH levels were 3.71-fold and 1.96-fold elevated at 18 weeks in female and males, respectively. Elevated GH levels persisted thereafter during the transition period to the development of adenomas. Both control and sAIPKO mice demonstrated higher mean GH levels in female vs male mice, as previously described in rodents [18], indicating a preservation of the sexually dimorphic GH response. GH levels of sAIPKO mice were  $42.4 \pm 8.1$ ,  $43.8 \pm 7.8$ , and  $51.5 \pm 5.7$  ng/mL in male and  $54.1 \pm 9.4$ ,  $51.7 \pm 7.8$ , and  $57.4 \pm 4.8$  ng/mL in female at 18, 24, and 30 weeks, respectively. Furthermore, elevated insulin-like growth factor 1 (IGF-1) levels were also found in sAIPKO mice. At 18 weeks,



**Figure 1.** Somatotroph-specific AIP knockout mice exhibit an accelerated growth phase beginning in adulthood in conjunction with hypersecretion of GH. (a) Photograph illustrating greater overall size of male and female sAIPKO mice as compared with control mice, all at 40 weeks of age. Scale bar represents 1 cm. (b) Body weights of sAIPKO mice are significantly greater than control mice, beginning at age 18 weeks throughout the remainder of their lifespan (for weeks beyond 18,  $*P < 0.01$ ). (c) Crown-rump lengths of sAIPKO knockout mice are significantly greater than control mice, beginning at age 18 weeks and continuing throughout lifespan (for weeks beyond 18,  $*P < 0.01$ ). Open circles, control females; darkened circles, sAIPKO females; open triangles, control males; darkened triangles, sAIPKO males. (d) Mean GH levels are 1.8- to 3.6-fold elevated in sAIPKO mice as compared with control mice. Statistically significant increases in peripheral GH levels in sAIPKO mice are evident at 18 weeks and beyond ( $**P < 0.05$ ). (e) Mean IGF-1 levels are 2.9-fold elevated in sAIPKO mice as compared with control mice at 18 weeks old. ( $*P < 0.01$ ). Vertical bars indicate standard deviation. Horizontal bars indicate mean levels. Open circles, control mice; darkened circles, sAIPKO mice. Age and sex-matched number of mice with each genotype is eight at age above 18 weeks old and four at age below 12 weeks old.

IGF-1 of sAIPKO mice and control mice were  $545.3 \pm 64.7$  (males:  $517.4 \pm 74.5$  and females:  $574.8 \pm 41.7$ ) ng/ml and  $188.0 \pm 10.2$  ng/ml, respectively ( $P$  values  $< 0.01$ ), and these increased levels persisted at ages thereafter. As a group, prolactin levels were similar in control vs sAIPKO mice. Peripheral prolactin level of control and sAIPKO mice were  $35.5 \pm 8.1$  ng/ml and  $32.9 \pm 7.8$  ng/ml at 18 weeks and also similar at 80 weeks of age.

We assayed random glucose levels and performed insulin tolerance testing to examine the metabolic consequences of GH hypersecretion associated with somatotroph *Aip* loss. Beginning at 30 weeks of age, sAIPKO mice displayed hyperglycemia with significantly higher blood glucose levels in both male and females [Supplemental Fig. 2(a)]. Insulin tolerance testing demonstrated a diminished response to intraperitoneal insulin and greater area under the curve in the sAIPKO mice [Supplemental Fig. 2(b)–(d)].

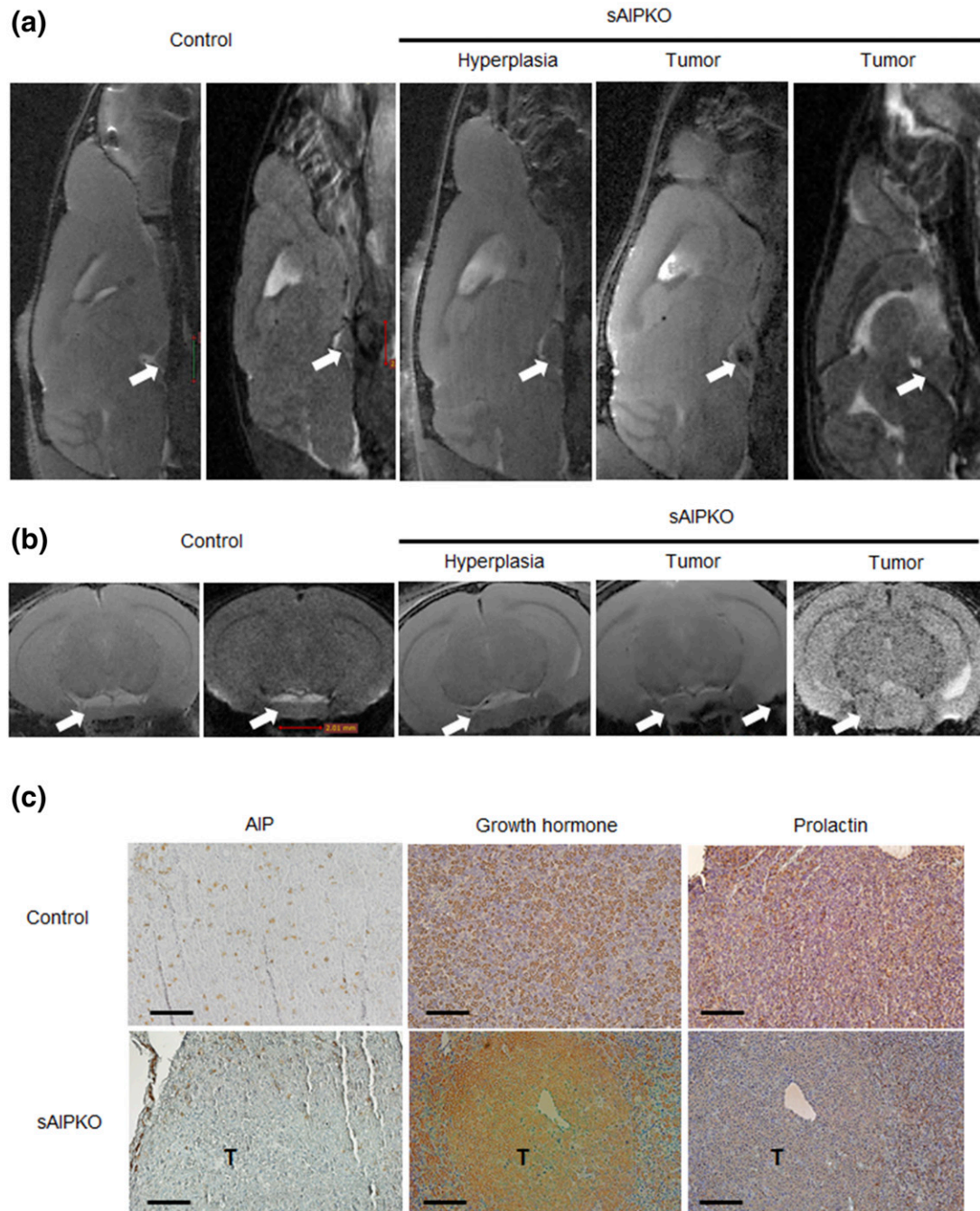
### *B. Conditional Disruption of Aip in Somatotrophs Triggers Pituitary Enlargement Followed by the Emergence of Adenomas*

