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Mouse Strain Susceptibility to Gonadectomy-Induced Adrenocortical Tumor Formation Correlates with the Expression of GATA-4 and Luteinizing Hormone Receptor

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Certain inbred strains of mice, including DBA/2J, develop adrenocortical tumors in response to gonadectomy. Spindle-shaped cells with limited steroidogenic capacity, termed A cells, appear in the subcapsular region of the adrenal gland, followed by sex steroid-producing cells known as B cells. These changes result from unopposed gonadotropin production by the pituitary, but the adrenocortical factors involved in tumorigenesis have not been characterized. GATA-4, a transcription factor normally expressed in fetal, but not adult, adrenocortical cells, was found in neoplastic cells that proliferate in the adrenal cortex of gonadectomized DBA/2J mice. GATA-4 mRNA was detected in the adrenal glands of female mice 0.5 months after ovariectomy and reached a maximum by 4 months. Castrated male mice developed adrenocortical tumors more slowly than gonadectomized females, and the onset of GATA-4 expression in the adrenal was delayed. *In situ* hybridization and immunohistochemistry revealed GATA-4 mRNA and protein in A and B cells, but not in normal adrenocortical cells. mRNA encoding another factor associated with adrenocortical tumorigenesis, LH receptor (LHR), was detected in A and B cells. In addition, transcripts for P450 17α-hydroxylase/C17-C20 lyase, an enzyme essential for the production of sex steroids, and inhibin-α were found in B cells. Unilateral ovarian regeneration, a phenomenon known to occur in gonadectomized mice, was observed in a subset of DBA/2J mice undergoing complete ovariectomy. In these animals, adrenocortical tumor progression was arrested; A cells and GATA-4 expression were evident, but there was no expression of LHR or P450 17α-hydroxylase/C17-C20 lyase. Strain susceptibility to adrenocortical tumorigenesis (DBA/2J $\gg$ FVB/N) correlated with the expression of GATA-4 and LHR, implicating these factors in the process of adrenocortical neoplasia in response to continuous gonadotropin stimulation. (*Endocrinology* 144: 4123–4133, 2003)

THE ADRENAL cortex is a major source of steroid hormones, which are synthesized from cholesterol through the sequential actions of a series of cytochrome P450 enzymes (1). The adrenal cortex of adult mice is divided into morphologically and functionally distinct layers or zones (2). Mineralocorticoids are synthesized in the outermost layer, the zona glomerulosa (zg), whereas glucocorticoids are produced in the zona fasiculata (zf). Murine adrenal cortex also contains a so-called X-zone, which develops after birth adjacent to the medulla and disappears at puberty in males and after the first pregnancy in females (2). In addition to this zonal organization, the adrenal cortex is arranged in radial cords of clonally related cells that extend from the zg to the zf (3). The adult adrenal cortex undergoes continual renewal; stem cells near the junction of the zg and zf give rise to daughter cells, which are displaced centripetally, where they undergo apoptosis (3, 4).

Abbreviations: E12.5, Embryonic d 12.5; LHR, LH receptor; P450c17, P450 17α-hydroxylase/C17-C20 lyase; P450scc, cytochrome P450 side-chain cleavage enzyme; RNase, ribonuclease; SF-1, steroidogenic factor-1; StAR, steroidogenic acute regulatory protein; SV40, simian virus 40; zf, zona fasiculata; zg, zona glomerulosa.

Certain inbred strains of mice develop adrenocortical tumors in response to gonadectomy (5–10). This process is strain dependent; adrenocortical adenomas form in DBA/2J and C3H mice, adrenocortical carcinomas develop in CE mice, whereas no adrenal tumors develop in C57BL/6J mice (5–10). When DBA/2J mice are gonadectomized, spindle-shaped cells, known as A cells, proliferate in the subcapsular region of the adrenal cortex (5–7). The function of these cells, which have limited steroidogenic capacity (9, 11, 12), is unknown. Foci of sex steroid producing cells, termed B cells, appear later within patches of A cells (13). This process is thought to represent metaplasia of competent cells in the adrenal cortex, which under the influence of unopposed pituitary gonadotropins transform into tissue resembling gonadal stroma (2, 10). Hypophysectomy, parabiosis, and adrenal transplantation experiments have confirmed that the adrenal glands of DBA/2J, C3H, CE, and other susceptible strains exhibit an inherent predisposition to tumor formation in response to gonadotropin stimulation (6–15). However, the molecular mechanisms underlying this process have not been elucidated.

Two members of the GATA transcription factor family,

GATA-4 and GATA-6, have been implicated in the regulation of gene expression and differentiation in steroidogenic cell types, including Sertoli, Leydig, granulosa, and theca-interstitial cells (16–22). High level GATA-4 expression accompanies periods of active cell proliferation in Sertoli and granulosa cells (16–18), and gonadotropin stimulation of immature ovaries results in increased levels of GATA-4 mRNA (16). In mice and humans, GATA-4 is expressed transiently in adrenocortical cells during fetal development, whereas GATA-6 is expressed in both fetal and adult adrenal cortex (23). These differing expression profiles suggest that GATA-4 and GATA-6 have distinct roles in adrenocortical cell differentiation and function. Recent studies have shown that increased expression of GATA-4 accompanies adrenocortical tumorigenesis in humans and in gonadectomized inhibin-α promoter simian virus 40 (SV40) T antigen transgenic mice (24, 25). In the adrenocortical tumors that develop in these transgenic mice, there is a concomitant increase in the expression of functional LH receptors (LHR), and the elevation in serum LH that accompanies gonadectomy is a prerequisite for tumor formation (26).

Here we show that GATA-4, a transcription factor normally expressed in somatic/interstitial cells of the mouse ovary and testes, but not in adult adrenal, defines a population of cells that proliferate in the adrenal cortex of DBA/2J mice following gonadectomy. In addition to GATA-4, some of these neoplastic adrenocortical cells express transcripts for LHR and P450 17α-hydroxylase/C17-C20 lyase (P450c17), an enzyme essential for the production of sex steroids, suggesting that these cells can respond directly to gonadotropin signals and synthesize sex steroids. These findings implicate GATA-4 and LHR in the process of gonadotropin-induced metaplasia in DBA/2J mice.

Materials and Methods

Experimental animals and treatments

All animal work was carried out in accordance with NIH Guidelines for the Care and Use of Laboratory Animals. Weanling DBA/2J or C57BL/6J mice were purchased from The Jackson Laboratory (Bar Harbor, ME), and weanling FVB/N mice were obtained from the Department of Pediatrics, CHRC Genetically Altered Mouse Core. The mice were specific pathogen free and were housed five per cage in controlled conditions of light (12 h of light, 12 h of darkness) and temperature (21 C). They were fed commercial mouse chow (Ralston Purina, St. Louis, MO) and tap water *ad libitum*. Mice were gonadectomized at 3–4 wk of age (27). Avertin anesthesia was used during surgical procedures, and buprenorphine was used for postoperative analgesia (27). At specified times the mice were killed by CO_2 inhalation. Blood samples were obtained at the time of death. At autopsy, adrenal glands were dissected, and adnexal regions were examined for evidence of ovarian regeneration (28). Tissues were frozen in OCT cryopreservation media (Tissue-Tek, Miles, Inc., Elkhart, IN), and then sectioned (10 μm). Cryosections were fixed in 4% paraformaldehyde in PBS before *in situ* hybridization or immunohistochemistry. Alternatively, harvested tissue was fixed overnight in 4% paraformaldehyde in PBS, embedded in paraffin, and then sectioned (4 μm) for immunohistochemistry or *in situ* hybridization. In some instances, harvested adrenal tissue was used to isolate total RNA (TRIzol reagent, Invitrogen, Carlsbad, CA).

