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Origin and Identity of Adrenocortical Tumors in Inhibin Knockout Mice: Implications for Cellular Plasticity in the Adrenal Cortex

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Inhibin knockout (*Inha*−/−) mice develop gonadal sex-cord tumors and—when gonadectomized—adrenocortical tumors. Previous reports demonstrated that adrenocortical tumors from *Inha*−/− mice produce estrogen and depend on gonadotropin signaling for initiation. Here we show that, in addition to producing estrogen, the adrenocortical tumors display a global change in cellular identity, composed of two unique cell types expressing differing arrays of genes normally restricted to theca and granulosa cells of the ovary. Many of these genes are also induced in wild-type adrenals after gonadectomy or upon chronic gonadotropin stimulation, suggesting that the adrenal cortex normally contains a population of pluripotent cells that can be driven toward an adrenal or gonadal identity given the appropriate pituitary stimuli. A central feature of this altered cellular identity is the switch from predominant expression of Gata6 (endogenous to the adrenal cortex) to Gata4, which defines cellular identity in the ovary. We show that stable transfection of Gata4 in cultured adrenocortical cells is sufficient to activate ovarian-specific genes of both theca and granulose lineages. Spatial analysis of Gata4 expression reveals a distinct pattern of localization to the supcapsular region of the adrenal, which contains undifferentiated progenitor cells that continuously populate the adrenocortical zones. Although both wild-type and *Inha*−/− mice display this pattern, only *Inha*−/− mice produce tumors composed of these Gata4-positive cells. These data suggest that *Inha*−/− adrenocortical tumors cells are derived from pluripotent adrenocortical progenitor cells that adopt a gonadal fate due to the convergent loss of inhibin and chronic exposure to elevated gonadotropins. (*Molecular Endocrinology* 20: 2848–2863, 2006)

THE ADRENAL CORTEX and gonad share a common embryonic origin from tissue of the urogenital ridge. A specific subset of urogenital cells, termed the adrenogonadal primordium, can first be detected at embryonic d 9.5 in mice by staining for the transcription factor steroidogenic factor-1 (Sf1), which is required for proper specification of both the gonad and adrenal cortex (1, 2). Shortly thereafter, the adrenogonadal primordium is divided into two distinct cellular compartments coincident with the ascension of the metanephric kidney and the inward migration of primordial germ cells into the caudal region of the urogenital ridge (3, 4). Positional cues derived from spatial separation of the two regions of the adrenogonadal primordium are thought to mediate differential patterns of gene expression, which ultimately result in the specification of distinct steroidogenic tissues of the adrenal cortex and gonad. Further development and differentiation of these tissues occurs upon maturation of the pituitary gland, which produces an array of peptide hormones that instructively direct the growth and differential steroidogenic profiles of the adrenal cortex and gonad (5–7).

Although the positional signals that direct the divergence of the adrenal cortex and gonad are poorly understood, several tissue-specific transcription factors that guide this process have been elucidated by *in vivo* loss-of-function studies and *in vitro* genetic screens (3, 8, 9). This group of factors includes Lim1, Dax1, Sry, and members of the Sox and Gata families of transcription factors. Of these factors, the GATA family transcription factors are particularly notable because they play several significant roles in development of the mammalian pituitary-adrenal-gonadal axis. In addition to helping specify the steroidogenic tissues of the adrenal and gonad, members of the Gata family are also critical for proper differentiation of the pituitary gonadotropes, specification of genitourinary ductal system, and sexual dimorphism of the male and female reproductive systems (10–12).

Within the Gata family, Gata4 and Gata6 are specifically involved in development and differentiation of the steroidogenic tissues of the adrenal and gonad.

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Abbreviations: Amh, Anti-Müllerian hormone; *Cyp17*, $P450_{c17\alpha}$; *Cyp19*, $P450_{aromatase}$; GC, granulosa cells; *Inha*, inhibin α; *Inhbb*, inhibin/activin β-B; Lhr, LH receptor; Sf1, steroidogenic factor-1; *Star*, steroidogenic acute regulatory protein; TC, theca cells.

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These two factors are variably expressed in the adrenal and gonad throughout fetal development and spatially correlate with the differential steroid production of these distinct tissues (10, 13, 14). Most notably, expression of Gata4 appears to positively correlate with the production of sex steroid hormones and is thus present in several gonadal cell lineages including male Leydig cells and female granulosa cells (GC) and theca cells (TC) (15). Gata4 is also present in the fetal murine adrenal cortex, which produces the androgen precursor DHEA, but is down-regulated in the postnatal adrenal cortex, which instead produces the steroid hormones aldosterone and corticosterone in response to angiotensin-II and pituitary ACTH, respectively (16). Gata6, on the other hand, is expressed in both the fetal and adult adrenal cortex and the ovarian interstitium, although it is not prominently expressed in the testis nor in the GC or TC compartments of the ovary (17, 18).

