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GATA4 Is a Critical Regulator of Gonadectomy-Induced Adrenocortical Tumorigenesis in Mice

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In response to gonadectomy certain inbred mouse strains develop sex steroidogenic adrenocortical neoplasms. One of the hallmarks of neoplastic transformation is expression of GATA4, a transcription factor normally present in gonadal but not adrenal steroidogenic cells of the adult mouse. To show that GATA4 directly modulates adrenocortical tumorigenesis and is not merely a marker of gonadal-like differentiation in the neoplasms, we studied mice with germline or conditional loss-of-function mutations in the *Gata4* gene. Germline *Gata4* haploinsufficiency was associated with attenuated tumor growth and reduced expression of sex steroidogenic genes in the adrenal glands of ovariectomized B6D2F1 and B6AF1 mice. At 12 months after ovariectomy, wild-type B6D2F1 mice had biochemical and histological evidence of adrenocortical estrogen production, whereas *Gata4*^{+/-} B6D2F1 mice did not. Germline *Gata4* haploinsufficiency exacerbated the secondary phenotype of postovariectomy obesity in B6D2F1 mice, presumably by limiting ectopic estrogen production in the adrenal glands. *Amhr2-cre*-mediated deletion of floxed *Gata4* (*Gata4*^f) in nascent adrenocortical neoplasms of ovariectomized B6.129 mice reduced tumor growth and the expression of gonadal-like markers in a *Gata4*^f dose-dependent manner. We conclude that GATA4 is a key modifier of gonadectomy-induced adrenocortical neoplasia, postovariectomy obesity, and sex steroidogenic cell differentiation. (*Endocrinology* 153: 0000–0000, 2012)

Adrenocortical neoplasms exist in approximately 5% of people over the age of 50 yr (1). The majority of these tumors are nonfunctioning adenomas, but some secrete steroid hormones that cause Cushing syndrome or other complications (2). Adrenocortical carcinomas are rare (~1 case per million per year) but carry a poor prognosis (3, 4). When functional, adrenocortical carcinomas tend to secrete cortisol or aldosterone; in rare cases these cancers produce androgens or estrogens (5). Despite extensive investigation, the factors accounting for the high incidence of adrenocortical adenomas and the low incidence of adrenocortical carcinomas are unknown (4).

The prevalence of adrenocortical neoplasia in certain animals can provide a foothold by which to study the molecular basis of tumorigenesis in humans (6). One genetically tractable model is postgonadectomy adrenocortical neoplasia in the mouse. In response to gonadectomy and the ensuing rise in serum gonadotropins, cells in the subcapsular region of the mouse adrenal cortex transform into sex steroid-producing neoplasms that are histologically and biochemically similar to normal gonadal tissue (7–12). Gonadectomy-induced adrenocortical neoplasia is strain dependent; susceptible strains include DBA/2J, CE/J, and hybrid C57Bl/6 × DBA/2J F1 (B6D2F1) mice

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Abbreviations: BrdU, Bromodeoxyuridine; Cyp17, cytochrome P450 17 α -hydroxylase/C17-C20 lyase; Cyp19, cytochrome P450 aromatase; E₁, estrone; E₂, estradiol; ER α , estrogen receptor α ; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; H&E, hematoxylin and eosin; Lhcgr, luteinizing hormone chorionic gonadotropin receptor; qRT-PCR, quantitative RT-PCR; SF1, steroidogenic factor-1; WT, wild type.

(7–12). Genome-wide linkage analysis of inbred strain crosses reveals that postgonadectomy adrenocortical neoplasia is a complex trait influenced by multiple genetic loci, but the underlying genes remain enigmatic (11).

GATA4, a transcription factor normally found in sex steroid-producing cells of the gonads but not corticoid-producing cells of the adult adrenal gland, is present in gonadectomy-induced adrenocortical neoplasms (13, 14). Targeted mutagenesis of *Gata4* has been linked to defects in the differentiation of sex steroidogenic lineages in the fetal mouse ovary and testis (15–22). By analogy, it has been hypothesized that GATA4 regulates the differentiation of gonadal-like sex steroidogenic cells in the adrenal glands of gonadectomized mice (7, 23).

Here, we assess the impact of loss-of-function mutations in murine *Gata4* on postovariectomy adrenocortical neoplasia and the secondary phenotype of ovariectomy-induced obesity. *Gata4*^{-/-} mice do not survive to term (24–28), precluding the use of these homozygotes in studies of adrenocortical tumorigenesis. Therefore we examine adrenocortical tumor formation in *Gata4*-haploinsufficient (29, 30) and conditional knockout (20, 21) mice. We show that constitutive and acquired mutations in *Gata4* mitigate the accumulation of neoplastic cells and the expression of sex steroidogenic markers in the adrenal cortex of ovariectomized mice. We also demonstrate that germline *Gata4* haploinsufficiency exacerbates postovariectomy obesity, presumably by constraining ectopic estrogen production by the adrenal glands. These results establish that GATA4 directly modulates postgonadectomy adrenocortical neoplasia and is not merely a marker of gonadal-like differentiation in the tumors.

Materials and Methods

Experimental mice

Procedures involving mice were approved by an institutional committee for laboratory animal care and were conducted in accordance with National Institutes of Health guidelines for the care and use of experimental animals.

C57Bl/6 *Gata4*^{+/-} mice (also termed *Gata4*^{+/ Δ ex2}) were generated and genotyped as described elsewhere (29, 30). *Gata4*^{F/F} mice (also termed *Gata4*^{tm1.1Sad/J}), which are homozygous for a floxed allele of *Gata4* (28, 31), and *Amhr2*^{cre/+} mice (also termed B6.129S7-*Amhr2*^{tm3(cre)Bhr/Mmnc}) (32, 33) were genotyped as described. Some of the *Amhr2*^{cre/+} mice were maintained on a mixed B6.129 genetic background, whereas others were backcrossed onto a C57Bl/6 background.

Gata4^{+/-} C57Bl/6 mice were crossed with DBA2/J or A/J mice to generate wild type (WT) and *Gata4*^{+/-} B6D2F1 or B6AF1 hybrids, respectively. *Amhr2*^{cre/+} C57Bl/6 mice were crossed with DBA2/J mice to generate WT and *Amhr2*^{cre/+} B6D2F1 hybrid mice. To generate conditional knockout mice,

Gata4^{F/F} mice were mated with B6.129 *Amhr2*^{cre/+} mice, and the resultant *Gata4*^{F/+}; *Amhr2*^{cre/+} mice were mated with *Gata4*^{F/F} mice to produce *Gata4*^{F/F}; *Amhr2*^{cre/+} mice (20, 21). To assess the pattern of expression of the *Amhr2*-cre transgene within the adrenal gland, B6.129 *Amhr2*^{cre/+} mice were crossed with ROSA26 flox-stop-flox *LacZ* reporter mice (also termed B6.129S4-*Gt(ROSA)26Sor*^{tm1Sor/J}) (34).

Mice were anesthetized and gonadectomized at 4 wk of age (12). At specified times, the mice were euthanized, blood was collected by cardiac puncture, and tissues were harvested. For the estrogen stimulation experiments, immature (19 d old) *Gata4*^{+/-} mice were implanted sc with a 1.5-mg 17 β -estradiol or placebo pellet (Innovative Research of America; Sarasota, FL) (35). Their uteri were harvested 7 d later after euthanasia. For the compensatory adrenal growth experiments, 6-wk-old male mice were anesthetized and subjected to left adrenalectomy (36). After 48 h, the mice were injected with bromodeoxyuridine (BrdU) (1 mg in 0.1 ml PBS ip). The right adrenal gland was harvested 1 d later after euthanasia.

Immunohistochemistry

Tissues were fixed overnight in 4% paraformaldehyde in PBS, embedded in paraffin, sectioned, and subjected to immunoperoxidase staining (37). The primary antibodies were: 1) goat anti-GATA4 (sc-1237, Santa Cruz Biotechnology, Inc., Santa Cruz, CA; 1:200), 2) goat anti-gremlin (sc-18274; 1:200), 3) goat anti-lactoferrin (sc-14434; 1:200), 4) mouse anti-BrdU (sc-51514; 1:200), 5) mouse antihuman estrogen receptor- α (ER α) (DAKO, Carpinteria, CA; 1:500), 6) mouse antihuman cytochrome P450 aromatase (Cyp19; sc-14245; 1:200), and 7) goat antihuman cytochrome P450 17 α -hydroxylase/C17-C20 lyase (Cyp17; sc-46081; 1:200). Secondary antibodies were: 1) donkey antigoat biotinylated IgG (Jackson ImmunoResearch Laboratories, West Grove, PA; dilution 1:1000) and 2) the MOM kit (Vector Laboratories, Burlingame, CA; prediluted). The avidin-biotin immunoperoxidase system (Vectastain Elite ABC Kit, Vector Laboratories, Inc.) and diaminobenzidine were used to visualize the bound antibody. Our analysis included control studies in which the primary antibody was omitted.

Morphological analyses

Adrenocortical tumor cross-sectional area was measured (38) using hematoxylin and eosin (H&E) stained tissue sections. The analysis included a minimum of four sections from each adrenal, spaced evenly (every 100 μ m) through the central portion of the gland; tangential sections lacking medulla were excluded. To detect extragonadal sex steroid production in ovariectomized mice, tissues sensitive to estrogens (uterus, vagina) and androgens (submaxillary gland) were analyzed (39).

X-gal staining

Adrenal glands were fixed with 0.2% glutaraldehyde in PBS for 15 min, permeabilized with 100 mM potassium phosphate (pH 7.4), 0.02% Nonidet P-40, and 0.01% sodium deoxycholate for 5 min, and then stained with X-gal at 37 C overnight (26). After staining, the glands were frozen in OCT cryopreservation media (Tissue-Tek, Torrance, CA), sectioned, and stained with nuclear fast red. Alternatively, the stained glands were post-fixed with Karnovsky solution and processed for transmission electron microscopy (20, 21).

Serum hormone levels

Measurements were made using ELISA kits for FSH, LH, and estrone (E_1) sulfate from Endocrine Technologies, Inc. (Newark, CA), estradiol (E_2) from BioCheck (Foster City, CA), and prolactin (PRL) from Cusabio Biotech Co. (Newark, DE).