To investigate the morphologic response to *Aip* disruption in pituitary somatotrophs, the macroscopic appearance of pituitaries were observed and compared in control vs sAIPKO mice using MRI [Fig. 2(a) and 2(b)]. Beginning at 18 weeks of age, diffuse enlargement of the pituitary glands of sAIPKO mice became evident on MRI, and low-intensity signaling adenomas appeared in older sAIPKO mice. Control and sAIPKO pituitaries appeared similar on a macroscopic level up until approximately 18 weeks of age, at which time pituitaries from sAIPKO mice appeared diffusely enlarged [Fig. 3(a)]. Macroscopically visible tumors were identified in the sAIPKO mice beginning at 24 weeks [Fig. 3(a)]. In measurements excluding regions encompassing visible macroscopic tumors, the mean pituitary volumes of sAIPKO mice and control mice were similar up until 12 weeks of age [Fig. 3(b)]. By 18 weeks of age, mean pituitary volumes of sAIPKO mice were significantly greater than those from control littermates in both sexes. Macroscopic tumors emerged in approximately 20% of male and female sAIPKO mice at 24 weeks, and by 30 weeks, 80% of sAIPKO mice had developed macroscopically visible tumors [Fig. 3(c)]. The predilection for a pituitary growth surge was particularly evident among female sAIPKO mice, whose pituitaries were significantly larger than their male counterparts at time points beyond 26 weeks.

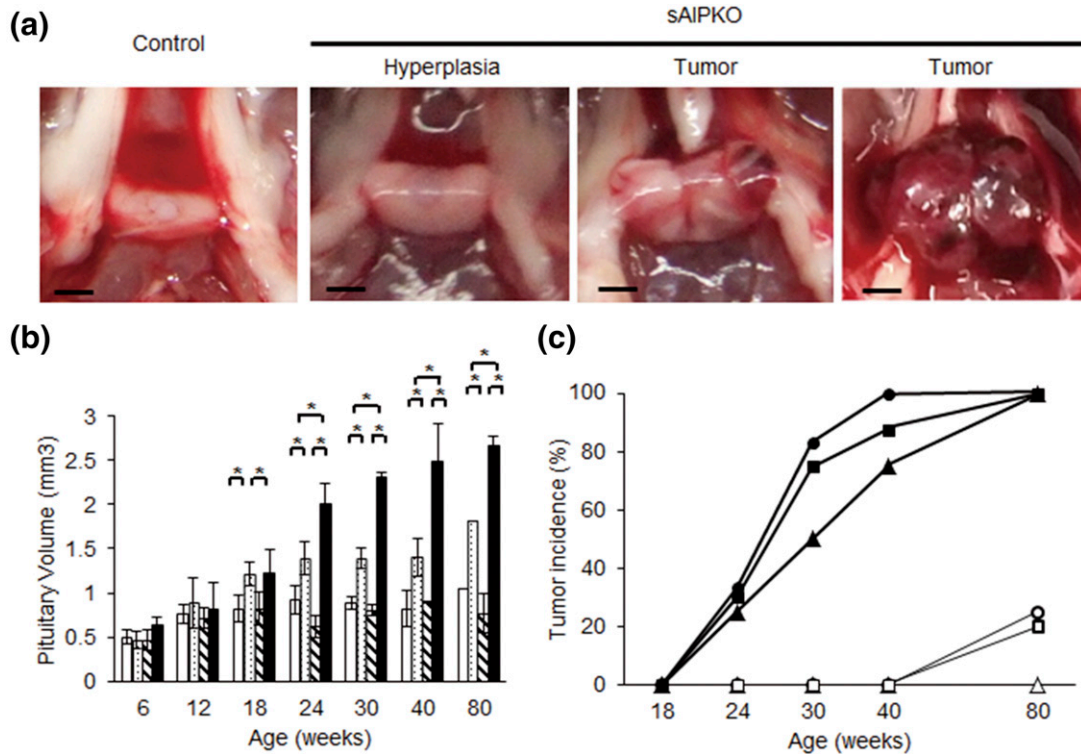
### *C. sAIPKO Pituitaries Display Diffuse Pathologic Hyperplasia Prior to Neoplastic Transformation*