Hormone measurements

Serum LH concentrations were measured using an immunofluorometric assay (Wallac Oy, Turku, Finland), as described previously (29).

Immunohistochemistry

Paraffin-embedded, paraformaldehyde-fixed tissue sections were deparaffinized, hydrated, and treated with 10 mM citric acid, pH 6, in a microwave oven for 10–30 min to facilitate antigen capture. Endogenous peroxidase was quenched with a methanol solution containing 0.5% H_2O_2. After incubation with blocking buffer (1.5% normal serum and 0.1% Tween-20 in PBS), sections were stained with the following primary antibodies: 1) polyclonal goat antimouse GATA-4 IgG (sc-1237, Santa Cruz Biotechnology, Inc., Santa Cruz, CA), or 2) rabbit antimouse GATA-6 IgG (sc-9055, Santa Cruz Biotechnology, Inc.). The sections were exposed to the antibody at dilutions of 1:200 (GATA-4) or 1:500 (GATA-6) for 1 h at 37 C. The avidin-biotin immunoperoxidase system (Vectastain Elite ABC Kit, Vector Laboratories, Inc., Burlingame, CA) and diaminobenzidine (Sigma-Aldrich Corp., St. Louis, MO) were used to visualize the bound antibody; slides were then counterstained with 100% hematoxylin.

Preparation of riboprobe templates for cytochrome P450 side-chain cleavage enzyme (P450scc) and P450c17

Partial-length cDNAs for mouse P450scc and P450c17 were generated via RT-PCR of mRNA isolated from the Leydig cell tumor line, mLTC-1 (30). Previously described primers (31) were used to generate a 536-bp PCR product spanning nucleotides 110–455 of mouse P450scc. A forward primer of 5′-CCAGATGGTGACTCTAGGCCTCTTGTC and a reverse primer of 5′-GGTCTATGGTAGTCAGTATGC were used to generate a 306-bp PCR product spanning nucleotides 331–631of mouse P450c17 (32). PCR reaction mixtures, which included *Taq* polymerase, were processed for 30 cycles, each consisting of 94 C for 1 min, 55 C for 1 min, and 72 C for 3 min. The resultant PCR products were ligated into the T-A cloning site of pCRII (Invitrogen, Carlsbad, CA).

Ribonuclease (RNase) protection assays

These assays were performed with a commercially available kit (Ambion, Austin, TX) and 5 μg total RNA. ^{32}P-Labeled antisense riboprobes were prepared by *in vitro* transcription in the presence of [^{32}P]CTP (800 Ci/mmol; ICN Biomedicals, Irvine, CA) using the following linearized plasmid templates and RNA polymerases: 1) GATA-4, *Not*I-digested pBluescript SK λG14A, T7 polymerase (33); 2) LHR, *Bgl*II-digested pGEM plasmid, T7 polymerase (34); 3) P450c17, *Eco*RV-digested pCRII plasmid described above, Sp6 polymerase; and 4) GATA-6, *Pst*I-digested pCRII plasmid, Sp6 polymerase (33). Antisense β-actin probe was prepared from template provided by the manufacturer. The sizes of the full-length and protected RNA probes (in nucleotides) were as follows: GATA-4, 491 and 430 bp; P450c17, 324 and 306 bp; GATA-6, 600 and 140 bp; β-actin, 300 and 250 bp; LHR, 450 bp; and multiple protected fragments of varying sizes, due to alternative splicing of this transcript (35, 36) (see Fig. 5). Protected fragments were separated by PAGE and quantified using phosphorimage analysis, normalizing to the expression of β-actin.

In situ hybridization

Frozen or paraffin-embedded tissue sections were subjected to *in situ* hybridization as previously described (33). Sections were incubated in a final volume of 80 μl with 10^6 cpm ^{33}P-labeled antisense riboprobe prepared using [^{33}P]UTP (3000 Ci/mmol; ICN Biomedicals) and the following linearized plasmid templates and RNA polymerases: 1) GATA-4, *Bam*HI-digested pBluescript SK λG14A, T7 polymerase (33); 2) GATA-6, *Eco*RV-digested pCRII plasmid, Sp6 polymerase (33); 3) 11β-hydroxylase, *Eco*RI-digested pBluescript plasmid, T3 polymerase (37); 4) P450 aldosterone synthase, *Eco*RI-digested pBluescript plasmid, T3 polymerase (37); 5) P450scc, *Hin*dIII-digested pCRII plasmid described above, T7 polymerase; 6) inhibin-α, *Eco*RI-digested pGEM-4Z plasmid, T7 polymerase (38); 7) steroidogenic acute regulatory protein (StAR), *Sal*I-digested pSport plasmid, SP6 polymerase (39); 8) P450c17, *Eco*RV-digested pCRII plasmid described above, Sp6 polymerase; 9) LHR, *Bgl*II-digested pGEM plasmid, T7 polymerase (34); and 10) steroidogenic factor-1 (SF-1), *Hin*dIII-digested plasmid, T7 polymerase (40).

Results

Morphological changes in the adrenal glands of gonadectomized DBA/2J mice

DBA/2J mice that undergo prepubertal gonadectomy develop nodular adenomas in the adrenal cortex by 6 months of age (5–7, 9, 12). We gonadectomized weanling female DBA/2J (a susceptible strain) or FVB/N mice (a nonsusceptible strain) and then examined adrenal glands 0.5–8 months later. Consistent with published reports (5–9), small, densely arranged A cells began to accumulate beneath the adrenal capsule of DBA/2J mice within weeks of gonadectomy (Fig. 1A). These subcapsular cells were polyhedral and had deeply staining nuclei with slightly basophilic cytoplasm. With time, the A cells extended between the epithelial cell columns of the zf and became spindle-shaped, resembling ovarian stroma (Fig. 1A). As these cells increased in number they disrupted the arrangement of the zg and zf, resulting in the formation of wedge-shaped areas. A few months after gonadectomy, nests of large, lipid-laden B cells were evident in these areas (Fig. 1B). Although small foci of A cells could be seen in the subcapsular region of older nongonadectomized DBA/2J mice, these cells did not invade deeply into the cortex, and no B cells were observed. Tumors are uncommon in the adrenal glands of intact FVB/N mice (41), and gonadectomy did not induce adrenocortical tumors in this strain (Fig. 1, C and D). Rare, small patches of A cells were observed in older FVB/N mice (>6 months of age), but these cells were not invasive, and no B cells were seen in these animals (data not shown).

To ensure that these strain differences in tumor development were not due to differences in gonadotropin levels, we measured serum LH levels in DBA/2J and FVB/N mice before and after ovariectomy (Table 1). Baseline and gonadectomy-induced changes in LH levels were comparable in the two strains.