Quantitative RT-PCR (qRT-PCR)

Total RNA was isolated from adrenal glands using TRIzol (Invitrogen, Carlsbad, CA). First-strand cDNA was produced with the SuperScript VILO cDNA Synthesis Kit (Invitrogen). An aliquot of cDNA was subjected to real time RT-PCR (20, 21) using the intron-spanning primers listed in Supplemental Table 1 published on The Endocrine Society's Journals Online web site at <http://endo.endojournals.org>. Target gene expression was normalized to the expression of three housekeeping genes: β -actin, ribosomal protein L19, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH).

Qualitative RT-PCR for detection of Cre-mediated recombination in adrenal glands

Total RNA was isolated from adrenal gland tissue using the Arcturus PicoPure RNA Isolation Kit (Applied Biosystems, Foster City, CA). cDNA was synthesized using a QuantiTect Reverse Transcription Kit (QIAGEN, Valencia, CA). cDNA was subjected to RT-PCR using a forward primer from exon 2 of the *Gata4* gene and a reverse primer from exon 7 (20, 21).

Statistical analysis

Numerical data are represented as mean \pm SD. Except where indicated, differences were assessed for statistical significance ($P < 0.05$) with the Student's *t* test.

Results

Germline *Gata4* haploinsufficiency impairs postgonadectomy adrenocortical tumor formation in B6D2F1 and B6AF1 mice

To determine the impact of GATA4 deficiency on adrenocortical tumorigenesis, we crossed *Gata4*^{+/-} C57Bl/6 mice, which harbor a deletion in exon 2 of *Gata4* that includes the translation start site (21, 29, 40), with DBA/2J mice and analyzed the resultant WT and *Gata4*^{+/-} B6D2F1 offspring. B6D2F1 mice, like the parental DBA/2J strain, develop adrenocortical neoplasia with complete penetrance by 6 months after ovariectomy (11). H&E-stained sections of adrenal glands from ovariectomized WT B6D2F1 mice confirmed the presence of subcapsular neoplasms (Fig. 1A). The tumors were composed of spindle-shaped type A cells (Fig. 1A, arrowhead), which resemble stromal cells of the postmenopausal ovary, and sex steroidogenic type B cells (Fig. 1A, arrow), which express the gonadal-like markers LH chorionic gonadotropin receptor (*Lhcgr*), *inhibin- α* , *Cyp17*, and *Cyp19* (7). Nuclear GATA4 antigen was evident in both the type A (Fig. 1C, arrowhead) and type B cells (Fig. 1C, arrow).

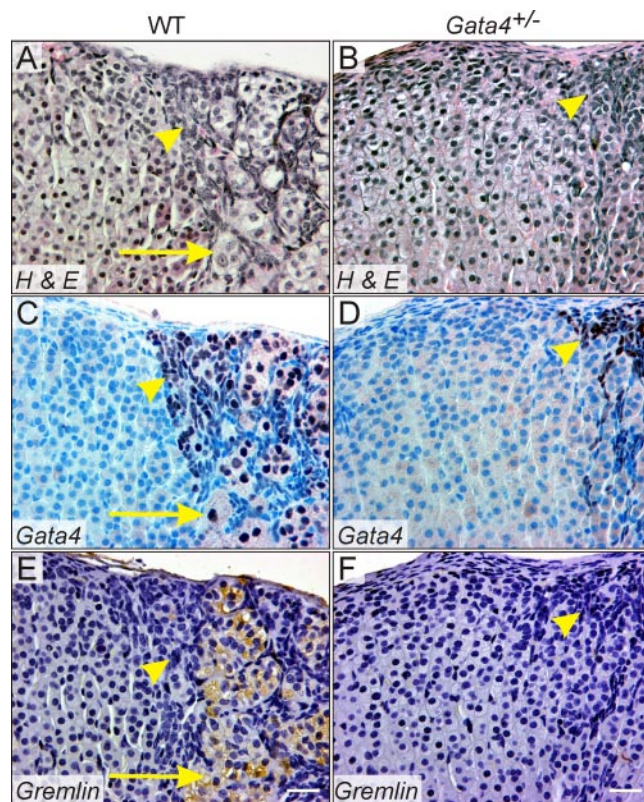


FIG. 1. Microscopic appearance of adrenal glands from ovariectomized WT (A, C, and E) or *Gata4*^{+/-} B6D2F1 mice (B, D, and F) were ovariectomized, and adrenal glands were harvested 3 months later. Adjacent tissue sections were stained with H&E (A and B), anti-GATA4 antibody (C and D), or antigremlin antibody (E and F). Neoplastic type A cells (yellow arrowheads) expressed nuclear GATA4, whereas neoplastic type B cells (yellow arrows) expressed both GATA4 and gremlin. Note the absence of type B cells in the *Gata4*^{+/-} adrenal gland. Bars, 40 μ m.

Antibody against gremlin, a bone morphogenetic protein antagonist implicated in granulosa cell function (41), stained sex steroidogenic type B cells (Fig. 1E, arrow) but not type A cells (Fig. 1E, arrowhead). Histological analysis of adrenal glands from ovariectomized *Gata4*^{+/-} B6D2F1 mice demonstrated only rare, small patches of GATA4-expressing type A cells that did not invade deeply into the cortex (Fig. 1, B and D). There was a paucity of morphologically recognizable type B cells in the adrenals of *Gata4*^{+/-} mice (Fig. 1B) and a concomitant reduction in gremlin immunoreactivity (Fig. 1F).

qRT-PCR confirmed the reduced expression of *Gata4* in the adrenal glands of gonadectomized B6D2F1 haploinsufficient mice. At 5 months after ovariectomy, the ratio of GATA4 mRNA levels in *Gata4*^{+/-} vs. WT mice was 0.22 ± 0.03 ($P < 0.05$; results normalized to expression of the housekeeping gene β -actin).

To quantify differences in adrenocortical tumor formation between the WT and *Gata4*^{+/-} B6D2F1 mice, we measured the cross-sectional area of cortex occupied by

neoplastic cells at varying times after ovariectomy (Fig. 2). By 6 months after ovariectomy, neoplastic cells occupied 27% of the cross-sectional area of adrenal cortex in WT mice. *Gata4* haploinsufficiency was associated with a reduction in the area occupied by neoplastic cells at all time points examined; at 6 months after ovariectomy the difference was highly significant ($P < 0.001$). The reduction in tumor size reflected decreases in both type A cells and type B cells, but the decrease in the latter was particularly striking. At 6 months after ovariectomy, sections of WT adrenal glands contained an average of 132 ± 104 type B cells, whereas sections of *Gata4*^{+/-} adrenal glands contained an average of 2 ± 1 type B cells ($P < 0.05$). The isolated type A cell hyperplasia seen in the adrenal glands of ovariectomized *Gata4*^{+/-} mice is a feature of older non-gonadectomized WT mice (42) and is not considered tumorous (43).

GATA factors have been implicated in the development and function of the anterior pituitary gland (44, 45). To ensure that *Gata4* haploinsufficient mice had intact gonadotrope function, we measured serum FSH and LH levels (Supplemental Fig. 1). *Gata4* haploinsufficiency had no significant impact on either basal or postovariectomy gonadotropin levels, so the reduction in tumor formation in *Gata4*^{+/-} mice cannot be ascribed to altered gonadotrope function.

To show that the *Gata4* haploinsufficiency effect was not limited to a single hybrid strain, we examined post-ovariectomy adrenocortical neoplasia in C57Bl/6 × A/J F1 (B6AF1) mice. We found that WT B6AF1 mice, like the

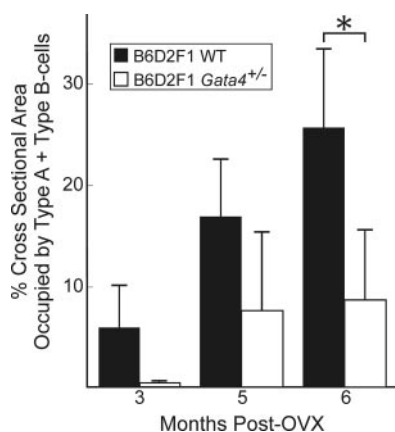


FIG. 2. Comparison of postovariectomy adrenocortical tumor size in WT and *Gata4*^{+/-} B6D2F1 mice. Weanling WT (black bars) or *Gata4*^{+/-} (white bars) B6D2F1 female mice were subjected to ovariectomy (OVX), and adrenal glands were harvested 3, 5, or 6 months later. Individual adrenal glands were fixed, sectioned, and stained with H&E. The average cross-sectional area of cortex occupied by tumor cells (type A plus type B cells) was quantified. Values represent the mean \pm SD of four to six adrenal glands. Note that the percentage of cortex occupied by tumor was greater in the WT than in the *Gata4*^{+/-} adrenals at all time points examined, and at 6 months this difference was highly significant (*, $P < 0.001$).

parental A/J strain (46), develop adrenocortical tumors after ovariectomy (Supplemental Fig. 2A). The tumors in adrenal glands of ovariectomized B6AF1 mice contained type A cells that expressed GATA4 antigen (Supplemental Fig. 2C) and type B cells that expressed both GATA4 (Supplemental Fig. 2C) and gremlin (Supplemental Fig. 2E). As in B6D2F1 mice, *Gata4* haploinsufficiency mitigated ovariectomy-induced adrenocortical neoplasia in B6AF1 mice (Supplemental Fig. 2, B, D, and F).

Germline *Gata4* haploinsufficiency impairs expression of gonadal-like markers in the adrenal glands of ovariectomized B6D2F1 and B6AF1 mice

The effect of *Gata4* haploinsufficiency on ovariectomy-induced changes in gonadal-like markers in the adrenal glands of B6D2F1 and B6AF1 mice was assessed by qRT-PCR 5 months after surgery. Results were normalized to expression of β -actin; normalization to two other housekeeping genes, *L19* and *GAPDH*, yielded similar results. Consistent with studies on other susceptible strains (7, 8, 11–13), ovariectomy of WT B6D2F1 (Fig. 3A) and WT B6AF1 (Fig. 3B) mice was associated with increased adrenal expression of the sex steroidogenic differentiation markers GATA4, *Cyp17*, *Lhcgr*, and *inhibin- α* (Fig. 3, A and B). In contrast, ovariectomy of WT mice had no significant impact on expression of the prototypical adrenocortical markers *M2CR* and *GATA6* (*i.e.* the ratio of mRNA levels in ovariectomized *vs.* intact mice was approximately 1) (Fig. 3, A and B). *Gata4* haploinsufficiency markedly reduced the ovariectomy-dependent expression of sex steroidogenic transcripts in both B6D2F1 (Fig. 3A) and B6AF1 (Fig. 3B) mice.