Microscopically, the pituitaries of neonatal and younger adult (18 weeks and under) control mice appeared identical to sAIPKO pituitaries on routine hematoxylin and eosin staining of adenohypophysial tissue. Hematoxylin and eosin pituitary sections from control mice showed of a mixture of polygonal acidophils and basophils arranged within a normal architecture of cords and nests [Fig. 4(a)]. The adenohypophyses of sAIPKO mice at 24 weeks of age displayed a slight predominance of cells with acidophilic cytoplasm, but were otherwise indistinguishable from control pituitary tissues on hematoxylin and eosin stained sections. The adenohypophyses from older sAIPKO mice at 40 weeks and beyond demonstrated irregularly shaped cells with atypical nuclei. No invasion of acinar tissue or extrapituitary metastasis was observed in any of the sAIPKO mice.

To precisely ascertain whether the processes of pituitary hyperplasia and/or neoplasia were evident, Gordon–Sweet silver staining of control and sAIPKO pituitary tissue was performed [Fig. 4(a)]. Whereas the adenohypophysial tissue from control mice consisted of small acini of pituitary cells surrounded by an intact reticulin network, adenohypophysial tissue from sAIPKO mice between the ages of 12 and 24 weeks showed larger acini with partial disruption of the reticulin fiber network. In sAIPKO mice older than 40 weeks of age, silver staining revealed expanded acini and complete disruption of the reticulin pattern, which are findings pathognomonic for adenomatous transformation. Detailed investigation into the timing of these changes revealed that the hyperplastic response observed in sAIPKO



**Figure 2.** Radiologic imaging showing pituitaries of somatotroph-specific AIP knockout mice progressively enlarge during adulthood prior to adenomatous transformation. Control and sAIPKO mice were studied at each time point. (a, b) MRI reveals change in radiographic appearance of the sAIPKO mice: (a) Sagittal and (b) coronal images demonstrate pituitary enlargement at 18 weeks, proceeding to findings on imaging consistent with adenoma at 80 weeks. Left two panels represent control mice pituitaries at 18 weeks and 80 weeks, middle panels represent pituitary enlargement in sAIPKO mice at 18 weeks, and right two panels represent pituitary adenomas in sAIPKO mice at 18 weeks and 80 weeks, respectively. (c) Representative photomicrograph of histologic sections from sAIPKO mouse pituitary adenomas demonstrating absent immunoreactivity for *AIP* (left), abundant immunoreactivity for GH (middle), and absent immunoreactivity for prolactin (right) at 40 weeks. Pituitary gland of age and sex matched control mouse was also demonstrated. Scale bar for each is 200  $\mu$ m. White arrow indicates the pituitary gland and T means the area of pituitary tumor.



**Figure 3.** Pituitary enlargement is followed sequentially by tumor development in somatotroph-specific AIP knockout mice. (a) Gross pituitary appearance in control vs sAIPKO knockout mice. A representative photograph of a control mouse pituitary at 40 weeks of age is compared with a homogeneously enlarged pituitary from a sAIPKO mouse evident at 18 weeks of age, an adenomatous pituitary from a sAIPKO mouse found at 40 weeks of age, and an invasive pituitary tumor from sAIPKO mouse at 80 weeks of age. Scale bar indicates 1 mm. (b) Pituitary volumes of sAIPKO and control mice are similar at 12 weeks. Beginning at 18 weeks, male and female sAIPKO pituitaries show significantly greater volume as compared with control littermates. Whereas pituitary size from control mice stabilizes after 12 weeks, the pituitaries from male sAIPKO mice exhibit further growth from 12 to 18 weeks, and pituitaries from female sAIPKO mice continue to grow through the lifespan. ( $*P < 0.01$ ). Open bars, male control; dotted, male sAIPKO; diagonal stripe, female control; black, female sAIPKO. (c) Chart of pituitary adenoma incidence in control vs sAIPKO mice shows that the tumors begin to appear at 24 weeks and beyond in both sexes, with a rapid increase between 24 and 30 weeks of age. Control mice develop microscopic tumors at a low overall prevalence ( $<20\%$ ) beyond 40 weeks of age. Open triangle, male control; open circle, female control; black triangle, male sAIPKO; black circle, female sAIPKO. Open rectangles represent mean of both sexes in controls and black rectangles represent mean of both sexes in sAIPKO mice. The number of age and sex-matched mice for each genotype is at eight at age above 18 weeks old and four at age below 12 weeks old.

pituitaries became pathologically evident at between 12 and 18 weeks, as it was at the latter time point that the mean number of pituitary cells per acina ( $28.34 \pm 7.24$  vs  $11.09 \pm 0.80$ ) and mean acinar area ( $2.01 \pm 0.45$ -fold compared with control from sAIPKO pituitary glands) became significantly larger than those of control litter mates [Fig. 4(b) and 4(c)]. The mean pituitary acinar area from sAIPKO mice continued to increase in size until stabilizing at its maximal size at 40 weeks [Fig. 4(c)]. Immunohistochemistry of adenomatous tissue showed a diffuse cytoplasmic GH immunoreactivity, and absence of immunoreactivity for Aip and prolactin [Fig. 2(c)].

