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General Article

GENERAL ARTICLE

Kisspeptin deficiency leads to abnormal adrenal glands and excess steroid hormone secretion

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Abstract

Knockout mice for the kisspeptin receptor, *Kiss1r* (*Kiss1r*$^{-/-}$) and its ligand kisspeptin, *Kiss1* (*Kiss1*$^{-/-}$) replicate the phenotype of isolated hypogonadotropic hypogonadism (IHH) associated with variants of these genes in humans. A recent report suggests that kisspeptin may be involved in human fetal adrenocortical development and function. Herein, we characterized the adrenal function and morphology in *Kiss1*$^{-/-}$ mice that do not go through normal puberty. Two fetal markers were expressed in eosinophilic cells potentially derived from the X-zone that should disappear at puberty in male mice and during the first pregnancy in female animals. Although the hypercorticosteronism observed in *Kiss1*$^{-/-}$ females corrected overtime, hyperaldosteronism persisted at 14 months and correlated with the overexpression of *Star*. To determine if *KISS1* and *KISS1R* genes are involved in the development of primary aldosteronism (PA) and hypercortisolism [Cushing's syndrome (CS)] in humans, we sequenced these 2 genes in 65 patients with PA and/or CS. Interestingly, a patient with CS presented with a germline *KISS1* variant (p.H90D, rs201073751). We also found three rare variants in the *KISS1R* gene in three patients with PA: p.C95W (rs141767649), p.A189T (rs73507527) and p.R229R (rs115335009). The two missense variants have been previously associated with IHH. Our findings suggest that *KISS1* may play a role in adrenal function in mice and possibly adrenocortical steroid hormone secretion in humans, beyond its recently described role in human fetal adrenocortical development.

Introduction

Almost two decades ago, inactivating variants in the kisspeptin receptor (*KISS1R*) gene were found in patients with idiopathic hypogonadotropic hypogonadism (IHH) (1). The essential role of *KISS1R* in the initiation of gonadotropin-releasing hormone (GnRH) signaling was shown in *Kiss1r* knockout (KO) mice (*Kiss1r*$^{-/-}$): indeed, *Kiss1r*$^{-/-}$ mice developed IHH similar to what is observed in patients carrying *KISS1R* inactivating variants, and like the human patients, responded to exogenous

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GnRH treatment (1). KO mice for the *KISS1R* ligand, kisspeptin (*Kiss1*$^{-/-}$) developed a phenotype similar to that of *Kiss1r*$^{-/-}$ mice, confirming that kisspeptin is the physiological ligand of *KISS1R* and that both molecules are needed for GnRH release (2,3).

Recently, it was described that kisspeptin treatment induced dehydroepiandrosterone sulphate (DHEAS) secretion by the human adrenocortical carcinoma cell line, H295R, and by cultured second-trimester human fetal adrenal (HFA) cells (4). Additional expression studies showed that kisspeptin is expressed widely in fetal adrenal cortex (4). These results supported a potential role of kisspeptin in the development of the adrenal and/or the regulation of adrenocortical function, at least during fetal development.

Following up on these reports, we studied adrenal pathology and secretion in *Kiss1* KO mice: we demonstrate here that the inactivation of *Kiss1* leads to persistence of the fetal X-zone in mice and that this was associated with hypersecretion of corticosterone and aldosterone. Although corticosterone levels normalized in older animals, hyperaldosteronism persisted. We then screened human patients with hypercortisolism (HC) or hyperaldosteronism caused by adrenal tumors. Interestingly, we identified one missense *KISS1* and three *KISS1R* variants (two missense and one synonymous) among the patients with steroid hormone hypersecretion; all had been previously described in patients with IHH or Kallmann syndrome.

Results

In *Kiss1*$^{-/-}$ mice, the X-zone persists

We analyzed the histology of adrenals from *Kiss1*$^{+/+}$ and *Kiss1*$^{-/-}$ females at 5 and 12 months (Fig. 1A and D). At 5 months of age, *Kiss1*$^{-/-}$mice had eosinophilic cells that accumulated at the border between the cortex and the medulla (Fig. 1A, females; and Supplementary Material, Fig. S1A, males). Similar cells were not observed in the adrenal glands of wild-type (WT) *Kiss1*$^{+/+}$animals.