The transcriptional control of Gata4 expression in steroidogenic tissues, as well as in other tissues, is poorly understood, but is likely to be highly complex based on the intricate spatial and temporal expression patterns of Gata4 throughout development and adulthood (11, 19, 20). Mechanisms that specify the initial expression of Gata4 in the gonads are unknown, although its expression is clearly regulated by the pituitary gonadotropins. FSH up-regulates Gata4 expression in the testis and ovary, whereas LH and chorionic gonadotropin have differential effects, which can be both stimulatory and inhibitory toward Gata4 expression (13, 14, 21). Receptors for these hormones are normally restricted to steroidogenic cells of the testis and ovary, and are not prominently expressed in the adult adrenal gland. In response to gonadotropins, Gata4 is both transcriptionally and posttranslationally induced to turn on an array of target genes necessary for cellular differentiation and steroidogenesis (12, 22, 23). Many targets of Gata4 have been identified *in vivo* and *in vitro*, and include such genes as anti-Müllerian hormone (*Amh*), steroidogenic acute regulatory protein (*Star*), inhibin α (*Inha*), inhibin/activin β-B (*Inhbb*), $P450_{c17\alpha}$ (*Cyp17*) and $P450_{aromatase}$ (*Cyp19*) (24–27). The latter two genes are particularly important for sex steroid production because they catalyze the synthesis of sex steroids from other steroid precursors in the gonad.

Whereas Gata4 is normally absent from the adult adrenal cortex, recent studies of adrenocortical tumors suggest that this key transcriptional regulator may be reactivated under pathological circumstances in the adrenal gland (28, 29). Most notably, a switch from predominant Gata6 expression to Gata4 expression has been reported in a subset of human and mouse adrenocortical carcinomas (30, 31). Although the relationship between the steroidogenic phenotype of adrenocortical tumors and Gata4 expression is unclear in human studies, several mouse models of adrenocortical tumorigenesis (*i.e.* $SF1^{S172}$ allele bearing mouse strains and inhibin α promoter-simian virus 40 T-antigen [Inhα/Tag] transgenic mice) demonstrate that induction of Gata4 in adrenocortical tumors correlates with sex steroid production (32–34). Gata4 misexpression in the adrenocortical tumors from these various mouse models is induced by gonadectomy and subsequent increased levels of pituitary gonadotropins, which occur as a result of perturbing the pituitary-gonadal axis by removing the negative feedback loop that gonad-derived sex steroids normally exert upon pituitary gonadotrope cells.

Current studies in our laboratory focus on the inhibin-α knockout mouse (*Inha*$-/-$) model of adrenocortical tumorigenesis, in which the α subunit of the heterodimeric TGFβ family ligand inhibin has been genetically ablated (35). Like the $SF1^{S172}$ and Inhα/Tag mice, these mice develop both gonadal and adrenocortical tumors, which are absolutely dependent upon gonadotropins for induction (36–38). Whereas adrenocortical tumors in these mice are also sex steroid-producing, the cellular identity of these tumors is not clear. Production of estrogen suggests that *Inha*$-/-$ adrenocortical tumors might represent a mixed gonadal identity because ovarian estrogen biosynthesis requires the presence and distinct enzymatic functions of both TC—which contain $P450_{c17\alpha}$ and catalyze androgen synthesis from steroid precursors—and GC—which contain $P450_{aromatase}$ and catalyze androgen aromatization to estrogens (39). It is not clear, however, whether this phenotype results from simple activation of the steroidogenic machinery for estrogen production in otherwise normal adrenocortical cells, or whether the *Inha*$-/-$ tumor cells have adopted a different cellular fate that includes a more global gonadal transcriptional program.

To address this question, we compared the expression profile of several gonad-restricted markers—both TC and GC-specific—in wild-type and *Inha*$-/-$ adrenal glands before and after surgical gonadectomy to determine the identity of these tumor cells. We found that several gonad-restricted markers were induced in the adrenal glands of *Inha*$-/-$ mice, and unexpectedly, also in the adrenal glands of wild-type littermates after gonadectomy. Expression of these genes correlated with the ectopic expression of the transcription factor Gata4, which is sufficient to induce both TC and GC-specific genes in adrenocortical Y1 cells. Further analysis of this observation suggests that a subset of subcapsular adrenocortical cells retain the developmental plasticity to respond to pituitary gonadotrope stimuli that normally specify gonadal cell fates, and that these cells—like those found in the gonad—become misregulated in the absence of inhibin, thus forming hyperproliferative clusters of gonad-like cells in the adrenal cortex.