#### D. Loss of Somatotroph AIP Disrupts Cell-Cycle Regulator Expression and Localization

Given the pivotal role of G1 cyclin dependent kinases (CDKs) and CDK inhibitors (CDKIs) in pituitary cell-cycle control and homeostasis, we hypothesized that altered expression of





pituitary sections from control mice at 40 weeks of age, on sections from pretumorigenic tissue at 18 weeks of age and on sections from macroscopically visible pituitary adenomas at 40 weeks of age in sAIPKO mice (Fig. 4). Immunohistochemistry of pituitary sections from control mice at 40 weeks of age revealed moderate p27 immunoreactivity. In pituitary sections from sAIPKO mice at 18 weeks of age, p27 immunoreactivity appeared abundant, but was diminished in adenomatous tissue (40 weeks of age). Immunohistochemistry of adenohypophysial tissue for CDK4 revealed a diffuse pattern with moderate level of expression in sections from control mice, and diffusely upregulated expression in sAIPKO mice at 18 weeks of age. In sections from adenomatous pituitary tissue from sAIPKO mice, the expression of CDK4 was roughly similar in intensity to sAIPKO pretumorigenic pituitary sections, but accumulated in the perinuclear region, as typical of cells traversing the G1-S transition (Fig. 5). In adenomas taken from 40-week-old sAIPKO mice, p27 expression was markedly reduced in both the nuclear and cytosolic compartments. Thus, the overall expression of p27 is greater in the pretumorigenic sAIPKO somatotrophs as compared with the controls somatotrophs, but the substantial accumulation of this CDKI in the cytoplasm suggests the possibility that cytoplasmic sequestration could impair its ability to induce growth arrest. Following adenomatous transformation, p27 expression is globally reduced. This altered pattern of cell-cycle regulator expression provides corroborative evidence that a loss of AIP function is characterized in early stages by deregulated growth that consequently becomes permissive for adenomatous transformation.

Regarding the expression of estrogen receptors (ERs), ER- $\alpha$  expression was more abundant in both the pretumorigenic and adenomatous pituitary tissue from the sAIPKO mice as compared with controls, as previously shown in the conventional AIP knockout mouse [14]. ER- $\beta$  expression in the pituitary was minimal in both genotypes (Fig. 5 and data not shown).

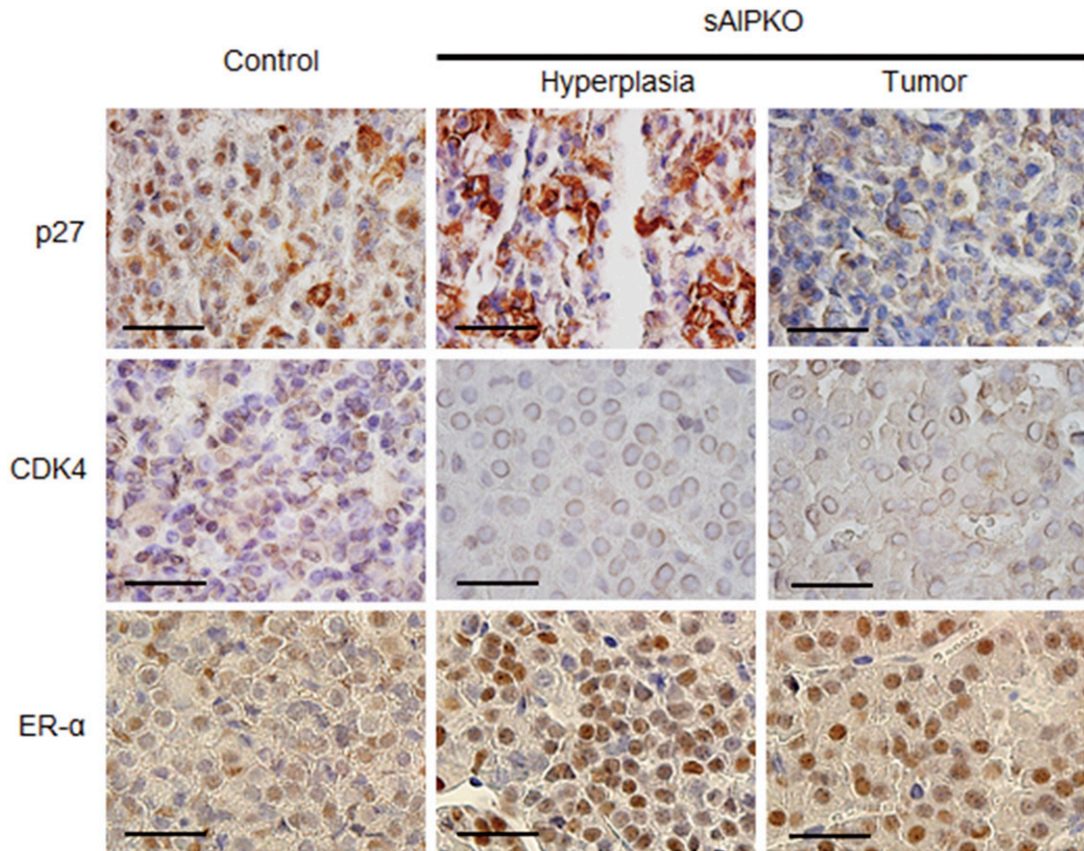
#### *E. sAIPKO Mice Present Diminished Response in Octreotide Acetate Suppression Test*

In real-time RT-PCR for mouse somatostatin receptors (SSTRs) 1 to 5, both control and sAIPKO mice presented the low expression of SSTR4. The expression of SSTR5 diminished significantly with the tumorous changes of pituitary gland in sAIPKO mice [Fig. 6(a)].