To characterize these cells, we used an antibody for the fetal marker, 20α-hydroxysteroid dehydrogenase (20αHSD). As shown in Figure 1B, 20αHSD-positive cells were present in the X-zone of *Kiss1*$^{-/-}$ mice but not in WT *Kiss1*$^{+/+}$ animals (Fig. 1B, females; and Supplementary Material, Fig. S1B, males). Accordingly, mRNA of several X-zone markers, such as *Akr1c18* (encoding for 20αHSD), *Pik3c2g*, *Inh*α and *Cyp17a1* (5–8) was significantly increased in the adrenal glands of*Kiss1*$^{-/-}$female compared with WT *Kiss1*$^{+/+}$ animals at 5 months of age (Fig. 1C). In males, only *Akr1c18* expression was significantly increased in the adrenal glands of *Kiss1*$^{-/-}$ animals (Supplementary Material, Fig. S1C) consistent with the immunostaining results (Supplementary Material, Fig. S1B). The higher expression of *Akr1c18* and *Pik3c2g* mRNA observed in the heterozygote, *Kiss1*$^{+/-}$ animals was not statistically significant (there was significant heterogeneity between the animals studied).

We did not quantify the number of 20αHSD-positive cells per adrenal examined, because these cells were only seen in the adrenals of KO animals at 5 and 12 months of age. For example, we did not observe any 20αHSD-positive cells in the 5-month-old WT males, whereas the four KO males studied had numerous cells positive for 20αHSD. Similarly, none of the five parous WT females at 5 and 12 months of age that we studied had any 20αHSD-positive cells, whereas all the six KO females at 5 months and four out of 5 at 12 months of age had 20αHSD-positive cells ($P < 0.005$).

Altogether, these results demonstrated that cells that retained the expression of the fetal marker 20αHSD persisted at the cortico-medullary junction of the adrenal glands of *Kiss1*$^{-/-}$ animals at 5 months of age. As previously described, cells retain fetal characteristics form the mouse X-zone (also called fetal zone), which is expected to degenerate after the first pregnancy in female mice and after puberty in male animals (9,10). Thus, the abnormal puberty and sterility previously described in *Kiss1*$^{-/-}$ mice (2) could explain, at least in part, the persistence of the X-zone observed in *Kiss1*$^{-/-}$ mice.

We therefore also studied the expression of X-zone markers in *Kiss1*$^{-/-}$females and compared it with that in nulliparous female *Kiss1*$^{+/-}$ and WT *Kiss1*$^{+/+}$ animals, in which the X-zone did not degenerate. The expression of three of the four markers studied, *Akr1c18*, *Pik3c2g* and *Cyp17a1* (Supplementary Material, Fig. S2), was significantly increased in the KO *Kiss1*$^{-/-}$ mice compared with nulliparous *Kiss1*$^{+/+}$ females, suggesting that the X-zone was expanded in the adrenal glands of the KO *Kiss1*$^{-/-}$ adrenals, even when compared with the X-zone found in age-matched WT adrenal glands with expected persistence of the X-zone. The persistence of the X-zone by histology (Fig. 1D) and 20αHSD staining (Fig. 1E) persisted too in the 12-month-old *Kiss1*$^{-/-}$female mice.

Kiss1$^{-/-}$mice had hypersecretion of glucocorticoids

We next analyzed the effect of *Kiss1* inactivation on glucocorticoid secretion. In contrast to human adrenal cortex, adult mouse adrenocortical cells do not secrete cortisol due to the absence of *Cyp17a1*, a steroidogenic enzyme that is essential for the biosynthesis of both androgens and cortisol (8). As *Cyp17a1* may be present in fetal adrenocortical cells and was slightly overexpressed in the adrenal glands of *Kiss1*$^{-/-}$animals at 5 months of age, we measured serum cortisol levels in these mice: cortisol was undetectable in all genotypes including, as expected, in WT animals. Serum corticosterone levels, on the other hand, were significantly increased in *Kiss1*$^{-/-}$females at 5 months of age, concomitantly with a decrease in ACTH levels (Fig. 2A), pointing to the adrenal origin of this hypersecretion. Like in other forms of mouse models of adrenocortical Cushing's syndrome (CS), this biochemical phenotype was limited to the female sex, and *Kiss1*$^{-/-}$ male mice had normal corticosterone and ACTH levels (Supplementary Material, Fig. S3A).