RESULTS

Inha$-/-$ Adrenocortical Tumors and Adrenals from Gonadectomized Mice Express Gonad-Restricted Transcripts Consistent with a Mixed Gonadal Lineage

The adrenal cortex and gonad share in common the expression of a large subset of genes, including

Cyp11a1 and *Star*, consistent with their common embryonic lineage and identity as steroidogenic organs (3). Many of these genes are under the transcriptional control of *Sf1/Nr5a1*, which is required both for the proper development and steroidogenic function of the adrenal, ovary, and testes (40). The identity of these tissues can be readily discriminated from nonsteroidogenic organs on the basis of *Sf1* expression (Fig. 1A). The inhibin-α subunit (*Inha*), which is a transcriptional target of Sf1, is also restricted to the gonads and adrenal gland (Fig. 1A). Genetic deletion of *Inha* in mice can be readily demonstrated by semiquantitative RT-PCR analysis of adrenal RNA because transcripts for this gene are completely undetectable. Using this method of analysis, we compared the expression profile of adrenal tissues before and after gonadectomy in wild-type and *Inha*$-/-$ mice to nonsteroidogenic tissues and gonads to determine the extent to which *Inha*$-/-$ adrenocortical tumors resemble gonadal tissue at the transcriptional level.

As reported previously, we found that both LH receptor (*Lhr*) and *Cyp17* are highly expressed in *Inha*$-/-$ adrenocortical tumors (Fig. 1B) (36). Transcripts for both of these molecules are only weakly detectable in the normal adult mouse adrenal, which lacks the capacity to produce androgens. In the ovary, both *Lhr* and *Cyp17* are initially restricted to the TC compartment, although during follicle maturation *Lhr* is also expressed in fully differentiated GC (41). The presence of these transcripts in *Inha*$-/-$ adrenocortical tumors suggests that a more extensive TC identity might also be present.

To explore this possibility, we compared the expression of several TC markers in *Inha*$-/-$ adrenocortical tumors to the normal adrenal cortex (Fig 1B). These markers include the transcriptional cofactor *Set*, which is required for optimal *Cyp17* expression, progesterone receptor (*Pr*), which functions as a regulator of terminal differentiation and growth in luteal TC, and the cell surface enzyme aminopeptidase-N (*Anpep*), whose function in follicular physiology is unclear (42–45). We also analyzed expression of the secreted peptides relaxin (*Rlx*) and relaxin-like factor (*Rlf*), which play important roles in remodeling follicular matrix proteins and in uterine conditioning during pregnancy (46, 47). Interestingly, several of these factors are expressed in the normal adrenal cortex, and are slightly increased in *Inha*$-/-$ adrenocortical tumors. This finding is consistent with the previously suggested hypothesis that the adrenocortical lineage is closely related to testicular Leydig cells, which are the testicular homologs of ovarian TC (8). Because the adrenal does not express significant levels of *Lhr* or *Cyp17*, however, it is clear that *Inha*$-/-$ adrenocortical tumors contain a population of TC-like cells that are distinct from the cells that normally populate the adrenal cortex.

In addition to containing cells with a theca-like identity, it would be predicted that *Inha*$-/-$ adrenocortical tumors also contain a population of cells that have the enzymatic capability of producing estrogen by aromatizing androgenic precursor steroids. Consistent with this prediction, the GC-specific enzyme $P450_{aromatase}$ (*Cyp19*) is highly expressed in *Inha*$-/-$ adrenocortical tumors, although absent from the adrenal gland before gonadectomy, suggesting that a granulosa-like cellular identity is also present in these tumors (Fig. 1C). In the ovary, *Cyp19* expression is tightly restricted to the GC compartment and is stimulated primarily by FSH-induced maturation of these cells (48). Consistent with the presence of *Cyp19* expression, we found that FSH receptor (*Fshr*) is highly expressed in these tumors, but not in the normal adrenal cortex (Fig. 1C). This observation is interesting because FSH is dispensable for gonadal tumor induction in *Inha*$-/-$ mice, although its loss significantly slows tumor growth and decreases estrogen output (49). Whereas the requirement for FSH in *Inha*$-/-$ adrenal tumor formation has not been explored, it is clear that *Inha*$-/-$ adrenocortical tumors are responsive to FSH and likely require FSH signaling for maximal steroidogenic output.

To further determine the extent to which *Inha*$-/-$ adrenocortical tumors contain a GC-like cellular identity, we analyzed the expression of several GC-specific markers in the same tissue panel as above (Fig. 1C). In addition to *Cyp19* and *Fshr*, we found significant expression of the inhibin/activin-β (*Inhba* and *Inhbb*) subunits and anti-Müllerian hormone (*Amh*) in these tumors (Fig. 1). These members of the TGFβ family of secreted ligands are specifically produced by GC and play critical roles in gonadal development, reproductive maintenance and hormonal cyclicity (50–52). Amh is normally present in immature and differentiated GC, whereas Inha/Actv-βA and -βB are primarily produced in response to FSH-induced follicle maturation. Notably, transcripts for all three of these peptide ligands are completely absent from the adrenal gland before gonadectomy.