As a control, we analyzed ovariectomy-dependent expression of steroidogenic genes in the adrenal glands of B6D2F1 mice haploinsufficient for another gonadal marker, *Amhr2*, the anti-Müllerian hormone receptor gene. *Amhr2* is expressed in murine postgonadectomy adrenocortical tumors but not in adjacent normal tissue (7, 8, 13). We performed qRT-PCR analysis on adrenal glands from ovariectomized *Amhr2*^{cre/+} mice, which harbor a knock-in *cre* allele that renders the mice *Amhr2* haploinsufficient. *Amhr2* haploinsufficiency had no impact on the expression of sex steroidogenic markers in the adrenal glands of ovariectomized B6D2F1 mice (Fig. 3C). The absence of a phenotype in the *Amhr2* haploinsufficient mice underscores the significance of the *Gata4* haploinsufficiency effect on sex steroidogenesis and constitutes an important negative control for the *Amhr2*-mediated conditional mutagenesis studies described below.

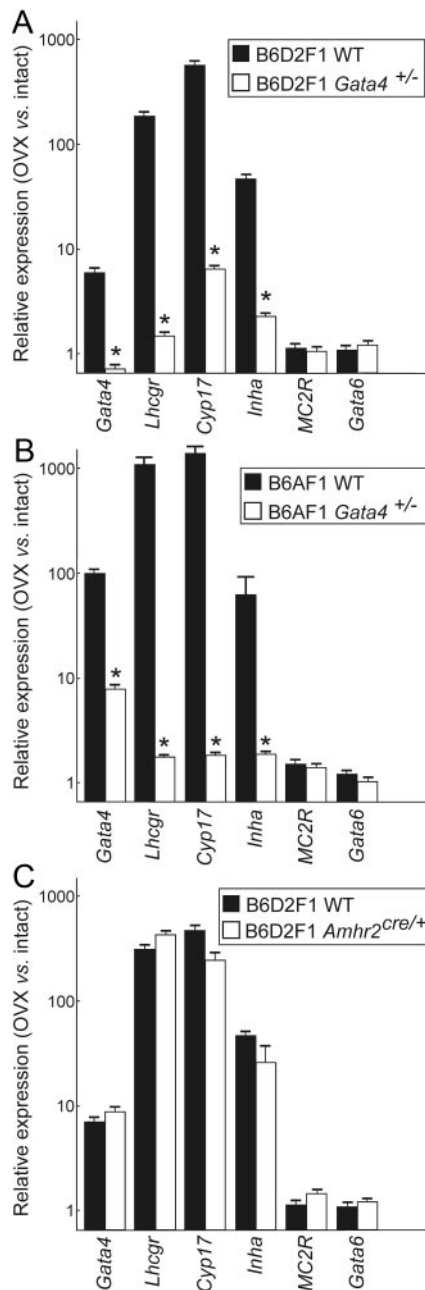


FIG. 3. Expression of steroidogenic differentiation markers in adrenal glands from ovariectomized WT, *Gata4* haploinsufficient, and *Amhr2* haploinsufficient mice. Weanling B6D2F1 (A and C) or B6AF1 (B) mice of the specified genotypes were subjected to ovariectomy (OVX) or left intact. Adrenal glands were harvested 5 months later and subjected to qRT-PCR analysis. *Graphs* show the relative expression (mean \pm SD) of various transcripts in ovariectomized ($n = 4$) vs. intact mice ($n = 4$). Results were normalized to expression of β -actin. The y-axis is plotted on a logarithmic scale. Note that *Gata4* haploinsufficiency attenuated the ovariectomy-dependent increase in expression of the sex steroidogenic differentiation markers *Lhcgr*, *Cyp17*, and *Inha* (*, $P < 0.001$). In contrast, loss of one functional *Amhr2* allele via knockin of *cre* had no significant impact on expression of steroidogenic cell markers in B6D2F1 mice.

Germline *Gata4* haploinsufficiency impairs adrenocortical estrogen production and exacerbates obesity in ovariectomized B6D2F1 mice

The examination of tissues that undergo morphological changes in response to circulating estrogens (uterus, vagina) or androgens (submaxillary gland) offers evidence for adrenocortical sex steroid production in gonadectomized mice. Older ovariectomized DBA/2J mice exhibit estrogenic stimulation of the uterus and vagina, whereas aged ovariectomized CE/J mice display both estrogenic and androgenic stimulation of target tissues (39).

Older ovariectomized WT B6D2F1 mice show a prominent estrogenic phenotype resembling that of the parental DBA/2J strain. At 12 months after ovariectomy, every WT B6D2F1 mouse ($n = 5$) had grossly enlarged uterine horns (Fig. 4A) with histological evidence of estrogenic changes in the uterus (Fig. 4B) and vagina (Fig. 4D), including uterine epithelial immunoreactivity for lactoferrin, an estrogen-responsive gene (Fig. 5A) (47). By comparison, none of the age-matched ovariectomized *Gata4*^{+/-} mice ($n = 4$) showed morphological evidence of extragonadal estrogen production ($P < 0.005$; two population proportions testing). Instead, the *Gata4*^{+/-} mice had grossly atrophic uteri (Fig. 4A) and lacked estrogen-induced histological changes in target tissues (Fig. 4, C and E), including lactoferrin immunoreactivity in the uterus (Fig. 5B). In contrast, nuclear estrogen receptor (ER) α immunoreactivity was evident in uterine epithelial and stromal cells of both the WT (Fig. 5C) and *Gata4*^{+/-} (Fig. 5D) mice. Uterine weights in the ovariectomized WT and *Gata4*^{+/-} B6D2F1 mice were 98 ± 5 mg and 13 ± 0.3 mg, respectively ($P < 0.01$).

Because GATA4 is not expressed in the uterus or its precursor, the Müllerian duct, the differences in uterine morphology between ovariectomized WT and *Gata4* haploinsufficient mice cannot be attributed to a direct effect of GATA4 on uterine responsiveness to estrogen (21). Reinforcing this premise, a robust estrogenic response, including increased lactoferrin immunoreactivity, was observed in the uteri of prepubertal *Gata4*^{+/-} mice implanted sc with an E₂ pellet (Fig. 5E) but not a placebo (Fig. 5F).

In addition to estrogenic changes, older ovariectomized WT mice (Fig. 4F), but not their *Gata4*^{+/-} counterparts (Fig. 4G), had histological evidence of androgenic stimulation in the submaxillary glands. Such masculinization of the female submaxillary gland occurs during pregnancy and lactation as a result of increased androgen secretion (48).

At 12 months after ovariectomy, serum E₂ and E₁ sulfate levels were greater in WT than *Gata4*^{+/-} B6D2F1 mice (Fig. 4, H and I). Estrogen induces PRL secretion (49,

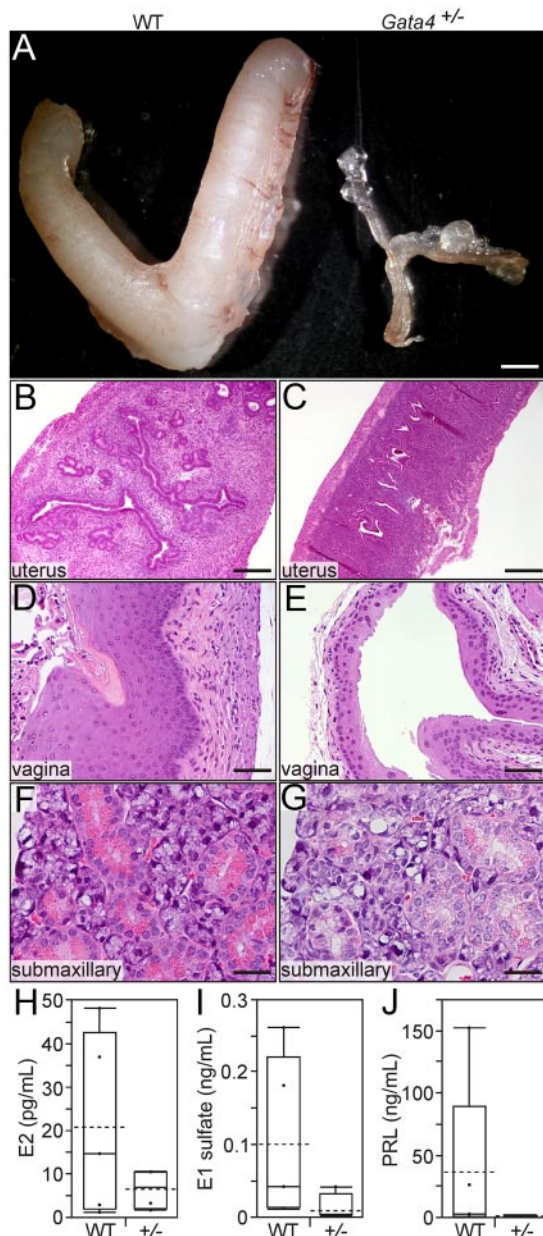


FIG. 4. *Gata4* haploinsufficiency abrogates extragonadal sex steroid production in older ovariectomized B6D2F1 mice. Weanling mice of the indicated genotypes were ovariectomized, and 12 months later uteri were analyzed for evidence of estrogen- or androgen-dependent stimulation. The uteri of WT mice were enlarged (A, left) and estrogenic (B), whereas the uteri of *Gata4*^{+/-} mice were atrophic (A, right) and hypoestrogenic (C). Consistent with estrogen stimulation, the vaginal epithelium of WT mice was thick and cornified (D). In contrast, the vaginal mucosa of *Gata4*^{+/-} mice appeared thin and hypoestrogenic (E). WT mice had evidence of androgen stimulation of the submaxillary gland, manifest as tall columnar acinar cells with basally located nuclei and abundant eosinophilic cytoplasmic granules (F). In *Gata4*^{+/-} mice, however, the submaxillary glands had the typical appearance of female mice, with centrally located epithelial cell nuclei and only a few cytoplasmic granules (G). Bars, 2 mm (A), 300 μ m (B and C), 75 μ m (D and E), and 40 μ m (F and G). Serum E₂, E₁ sulfate, and PRL levels in WT and *Gata4*^{+/-} mice are shown in panels H, I, and J, respectively. In the *box-and-whisker* plots, *dots* represent individual measurements, *boxes* represent the interquartile range, *whiskers* indicate the first and fourth quartiles, *solid lines* indicate the median, and *dashed lines* represent the mean (63).