Increased corticosterone levels were associated with the upregulation of three members of the steroidogenic pathway, *Star*, *Cyp21* and *Cyp11b1* (Fig. 2B). However, by histology, zona fasciculata looked normal with the expected expression of Akr1b7 at this stage (Fig. 2C). In the absence of any hyperplasia or tumors, both corticosterone and ACTH levels normalized in *Kiss1*$^{-/-}$ females by 12 months of age (Supplementary Material, Fig.S4).

Kiss1 $^{-/-}$ mice developed hyperaldosteronism

Aldosterone levels in *Kiss1*$^{-/-}$ mice were significantly elevated in both female and male animals at 5 months of age (Fig. 2D; females and Supplementary Material, Fig. S3C; males), and they remained increased at 12 months of age (Fig. 2D). The higher aldosterone levels observed in *Kiss1*$^{-/-}$ mice were not associated with an increased expression of aldosterone synthase (*Cyp11b2*), neither at 5 nor at 12 months of age, and there was no hyperplasia or tumors histologically at either age (Fig. 1D).

At 5 months of age, the overexpression of *Star* and *Cyp21* involved in the synthesis of both corticosterone and aldosterone

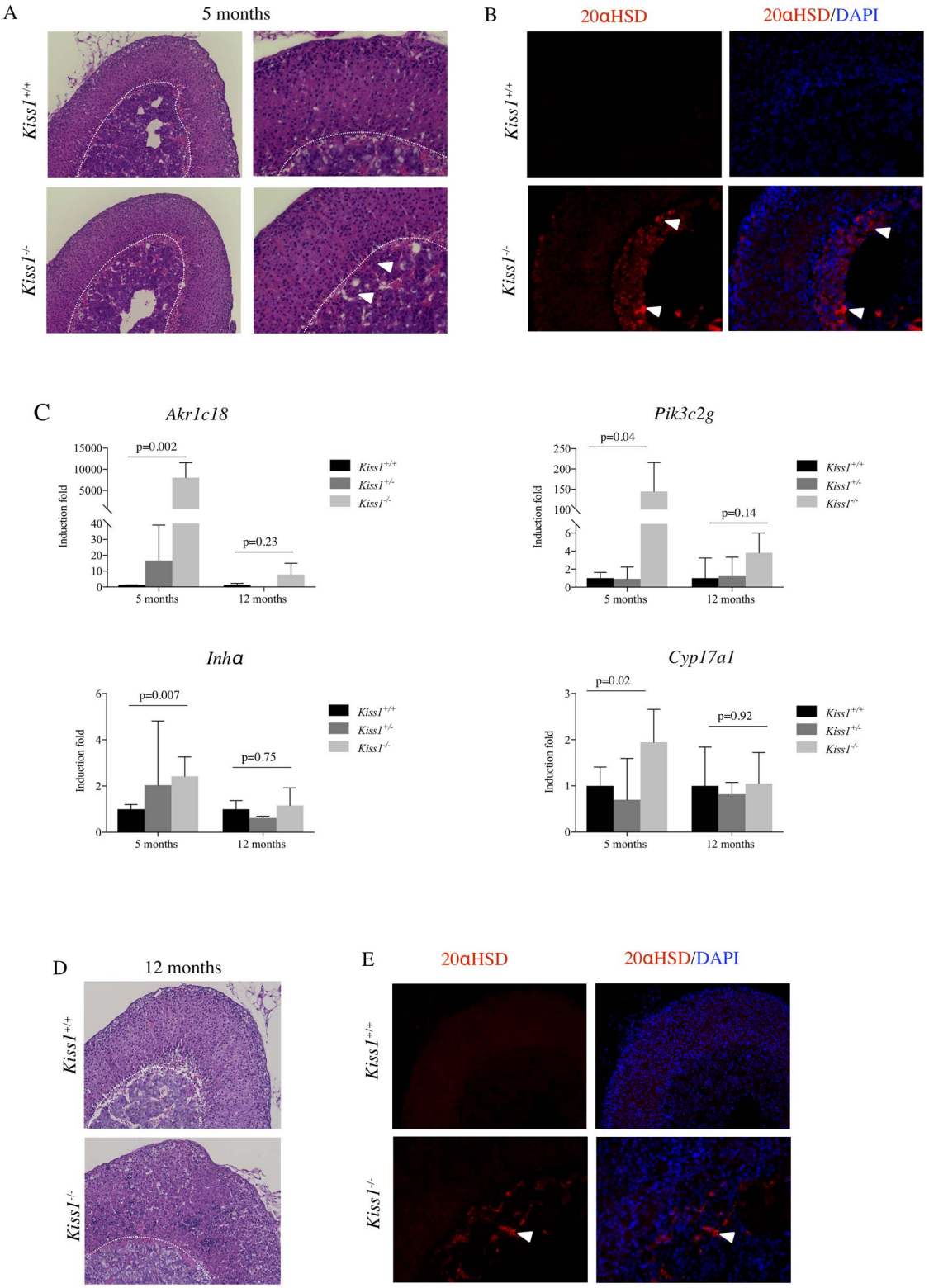