Estrogen receptor-β (*Esr2*) and oxytocin (*oxt*) are other GC-specific markers also present in *Inha*$-/-$ adrenocortical tumors, but not the adrenal gland before gonadectomy (Fig. 1C). Unlike ERα, which is ubiquitously expressed in a large range of tissues including the adrenal and liver, ERβ is specifically induced in GC during gonadotropin-induced maturation (53). Expression of this factor in GC is critical because it mediates both the differentiation and growth-promoting effects of estrogens in this compartment of the ovary and permits appropriate GC responsiveness to ovulatory surges of LH (54). The secreted hormone oxytocin is produced specifically in GC in response to gonadotropins, although the role of gonadally derived oxytocin is unclear (55). Together with the expression of several TC-specific markers and other GC-specific markers, these data support the hypothesis that *Inha*$-/-$ adrenocortical tumors are not simply composed of normal adrenal cells that express sex steroid-producing enzymes but are instead composed of one or more cell types that have adopted a mixed ovarian identity.

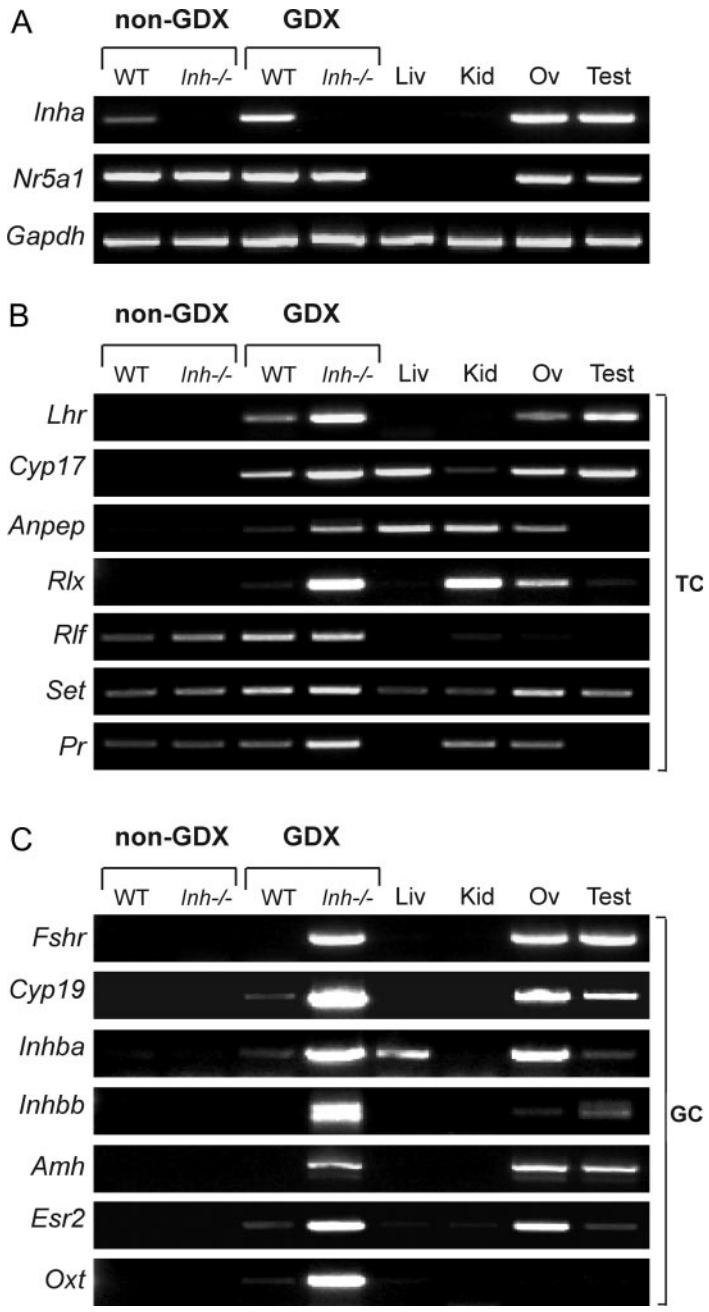

Fig. 1. *Inha*−/− Adrenocortical Tumors and the Post-Gonadectomy (GDX) Adrenal Cortex Express Gonad-Specific Transcripts

Semiquantitative RT-PCR was performed on cDNA libraries that were reverse- transcribed from 1.0 μg of total RNA harvested from indicated tissues. A, Expression of steroidogenic factor-1 (*Nr5A1*) defines steroidogenic tissues of the gonad and adrenal, and distinguishes them from nonsteroidogenic tissues, including the liver and kidney, whereas *Gapdh* is universally expressed in all tissue types. Both the adrenal and gonad express the inhibin-α subunit, which is completely lost in *Inha*−/− mice. B, Both *Lhr* and *Cyp17*, which are normally localized to follicular TC and testicular Leydig cells, are absent from the normal adrenal gland but are found both in *Inha*−/− and wild-type (WT) tissues after gonadectomy, consistent with their role in sex steroidogenesis. Several genes that have been reported to be theca-specific in the ovary (*Pr*, *Set*, *Rlx*, and *Rlf*) are also expressed in the adrenal cortex, supporting previous assertions that adrenocortical cells are developmentally closest to the theca/Leydig steroidogenic lineage. C, All GC-specific markers we tested are absent from the normal adrenal, but are all expressed at high levels in *Inha*−/− adrenal tumor cells. Most are also expressed at very low levels in the WT adrenal cortex after gonadectomy as well, implying responsiveness of the normal adrenal to gonadotropins after prolonged exposure.