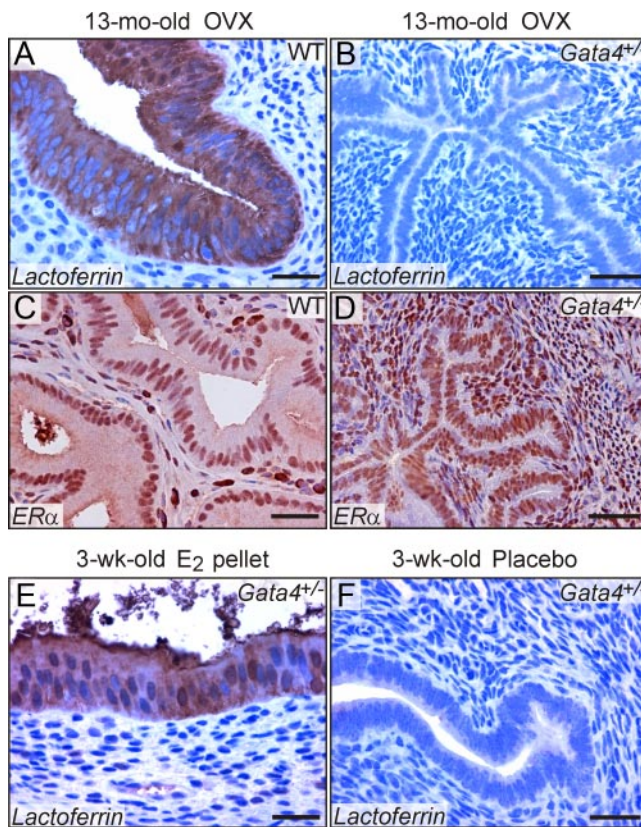


FIG. 5. Lactoferrin and ER α immunoreactivity in the uteri of WT and *Gata4*^{+/-} B6D2F1 mice. Weanling WT or *Gata4*^{+/-} B6D2F1 mice were ovariectomized (OVX), and 12 months later uteri were subjected to immunoperoxidase staining for the estrogen response marker lactoferrin or for ER α . Lactoferrin immunoreactivity was seen in the uterine epithelial cells of ovariectomized WT mice (A) but not *Gata4*^{+/-} mice (B). In contrast, nuclear immunoreactivity for ER α was evident in uterine epithelial and stromal cells of ovariectomized WT (C) and *Gata4*^{+/-} (D) mice. To show that the uteri of *Gata4*^{+/-} mice were capable of responding to estrogen, prepubertal *Gata4*^{+/-} mice were implanted sc with an E₂ pellet or a placebo. Lactoferrin immunoreactivity and other estrogenic changes were observed in the uteri of immature mice implanted with E₂ (E) but not the placebo (F). Bars, 75 μ m.

50), and ovariectomized WT mice had higher serum PRL levels than their *Gata4*^{+/-} counterparts (Fig. 4J). PRL can have direct effects on the adrenal gland (51) and estrogen-sensitive organs such as the uterus (49), so some of the changes in the ovariectomized WT mice could reflect PRL effects. Arguing against a direct effect of PRL on the uterus, Kiss *et al.* (52) found that administration of PRL + E₂ to ovariectomized B6D2F1 mice elicited no more uterine epithelial proliferation than E₂ alone.

Gross inspection of older ovariectomized WT B6D2F1 mice demonstrated large adrenal tumor nodules that distorted the surface of the glands (data not shown), supporting the notion that adrenocortical tumors were the source of extragonadal sex steroids. Such adrenal tumor nodules were not evident in aged ovariectomized *Gata4*^{+/-} mice.

Estrogenic changes were not observed in reproductive organs of younger ovariectomized WT B6D2F1 mice (< 6 months after ovariectomy; data not shown). qRT-PCR analysis of adrenal glands from WT B6D2F1 mice of varying ages showed that the up-regulation of *Cyp19* expression lagged that of other gonadal-like markers, such as *Lhcgr* and *Cyp17* (Supplemental Fig. 3A). A marked increase in *Cyp19* expression occurred between 5 and 6 months after ovariectomy. The late onset of *Cyp19* expression may explain why estrogenic changes in the uterus and vagina are limited to older ovariectomized mice. Immunohistochemistry on adrenal glands from WT B6D2F1 mice 6 months after ovariectomy demonstrated *Cyp17* (Supplemental Fig. 3B) and *Cyp19* (Supplemental Fig. 3C) immunoreactivity in type B neoplastic cells, in agreement with studies of other ovariectomized strains of mice (7).

In addition to triggering adrenocortical tumorigenesis, ovariectomy is known to exacerbate obesity in B6D2F1 and other strains of mice (11). Postovariectomy obesity has been attributed to reduced energy expenditure (53), and estrogen repletion of ovariectomized mice has been shown to prevent gains in visceral adiposity (53). At 12 months after ovariectomy, WT B6D2F1 mice were significantly less obese than their *Gata4*^{+/-} counterparts [body weights = 40.4 ± 4.7 g and 51.7 ± 4.9 g, respectively (*P* < 0.01)], consistent with extragonadal estrogen production in the former. Visual inspection confirmed that the differences in weight between WT and *Gata4*^{+/-} mice reflected differences in abdominal adiposity.

Collectively, these observations suggest that *Gata4* haploinsufficiency abrogates extragonadal sex steroid production in ovariectomized B6D2F1 mice by inhibiting adrenocortical tumor formation and that absence of ectopic estrogen production exacerbates postovariectomy obesity.

Germline *Gata4* haploinsufficiency has no effect on the growth of nonneoplastic adrenocortical cells in B6D2F1 mice

Having demonstrated that constitutive *Gata4* haploinsufficiency decreases postgonadectomy adrenocortical tumor development, we next assessed the impact of *Gata4* haploinsufficiency on the growth of nonneoplastic adrenocortical cells, using the established model of compensatory adrenal hyperplasia/hypertrophy in mice subjected to left adrenalectomy (36). There was no difference in right adrenal gland weight between 6-wk-old WT and *Gata4*^{+/-} B6D2F1 mice subjected to sham adrenalectomy (1.1 ± 0.2 mg vs. 1.2 ± 0.1 mg, respectively). Thus, even though *Gata4* is transiently expressed in the adrenal during fetal development (54), *Gata4* haploinsufficiency had no effect on adrenal gland size in intact adult mice. Like-

wise, *Gata4* haploinsufficiency had no impact on compensatory adrenal growth after unilateral adrenalectomy (Supplemental Fig. 4A). Histological analysis demonstrated that the increase in adrenal gland weight reflected enlargement of the cortex (Supplemental Fig. 4, B–E), and BrdU-labeling studies showed that unilateral adrenalectomy elicited similar increases in adrenocortical cell proliferation in WT and *Gata4*^{+/-} mice (Supplemental Fig. 4, B–E). We conclude that *Gata4* haploinsufficiency, unlike steroidogenic factor-1 (*Sf1*) (*Nr5a1*, *Ad4BP*) haploinsufficiency (36, 55), has no effect on the growth of normal adrenocortical cells.

Conditional deletion of *Gata4* in nascent adrenocortical neoplasms of ovariectomized B6.129 mice impairs sex steroidogenesis in a *Gata4* dose-dependent manner

The Cre-loxP recombination system was used to conditionally ablate the *Gata4* gene in nascent adrenocortical tumor cells of ovariectomized B6.129 mice. Like other mouse strains, the mixed B6.129 strain develops postgonadectomy adrenocortical tumors composed of type A and type B cells (Fig. 6A), although the postovariectomy adrenocortical tumors are smaller than those found in comparably aged B6D2F1 or B6AF1 mice. To generate conditional knockout mice, we crossed mice harboring a floxed allele of (*Gata4*^F) with mice bearing the *Amhr2*-cre knock-in allele. Cre-mediated recombination deletes exons 3–5 of *Gata4*, resulting in a null allele (28, 31). The *Amhr2*-cre transgene has been used extensively to target gene deletion in gonadal somatic cells (32, 33, 56–61). *Gata4* conditional knockout mice generated using *Amhr2*-cre have reduced fertility due to defects in gonadal somatic cell function (20, 21). By analogy, we hypothesized that recombination of *Gata4*^F by *Amhr2*-cre would impair the development of gonadal-like tumors in the adrenal glands of gonadectomized mice in a *Gata4*^F dose-dependent manner.