Figure 1. The X-zone does not regress in *Kiss1*$^{-/-}$ adrenals. (**A**) Histology analysis showed an accumulation of cells at the border of the cortex and the medulla that may be fetal X-zone cells in the adrenal glands of female *Kiss1*$^{-/-}$ mice that were not observed in *Kiss1*$^{+/+}$ animals at 5 months of age. C: Cortex, M: Medulla. A white dashed line is indicating the border between the cortex and the medulla. Arrowhead points to the fetal cells composing the X-zone. (**B**) 20α*HSD* staining confirmed the fetal-like differentiation of the cells. The white arrowhead points to the fetal cells composing the X-zone. (**C**) The expression of X-zone markers, *Akr1c18*, *Pik3c*, *Inha* and *Cyp17a1* was increased in the adrenal glands of female *Kiss1*$^{-/-}$ mice (compared with *Kiss1*$^{+/+}$) both at 5 and 12 months of age, by RT qPCR. Bars represent the induction fold of at least four individual adrenals ± standard deviation. *P*-value was calculated using Student's *t*-test. (**D–E**) Histology by hematoxylin and eosin (D) and 20α*HSD* staining (E) demonstrate that fetal cells were still observed in the adrenal glands of*Kiss1*$^{+/+}$and *Kiss1*$^{-/-}$ animals at 12 months.