In octreotide acetate suppression test, glucose level decreased significantly at both doses of 30  $\mu$ g/kg and 500  $\mu$ g/kg [Fig. 6(b)]. At suppression test with 30  $\mu$ g/kg octreotide acetate, control and sAIPKO mice with pituitary hyperplasia showed substantial decrease in GH level, which were recovered at 120 minutes after injection [Fig. 6(c)]. With 500  $\mu$ g/kg of octreotide acetate, GH level was significantly suppressed to a similar level in both control mice and sAIPKO mice with pituitary adenoma, which was persisted for 2 hours [Fig. 6(d)].

### **3. Discussion**

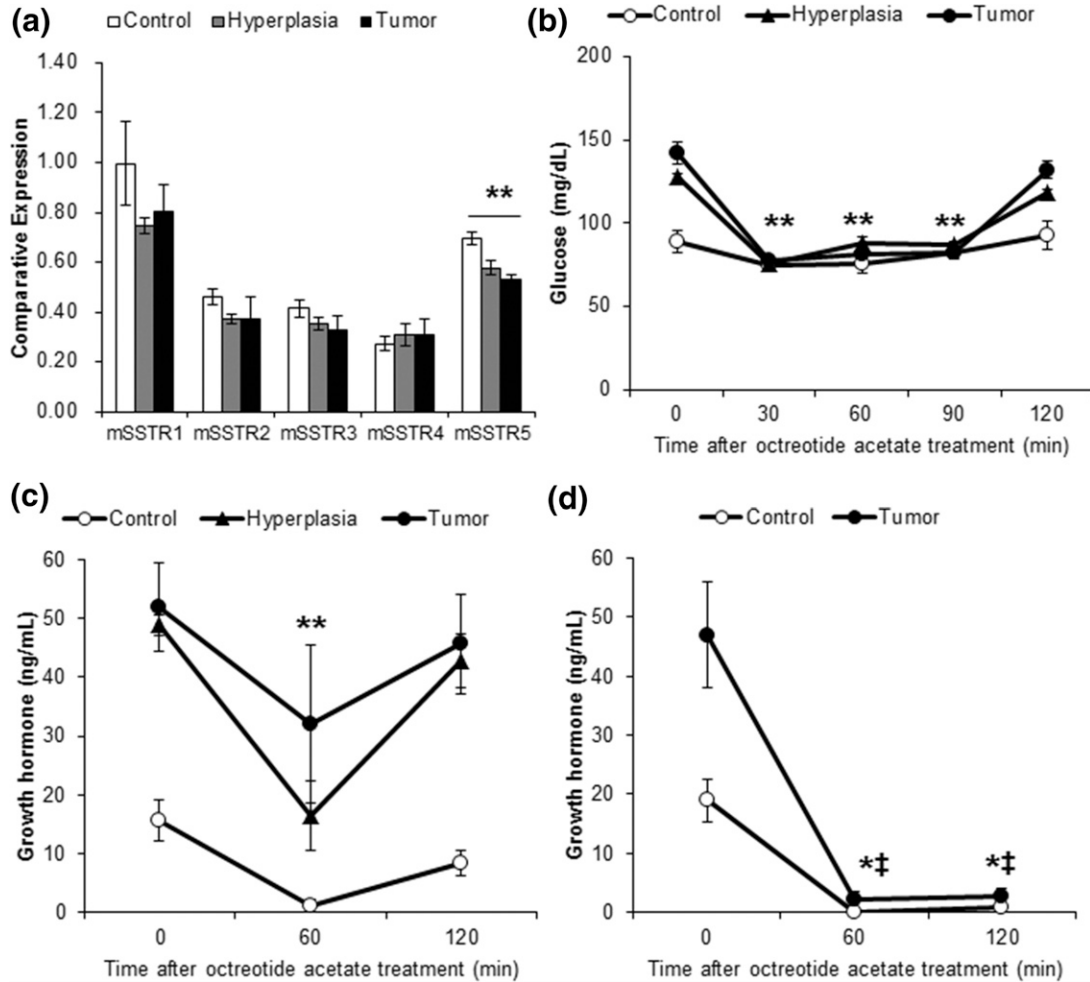
In humans, germline mutations in the *AIP* gene confer an increased risk for the development of pituitary adenomas, though the phenotypes of familial isolated pituitary adenoma and PAP display incomplete penetrance [21]. It is unknown whether incomplete penetrance is a result of somatic maintenance of the wild-type *AIP* allele in unaffected carriers, or whether an insufficient number of transforming genetic events have yet to occur in the somatic tissue of these individuals. Conventional heterozygous *Aip* knockout mice have been shown to develop pituitary adenomas with almost complete penetrance in later adulthood, a finding that has validated AIP as a pituitary tumor suppressor gene in this predisposition syndrome [14]. Heterozygous *Aip* mice also exhibit a mildly altered secretory pattern of GH even before the development of pituitary adenoma [22]. Furthermore, microarray analysis of mouse embryonic fibroblasts revealed that dysfunctional *Gai* signaling increases cyclic adenosine monophosphate synthesis leading to GH-secreting pituitary adenoma in conventional *Aip* knockout mice [23]. In that study, *Gai*-2 protein, phosphorylated extracellular signal-regulated kinases 1 and 2, and cyclic adenosine monophosphate response element-binding protein expression levels were decreased both in human and mouse GH-secreting AIP mutant pituitary adenomas. However, because the functional studies were performed in cell lines and