Because only *Inha*−/− mice develop estrogen-producing adrenocortical tumors after gonadectomy, we did not expect that their wild-type counterparts would express any gonad-restricted transcripts after gonadectomy. Contrary to this expectation, we found that several of these transcripts, both GC and TC specific,

were also expressed in the adrenals of wild-type mice after gonadectomy, albeit at lower levels than those observed in *Inha*−/− adrenocortical tumors (Fig. 1). This observation has several important implications. First, it suggests that the specification of gonadal cells in the adrenal cortex after gonadectomy is a result of chronic gonadotropin up-regulation as opposed to the loss of inhibin. This distinction is critical because it implies that the mechanistic role of inhibin in the adrenal is not to prevent the misspecification of adrenal cells in response to elevated gonadotropins, but rather to control the growth of these cells once they are specified. Second, it suggests that the normal adult adrenal cortex contains a population of developmentally plastic cells that given the improper hormonal stimuli, will respond by adopting a gonadal identity.

Constitutive Overexpression of LH Is Sufficient to Specify the Thecal Lineage in the Adrenal Gland

The observation that surgical gonadectomy is sufficient to specify gonadal cellular identities in the normal adrenal cortex prompted us to ask whether simply elevating gonadotropin levels was sufficient to specify either the TC or GC identity as well. Because elevated LH is required for adrenal tumor formation in *Inha*−/− mice, we examined the adrenals of bLH-βCTP mice (*LH-CTP*), which display constitutively elevated serum LH levels comparable to wild-type mice after gonadectomy (36, 56). As previously reported in these mice, both *Lhr* and *Cyp17* were expressed in the adrenal in absence of gonadectomy (Fig 2A) (57). All of the TC-specific markers we previously analyzed and found up-regulated in *Inha*−/− tumors were also up-regulated in *LH-CTP* mice (Fig 2B), consistent with the sufficiency of LH to ectopically induce the TC identity in the adrenal.

In contrast to their ectopic adrenal expression or up-regulation of TC markers, *LH-CTP* mice do not demonstrate expression of *Cyp19* or *Fshr* (Fig. 2A), nor do they express any of the other GC markers found in the adrenals of wild-type mice post-gonadectomy (data not shown). As such, elevated FSH levels appear to be absolutely required to specify the GC identity in the mouse adrenal cortex after gonadectomy. This observation is interesting, as it suggests that TC and GC cellular fates may be induced separately by LH and FSH, respectively. In the absence of data from mice that overexpress only FSH, however, we are unable to determine whether elevated FSH is sufficient to induce the GC identity in the adrenal.

Inha−/− Adrenocortical Tumors Are Composed of Multiple, Distinct Steroidogenic Cell Types

Although both the TC and GC cellular identities are presumably specified from the same population of developmentally plastic cells in the adrenal cortex, it is not clear whether these identities are contained in a single differentiated cell or two separate cell populations. Because ovarian expression of several genetic markers is compartmentalized into TC and GC populations respectively, the mixed cellular phenotype of *Inha*−/− adrenocortical tumors suggests that two separate cell populations might be specified within the tumors. Gene expression data from *LH-CTP* mice support this hypothesis, as they suggest that each cellular identity in the adrenal is separately specified by a different gonadotropin. Because the expression data from *Inha*−/− tumors are taken from whole tumors and not individual cells, however, they do not rule out the possibility that the tumors are composed of a single cellular lineage that contains the cumulative differentiating effects of LH and FSH. To address this question, we analyzed the spatial expression of Lhr and Amh to determine whether the different cellular identities were mixed in a single cell type, or specified

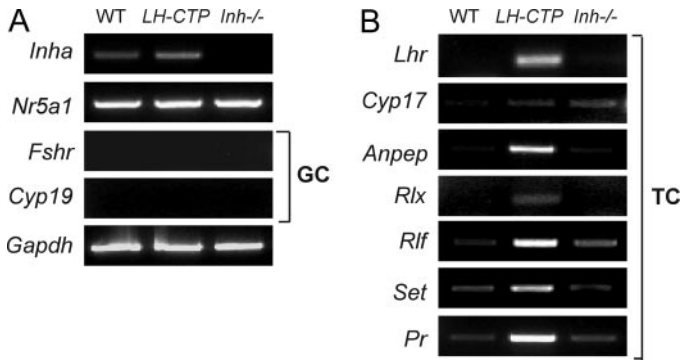

Fig. 2. Constitutive Up-Regulation of LH Induces Theca-Specific Gene Expression in the Adrenal Cortex