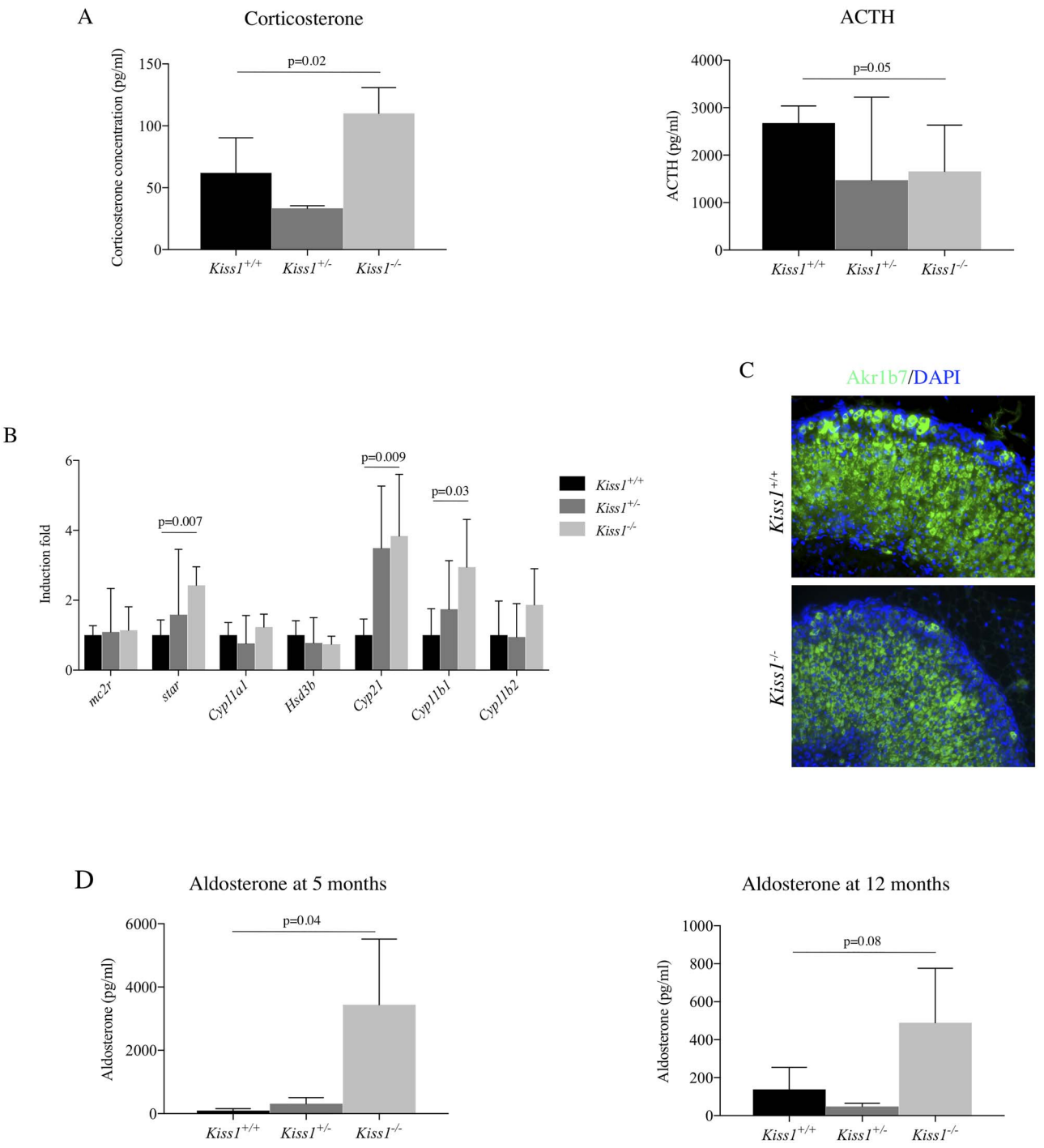

Figure 2. Female Kiss1$^{-/-}$ animals have primary hyperaldosteronism. (**A**) Serum corticosterone and ACTH concentration were up- and downregulated, respectively, in Kiss1$^{-/-}$ female compared with Kiss1$^{+/+}$ animals at 5 months of age. Bars represent the mean of either corticosterone or ACTH level of at least six individual adrenals ± standard deviation. (**B**) RTqPCR for *mc2r*, *Star*, *Cyp11a1*, *Hsd3b*, *Cyp21*, *Cyp11b1* and *Cyp11b2* in Kiss1$^{+/+}$, Kiss1$^{+/-}$ and Kiss1$^{-/-}$ from the adrenal glands of 5-month-old female mice. Bars represent the mean of the hormone level of at least six individual glands ± standard deviation. *P*-value was calculated using Student's *t*-test. (**C**) Immunofluorescence staining of Akr1b7 on Kiss1$^{+/+}$ and Kiss1$^{-/-}$ adrenals at 5 months. (**D**) Serum aldosterone concentration was significantly increased in Kiss1$^{-/-}$ females at both 5 and 12 months. Bars represent the mean of aldosterone level of at least six individual samples ± standard deviation.

(Fig. 2B) could, at least in part, explain the elevation of aldosterone in Kiss1$^{-/-}$ female mice; however, it does not explain hyperaldosteronism in male mice and both *Star* and *Cyp21* expression normalized, along with all other molecules of the steroidogenic pathway that we tested by 12 months of age in animals of both sexes (Supplementary Material, Fig. S3).