A ROSA26 flox-stop-flox *lacZ* reporter (R26R), which indelibly expresses β-galactosidase in response to Cre recombinase (34), was used to assess the pattern of expression of the *Amhr2*-cre transgene within the adrenal glands of ovariectomized mice. Whole-mount X-gal staining of adrenal glands from ovariectomized R26R; *Amhr2*^{cre/+} mice demonstrated β-galactosidase activity in discrete patches of cells near the surface of the gland (Fig. 6B). Histological analysis of these adrenal glands showed X-gal staining in subcapsular neoplasms but not in adjacent normal adrenocortical cells (Fig. 6C). Transmission electron microscopy demonstrated the presence of electron-dense crystalloids indicative of the X-gal reaction product in both type A (Fig. 6D) and type B cells (Fig. 6E). Con-

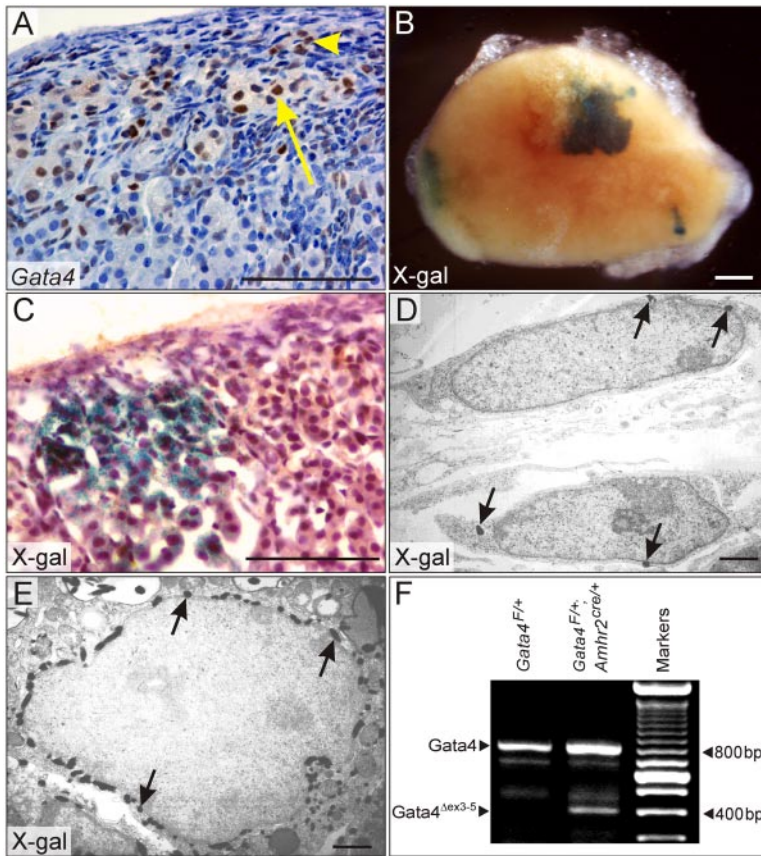


FIG. 6. Ovariectomized B6.129 mice develop adrenocortical neoplasms that express GATA4 and *Amhr2*-cre. B6.129 mice were ovariectomized, and adrenal glands were harvested 5 months later. Immunohistochemical staining of adrenal tissue sections (A) showed nuclear GATA4 antigen in type A (yellow arrowhead) and type B (yellow arrow) cells. B–E, Mice harboring *Amhr2*-cre and *R26R* transgenes were ovariectomized, and after 5 months adrenal glands were subjected to whole-mount X-gal staining (B) to highlight cells expressing Cre. Sections of X-gal-stained adrenal tissue were processed for light (C) or transmission electron (D and E) microscopy. Note the presence of subcapsular neoplastic cells expressing Cre (C). Electron-dense crystalloids (black arrows) indicative of the X-gal reaction product were seen in both small, spindle-shaped, type A cells (D) and large, mitochondria-rich, type B cells (E). Weanling mice of the specified genotypes were ovariectomized, and adrenal RNA was isolated 3 months later. The RNA was subjected to qualitative RT-PCR analysis with primers that distinguish transcripts derived from the intact *Gata4*^F allele vs. the recombined allele lacking exons 3 to 5 (*Gata4*^{Δex3–5}) (F). Note that a transcript derived from the recombined allele is present in the adrenal glands of *Gata4*^{F/+}; *Amhr2*^{cre/+} mice. Bars, 100 μm (A–C), 2 μm (D and E).

sistent with prior ultrastructural studies (62), type A cells contained abundant rough endoplasmic reticulum but few mitochondria and no lipid droplets (Fig. 6D), whereas type B cells contained prominent smooth endoplasmic reticulum, lipid droplets, and mitochondria (Fig. 6E). Control whole-mount X-gal stains of adrenal glands from nonovariectomized *R26R*; *Amhr2*^{cre/+} mice showed little or no staining (data not shown). These *R26R* lineage tracing experiments establish that the *Amhr2*-cre transgene is induced after ovariectomy and is expressed in type A and type B cells or their progenitor(s).

To verify *Amhr2*-cre-mediated deletion of the *Gata4*^F allele in the adrenal gland of gonadectomized mice, we

employed a qualitative RT-PCR assay that distinguishes transcripts derived from the intact and recombined alleles (20). RT-PCR analysis of adrenal glands from ovariectomized *Gata4*^{F/+} mice yielded a 782-bp fragment reflecting transcription of the intact allele (Fig. 6F). In contrast, the adrenal glands of ovariectomized *Gata4*^{F/+}; *Amhr2*^{cre/+} mice contained both the 782-bp fragment and a 401-bp fragment derived from the recombined allele lacking exons 3–5 (*Gata4*^{Δex3–5}).

Adrenal glands from ovariectomized *Gata4*^{F/F} mice contained tumors that invaded deeply into the cortex, as highlighted by GATA4 immunohistochemistry (Fig. 7, A and C). Both type A (Fig. 7C, arrowhead) and type B (Fig. 7C, arrow) cells were evident in the tumors. In contrast, adrenal glands from *Gata4*^{F/F}; *Amhr2*^{cre/+} (conditional knockout) mice contained only small neoplasms composed exclusively of type A cells that did not invade deeply into the cortex (Fig. 7, B and D). We conclude that up-regulation of *Amhr2*-cre in nascent adrenocortical tumors reduces but does not entirely block the accumulation of GATA4-positive neoplastic cells. To confirm reduced expression of *Gata4* in the adrenal glands of gonadectomized conditional knockout mice, we performed qRT-PCR using primers specific for the WT GATA4 mRNA. The ratio of GATA4 mRNA levels in ovariectomized *Gata4*^{F/F}; *Amhr2*^{cre/+} vs. *Gata4*^{F/F} mice was 0.20 ± 0.02 ($P < 0.05$, normalized to β -actin mRNA levels).

Previous studies have documented leaky expression of *Amhr2*-cre in the pituitary gland (63). To assess pituitary gonadotrope function in the conditional knockout animals, we measured basal and postovariectomy serum gonadotropin levels in mice of the following four genotypes: 1) *Gata4*^{F/+}, 2) *Gata4*^{F/+}; *Amhr2*^{cre/+}, 3) *Gata4*^{F/F}, and 4) *Gata4*^{F/F}; *Amhr2*^{cre/+} (Supplemental Fig. 5, A and B). There were no significant differences in gonadotropin secretion among the four genotypes, so the reduction in tumor formation in the conditional knockout mice cannot be attributed to altered pituitary function.

qRT-PCR was used to assess ovariectomy-induced changes in expression of steroidogenic markers in the ad-

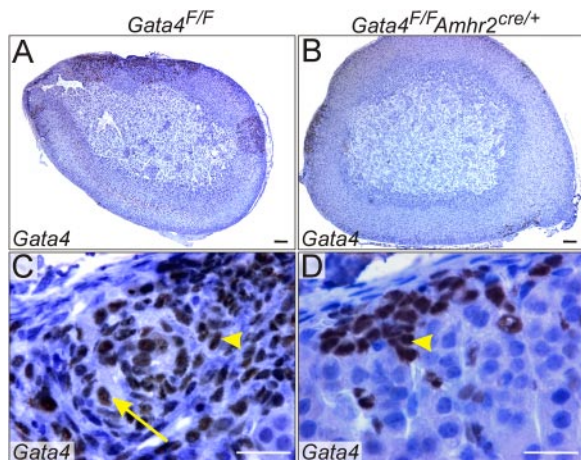


FIG. 7. Microscopic appearance of adrenal glands from ovariectomized *Gata4^{F/F}* and *Gata4^{F/F};Amhr2^{cre/+}* B6.129 mice. Weanling *Gata4^{F/F}* (A and C) or *Gata4^{F/F};Amhr2^{cre/+}* (B and D) B6.129 mice were ovariectomized, and adrenal glands were harvested 3 months later. Tissue sections were stained with GATA4 antibody. Shown are low- (A and B) and high-magnification (C and D) views. Tumors composed of type A cells (yellow arrowheads) and type B cells (yellow arrows) invade deeply into the adrenal cortex of ovariectomized *Gata4^{F/F}* mice. Note the absence of type B cells in the *Gata4^{F/F};Amhr2^{cre/+}* adrenal gland. Bars, 200 μ m (A and B); 25 μ m (C and D).

renal glands of conditional knockout and control mice (Fig. 8). Results were normalized to β -actin; the outcome was similar when results were normalized to L19 or GAPDH. In general, ovariectomy-induced changes in gene expression were more variable in B6.129 (mixed genetic background) mice (Fig. 8) than in B6D2F1 or B6AF1 mice (Fig. 3). As expected, expression of the prototypical adrenocortical marker, melanocortin 2 receptor, was similar in conditional knockout and control mice, whether the mice were ovariectomized or intact (Fig. 8A). In contrast, expression of the gonadal markers *Lhcgr* (Fig. 8B) and inhibin- α (Fig. 8C) was reduced in the ovariectomized *Gata4^{F/F};Amhr2^{cre/+}* mice vs. control *Gata4^{F/F}* mice ($P < 0.05$). Similarly, expression of *Cyp17* was decreased in the ovariectomized *Gata4^{F/F};Amhr2^{cre/+}* mice, although this did not reach statistical significance ($P = 0.08$; data not shown). We conclude that *Amhr2*-cre-mediated deletion of *Gata4^F* in nascent adrenocortical neoplasms of ovariectomized mice reduces expression of gonadal-like markers in a *Gata4^F* dose-dependent manner.