Semiquantitative RT-PCR was performed on cDNA libraries that were reverse-transcribed from 1.0 μg of total RNA harvested from the adrenals of nongonadectomized 10-wk-old mice. A, Adrenals from bLH-βCTP (*LH-CTP*) mice express normal levels of *Nr5a1* and *Inha*, although they lack any expression of GC markers including *Fshr* and *Cyp19*. B, In contrast to the lack of GC markers, theca markers are prominently expressed or up-regulated in *LH-CTP* mice. Notably, aminopeptidase-N (*Anpep*) and *Lhr* are completely absent from both wild-type (WT) and *Inha*−/− animals but are prominently expressed in *LH-CTP* mice, suggesting that constitutively elevated LH is sufficient to induce a thecal lineage of differentiation in the adrenal.

as two distinct cellular populations within adrenocortical tumors.

Immunofluorescent detection of Lhr and Amh in normal ovarian follicles clearly demonstrates that Lhr and Amh specifically mark TC and GC, respectively, as the staining for these markers is mutually exclusive (Fig. 3A). Interestingly, small adrenocortical tumors from *Inha*−/− mice, which have an adenomatous structure reminiscent of ovarian follicles, also show distinct staining for Lhr and Amh. Lhr expression is restricted to the outer part of tumor nodules, whereas Amh is expressed in a more central pattern (Fig. 3B). As these tumors become increasingly larger, they appear to lose this organization, and become more structurally heterogeneous. In the larger tumors, Lhr-positive and Amh-positive regions of cells are mixed together, although staining for the two markers in cells of these tumors still appears to be mutually exclusive (Fig. 3C). Together, these data suggest that *Inha*−/− adrenocortical tumors are cellularly heterogenous and composed of at least two cell types that appear to be directed toward either a TC or GC fate in a mutually exclusive fashion.

The Specification of Gonadal Cellular Identities in the Adrenal Correlates with Ectopic Expression of Gata4

Within the ovary, both GC and TC express the transcription factor Gata4, which is directly involved in the differentiation and steroidogenic function of these cells in mammalian reproduction (12). Several of the gonad-specific transcripts that we analyzed in *Inha*−/− tumors are direct transcriptional targets of Gata4, including *Cyp17*, *Cyp19*, *Amh*, *Inhbb*, and *Inha* itself (27). As predicted by these data, Gata4 can be readily detected at both the mRNA and protein levels in *Inha*−/− adrenocortical tumors, but not in the adrenals of *Inha*−/− or wild-type mice before gonadectomy (Fig. 4, A and C). Gata4 transcripts are also detectable in the wild-type adrenal gland after gonadectomy and in the adrenals of *LH-CTP* mice before gonadectomy, although only at the mRNA level (Fig 4A). As such, Gata4 expression in the adrenal clearly correlates with elevated levels of gonadotropins and the specification of gonadal cells after gonadectomy.

Due to the fact that gonadectomized female *Inha*−/− mice develop adrenocortical tumors more rapidly than their male counterparts, we chose to focus the bulk of our studies on female *Inha*−/− adrenocortical tumors. Because adrenocortical tumors from both sexes primarily produce estrogens, it would be predicted that male adrenocortical tumors are phenotypically similar to female tumors at the molecular level, and therefore more representative of ovarian cells rather than testicular cells. To ensure that this is true and that the conclusions drawn from these studies are applicable to both sexes of *Inha*−/− mice, we screened *Inha*−/− tumors from both male and female mice for expression of several markers