KISS1 and *KISS1R* genes were sequenced in a cohort of patients with adrenal tumors

We sequenced the *KISS1* and *KISS1R* genes in a cohort of 66 patients with adrenal steroid hypersecretion syndromes due to a variety of tumors, including 36 patients with primary aldosteronism (PA), 20 with combined aldosteronism and

Table 1. Cohort of patients sequenced at germ line level for *KISS1* and *KISS1R* genes

Patients' diagnosis	$n = 66$		Tumor location		
	Female	Male	Bilateral	Unilateral	N/A
PA	21	15	13	13	10
PA/HC	18	2	11	4	15
CS	10	-	6	1	3

Patients were divided into three groups based on their hormonal phenotype: patients with PA alone or combined with HC (PA/HC) and those with CS. The number and gender of patients as well as the tumor location are shown.

Table 2. Allele frequency of *KISS1* and *KISS1R* sequence variants identified in this study versus gnomAD exomes controls

Gene	DNA change	Protein change	SNP identification	*In silico* prediction	Patients	Allele frequency controls	References
KISS1	c.268C > G	p.H90D	rs201073751	Likely benign	1 PA/HC	0.00137	Silveira *et al.*, 2010 (11); Chan *et al.*, 2011 (12); Huijbregts *et al.*, 2012 (13)
KISS1R	c.565G > A	p.A189T	rs73507527	Benign	1 PA	0.00456	Sykiotis *et al.*, 2010 (14); Miraoui *et al.*, 2013 (16)
KISS1R	c.285C > G	p.C95W	rs141767649	Uncertain significance	1 PA	0.0000192	Francou *et al.*, 2016 (15)
KISS1R	c.687C > T	p.R229R	rs115335009	Likely benign	1 PA	0.00239	-

Abbreviations: PA: primary aldosteronism; PA/HC: primary aldosteronism/hypercortisolism and CS: Cushing's syndrome.

hypercorticolism, and 10 with ACTH-independent CS (Table 1). One *KISS1* variant, pH90D (c.268C > G, rs201073751) that is relatively frequent in the African population (AF: 0.022 in gnomAD) was identified in a patient with combined PA and HC (Table 2). This variant has been previously seen in a patient with central precocious puberty (CPP), leading investigators to believe that it may be activating *KISS1* signaling; *in silico* it is predicted to be likely benign and *in vitro* studies that have been performed elsewhere did not show an increase of *KISS1* activity or stability (11). In our investigation, we saw some decrease of the *KISS1* expression by immunohistochemistry in the tumor of the patient carrying the variant; however, *KISS1* gene sequencing in tumor cells failed to identify additional genetic changes or loss of heterozygosity (LOH) (data not shown).

Three *KISS1R* variants, two missense and one synonymous, were identified specifically among patients with PA. The two missense variants, p.A189T (c.565G > A, rs141767649) that are relatively frequent in African population (AF: 0.065 in gnomAD) and p.C95W (c.285C > G, rs141767649), were previously published in IHH cohorts; they have been tested *in vitro* (15,16). Only the tumor harboring the p.A189T variant was available to us; analysis of the tumor DNA did not reveal any LOH or other somatic events in *KISS1R* defect but, again, by immunohistochemistry, there was some decrease of *KISS1R* expression in tumor cells (data not shown).

Beyond adrenocortical hyperplasia, three of the patients with sequence variants had multinodular thyroid disease and/or kidney cysts. There was no history of malignancies and all patients to date have not developed any other tumors and remain free of any cancers.

Discussion

In this work, we report that in *Kiss1* KO mice the X-zone persists in both male and female animals, as shown by the continuing expression of the *Akr1c18*, *Pik3c8*, *Inha* and *Cyp17a1* markers. Interestingly, not all these molecules showed identical profiles in both sexes and across ages, pointing perhaps to a model of molecular regulation in *Kiss1* KO mice that is different from the other animals showing X-zone persistence.