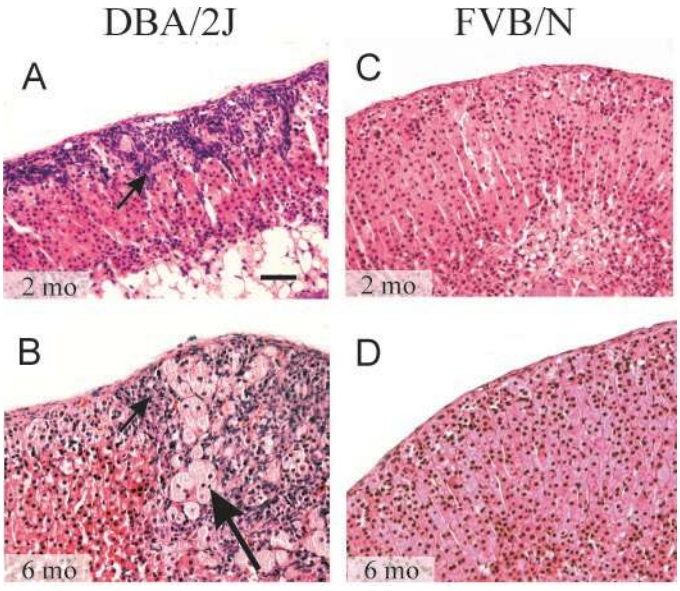

FIG. 1. Morphological changes in the adrenal glands of gonadectomized DBA/2J and FVB/N mice. Weanling female DBA/2J (A and B) or FVB/N (C and D) mice were subjected to gonadectomy, and adrenal glands were harvested 2 months (A and C), or 6 months (B and D) later. Tissues were fixed in paraformaldehyde, paraffin-embedded, sectioned, and stained with hematoxylin-eosin. Note that spindle-shaped A cells (*small arrows*) and lipid-laden B cells (*large arrow*) accumulate in the subcapsular region of adrenal cortex of DBA/2J, but not FVB/N, mice. *Bar*, 50 μm.

GATA-4 protein is expressed in neoplastic cells that accumulate in the adrenal cortex of gonadectomized female DBA/2J mice

Based on previous studies with inhibin-α promoter SV40 T antigen transgenic mice, which develop GATA4-expressing adrenocortical carcinomas after gonadectomy (24), we hypothesized that changes in GATA factor expression might accompany adrenal tumor formation in gonadectomized DBA/2J mice. To assess the impact of gonadectomy on adrenocortical expression of GATA-4 protein, we performed immunoperoxidase staining on intact female DBA/2J mice or littermates that had been subjected to gonadectomy 0.5–1 months earlier. In nongonadectomized DBA/2J mice, small foci of GATA-4-positive cells were seen in the subcapsular region (Fig. 2, A and C). These cells, which were histologically identical to A cells, did not invade deeply into the adrenal cortex. In contrast, large patches of GATA-4-positive A cells were evident in gonadectomized animals (Fig. 2, B and D), and these cells penetrated deeply into the zf within 1 month of ovariectomy (Figs. 2D and 3A). Within A cells, GATA-4 antigen was confined to the nucleus (Fig. 3A). Nests of B cells expressing GATA-4 antigen in their nuclei began to appear 1 month after gonadectomy (Fig. 3B). No GATA-4-positive cells were evident in the adrenal glands of intact or gonadectomized FVB/N mice (Fig. 2, E and F).

Next we used immunohistochemistry to compare the expressions of GATA-4 and GATA-6 in the adrenal glands of DBA/2J females that had been gonadectomized 4 months earlier. While no GATA-4 antigen was evident in normal adrenocortical cells, there was intense GATA-4 immunostaining in the nuclei of both A and B cells in the subcapsular tumors of gonadectomized DBA/2J mice (Fig. 4, A and B). GATA-6 protein was detected in the nucleus of normal adrenocortical cells, but not in A cells of gonadectomized DBA/2J mice (Fig. 4C). Faint expression of GATA-6 protein was evident in B cells of ovariectomized mice (Fig. 4C). We conclude that GATA-4 is a marker of the neoplastic A and B cells that accumulate in the adrenal glands of female DBA/2J mice after gonadectomy, whereas GATA-6 expression is down-regulated in A cells.

TABLE 1. Serum LH measurements in female DBA/2J and FVB/N mice subjected to sham surgery or gonadectomy

	Serum LH (ng/ml)			
	Sham		Gdx	
	2 months	6 months	2 months	6 months
DBA/2J	0.55 ± 0.14	0.73 ± 0.21	3.2 ± 0.8	9.7 ± 1.8
FVB/N	0.53 ± 0.09	1.1 ± 0.3	3.5 ± 0.9	9.1 ± 0.7

Weanling DBA/2J or FVB/N mice were subjected to sham surgery or gonadectomy (Gdx). At the indicated times, serum LH levels were measured as described in *Materials and Methods*. Values represent the mean ± SEM of four to six measurements.

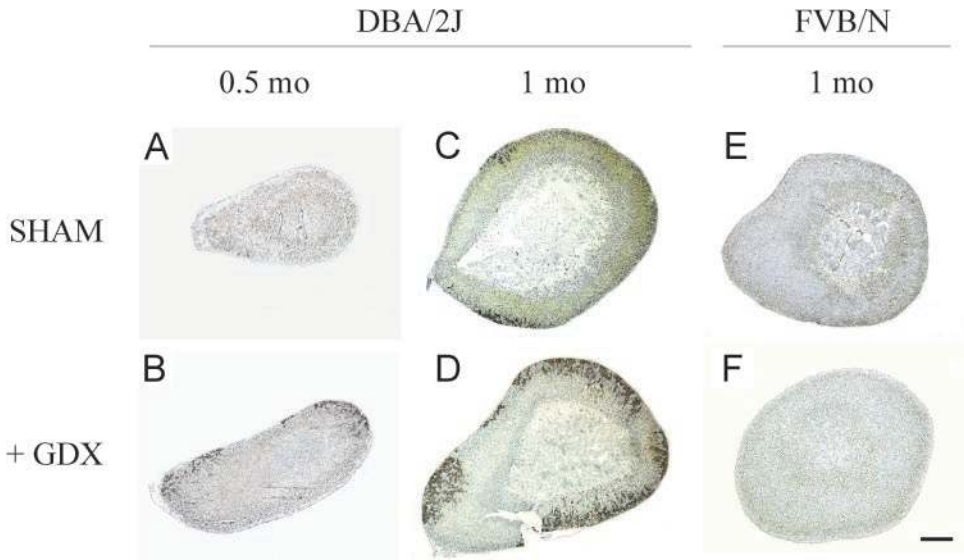

FIG. 2. Expression of GATA-4 protein in the adrenal cortex of intact *vs.* gonadectomized DBA/2J mice. Weanling female DBA/2J (A–D) or FVB/N (E and F) mice were subjected to sham surgery (A, C, and E) or gonadectomy (+ GDX; B, D, and F), and adrenal glands were harvested 0.5 months (A and B) or 1 month (C–F) later. The glands were fixed in paraformaldehyde, embedded in paraffin, sectioned, and subjected to immunoperoxidase staining with antibodies against GATA-4. Sections were counterstained with hematoxylin. Cells expressing GATA-4 antigen stain *dark brown*. In nongonadectomized DBA/2J mice, rare GATA-4-positive cells are evident in the subcapsular region (A and C), but these cells do not deeply invade the adrenal cortex. On the other hand, large patches of GATA-4 positive A cells are evident in gonadectomized DBA/2J mice (B and D), and these cells penetrate deeply into the zf. GATA-4-positive cells are not evident in intact or gonadectomized FVB/N mice. *Bar*, 100 μm.