Discussion

Gonadectomy-induced adrenocortical neoplasia has been attributed to continuous gonadotropin secretion by the pituitary gland (7). Adrenal transplantation and parabiosis experiments have established that the adrenal glands of susceptible mouse strains exhibit an inherent predispo-

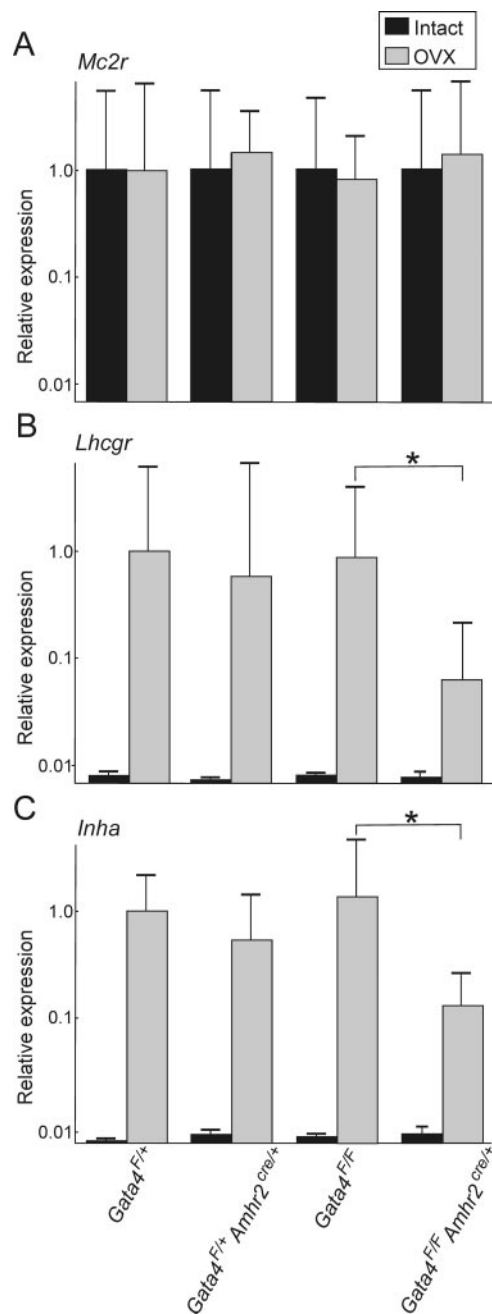


FIG. 8. Expression of steroidogenic differentiation markers in adrenal glands from ovariectomized *Gata4* conditional knockout mice. Weanling mice of the specified genotypes were subjected to ovariectomy (OVX, gray bars) or left intact (black bars). Adrenal glands were harvested 3 months later and subjected to qRT-PCR analysis for *Mc2r* (A), *Lhcgr* (B), or *Inha* (C). The graphs show the relative expression (mean \pm SD) of different transcripts in ovariectomized ($n = 4-8$) vs. intact mice ($n = 7-14$) of the indicated genotypes. Results were normalized to expression of β -actin. The y-axis is plotted on a logarithmic scale. For each marker, the average expression in ovariectomized *Gata4^{F/F}* mice was arbitrarily set at 1. Note that *Lhcgr* and *Inha* transcripts are less abundant in the adrenals of ovariectomized *Gata4^{F/F};Amhr2^{cre/+}* mice than *Gata4^{F/F}* mice (*, $P < 0.05$).

sition to sex steroidogenic tumor formation in response to persistently elevated serum gonadotropin levels (7, 9–11, 64). However, the molecular mechanisms underlying ad-

renal gland susceptibility have remained elusive. Our findings offer genetic proof that GATA4 is a key player insofar as loss-of-function mutations in *Gata4* impair ovariectomy-induced adrenocortical neoplasia in multiple susceptible strain backgrounds (B6D2F1, B6AF1, and B6.129). The consistent GATA4 deficiency phenotype in germline haploinsufficient mice bearing a deletion in exon 2 and in conditional knockout mice with an acquired deletion in exons 3–5 suggests that: 1) *Gata4* is a dose-dependent modifier of postgonadectomy adrenocortical neoplasia, and 2) the *Gata4* effect is not the result of a particularly engineered allele but rather a general property of loss-of-function mutations. We conclude that the hormonal changes that accompany gonadectomy cannot drive adrenocortical tumorigenesis in susceptible strains of mice without the proper dosage of *Gata4*.

The present results offer insights into the mechanism by which GATA4 deficiency attenuates postgonadectomy adrenocortical neoplasia. In theory, GATA4 could promote tumor initiation, sex steroidogenic differentiation, or tumor cell growth/survival. The conditional knockout model, in which recombination of the *Gata4^F* gene occurs after up-regulation of *Ambr2-cre* in nascent tumors, dissociates the effects of GATA4 on tumor initiation from its effects on the subsequent stages of tumor development (gonadal-like differentiation and neoplastic cell proliferation/survival). That features of the haploinsufficiency phenotype are conserved in the conditional knockout model suggests that GATA4 deficiency has direct effects on sex steroidogenic differentiation or tumor proliferation/survival. In preliminary experiments, we found no evidence for decreased tumor proliferation or increased tumor apoptosis in the adrenal glands of gonadectomized GATA4-deficient mice (data not shown). These findings, together with the paucity of type B cells in the germline haploinsufficiency and conditional knockout mouse models and the absence of extragonadal estrogen production in the haploinsufficiency model, lead us to surmise that GATA4 deficiency disrupts postgonadectomy tumorigenesis principally via inhibition of sex steroidogenic differentiation in the neoplastic cells.

During normal gonadal development, GATA4 promotes sex steroidogenic cell differentiation by repression of genes that maintain stem/progenitor cells in an undifferentiated state, such as genes in the Wnt/ β -catenin signaling pathway (16, 65) and by subsequent activation of genes involved in sex steroid biosynthesis (e.g. *Lhcgr*, *Cyp17*, *Cyp19*) (14, 21, 66–68). Mice homozygous for the *Gata4^{ki}* allele, a mutation that blocks the interaction between GATA4 and its cofactor FOG2, fail to properly repress *Dkk1*, a secreted inhibitor of the canonical Wnt signaling pathway, leading to impaired ovarian develop-

ment (16, 65). Activation of β -catenin is common in human adrenocortical tumors (69), and constitutive activation of β -catenin in the adrenal cortex of transgenic mice promotes adrenocortical neoplasia (70). It is unclear whether disruption of the Wnt/ β -catenin signaling pathway accounts for the lack of adrenocortical tumor formation in gonadectomized *Gata4* haploinsufficient or conditional knockout mice. In preliminary studies we did not detect significant differences in expression of *Dkk1* or other components of this signaling pathway (data not shown). Identification of the GATA4 target genes essential for postgonadectomy adrenocortical neoplasia awaits further study.

Like *Gata4*, the *Sf1* (*Nr5a1*, *Ad4BP*) gene has been shown to be a dosage-sensitive modifier of steroidogenic cell differentiation and adrenocortical tumorigenesis. *Sf1* null mouse embryos exhibit aberrant adrenal and gonadal development (71), and haploinsufficiency for this factor disrupts the organization, growth, and function of the adrenal gland (36, 55). Transgenic expression of SF1 in fetal adrenal progenitor cells of mice leads to ectopic adrenal formation (72), and increased *Sf1* dosage in mice leads to subcapsular adrenocortical tumors that express GATA4 and other gonadal markers (73, 74). GATA4 and SF1 have been shown to cooperate in the expression of gonadal genes (14, 75), implying that synergistic interactions between these two transcription factors might contribute to gonadectomy-induced adrenocortical neoplasia. Arguing against a possible genetic interaction, combined haploinsufficiency of *Sf1* and *Gata4* does not cause abnormalities in mouse gonadal development beyond that of *Sf1* haploinsufficiency alone (76).

Linkage analysis of crosses between susceptible DBA/2J and nonsusceptible C57Bl/6 mice has shown that postgonadectomy tumorigenesis is a complex trait and that a major locus for tumorigenesis resides on chromosome 8 (11). One of the candidate genes in the linkage region is secreted frizzled related protein 1 (*Sfrp1*), a tumor suppressor that inhibits Wnt/ β -catenin signaling. Although *Sfrp1* is an attractive candidate gene for tumorigenesis, no causal mutations have been identified in the coding or noncoding regions of the gene in DBA/2J mice (11). This same genome-wide scan found no evidence for linkage to *Gata4* (chromosome 14) or *Sf1* (chromosome 2), implying that any polymorphisms between DBA/2J and C57Bl/6 in the *Gata4* and *Sf1* genes are not functionally significant. Our haploinsufficiency and conditional knockout models provide a complementary candidate gene approach to genetic linkage analyses.

Our findings have implications for gonadotropin-dependent adrenocortical tumors in other species. The phenomenon of postgonadectomy adrenocortical neoplasia is

not limited to mice; subcapsular sex steroidogenic tumors have been reported in the adrenal glands of gonadectomized ferrets, hamsters, cats, and goats (69, 77). GATA4, Lhcgr, inhibin- α , and other gonadal-like markers are hallmarks of postgonadectomy adrenocortical tumors in ferrets (7, 64, 78, 79). The human adrenal cortex constitutively expresses low levels of Lhcgr, and this receptor has been shown to be functionally active in the adrenal during pregnancy and other high gonadotropin states (9). Therefore, it has been proposed that adrenal responsiveness to LH, influenced by modifier genes, may contribute to adrenocortical tumorigenesis in humans (6, 9). Although rare, adrenocortical neoplasms with histological features resembling luteinized ovarian stroma (thecal metaplasia) have been reported in postmenopausal women (80, 81) and men with acquired testicular atrophy (82).

In addition to triggering adrenocortical tumorigenesis, ovariectomy elicits obesity in mice. Consequently, ovariectomized mice have been used to model the metabolic consequences of human menopause (53). In the mouse, postovariectomy weight gain is strain dependent (B6D2F1 > DBA/2J > C57Bl/6) (11), and ovariectomy-induced gains in visceral adiposity can be offset by estrogen repletion (53). Data presented here indicate that *Gata4* is a genetic modifier of not only adrenocortical tumorigenesis but also postovariectomy weight gain in B6D2F1 mice. We propose that *Gata4* haploinsufficiency exacerbates postovariectomy obesity in aged B6D2F1 mice by limiting adrenocortical estrogen production. Changes in adrenal glucocorticoid production can also impact obesity in mice, but studies suggest that postovariectomy adrenocortical neoplasia is not associated with alterations in the ACTH/glucocorticoid axis (7, 9, 10).

Although structurally and functionally distinct, the major steroid-producing organs, *i.e.* the adrenal cortex and gonads, arise from a common pool of progenitors in the adrenogonadal primordium (7, 9, 83–85). Despite extensive investigation, the factors that determine whether a steroidogenic cell precursor adopts an adrenocortical or gonadal phenotype are not fully understood. The phenotypic switch from production of corticoids to sex steroids makes postgonadectomy adrenocortical neoplasia an attractive model for the study of not only tumorigenesis but also steroidogenic lineage specification. Our results support the premise that GATA4 is a key regulator of a sex steroidogenic cell specification. We propose that GATA4, acting in concert with gonadotropins and other hormones, determines the functional identity of sex steroidogenic cells.