Cyp17a1 gene encodes for a steroidogenic enzyme essential for the production of cortisol and androgens in the adrenal cortex. *Cyp17a1* expression is limited at the zona fasciculata and reticularis in the human cortex, but it is absent in the mouse definitive zone. *Kiss1*$^{-/-}$ mice had *Cyp17a1* overexpressed at the RNA level at 5 months of age, as one would expect from their persisting X-zone. It is noteworthy, however, that *Cyp17a1* expression normalized at 12 months despite the X-zone still being present. This could be due to either the number of cells expressing *Cyp17a1* being too low in the total RNA extracted from the whole adrenal gland (too low to be detected by RTqPCR at this stage), or that fetal cells of the type we observed elsewhere (see following text) may not be identical to those that express *Cyp17a1* in *Kiss1*$^{-/-}$ mice. In animals with adrenal cortex-specific complete inactivation of the main regulatory subunit type 1α of the PKA, *Prkar1a* (the AdKO mouse), fetal markers such as *Akr1c18* and *Cyp17a1* continue to be expressed highly across the mouse's life span (7), but the phenotype of these mice is far more dramatic than the one we observed in the *Kiss1*$^{-/-}$ mice: they developed CS due to an expansion of cells that were zona reticularis-like arising from the definitive cortex (17). In *Kiss1*$^{-/-}$mice, the increase in *Cyp17a1* expression is not associated with hyperplasia, tumors or any form of cellular expansion, and consequently cortisol secretion, if any, remained below the detection level for any assay.

Our mouse data supported the observations made in human tissues and cells: *KISS1R* is widely expressed in the HFA cortex and may regulate adrenal steroid hormone secretion (4). Thus, we then investigated the possibility that *KISS1* and *KISS1R* genetic variants may be present among human patients with various adrenocortical gluco- and mineralo-corticoid-secreting tumors. We identified three missense variants, one *KISS1*, two *KISS1R* and one *KISS1R* synonymous variant, among 66 patients with PA alone or combined with hypercortisolemia. Two of these

variants, p.C95W (rs141767649) and p.A189T (rs73507527), have previously been described in patients with IHH (15,16). The functional consequences of the p.H90D variant remain unclear as it has previously been identified both in the homozygote state in a patient with CPP, as well as in IHH, in the heterozygote state (11), and it is relatively common in Africans, suggesting it may be a non-functional polymorphism. The carrier of this variant was also diagnosed with central hypogonadism at the age of 53, a year after undergoing adrenalectomy for his PA; at the time, he had neither CS nor hyperaldosteronism (data not shown).

These data overall pointed to the possibility that *KISS1* and/or *KISS1R* are involved in the adrenocortical development and hormonal secretion. In older adrenal cortex, in both humans and mice, hyperaldosteronism may be the consequence of *KISS1/KISS1R* deficiency, although significantly stronger data need to be collected to conclude a definite causative effect. It should be noted that the first study that pointed to this possibility was by Nakamura *et al.* (18) when *KISS1* was still known as 'metastin', a molecule with an effect in thyroid and other cancer metastases (19). It is noteworthy that out of four patients with *KISS1/KISS1R* variants, two also developed either thyroid multinodular disease (rs141767649) or kidney cysts (rs201073751), and a third one had both conditions (rs115335009). However, neither they, nor the mice we studied, have developed any other tumors to date, and their type of adrenocortical hyperplasia almost never develops into malignancy. Aging the *Kiss1*$^{-/-}$ mice further in our laboratory did not lead to any differences in malignancies from their normal littermates (data not shown) despite the described associations between *KISS1/KISS1R* expression and their deregulation in cancer (20). Finally, these data are also supportive of *KISS1/KISS1R* signaling involvement in obesity, metabolic disorders and even gonadal steroid hormone perturbations (i.e. in polycystic ovarian syndrome) in a sexually dimorphic manner (21–23), as is the case in the *Kiss1*$^{-/-}$ mice we studied here.

Materials and Methods

Patients and sequencing

All the patients signed consent forms and were admitted to the National Institute of Health (NIH) Clinical Center under research protocols approved by the Institutional Review Boards at NIH. Germ line and adrenal tumor DNA from patients were sequenced for *KISS1* and *KISS1R* genes using the primers previously published (1).