Changes in GATA-4, LHR, and P450c17 mRNA levels in the adrenal cortex of DBA/2J mice after gonadectomy

To show correlation between GATA-4 expression and adrenocortical tumor development, we used RNase protection assays to measure GATA-4 mRNA in the adrenal glands of DBA/2J mice at various time points after gonadectomy (Fig. 5). In light of earlier reports demonstrating that GATA-4 and LHR are coexpressed in another mouse model of adrenocortical tumorigenesis (24), we also performed RNase protection assays for LHR mRNA. In agreement with published studies on other mouse strains (23, 24, 34), neither GATA-4 nor LHR mRNA was detected in the adrenal glands of intact, weanling, female DBA/2J mice (Fig. 5, *left panel*). There was only weak expression of GATA-4 and LHR mRNA in the adrenals of older intact adult DBA/2J females (Fig. 5, *left panel*), and neither of these transcripts was evident in older gonadectomized FVB/N females (Fig. 5, *right panel*). Compared with intact animals of the same age, gonadectomized female DBA/2J mice exhibited a marked increase (10- to 15-fold by phosphorimage analysis) in the steady state levels of GATA-4 and LHR mRNA within the adrenal gland (Fig. 5, *left panel*). These increases in the levels of GATA-4 and LHR mRNA were evident as early as 0.5 months after ovariectomy and reached a maximum by 4 months. In castrated male DBA/2J mice, more modest increases in GATA-4 and LHR mRNA levels were noted and only after a longer latency period (Fig. 5, *center panel*). This correlates with the observation that gonadectomized female DBA/2J mice develop adrenocortical tumors more readily than their male counterparts (9), which may be related to lower levels of serum LH that accompany gonadectomy in males *vs.* females (42). In contrast to GATA-4 and LHR, the level of GATA-6 mRNA remained relatively constant after gonadectomy, with the exception of a transient decrease in the female adrenal 1 month after gonadectomy (Fig. 6, *upper panel*).

P450c17, an enzyme required for the synthesis of sex steroids, is expressed in the fetal adrenal, but is normally absent from the adrenals of postnatal mice (43). Hence, the developmental expression pattern of P450c17 in the adrenal cortex coincides with that of GATA-4 (23). RNase protection assays demonstrated that adrenal expression of P450c17 mRNA increased dramatically after gonadectomy in female and male DBA/2J mice (Fig. 6, *upper panel*), similar to the pattern observed for GATA-4 and LHR transcripts. As expected, gonadectomized FVB/N mice did not exhibit an increase in P450c17 expression (Fig. 6, *lower panel*). We conclude that gonadectomy of female or male DBA/2J mice is accompanied by increased expression of GATA-4, LHR, and P450c17 within the adrenal gland, and that there is a tight temporal correlation between the expression of these transcripts and the appearance of neoplastic cells in the adrenal cortex. Moreover, strain susceptibility to adrenocortical tumorigenesis (DBA/2J $\gg$ FVB/N) correlates with expression of GATA-4, LHR, and P450c17 after gonadectomy.

Localization of transcripts for GATA-4, LHR, P450c17, and other steroidogenic markers in the adrenocortical tumors that develop in gonadectomized DBA/2J mice

In situ hybridization was used to localize the expression of GATA-4, LHR, and other key markers in the adrenal glands from intact and gonadectomized female DBA/2J mice (Fig. 7). A cells and B cells expressed abundant GATA-4 (Fig. 7, E and I) and LHR (Fig. 7, F and J) mRNA. In contrast, P450c17 showed a patchy expression that localized exclusively to B cells (Fig. 7, H and L), the presumed source of sex steroids in the tumors of gonadectomized mice. Inhibin-α is ex-

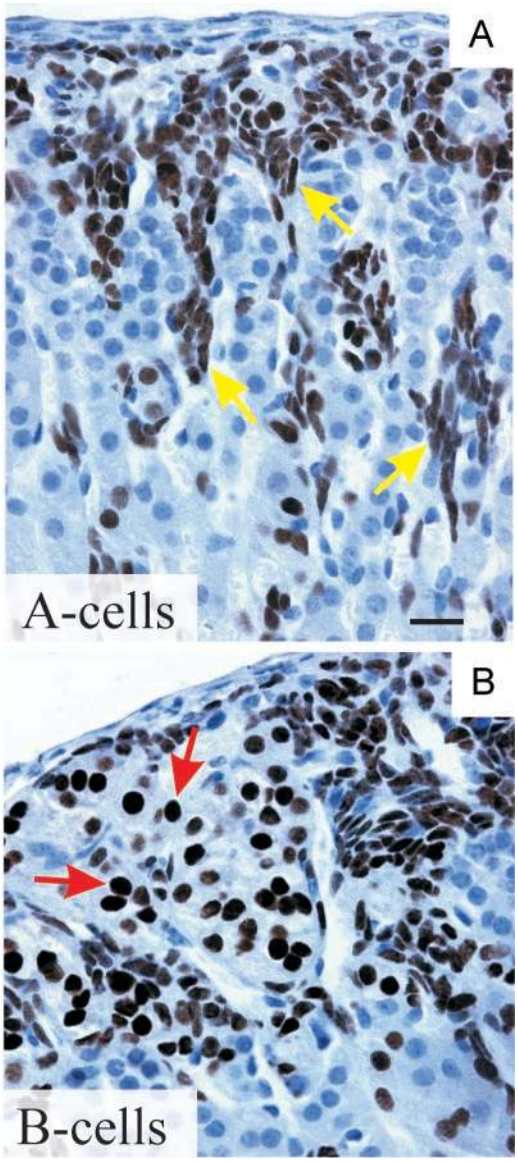

FIG. 3. High magnification view of GATA-4 protein in neoplastic A and B cells in the adrenal gland of a gonadectomized DBA/2J mouse. A weanling female DBA/2J mouse was gonadectomized, and adrenal glands were harvested 1 month later. The glands were fixed in paraformaldehyde, embedded in paraffin, sectioned, and subjected to immunoperoxidase staining with antibodies against GATA-4. Sections were counterstained with hematoxylin. Nuclei expressing GATA-4 antigen stain *dark brown*, whereas nuclei lacking this antigen stain *blue*. Note that abundant GATA-4 antigen is present in neoplastic A cells (*yellow arrows*) and B cells (*red arrows*), but not in normal epithelial cells of the adrenal cortex. *Bar*, 20 μm.

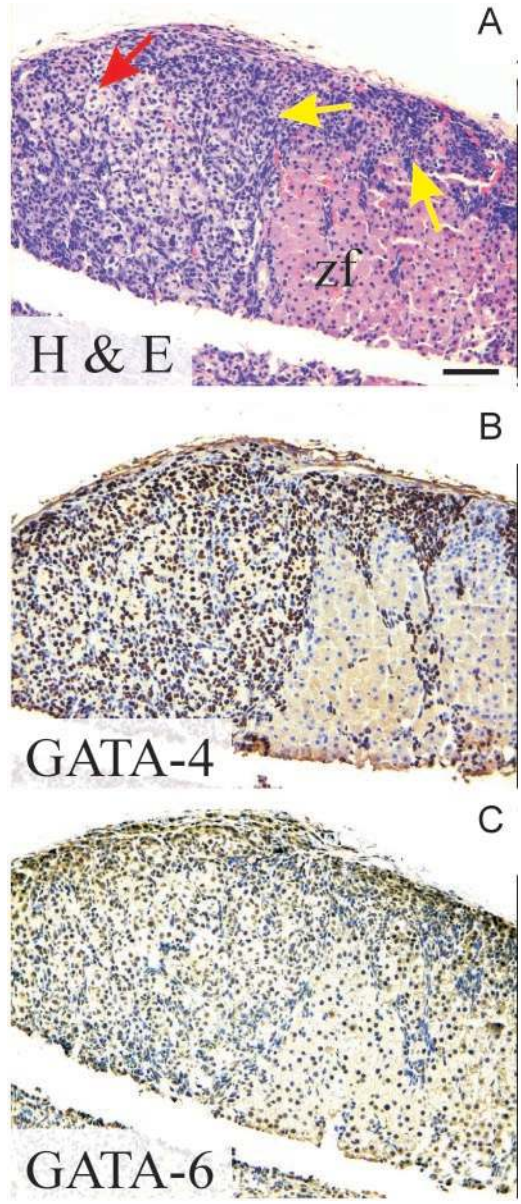

FIG. 4. Comparison of GATA-4 and GATA-6 protein expression in the adrenal cortex of gonadectomized DBA/2J mice. Weanling female DBA/2J mice were gonadectomized, and adrenal glands were harvested 4 months later. The glands were fixed in paraformaldehyde, embedded in paraffin, sectioned, and subjected to hematoxylin-eosin staining (A) or immunoperoxidase staining with antibodies against GATA-4 (B) or GATA-6 (C). The immunostained sections were counterstained with hematoxylin. Nuclei expressing antigen stain *brown*, while negative nuclei stain *blue*. Note that abundant GATA-4 antigen is seen in the nuclei of A cells (*yellow arrows*) and B cells (*red arrow*), but not in normal epithelial cells of the zf. GATA-6 antigen is evident in normal cells of the zf and in B cells, but not A cells. *Bar*, 50 μm.