**Figure 5.** Immunohistochemistry analysis of control and somatotroph-specific AIP knockout pituitary tissue indicates upregulation of ER- $\alpha$  and altered localization of selected cell cycle regulators. Sections from paraffin embedded sAIPKO pituitaries show increased immunoreactivity of the cyclin dependent kinase inhibitor p27 at 18 weeks, corresponding temporally to the stage of hyperplasia as demonstrated by reticulin staining, as depicted in Fig. 3(A). p27 immunoreactivity markedly diminishes in the adenomatous gland at 40 weeks. CDK4 immunoreactivity is largely cytoplasmic in the control pituitary, but displays enhanced perinuclear immunoreactivity in sAIPKO pituitaries at 18 weeks, which appears more distinct in the adenomatous gland at 40 weeks. ER- $\alpha$  immunoreactivity is also increased in sAIPKO pituitaries at both the hyperplastic and adenomatous stages. Representative results are from triplicate samples in at least two independent experiments. Scale bar represents 500  $\mu$ m; original magnification:  $\times 400$ .

mouse embryonic fibroblasts, it remains unproven whether downregulation of this pathway plays a primary tumor initiating role in transformation of somatotrophs. Furthermore, due to the embryonic lethal nature of biallelic *Aip* deficiency, the molecular process of tumorigenesis in pituitary itself is difficult to study in heterozygous *Aip*<sup>+/-</sup> mice as a result of the random timing with which the wild-type allele is inactivated [13].

Using sAIPKO mice, we examined the precise sequence of cellular and molecular alterations that occur in the process of pituitary tumorigenesis associated with AIP deficiency. An analysis of the temporal sequence of pathological events identified pituitary hyperplasia as a precursor lesion for the development of pituitary adenoma. Our conditional, sAIPKO mice develop pituitary adenomas earlier than conventional heterozygous *Aip* knockout mice supporting the premise that functional inactivation of both *Aip* alleles is necessary for tumorigenesis. However, although somatotroph tumors in our sAIPKO mice develop earlier than those in conventional *Aip*<sup>+/-</sup> mice, they did not arise until middle adulthood, after a period of proliferative growth. The relatively indolent course of neoplastic transformation of pituitary somatotrophs suggests that loss of function of *AIP* alone is not sufficient for tumorigenesis. As applicable for virtually all forms of cancer, additional somatic mutations in



**Figure 6.** Somatotroph specific AIP knockout mice exhibit diminished responsiveness in octreotide acetate suppression test. (a) mRNA expression of somatostatin receptor subtypes in three conditions of control, hyperplasia, and tumor. Relative expression values were measured compared with somatostatin receptor subtype 1 of control mice. (b) Changes of glucose level after octreotide acetate injection at a dose of 30 µg/kg. (c, d) Changes of serum GH level during octreotide suppression test at doses of 30 µg/kg and 500 µg/kg, respectively. \*\* $P < 0.05$  compared with the values before octreotide acetate injection in each control, sAIPKO mice with hyperplastic pituitary gland, and sAIPKO mice with adenomatous pituitary gland, respectively. Open bars and open circle, control; gray bars and black rectangles, sAIPKO mice with hyperplasia; black bars and black circle, sAIPKO mice with tumor. Number of mice in each group is five.

genes that provide a selective advantage and/or generate genomic instability are probably essential for tumor formation in this predisposition syndrome [24]. Given the delayed nature with which the tumors emerge even with both *Aip* alleles inactivated from birth in mice, it is our speculation that in humans with germline heterozygous *AIP* mutations, tumors arise after loss of the somatic wild-type allele, due to accumulation of nucleotide substitutions, small deletions or insertions, or epigenetic processes that result in gene silencing or activation.

In accordance with the predilection for pituitary tumorigenesis, the biallelic loss of this tumor suppressor in sAIPKO mice causes a postnatal stimulation of somatotroph proliferation, manifesting pathologically as hyperplasia, and physiologically as GH and IGF-1 excess. Among the cell-cycle proteins that regulate the G1-S transition, CDK4, p27, and Rb are particularly critical components in neuroendocrine tissues. The pituitary gland is particularly sensitive to genetic alteration of genes involved in the CDK4, p27, and Rb pathway