Animal studies

Kiss1 $^{-/-}$ mice that have been previously described in the literature (1,2) have been kindly provided by Dr S. Seminara (Reproductive Endocrine Unit, Massachusetts General Hospital, Harvard Medical School, Boston, MA). Mice were maintained in a 14 h light (6 a.m.–8 p.m.)/10 h dark cycle. Both male and female mice at 5 and 12 months of age were sacrificed for the RNA, protein and blood collection as we have previously described (24). Procedures were approved by the *Eunice Kennedy Shriver* National Institute for Child Health and Human Development Institutional Animal Care and Use Committee. Mice were euthanized by slow replacement of air with CO_2 followed by cervical dislocation. Blood was collected by intracardiac puncture and placed in serum separator tubes (365956, BD microtainer). After centrifugation (4500 g, 3 min., 4°C), serum was stored at −80°C. Tissues were snap frozen on dry ice and stored at −80°C until use or fixed in 4% paraformaldehyde (PFA) for 24 h at 4°C for immunohistochemistry (see following).

Immunohistochemistry

Fixed tissues were dehydrated, paraffinized and sectioned by Histoserv Inc. (Germantown, MD, USA). Hematoxylin and eosin staining (H&E) was performed by Histoserv. After deparaffinization in Histoclear (HS-202, National diagnostics, USA), 5 µ thick sections were rehydrated using ethanol gradient before the epitope retrieval in Vector antigen retrieval solution (H3300, Vector Labs, USA) at 95°C. PBS/BSA 1% was used as blocking solution before overnight incubation at 4°C with primary antibody. 20αHSD (591009, Antibody Research) and Akr1b7 (sc-27763, Santa Cruz) primary antibodies were detected using the appropriate secondary antibody coupled to biotin (Jackson Immuno-Research Laboratory, USA). The signal was then amplified by incubation with a streptavidin-horseradish peroxidase (HRP) amplification (016-030-084, Jackson Immuno-Research Laboratory). The substrate, 3,3′-diaminobenzidine tetrahydrochloride (DAB) was incubated to detect the HRP activity (SK-4105, Vector Labs). The slides were counterstained with hematoxylin (K8008, Dako).

Reverse transcription quantitative polymerase chain reaction (PCR)

Five hundred nanograms of total RNA extracted from frozen adrenals using RNA kit (12183018A, Life Technologies, USA) were reverse transcribed using the superscript III first strand synthesis supermix (11752050, ThermoFischer, USA). Quantitative PCR reactions were performed on 2 µl of one-twentieth of dilution using either Taqman probes or specific primers (see following text). Each reaction was performed in duplicate with Taqman Fast Advanced Mastermix (4444963, Life Technologies) or SYBR green mix (4309155, Applied Biosystems, USA). Results were expressed as an induction fold calculated using $\Delta\Delta C_T$ method. All measurements were normalized to a housekeeper gene and represent the mean value of at least six adrenals per genotype ± standard deviation. Statistical analysis was performed with Student's *t*-test when results followed a normal distribution. Taqman Gene Expression Assay Probes used in this study were as follows: Mm00490735_m1 (*Cyp11a1*), Mm01159156_g1 (*hsd3b7*), Mm00441558_m1 (*Star*), Mm99999915_g1 (*gapdh*), Mm01262510_m1 (*mc2r*), Mm00484040_m1 (*Cyp17a1*), Mm00439683_m1 (*Inha*) and Mm00506289_m1 (*Akr1c18*). Primers used in this study were previously described (25).

Biochemical measurements

Blood sampled by intracardiac puncture at 9 a.m. was placed in serum separator tubes (365967, BD Microtainer) and centrifuge at 7000 rpm. The supernatant was then collected and stored at −80°C. Serum concentrations were measured using commercially available assays: corticosterone (55-CORMS-E01, ALPCO Diagnostics), ACTH (KT-6010, KAMIYA) and aldosterone (ADI-900-173, Enzo Life Sciences).

Statistical analysis

Data were presented as mean ± standard deviation; relative changes were described as an induction fold. All data distributions were assessed for approximate normality. Continuous data were compared between two independent groups using

t-tests using Graphpad Prism (Graphpad, San Diego). For the comparison between mice that had or did not have X-zone, Fisher's exact test was used for the comparisons, given the small numbers.

Supplementary Material

Supplementary Material is available at *HMG* online.

Mouse protocol registration number

ASP#18-033.

Human clinical protocol

00-CH-0160.

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Conflict of Interest statement. Dr Stratakis reports that he has patents on the function of the PRKAR1A, PDE11A and GPR101 genes. His laboratory has also received research funding from Pfizer, Inc. for a research subject different than what is being reported here.

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