pressed in human adrenocortical tumors (44) and in a transgenic mouse model of adrenocortical tumorigenesis (25, 26). Based on gene disruption studies in mice, inhibin-α is considered to be a tumor suppressor in gonads (45), and gonadectomy of inhibin-α−deficient mice results in the appearance of adrenocortical tumors (46). In ovariectomized DBA/2J mice, inhibin-α mRNA was detected in B cells and, to a lesser extent, in A cells in adrenocortical tumors (Fig. 7, G and K). Little inhibin-α mRNA was observed in normal adrenocortical cells (Fig. 7G). The expression of other factors involved in steroidogenesis was altered profoundly in the adrenocortical tumors found in gonadectomized mice. mRNA for SF-1, a transcription factor that regulates a variety of genes in steroidogenic cells (47), was reduced in areas rich in A cells (Fig. 7D). Compared with normal cells of the zg or zf, A cells expressed little mRNA for either P450scc, an enzyme that converts cholesterol to pregnenolone (1) (Fig. 7B),

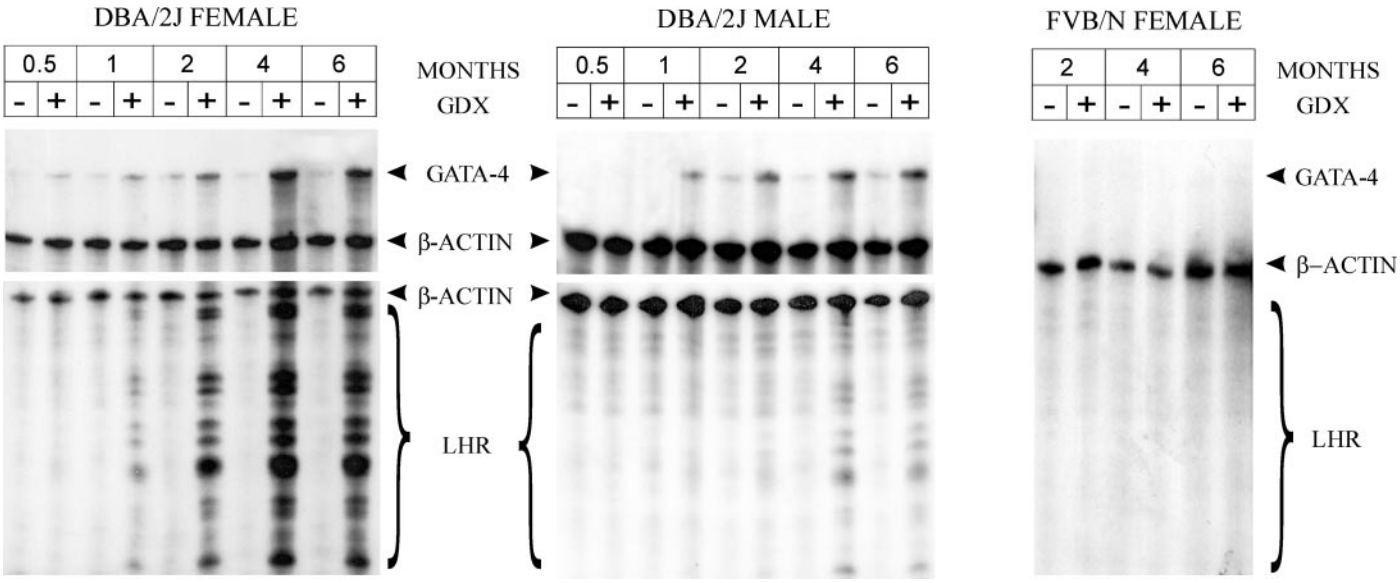

FIG. 5. GATA-4 and LHR mRNA levels in the adrenal glands of intact *vs.* gonadectomized DBA/2J and FVB/N mice. Weanling mice were subjected to gonadectomy (GDX) or sham surgery, and adrenal glands were harvested 0.5–6 months later. RNase protection assays for GATA-4 and LHR were performed as described in *Materials and Methods*. β-Actin mRNA levels were measured simultaneously to control for RNA recovery and loading. The *left*, *center*, and *right panels* show female DBA/2J, male DBA/2J, and female FVB/N mice, respectively. The GATA-4 riboprobe protects a fragment of 430 nucleotides, whereas the β-actin probe protects a fragment of 250 nucleotides. The LHR riboprobe protects a series of small fragments due to complex alternative splicing of this transcript (35, 36). This experiment was repeated on three separate occasions with identical results. In separate experiments (not shown), we confirmed that neither GATA-4 nor LHR mRNA was detectable in gonadectomized or sham-operated FVB/N mice at earlier time points (0.5 or 1 month).

or StAR, a factor that mediates the delivery of cytosolic cholesterol to the mitochondrial inner membrane (48) (Fig. 7C). B cells did express transcripts for SF-1, GATA-6, P450scc, and StAR in amounts comparable to normal adrenocortical epithelial cells (data not shown). Collectively, the expression patterns of these key steroidogenic markers confirm the long-standing idea that A cells have limited steroidogenic capacity (9, 11, 12), whereas B cells have the capacity to produce sex steroids (9, 12, 13). The overlapping patterns of expression of GATA-4 and LHR emphasize the relationship between these factors and gonadotropin-associated adrenocortical neoplasia.

Ovarian regeneration limits adrenocortical tumor development in ovariectomized DBA/2J mice

Unilateral regeneration of ovarian tissue occurs in some inbred mice (<15%) undergoing complete bilateral ovariectomy (28). This regenerated tissue typically arises in the vicinity of the distal oviduct and has a distinctive appearance, characterized by a small number of follicles and luteinized ovarian stromal tissue (28). Despite this unusual appearance, regenerated ovarian tissue is capable of sex steroid production, and estrous may resume within weeks of complete ovariectomy (28). We observed regenerated ovarian tissue in a subset of female mice that had undergone gonadectomy and then resumed estrous (Fig. 8, A and B). This regenerated tissue contained spindle-shaped stromal cells that expressed GATA-4 (Fig. 8C). Two months after surgery, animals with regenerated ovarian tissue had serum LH levels (1.1 ± 0.3 ng/ml; n = 4) comparable to those in sham-operated DBA/2J mice (see Table 1), confirming that the regenerated ovarian tissue was functional and produced hormones capable of feedback inhibition of the pituitary gland.