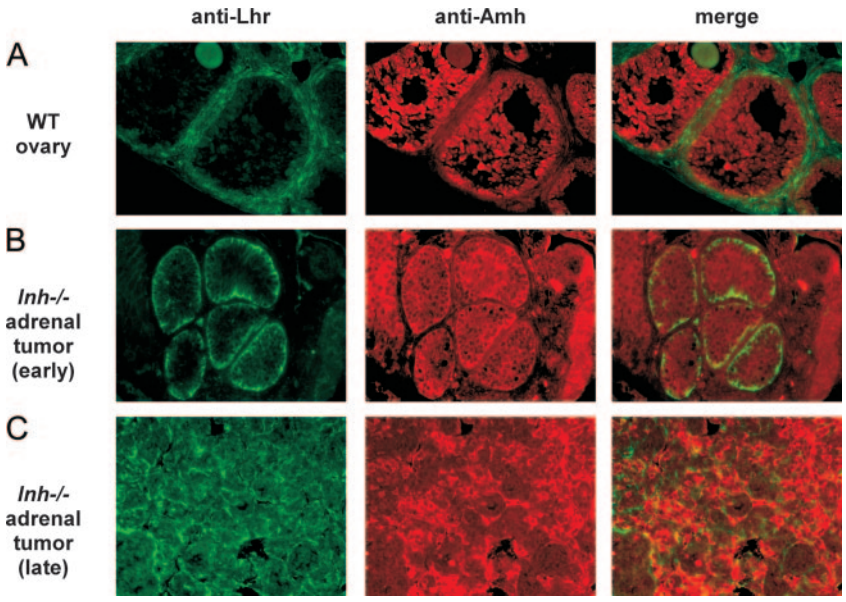

Fig. 3. *Inha*−/− Adrenocortical Tumors Are Composed of Two Distinct Cell Populations of Gonadal Identity

Immunofluorescent costaining for Lhr (*green*) and Amh (*red*) was performed on tissue sections from wild-type and *Inha*−/− mice. A, In the normal preovulatory follicle of wild-type (WT) mice, Lhr is a molecular marker for TC, which surround the GC and oocyte of the follicle. Amh is specifically expressed by GC. B, Small adrenocortical tumors taken from *Inha*−/− mice display an adenomatous structure similar to the follicle. Peripheral cells in these small tumor nodules display specific staining for Lhr, similar to that seen in TC of the normal follicle. Conversely, the innermost cells of the tumor are positive for Amh, and do not express Lhr, similar to that seen in GC. C, Although larger *Inha*−/− adrenocortical tumors lose this organization, they still continue to express both Amh and Lhr in populations of cells in mutually exclusive fashion.

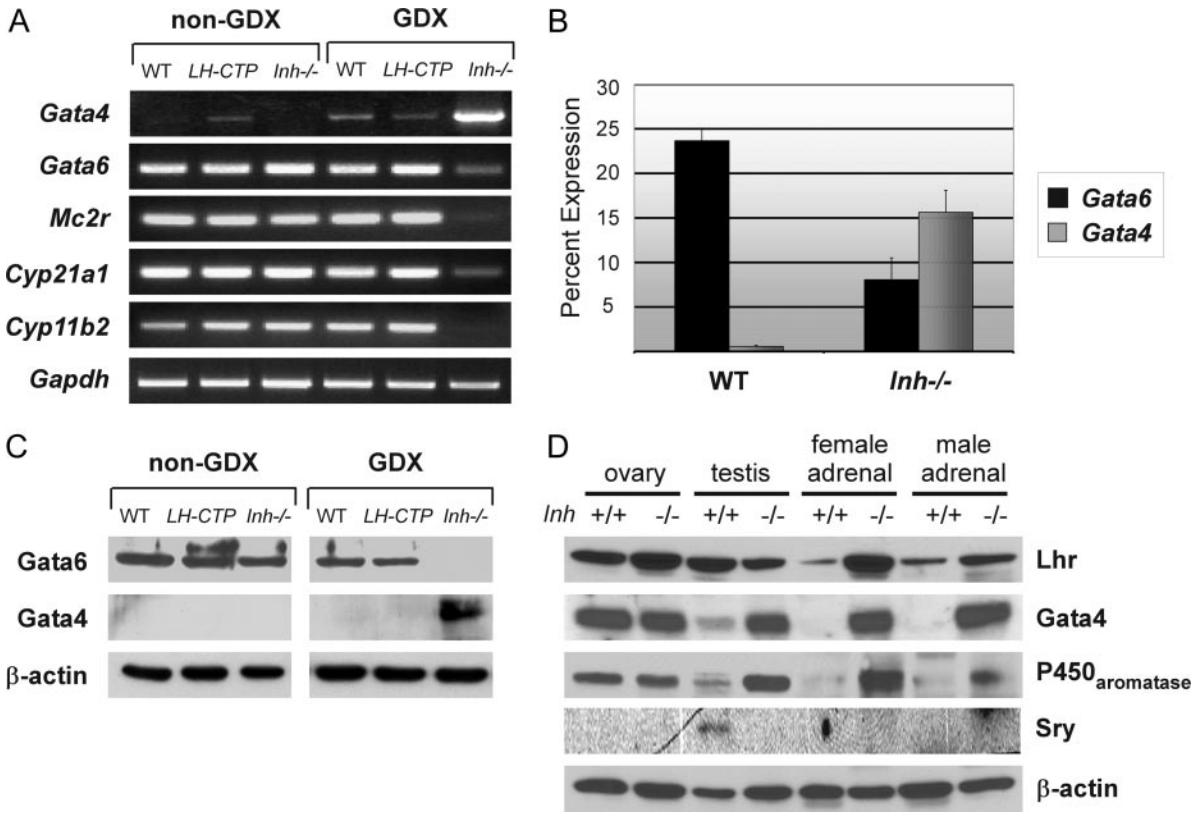

Fig. 4. Gata4 Expression Correlates with Gonad-Specific Gene Expression in *Inha*−/− Adrenocortical Tumors and the Post-Gonadectomy (GDX) Adrenal Cortex