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References

1. Thompson GB, Young Jr WF 2003 Adrenal incidentaloma. *Curr Opin Oncol* 15:84–90
2. Bertherat J, Mosnier-Pudar H, Bertagna X 2002 Adrenal incidentalomas. *Curr Opin Oncol* 14:58–63
3. Barlaskar FM, Hammer GD 2007 The molecular genetics of adrenocortical carcinoma. *Rev Endocr Metab Disord* 8:343–348
4. Volante M, Buttigliero C, Greco E, Berruti A, Papotti M 2008 Pathological and molecular features of adrenocortical carcinoma: an update. *J Clin Pathol* 61:787–793
5. Cavlan D, Bharwani N, Grossman A 2010 Androgen- and estrogen-secreting adrenal cancers. *Semin Oncol* 37:638–648
6. Beuschlein F, Galac S, Wilson DB 2012 Animal models of adrenocortical tumorigenesis. *Mol Cell Endocrinol* 351:78–86
7. Bielinska M, Kiiveri S, Parviainen H, Mannisto S, Heikinheimo M, Wilson DB 2006 Gonadectomy-induced adrenocortical neoplasia in the domestic ferret (*Mustela putorius furo*) and laboratory mouse. *Vet Pathol* 43:97–117
8. Johnsen IK, Slawik M, Shapiro I, Hartmann MF, Wudy SA, Looyenga BD, Hammer GD, Reincke M, Beuschlein F 2006 Gonadectomy in mice of the inbred strain CE/J induces proliferation of subcapsular adrenal cells expressing gonadal marker genes. *J Endocrinol* 190:47–57
9. Bernichtein S, Alevizaki M, Huhtaniemi I 2008 Is the adrenal cortex a target for gonadotropins? *Trends Endocrinol Metab* 19:231–238
10. Bernichtein S, Peltoketo H, Huhtaniemi I 2009 Adrenal hyperplasia and tumours in mice in connection with aberrant pituitary-gonadal function. *Mol Cell Endocrinol* 300:164–168
11. Bernichtein S, Petretto E, Jamieson S, Goel A, Aitman TJ, Mangion JM, Huhtaniemi IT 2008 Adrenal gland tumorigenesis after gonadectomy in mice is a complex genetic trait driven by epistatic loci. *Endocrinology* 149:651–661
12. Bielinska M, Genova E, Boime I, Parviainen H, Kiiveri S, Lepälüoto J, Rahman N, Heikinheimo M, Wilson DB 2005 Gonadotropin-induced adrenocortical neoplasia in NU/J nude mice. *Endocrinology* 146:3975–3984
13. Bielinska M, Parviainen H, Porter-Tinge SB, Kiiveri S, Genova E, Rahman N, Huhtaniemi IT, Muglia LJ, Heikinheimo M, Wilson DB 2003 Mouse strain susceptibility to gonadectomy-induced adrenocortical tumor formation correlates with the expression of GATA-4 and luteinizing hormone receptor. *Endocrinology* 144:4123–4133
14. Viger RS, Guittot SM, Anttonen M, Wilson DB, Heikinheimo M 2008 Role of the GATA family of transcription factors in endocrine development, function, and disease. *Mol Endocrinol* 22:781–798
15. Manuylov NL, Fujiwara Y, Adameyko II, Poulat F, Tevosian SG 2007 The regulation of Sox9 gene expression by the GATA4/FOG2

- transcriptional complex in dominant XX sex reversal mouse models. *Dev Biol* 307:356–367
16. Manuylov NL, Smagulova FO, Leach L, Tevosian SG 2008 Ovarian development in mice requires the GATA4-FOG2 transcription complex. *Development* 135:3731–3743
 17. Tevosian SG, Albrecht KH, Crispino JD, Fujiwara Y, Eicher EM, Orkin SH 2002 Gonadal differentiation, sex determination and normal Sry expression in mice require direct interaction between transcription partners GATA4 and FOG2. *Development* 129:4627–4634
 18. Bielinska M, Seehra A, Toppari J, Heikinheimo M, Wilson DB 2007 GATA-4 is required for sex steroidogenic cell development in the fetal mouse. *Dev Dyn* 236:203–213
 19. Bouma GJ, Washburn LL, Albrecht KH, Eicher EM 2007 Correct dosage of Fog2 and Gata4 transcription factors is critical for fetal testis development in mice. *Proc Natl Acad Sci USA* 104:14994–14999
 20. Kyrönlahti A, Euler R, Bielinska M, Schoeller EL, Moley KH, Toppari J, Heikinheimo M, Wilson DB 2011 GATA4 regulates Sertoli cell function and fertility in adult male mice. *Mol Cell Endocrinol* 333:85–95
 21. Kyrönlahti A, Vetter M, Euler R, Bielinska M, Jay PY, Anttonen M, Heikinheimo M, Wilson DB 2011 GATA4 deficiency impairs ovarian function in adult mice. *Biol Reprod* 84:1033–1044
 22. Manuylov NL, Zhou B, Ma Q, Fox SC, Pu WT, Tevosian SG 2011 Conditional ablation of Gata4 and Fog2 genes in mice reveals their distinct roles in mammalian sexual differentiation. *Dev Biol* 353:229–241
 23. Parviainen H, Kiiveri S, Bielinska M, Rahman N, Huhtaniemi IT, Wilson DB, Heikinheimo M 2007 GATA transcription factors in adrenal development and tumors. *Mol Cell Endocrinol* 265–266:17–22
 24. Molkenin JD, Lin Q, Duncan SA, Olson EN 1997 Requirement of the transcription factor GATA4 for heart tube formation and ventral morphogenesis. *Genes Dev* 11:1061–1072
 25. Kuo CT, Morrisey EE, Anadappa R, Sigrist K, Lu MM, Parmacek MS, Soudais C, Leiden JM 1997 GATA4 transcription factor is required for ventral morphogenesis and heart tube formation. *Genes Dev* 11:1048–1060
 26. Narita N, Bielinska M, Wilson D 1997 Cardiomyocyte differentiation by GATA-4-deficient embryonic stem cells. *Development* 122:3755–3764
 27. Narita N, Bielinska M, Wilson DB 1997 Wild type endoderm abrogates the ventral developmental defects associated with GATA-4 deficiency in the mouse. *Dev Biol* 189:270–274
 28. Watt AJ, Battle MA, Li J, Duncan SA 2004 GATA4 is essential for formation of the proepicardium and regulates cardiogenesis. *Proc Natl Acad Sci USA* 101:12573–12578
 29. Pu WT, Ishiwata T, Juraszek AL, Ma Q, Izumo S 2004 GATA4 is a dosage-sensitive regulator of cardiac morphogenesis. *Dev Biol* 275:235–244
 30. Zeisberg EM, Ma Q, Juraszek AL, Moses K, Schwartz RJ, Izumo S, Pu WT 2005 Morphogenesis of the right ventricle requires myocardial expression of Gata4. *J Clin Invest* 115:1522–1531
 31. Oka T, Maillot M, Watt AJ, Schwartz RJ, Aronow BJ, Duncan SA, Molkenin JD 2006 Cardiac-specific deletion of Gata4 reveals its requirement for hypertrophy, compensation, and myocyte viability. *Circ Res* 98:837–845
 32. Jamin SP, Arango NA, Mishina Y, Hanks MC, Behringer RR 2002 Requirement of Bmpr1a for Müllerian duct regression during male sexual development. *Nat Genet* 32:408–410
 33. Jayasuria P, Ikeda Y, Jamin SP, Zhao L, De Rooij DG, Themmen AP, Behringer RR, Parker KL 2004 Cell-specific knockout of steroidogenic factor 1 reveals its essential roles in gonadal function. *Mol Endocrinol* 18:1610–1619
 34. Soriano P 1999 Generalized lacZ expression with the ROSA26 Cre reporter strain. *Nat Genet* 21:70–71
 35. Alexander BM, Van Kirk EA, Naughton LM, Murdoch WJ 2007 Ovarian morphometrics in TP53-deficient mice. *Anat Rec (Hoboken)* 290:59–64
 36. Beuschlein F, Mutch C, Bavers DL, Ulrich-Lai YM, Engeland WC, Keegan C, Hammer GD 2002 Steroidogenic factor-1 is essential for compensatory adrenal growth following unilateral adrenalectomy. *Endocrinology* 143:3122–3135
 37. Anttonen M, Ketola I, Parviainen H, Pusa AK, Heikinheimo M 2003 FOG-2 and GATA-4 are coexpressed in the mouse ovary and can modulate Müllerian-inhibiting substance expression. *Biol Reprod* 68:1333–1340
 38. Fahle M, Palm G 1983 Calculation of surface areas from serial sections. *J Neurosci Methods* 9:75–85
 39. Woolley GW 1949 The adrenal cortex and its tumors. *Ann NY Acad Sci* 50:616–626
 40. Jay PY, Bielinska M, Erlich JM, Mannisto S, Pu WT, Heikinheimo M, Wilson DB 2007 Impaired mesenchymal cell function in Gata4 mutant mice leads to diaphragmatic hernias and primary lung defects. *Dev Biol* 301:602–614
 41. Pangas SA, Jorgez CJ, Matzuk MM 2004 Growth differentiation factor 9 regulates expression of the bone morphogenetic protein antagonist gremlin. *J Biol Chem* 279:32281–32286
 42. Ward JM, Anver MR, Mahler JF, Devor-Henneman DE, Maronpot RR, Sundberg JP, Frederickson RM 2002 Pathology of mice commonly used in genetic engineering (C57BL/6; B6,129; and FVB/n). In: *Pathology of genetically engineered mice*. Ames, IA: Iowa State University Press; 161–179
 43. Frith CH, Dunn TB 1994 Tumours of the adrenal gland. *IARC Sci Publ* 111:595–609
 44. Charles MA, Saunders TL, Wood WM, Owens K, Parlow AF, Camper SA, Ridgway EC, Gordon DF 2006 Pituitary-specific Gata2 knockout: effects on gonadotrope and thyrotrope function. *Mol Endocrinol* 20:1366–1377
 45. Lo A, Zheng W, Gong Y, Crochet JR, Halvorson L 2011 GATA transcription factors regulate luteinizing hormone β (LH β) gene expression. *J Mol Endocrinol* 47:45–58
 46. Kim JS, Kubota H, Kiuchi Y, Doi K, Saegusa J 1997 Subcapsular cell hyperplasia and mast cell infiltration in the adrenal cortex of mice: comparative study in 7 inbred strains. *Exp Anim* 46:303–306
 47. Teng CT 2002 Lactoferrin gene expression and regulation: an overview. *Biochem Cell Biol* 80:7–16
 48. Rossi JA, Bruschi LC 1989 Alteration of the submandibular glands of female mice during pregnancy and lactation. A histological, histometric and histochemical study. *Acta Morphol Hung* 37:135–145
 49. Freeman ME, Kanyicska B, Lerant A, Nagy G 2000 Prolactin: structure, function, and regulation of secretion. *Physiol Rev* 80:1523–1631
 50. Rulli SB, Kuorelahti A, Karaer O, Pelliniemi LJ, Poutanen M, Huhtaniemi I 2002 Reproductive disturbances, pituitary lactotrope adenomas, and mammary gland tumors in transgenic female mice producing high levels of human chorionic gonadotropin. *Endocrinology* 143:4084–4095
 51. Kero J, Poutanen M, Zhang FP, Rahman N, McNicol AM, Nilson JH, Keri RA, Huhtaniemi IT 2000 Elevated luteinizing hormone induces expression of its receptor and promotes steroidogenesis in the adrenal cortex. *J Clin Invest* 105:633–641
 52. Kiss R, de Launoit Y, L'Hermite-Balériaux M, L'Hermite M, Paridaens RJ, Danguy AJ, Pasteels JL 1987 Effect of prolactin and estradiol on cell proliferation in the uterus and the MXT mouse mammary neoplasm. *J Natl Cancer Inst* 78:993–998
 53. Rogers NH, Perfield II JW, Strissel KJ, Obin MS, Greenberg AS 2009 Reduced energy expenditure and increased inflammation are early events in the development of ovariectomy-induced obesity. *Endocrinology* 150:2161–2168
 54. Kiiveri S, Liu J, Westerholm-Ormio M, Narita N, Wilson DB, Vuottilainen R, Heikinheimo M 2002 Differential expression of GATA-4