Ovarian regeneration and the resumption of sex steroid production had a profound influence on adrenal tumor development (Fig. 8D *vs.* 8G). The adrenal glands from mice with ovarian regeneration contained small collections of A cells (Fig. 8D, *black arrow*), but lacked B cells. The mRNA expression patterns of the steroidogenic enzymes P450 11β-hydroxylase (11β-OHase) and P450 aldosterone synthase followed the distribution observed in normal adrenal glands (47) and differed from those of gonadectomized females in which ovaries were completely absent (Fig. 8, E and F *vs.* H and I). RNase protection assays at 2 months postgonadectomy showed that GATA-4 mRNA expression was reduced in the adrenals of animals with regenerated ovarian tissue compared with animals without ovarian regeneration (Fig. 9). Furthermore, the adrenal glands from animals with regenerated ovarian tissue did not express LHR or P450c17 (Fig. 9). Thus, in the presence of ovarian regeneration, limited A cell proliferation occurs, but B cell differentiation is halted.

Discussion

Woolley and collaborators (5) first demonstrated that gonadectomy caused undifferentiated cells in the subcapsular region of the female mouse adrenal cortex to be transformed into sex steroid-producing cells that are histologically and functionally similar to ovarian tissue. Subsequent studies established that gonadectomy-induced adrenocortical tumor formation occurs in both female and male mice, but is highly strain dependent (6–9). The most susceptible strain, CE, develops adrenocortical carcinomas (7). DBA/2J, C3H, and BALB/c mice develop adrenocortical adenomas (6–10),

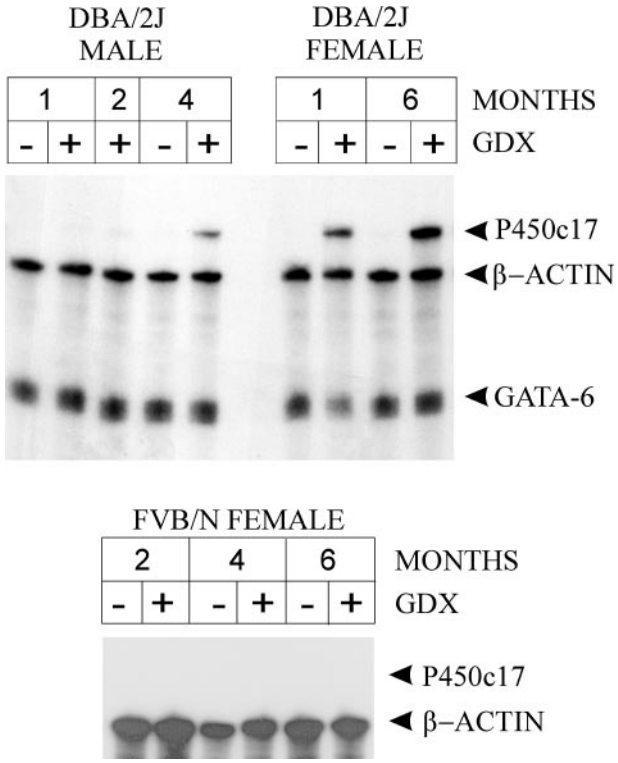

FIG. 6. P450c17 and GATA-6 mRNA levels in the adrenal glands of intact *vs.* gonadectomized DBA/2J and FVB/N mice. Weanling mice were subjected to gonadectomy (GDX) or sham surgery, and adrenal glands were harvested 1–6 months later. RNase protection assays for P450c17 and GATA-6 were performed as described in *Materials and Methods*. β-Actin mRNA levels were measured simultaneously to control for RNA recovery and loading. The *upper* and *lower panels* show DBA/2J and FVB/N mice, respectively. The P450c17 riboprobe protects a fragment of 324 nucleotides, the β-actin probe protects a fragment of 250 nucleotides, and the GATA-6 riboprobe protects a fragment of 140 nucleotides. This experiment was repeated twice with identical results.

whereas other stains, including C57BL/6J (10) and FVB/N (this study), are resistant to tumor formation.

The phenomenon of gonadotropin-induced adrenocortical tumorigenesis is not unique to mice. Subcapsular, spindle cell tumors have been reported in the adrenal glands of other gonadectomized rodents, such as rats, guinea pigs, hamsters, and ferrets (10, 49). Often these tumors produce ectopic sex steroids. Moreover, spindle cell neoplasms have been described in the subcapsular region of the adrenal cortex in humans with high serum gonadotropin levels, including postmenopausal women (50–54) and men with acquired testicular atrophy (55). In women, these adrenocortical changes have been termed thecal metaplasia because of the histological similarities with theca-interstitial cells of the ovary. In some instances these subcapsular human adrenocortical tumors have been associated with increased sex steroid production (52, 56, 57). The overall incidence of gonadotropin-induced adrenal neoplasms in humans is difficult to quantify, although autopsy series suggest that incidental adrenocortical tumors are present in approximately 5% of older women and men (58).

That a variety of species exhibit the phenomenon of gonadotropin-induced adrenocortical neoplasia suggests that mammalian adrenal glands contain progenitor cells that can proliferate and differentiate into sex steroid-producing stroma in response to continuous gonadotropin signaling. The origin of these neoplastic adrenocortical cells, which share properties with somatic/interstitial cells of the gonads, remains elusive. Whether a common stem cell gives rise to epithelial cells in the zg or zf and also to these stromal progenitors is unknown. Rare isolated cells with morphological features of Leydig cells have been reported in human adrenal cortex (59–61). In rodents there is evidence that steroidogenic cells of the gonads and adrenals originate from a common set of progenitors near the anterior end of the mesonephros (62). Alternatively, these cells may be derived from pluripotent cells that arise from coelomic epithelium (63).

Studies of the origin of adrenocortical stromal cells have been hampered in part by a paucity of suitable markers. We have found that gonadectomy of DBA/2J mice results in a marked increase in GATA-4 mRNA and protein in a sensitive population of subcapsular adrenocortical cells corresponding to the previously described A cells (5–7). These cells, which also express LHR, are not steroidogenic since they lack expression of markers such as SF-1, StAR, p450scc, P450 11β-OHase, or P450 aldosterone synthase. Instead they proliferate and differentiate presumably giving rise to B cells within developing tumors (5–9). B cells express a range of steroidogenic markers including p450c17, an enzyme capable of synthesizing sex steroids. Ectopic sex steroids produced by B cells elicit physiological effects, such as estrogen-induced changes in the endometrial lining or androgen-induced changes in the epithelial lining of Bowman's capsule (5–9). The presence of GATA-4 and LHR in both A and B cells supports histological evidence (5–9) that nonsteroidogenic A cells transform into sex steroid-producing B cells.

Previous studies have shown that GATA-4 is expressed in a variety of steroidogenic cells in the normal mouse, including Sertoli, Leydig, granulosa, thecal, and fetal adrenal cells (16–20, 23, 24). GATA-4 is also expressed in interstitial cells of the ovary and testes. In gonadal cells, GATA-4 has been implicated in the regulation of both tissue-specific gene expression (64, 65) and cellular proliferation (16–18, 66, 67). These findings coupled with our observation that cells expressing GATA-4 accumulate in the adrenal glands of gonadectomized DBA/2J mice suggest that GATA-4 plays a role in both differentiation and gonadotropin-induced proliferation of certain cell populations in the gonads and adrenals.