[25]. CDK4 is uniquely essential for the postnatal proliferation of the anterior pituitary [26], whereas knock-in or transgenic mice expressing a constitutively active CDK4 allele (resistant to p16 inhibition) display the reciprocal phenotype of anterior lobe pituitary tumors [27, 28]. Moreover, transgenic overexpression of GH releasing hormone normally causes hyperplasia and adenoma formation in the somatotroph, whereas it is ineffective in the *Cdk4*<sup>-/-</sup> genetic background. In contrast, mice deficient for the cyclin dependent kinase p27<sup>kip1</sup> (encoded by *Cdkn1b*) exhibit pituitary adenomas and anterior pituitary lobe hyperplasia with nearly complete penetrance by 10 months [29, 30]. Thus, in the face of abundant genetic evidence indicating that the normal proliferation of the anterior pituitary requires normal CDK4 and p27 activity, we chose to analyze their expression in the somatotrophs of the conditional *Aip* knockout. On a molecular level, the pretumorigenic AIP deficient somatotrophs exhibit alterations in cell-cycle regulator expression consistent with one of a hyperproliferative state. These changes include the redistribution of p27 from the nucleus to cytosol, perinuclear accentuation of CDK4 localization, and general increase in ER- $\alpha$  expression, the latter of which was also described in conventional *Aip* knockout mice pituitary tumors [14]. An upregulation of CDK4 expression coinciding with transformation from the pretumorigenic to the adenomatous state is consistent conceptually with prior evidence that this kinase plays a proliferative role in pituitary cell growth [28, 31]. Perinuclear CDK4 accumulation on the cytosolic surfaces of the nuclear pores is a hallmark of the G1-S transition in mammalian cells [32], and this particular pattern of CDK4 expression has been identified in the progression of colonic neoplasia from normal epithelia to adenomas or carcinomas [33]. The CDKI p27, encoded by *CDKN1B*, is a well-documented pituitary tumor suppressor, as it is underexpressed or absent in most human pituitary tumors [34], germline mutations in *CDKN1B* cause a multiple endocrine neoplasia type 4 condition heralded by pituitary adenomas [20], and *Cdkn1b* disruption causes highly penetrant pituitary neoplasms in mice [29]. An unusual finding in our study was the apparent upregulation and redistribution of p27 from the nucleus to cytosol in the pretumorigenic sAIPKO pituitaries. Despite its widely accepted tumor suppressive role, some nonpituitary tumors exhibit prominent p27 expression; in such cases, the protein is often mislocalized to the cytoplasm [35–37]. Because the growth-restraining activity of p27 depends on its nuclear localization, the aberrant cytosolic accumulation of p27 in pretumorigenic sAIPKO pituitaries may be permissive for the proliferative response observed [38]. By the time the adenomatous state evolves in the sAIPKO mice, p27 expression is diminished altogether. Whether AIP directly participates in p27 cytoplasmic redistribution as part of a molecular chaperone complex, or these changes result alterations in other signaling pathways that converge upon p27 phosphorylation is not known, but it is relevant to note that AIP plays a role as an adaptor protein in shuttling the AhR and other proteins to the nucleus. Thus, taken together, the cell-cycle protein changes in the pretumorigenic state portray a replicative signature, whereas those observed in the adenomatous state are reflective of neoplasia.

The AIP gene encodes the AhR–interacting protein, which is a cochaperone for the AhR in a complex containing heat-shock proteins [39]. *Aip* has a broad range of interacting partners, which participate in a diverse array of signaling pathways [39]. Which of these pathways participates in somatotroph tumorigenesis as a result of AIP mutation has not been elucidated. In *in vitro* studies of nonendocrine cells, AIP either prevents AhR-mediated transcription or promotes its signaling, depending upon species and cell type [40]. AhR also alternatively inhibits or stimulates or inhibits cell proliferation in a tissue and context dependent manner. The latter inhibitory effects have been shown to occur in association with an increase in p27 levels [41]. Moreover, like AIP, CDK4 exists in chaperone complexes with heat shock protein 90 [42]. On the other hand, it is possible that the tumor suppressor properties of AIP occur entirely independent of AhR actions [43]. Regardless of the significance of the relationship between *Aip* and AhR, the results shown in this study of higher CDK4 and lower p27 expression in the somatotroph *Aip* conditional knockout are conceptually consistent with the loss of *Aip* tumor suppression advancing cell-cycle progression through enhanced *Cdk4* function and diminished p27 activity, followed by cell proliferation and as yet undefined

processes that promote adenomatous transformation. Whether this sequence of molecular events occurs via AhR, or through one of many other interacting proteins in pituitary somatotrophs, remains to be determined.

ER- $\alpha$  plays a pivotal role in the development of the pituitary gland, especially in trans-differentiation of mammosomatotroph [2]. Because AhR, for which AIP acts as a cochaperone, has been shown to exhibit substantial cross-talk with ER- $\alpha$ , *Aip* disruption could influence AhR signaling, ER- $\alpha$  function, or both [44]. Although ER- $\alpha$  transcripts have not been demonstrated in one study of human somatotroph adenomas [45], the sAIPKO mice display increased expression of ER- $\alpha$  both at the hyperplastic and adenomatous stages. This increased expression in the adult sAIPKO somatotrophs might occur as a consequence of *Aip* interference with normal AhR expression, localization, and/or function, any of which might explain the apparent sex-related phenotypic differences (exaggerated growth in females) observed in the sAIPKO mice.

In the clinical aspect of AIP on medical responsiveness, acromegalic patients with AIP mutation showed the resistance to somatostatin analogs [46]. Furthermore, pretreatment of somatostatin analogs increased the expression of AIP in both *in vivo* and *in vitro* conditions [47]. Both studies suggest that AIP plays an important role in the action of somatostatin analogs in both familial and sporadic GH secreting pituitary adenoma [48]. In the sAIPKO model, they exhibited the similar expression pattern of SSTR subtypes. As shown in human studies [49], SSTR4 mRNA expression levels were low in both control and sAIPKO model. Furthermore, with progression of pituitary tumorigenesis from hyperplasia to adenoma, the expression of SSTR5 mRNA decreased significantly. The changes in SSTR5 suggest that there exist substantial pathologic changes even between pituitary hyperplasia and adenomatous transformation. In a relatively low-dose octreotide suppression test, sAIPKO mice showed diminished response in GH suppression, which were overcome by high dose octreotide treatment. This result suggest that initiation of high-dose medical treatment should be considered in acromegalic patients with mutation or decreased expression of AIP, although the side effects of high-dose somatostatin analogs might be matter. To our knowledge, most animals with pituitary adenoma had failed in proving the responsiveness for conventional medications. However, sAIPKO mice showed the potentiality as an animal model in development for new therapeutics for pituitary adenoma [50].