- and GATA-6 in fetal and adult mouse and human adrenal tissue. *Endocrinology* 143:3136–3143
55. Bland ML, Jamieson CA, Akana SF, Bornstein SR, Eisenhofer G, Dallman MF, Ingraham HA 2000 Haploinsufficiency of steroidogenic factor-1 in mice disrupts adrenal development leading to an impaired stress response. *Proc Natl Acad Sci USA* 97:14488–14493
 56. Xu Q, Lin HY, Yeh SD, Yu IC, Wang RS, Chen YT, Zhang C, Altuwajri S, Chen LM, Chuang KH, Chiang HS, Yeh S, Chang C 2007 Infertility with defective spermatogenesis and steroidogenesis in male mice lacking androgen receptor in Leydig cells. *Endocrine* 32:96–106
 57. Pangas SA, Li X, Umans L, Zwijsen A, Huylebroeck D, Gutierrez C, Wang D, Martin JF, Jamin SP, Behringer RR, Robertson EJ, Matzuk MM 2008 Conditional deletion of Smad1 and Smad5 in somatic cells of male and female gonads leads to metastatic tumor development in mice. *Mol Cell Biol* 28:248–257
 58. Boyer A, Hermo L, Paquet M, Robaire B, Boerboom D 2008 Seminiferous tubule degeneration and infertility in mice with sustained activation of WNT/CTNNB1 signaling in Sertoli cells. *Biol Reprod* 79:475–485
 59. Archambeault DR, Yao HH 2010 Activin A, a product of fetal Leydig cells, is a unique paracrine regulator of Sertoli cell proliferation and fetal testis cord expansion. *Proc Natl Acad Sci USA* 107:10526–10531
 60. Tanwar PS, Kaneko-Tarui T, Zhang L, Rani P, Taketo MM, Teixeira J 2010 Constitutive WNT/ β -catenin signaling in murine Sertoli cells disrupts their differentiation and ability to support spermatogenesis. *Biol Reprod* 82:422–432
 61. Ren Y, Cowan RG, Harman RM, Quirk SM 2009 Dominant activation of the hedgehog signaling pathway in the ovary alters theca development and prevents ovulation. *Mol Endocrinol* 23:711–723
 62. Sharawy MM, Liebelt AG, Dirksen TR, Penney DP 1980 Fine structural study of postcastrational adrenocortical carcinomas in female CE-mice. *Anat Rec* 198:125–133
 63. Hernandez Gifford JA, Hunzicker-Dunn ME, Nilson JH 2009 Conditional deletion of β -catenin mediated by Amhr2cre in mice causes female infertility. *Biol Reprod* 80:1282–1292
 64. Schoemaker NJ, Teerds KJ, Mol JA, Lumeij JT, Thijssen JH, Rijnberk A 2002 The role of luteinizing hormone in the pathogenesis of hyperadrenocorticism in neutered ferrets. *Mol Cell Endocrinol* 197:117–125
 65. Tevosian SG, Manuylov NL 2008 To β or not to β : Canonical β -catenin signaling pathway and ovarian development. *Dev Dyn* 237:3672–3680
 66. LaVoie HA, Singh D, Hui YY 2004 Concerted regulation of the porcine steroidogenic acute regulatory protein gene promoter activity by follicle-stimulating hormone and insulin-like growth factor I in granulosa cells involves GATA-4 and CCAAT/enhancer binding protein β . *Endocrinology* 145:3122–3134
 67. Kwintkiewicz J, Cai Z, Stocco C 2007 Follicle-stimulating hormone-induced activation of Gata4 contributes in the up-regulation of Cyp19 expression in rat granulosa cells. *Mol Endocrinol* 21:933–947
 68. Rahman NA, Kiiveri S, Rivero-Müller A, Levallet J, Vierre S, Kero J, Wilson DB, Heikinheimo M, Huhtaniemi I 2004 Adrenocortical tumorigenesis in transgenic mice expressing the inhibin α -subunit promoter/SV40 virus T-antigen transgene: relationship between ectopic expression of luteinizing hormone receptor and transcription factor GATA-4. *Mol Endocrinol* 18:2553–2569
 69. Bielinska M, Parviainen H, Kiiveri S, Heikinheimo M, Wilson DB 2009 Origin and molecular pathology of adrenocortical neoplasms. *Vet Pathol* 46:194–210
 70. Berthon A, Sahut-Barnola I, Lambert-Langlais S, de Jousineau C, Damon-Soubeyrand C, Louiset E, Taketo MM, Tissier F, Bertherat J, Lefrançois-Martinez AM, Martinez A, Val P 2010 Constitutive β -catenin activation induces adrenal hyperplasia and promotes adrenal cancer development. *Hum Mol Genet* 19:1561–1576
 71. Zhao L, Bakke M, Hanley NA, Majdic G, Stallings NR, Jayasuria P, Parker KL 2004 Tissue-specific knockouts of steroidogenic factor 1. *Mol Cell Endocrinol* 215:89–94
 72. Zubair M, Oka S, Parker KL, Morohashi K 2009 Transgenic expression of Ad4BP/SF-1 in fetal adrenal progenitor cells leads to ectopic adrenal formation. *Mol Endocrinol* 23:1657–1667
 73. Doghman M, Cazareth J, Lalli E 2008 The Tcf/ β -catenin antagonist PKF115–584 inhibits proliferation of adrenocortical carcinoma cells. *J Clin Endocrinol Metab* 93:3222–3225
 74. Doghman M, Karpova T, Rodrigues GA, Arhatte M, De Moura J, Cavalli LR, Virolle V, Barbry P, Zambetti GP, Figueiredo BC, Heckert LL, Lalli E 2007 Increased steroidogenic factor-1 dosage triggers adrenocortical cell proliferation and cancer. *Mol Endocrinol* 21:2968–2987
 75. Tremblay JJ, Viger RS 2003 A mutated form of steroidogenic factor 1 (SF-1 G35E) that causes sex reversal in humans fails to synergize with transcription factor GATA-4. *J Biol Chem* 278:42637–42642
 76. Pelusi C, Zhao L, Stallings NR, Parker KL 2007 Combined haploinsufficiency of SF-1 and GATA4 does not reveal a genetic interaction in mouse gonadal development. *Sex Dev* 1:152–160
 77. Meler EN, Scott-Moncrieff JC, Peter AT, Bennett S, Ramos-Vara J, Salisbury SK, Naughton JF 2011 Cyclic estrous-like behavior in a spayed cat associated with excessive sex-hormone production by an adrenocortical carcinoma. *J Feline Med Surg* 13:473–478
 78. Wagner S, Kiupel M, Peterson II RA, Heikinheimo M, Wilson DB 2008 Cytochrome b5 expression in gonadectomy-induced adrenocortical neoplasms of the domestic ferret (*Mustela putorius furo*). *Vet Pathol* 45:439–442
 79. Peterson II RA, Kiupel M, Bielinska M, Kiiveri S, Heikinheimo M, Capen CC, Wilson DB 2004 Transcription factor GATA-4 is a marker of anaplasia in adrenocortical neoplasms of the domestic ferret (*Mustela putorius furo*). *Vet Pathol* 41:446–449
 80. Fidler WJ 1977 Ovarian thecal metaplasia in adrenal glands. *Am J Clin Pathol* 67:318–323
 81. Wont TW, Warner NE 1971 Ovarian thecal metaplasia in the adrenal gland. *Arch Pathol* 92:319–328
 82. Romberger CF, Wong TW 1989 Thecal metaplasia in the adrenal gland of a man with acquired bilateral testicular atrophy. *Arch Pathol Lab Med* 113:1071–1075
 83. Kim AC, Barlaskar FM, Heaton JH, Else T, Kelly VR, Krill KT, Scheys JO, Simon DP, Trovato A, Yang WH, Hammer GD 2009 In search of adrenocortical stem and progenitor cells. *Endocr Rev* 30:241–263
 84. Zubair M, Parker KL, Morohashi K 2008 Developmental links between the fetal and adult zones of the adrenal cortex revealed by lineage tracing. *Mol Cell Biol* 28:7030–7040
 85. Val P, Jeays-Ward K, Swain A 2006 Identification of a novel population of adrenal-like cells in the mammalian testis. *Dev Biol* 299:250–256