The expression of GATA-4 and LHR has been observed in another mouse model of adrenocortical tumorigenesis, namely that of inhibin-α promoter-SV40 T antigen transgenic mice, which develop adrenocortical carcinomas after gonadectomy (25, 26). Thus, enhanced GATA-4 expression and LH signaling have been linked to adrenocortical tumorigenesis in two independent mouse models, including one that is not reliant on the potent oncogene large T antigen. Although there are similarities in adrenocortical tumorigenesis in the inhibin-α promoter-SV40 T antigen transgenic and DBA/2J mouse models, there are some differences. For example, tumors in the transgenic mouse model develop later in life and are carcinomas rather than adenomas (24–26). The inter-

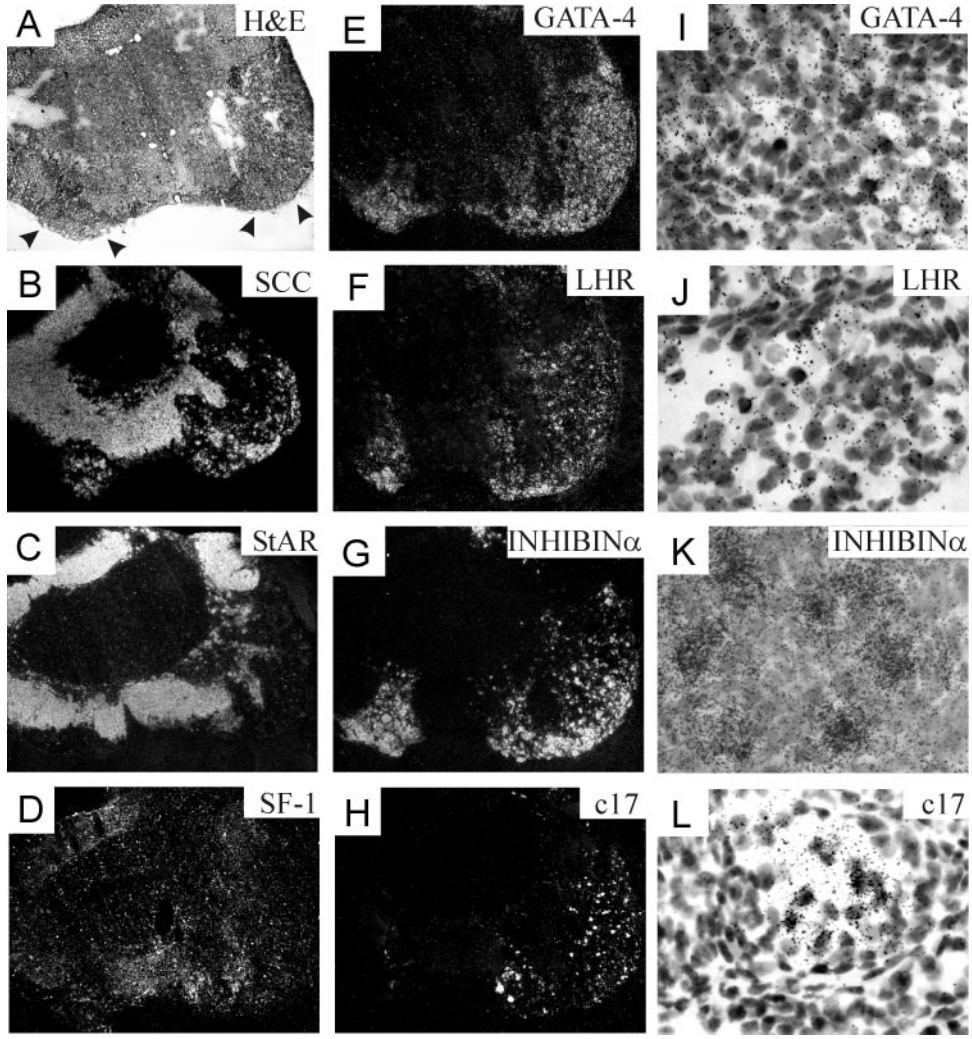

FIG. 7. Expression of steroidogenic differentiation markers in the adrenal cortex of a gonadectomized female DBA/2J mouse. A weanling female DBA/2J mouse was ovariectomized, and adrenal glands were harvested 8 months later. The glands were frozen in OCT, sectioned, and then stained with hematoxylin-eosin (A) or subjected to *in situ* hybridization with riboprobes for P450scc (B), StAR (C), SF-1 (D), GATA-4 (E and I), LHR (F and J), inhibin-α (G and K), and P450c17 (H and L). Low magnification views (×40) of one of the glands are shown in A–H, whereas high magnification views (×400) are shown in I–L. Darkfield views are shown in B–H, whereas brightfield views are shown in A and I–L. The *arrows* in A highlight areas containing neoplastic cells. Note that the large B cells express large amounts of mRNA for inhibin-α and P450c17.

relationship between GATA-4 and LHR in gonadectomy-associated adrenocortical tumors has not been fully elucidated and is the subject of ongoing investigation.

In contrast to GATA-4, there is little GATA-6 expression in the A cells that accumulate in the adrenal glands of gonadectomized DBA/2J mice or in the adenocarcinoma cells that form in gonadectomized inhibin-α promoter-SV40 T antigen transgenic mice (24). GATA-6 expression decreases when certain other cell types, including vascular smooth muscle cells and glomerular mesangial cells, are induced to proliferate (68). Transient transfection with GATA-6 induces growth inhibition in smooth muscle cells, fibroblasts, and glomerular mesangial cells, and that inhibition is dependent on the presence of the cyclin-dependent kinase inhibitor $p21^{cip1}$ (69, 70). Hence, down-regulation of GATA-6 expression may be critical for the cellular proliferation that accompanies A cell accumulation in gonadectomized DBA/2J adrenals. On the other hand, GATA-6 expression is evident in B cells found in the adrenal glands of gonadectomized DBA/2J mice, suggesting that this transcription factor is involved in steroidogenesis in these cells.

Adrenocortical tumors often show dysregulation of P450c17 expression (71–73). We found that adrenal tumor formation in gonadectomized DBA/2J mice is accompanied by increased expression of P450c17 from early stages onward. During fetal mouse development there is a transient expression of P450c17 in a subset of adrenocortical cells between embryonic d 12.5 (E12.5) and E14.5 (43). P450c17 expression ceases between 16.5 and term (E18.5) and is absent from the adrenal glands of adult mice (43). Consequently, adrenal glands from adult mice produce corticosterone and aldosterone, but not cortisol or sex steroids. The spatial and temporal expression patterns of different steroid hydroxylases in the adrenal suggest that multiple factors are required to program cell type and species-specific expression. GATA-4 is expressed at the same time as P450c17 in fetal mouse adrenal (23), and LHR appears transiently in human fetal adrenal at the same time as GATA-4 (23). As the P450c17 promoter contains multiple GATA elements, and its *in vitro* expression is enhanced by cAMP (74), it is tempting to speculate that the presence of GATA-4 and the activation of LHR are necessary or sufficient to promote P450c17 steroidogenic activity in the adrenal tumors of gonadectomized DBA mice.

As discussed earlier, only certain inbred mouse strains, DBA/2J included, respond to gonadectomy by developing