Strikingly, the expansion of the somatotroph population and ensuing state of GH excess is not manifested until well into murine adulthood, showing a distinct onset at 18 weeks in the sAIPKO mice. To our knowledge, detailed analyses of the timing of AIP expression levels have not been reported in the adeno-hypophysis of mice; however, there is a clear differential response of the pituitary somatotrophs in sAIPKO vs control mice with respect to GH secretion at a stage beyond that of the murine pubertal growth spurt, coinciding with enhanced physical growth. Such findings are consistent with the concept that pituitary somatotrophs exhibit an adaptive ability to respond to trophic stimuli with changes in cell mass and hormone secretion, and that there are unique mechanisms regulating their inherent plasticity in a precise, lineage-specific manner [51]. Notably, the phenotypic characterization of a conditional knockout of *Aip* in the liver did not report an increase in hepatocyte proliferation or neoplasia, providing additional supporting evidence of the tissue-specific role of AIP in regulating pituitary cell growth [52]. However, there was a limitation on analysis of serum GH level. Pulsatile secretory pattern and longitudinal changes of serum GH in an identical mouse was not evaluated in this study. Because GH is secreted in ultradian manner with secretory bursts with 3-hour intervals [53], further evaluation on the rhythm of GH secretion could suggest the important information about the role of AIP.

Finally, on a clinical level, the sAIPKO mice exhibit aggressive tumors that are relatively resistant to the suppressive effects of octreotide, in parallel with observations of tumor invasion and poor somatostatin analog responsiveness in humans with acromegaly who harbor AIP mutations [46]. One study has shown that pretreatment of somatostatin analogs increases the expression of AIP in both *in vivo* and *in vitro* conditions [47]. These findings suggest that AIP is involved in somatostatin receptor signaling, and mediates the

antisecretory or antiproliferative effects of somatostatin analogs in GH-secreting pituitary adenomas [48]. SSTR2 has a 10-fold higher affinity for octreotide than SSTR5. Furthermore, SSTR5 can form homo- or heterodimers with other G-protein-coupled receptors including SSTRs and dopamine receptors, which modify the response to its ligand [54, 55]. Consequently, altered SSTR subtype expression also influences medical responsiveness to somatostatin analogs. The SSTR subtype expression data presented here in the sAIPKO model are mostly similar to controls, as has been shown in studies of human somatotroph tumors that are resistant to therapy [56]. However, in the sAIPKO model, with progression of pituitary tumorigenesis from hyperplasia to adenoma, the expression of SSTR5 mRNA decreases significantly. Taboada *et al.* [49] reported that an increase in SSTR2 mRNA expression and decrease SSTR2/SSTR5 mRNA ratio positively correlated with drug responsiveness of acromegalic patients. The sAIPKO mice display a similar SSTR2 mRNA and decreased SSTR5 mRNA expression, as the control, illustrating that the effect of shifted ratio of SSTR2/SSTR5 could be different from that of human acromegalic patients. In a relatively low-dose octreotide suppression test, sAIPKO mice showed diminished response in GH suppression, which was overcome by high-dose octreotide treatment. This result suggests that rapid escalation of somatostatin therapy might be considered in acromegalic patients with known *AIP* mutations who are likely to exhibit somatostatin analog resistance [46].

There were several limitations in this study. First, the proliferation index of pituitary adenoma such as Ki67 was not evaluated in this study. Because histopathologic changes could only occur at a single time point, it was not able to be stated with certainty that foci of increased proliferation existed prior to transformation to adenoma. However, the consistent findings of hyperplasia in the mutant pituitaries at the 18-week mark, with adenomas found at later time points, supports theoretically for hyperplasia preceding adenomatous transformation. Second, there was not enough number of mice in the octreotide suppression test. Although there were consistent changes of GH level in each animal group, further studies evaluating the effect of octreotide on sAIPKO mice should be necessary with more number of mice.

In conclusion, in view of our findings using the conditional sAIPKO model, we propose that *AIP* deficiency as it occurs in the human condition of familial isolated pituitary adenoma predisposes to pituitary neoplasia through the following series of events: (1) genetic loss of the wild-type allele; (2) sustained cellular proliferation, characterized by an insensitivity to inhibitory hormonal feedback; and (3) a consequential greater rate of cell cycling leading to the acquisition of somatic mutations or other genetic events that drive neoplastic transformation. The conditional sAIPKO model has furthermore uncovered a novel potential physiological role in the negative regulation of somatotroph proliferation and GH release that may have implications for the understanding and treatment of adult-onset GH deficiency in humans.

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Address all correspondence to: Eun Jig Lee, MD, PhD, Division of Endocrinology, Department of Internal Medicine, Yonsei University College of Medicine, 50-1 Yonseiro, Seodaemun-gu, Seoul Korea 03722. E-mail: [ejlee423@yuhs.ac](mailto:ejlee423@yuhs.ac).

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C.R.K. wrote the first draft of the manuscript. C.R.K., Y.J.L., S.H.K., and S.J.L. conducted the phenotype analysis of mice model for GH secreting pituitary adenoma. B.H. and J.H.K. analyzed the result of high throughput RNA sequencing. R.D.K. and H.K. provided and key animal for development of mice model and made concept for Fig. 5. All authors have contributed to the final draft.

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