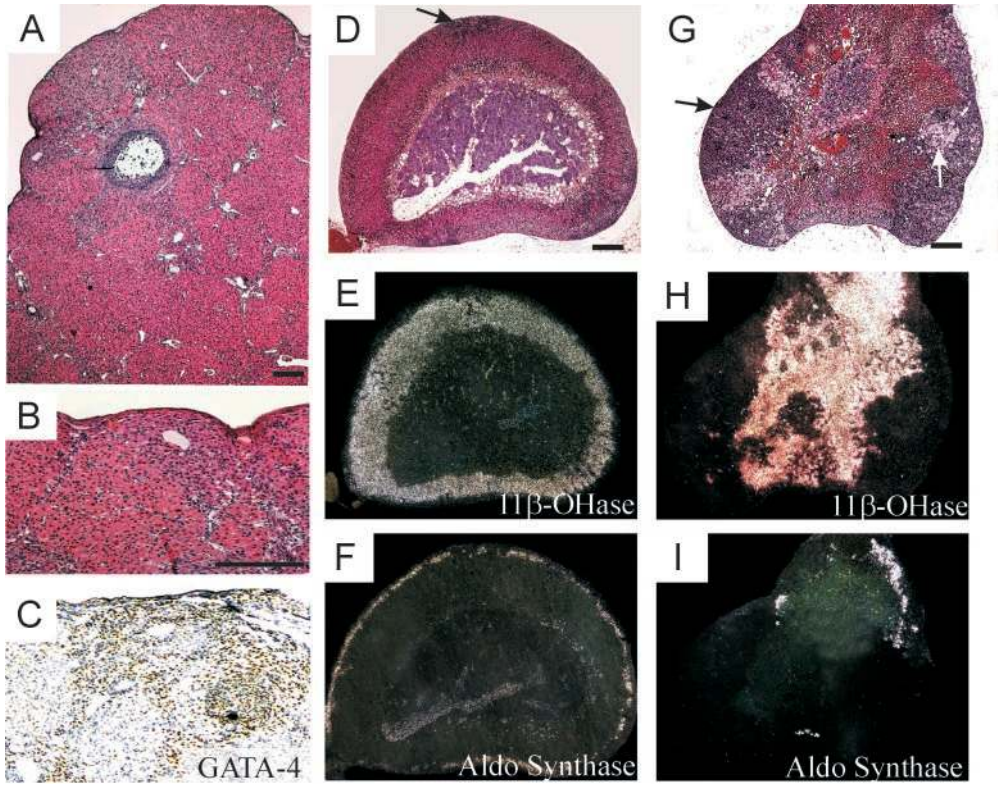

FIG. 8. Effect of ovarian regeneration on the accumulation of neoplastic cells in the adrenal glands of gonadectomized female DBA/2J mice. Weanling female DBA/2J mice were subjected to complete ovariectomy and then killed 6 months later. Ovarian regeneration was observed in a subset of these animals. Tissues from mice with (A–F) or without (G–I) ovarian regeneration were fixed in paraformaldehyde, paraffin-embedded, sectioned, and subjected to hematoxylin-eosin staining (A, B, D, and G), immunoperoxidase staining with antibody against GATA-4 (C), or *in situ* hybridization with probes for 11β-OHase (E and H) or aldosterone synthase (F and I). Low (A) and high (B and C) magnification views of a regenerated ovary show luteinized stromal tissue, a few follicles, and spindle-shaped cells that express GATA-4 antigen (C). Adrenal glands from mice with ovarian regeneration have small, superficial patches of A cells (D, *black arrow*), but no B cells. The patterns of expression of 11β-OHase (E) and aldosterone synthase (F) are essentially normal in adrenal glands from mice with ovarian regeneration. In contrast, the adrenal glands from mice lacking ovarian regeneration are infiltrated with large patches of A cells (G, *black arrow*) and B cells (G, *white arrow*), which distort the normal adrenal architecture. These neoplastic cells express negligible 11β-OHase (H) or aldosterone synthase (I) mRNA. *Bars*, 200 μm.

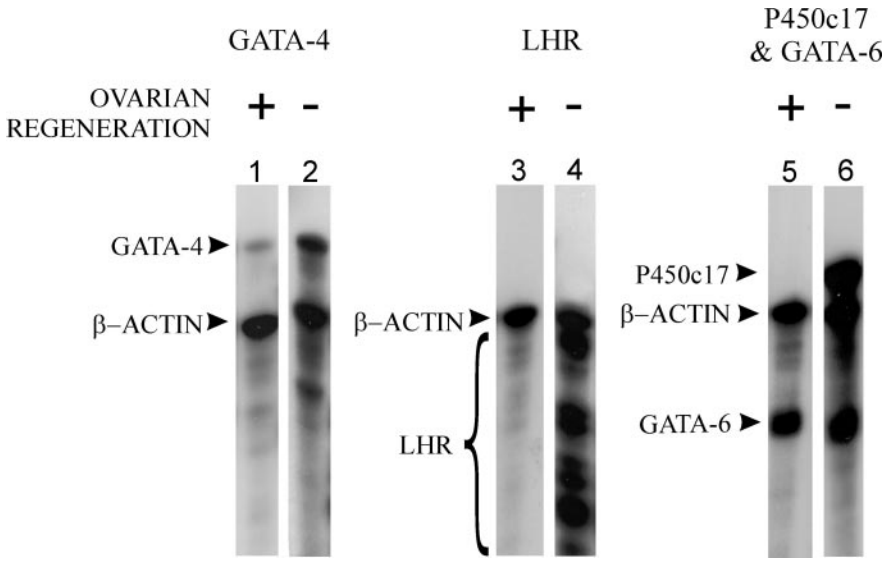

FIG. 9. Effect of ovarian regeneration on the expression of GATA-4, LHR, P450c17 and GATA-6 mRNAs in gonadectomized female DBA/2J mice. Weanling female mice were subjected to gonadectomy, and then adrenal glands were harvested 2 months later. Mice with and without ovarian regeneration were identified by careful inspection. RNase protection assays for GATA-4 (lanes 1, 2), LHR (lanes 3, 4), P450c17 (lanes 5, 6), and GATA-6 (lanes 5, 6) were performed as described in *Materials and Methods*. β-actin mRNA levels were measured simultaneously to control for RNA recovery and loading. This experiment was repeated twice with identical results.

adrenocortical tumors. It has been shown recently that DBA/2J mice carry a polymorphism in the gene encoding SF-1, which results in an Ala→Ser substitution at residue 172 of the protein (75). This polymorphism appears to influence steroidogenesis; the $SF1^{A172}$ allele is present in mouse strains with high steroidogenic capacity (*e.g.*

C57BL/6J), whereas the $SF1^{S172}$ allele is found in strains with low steroidogenic capacity (*e.g.* DBA/2J and C3H/HeJ) (75). Mutant Y1 adrenocortical tumor cell lines expressing the $SF1^{S172}$ allele are ACTH resistant, although the $SF1^{S172}$ allele appears to not be directly responsible for this effect (75, 76). Isolated Leydig cells from strains that contain the $SF1^{A172}$ (C57BL/6J) produce twice as much testosterone in response to hCG stimulation as cells from strains carrying the $SF1^{S172}$ allele (DBA/2J) (77). It is intriguing that strains with the $SF1^{S172}$ allele are those that develop postgonadectomy adrenocortical tumors, whereas a strain with the $SF1^{A172}$ allele (C57BL/6J) is resistant to tumor formation. The *SF1* polymorphism and its hormonal consequences may render gonadectomized DBA/2J mice prone to adrenal tumor development.

There is a delay in the onset of tumorigenesis and in the expression of GATA-4 and LHR after gonadectomy in male *vs.* female DBA/2J mice. Postgonadectomy levels of gonadotropins and other circulating hormones differ between genders (42), which may account for these differences in the rate of tumor progression. Once started, the process of neoplasia appears to follow similar paths in males and females, judging from the expression of GATA-4, LHR, and p450c17 in the adrenal tumors of both sexes.

Previous studies have shown that various signaling pathways are disrupted in adrenocortical tumors (78–80). Ectopic expression of LHR has been reported in adrenocortical neoplasms of mice (24–26) and humans (79), and a concomitant increase in GATA-4 expression has been noted in some of these cases (Ref. 24 and this study). We postulate that ectopic expression of LHR in adrenocortical cells confers inappropriate sensitivity to LH, which, in conjunction with GATA-4, leads to changes in cell proliferation, differentiation, or steroid production. Future studies will focus on how GATA-4 and LHR interact with one another and with other signaling molecules, such as activin receptors and Smad2 (80), during the process of adrenocortical tumorigenesis.

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