Expression of Gata4 and Gata6 was measured in adrenal and/or adrenal tumor tissue from WT, *LH-CTP*, and *Inha*−/− mice. A, Semiquantitative RT-PCR performed from adrenal cDNA libraries shows that *Gata6* is prominently expressed in the adrenal gland across genotypes but is decreased in *Inha*−/− adrenocortical tumors. *Gata4* is not normally expressed in the adrenal gland before gonadectomy, except in *LH-CTP* mice, which have elevated serum LH. After gonadectomy, *Gata4* is found in the adrenal across genotypes but is most highly expressed in *Inha*−/− tumors. Up-regulation of *Gata4* and loss of *Gata6* expression is concomitant with loss of other adrenocortical-specific markers including the melanocortin-2 receptor (*Mc2r*), 21-hydroxylase (*Cyp21a1*), and aldosterone synthase (*Cyp11b2*). B, Quantitative analysis of *Gata4* and *Gata6* transcript abundance was performed in WT adrenals after gonadecomty and *Inha*−/− adrenocortical tumors. Levels of abundance are displayed as a percentage of *Arbp*, with error being calculated by the standard deviation of triplicate measurements from four separate tissue samples for each genotype. *Inha*−/− mice display both an increase in *Gata4* expression and a decrease in *Gata6* expression compared with WT mice, consistent with the semiquantitative data for these tissues. C, Protein levels of Gata4 and Gata6 were measured by immunoblotting from adrenal and adrenal tumor lysates. Whereas Gata6 is broadly expressed in adrenal tissue across genotypes, it is either absent or weakly detectable in *Inha*−/− adrenocortical tumors. Gata4 protein is exclusively detected in *Inha*−/− adrenocortical tumors and is not found at measurable levels in any adrenal lysates. D, Expression of ovary and testis-specific markers were analyzed by immunoblotting to determine the identity of male *Inha*−/− adrenocortical tumors. These tumors express high levels of Gata4, Lhr and $P450_{aromatase}$, and lack expression of Sry, consistent with an ovarian phenotype in both male and female adrenocortical tumors.

that discriminate between the male and female gonad (Fig. 4D). Whereas both Gata4 and Lhr are normally found in the testis and ovary, they are more highly expressed in the latter. Male *Inha*−/− testicular and adrenocortical tumors show increased expression of both of these proteins, consistent with a switch toward a more ovarian phenotype. An even more specific ovarian marker, $P450_{aromatase}$, is also abundant in male *Inha*−/− tumors, which is expected given their high level of estrogen production. In contrast to these markers, the Sox-family transcription factor Sry is only found in the male testis, and is normally absent from both the ovary and adrenal cortex. Both testicular and adrenocortical tumors from male *Inha*−/− mice lack expression of Sry, confirming that these tumors adopt an ovarian cellular identity as opposed to a testicular identity. These findings are interesting as they suggest a role for inhibin not only in the regulation of cellular proliferation, but also in the cell fate determination of bipotential gonadal progenitor cells.

Expression of Gata4 in the Adrenal Cortex Is Limited to the Subcapsular Zone

Our inability to detect significant levels of Gata4 protein by immunoblotting in any adrenal tissue besides *Inha*−/− tumors (Fig. 4C), even when mRNA transcripts are present (Fig. 4A), suggests either that very little pro-

tein is being produced, or that its production is limited to a very small subset of cells in the adrenal that represent only a fraction of the total cellular mass. To differentiate between these two possibilities, we performed immunohistochemical staining for Gata4 on paraffin sections of adrenals from wild-type, *LH-CTP* and *Inha*−/− mice before and after gonadectomy. Consistent with our RT-PCR analysis, Gata4 expression was not detected by immunohistochemistry in the adrenal cortex of wild-type or *Inha*−/− mice before gonadectomy, although it was weakly expressed in a narrow rim of subcapsular adrenocortical cells in *LH-CTP* mice (Fig. 5A). Gata4 expression was more prominent in all three genotypes after gonadectomy and was similarly restricted to the subcapsular zone of the adrenal (Fig. 5A). Because this population of cells composes only a fraction of the entire cortex, it is likely that we cannot detect Gata4 protein in the adrenal by immunoblotting because it is diluted beyond the limit of detection in the total protein mass. Interestingly, cellular proliferation in the adrenal cortex is restricted to the same subcapsular zone where Gata4 expression was detected, suggesting that the adrenocortical progenitor cells that reside in this region may be the cellular targets of gonadotropin signaling, which eventually become Gata4 positive under chronic stimulation (58, 59).

Gata6 Is Excluded from the Adrenal Tumor Tissue of *Inha*−/− Mice

Previous reports of Gata4 expression in adrenocortical tumors have indicated that activation of Gata4 occurs

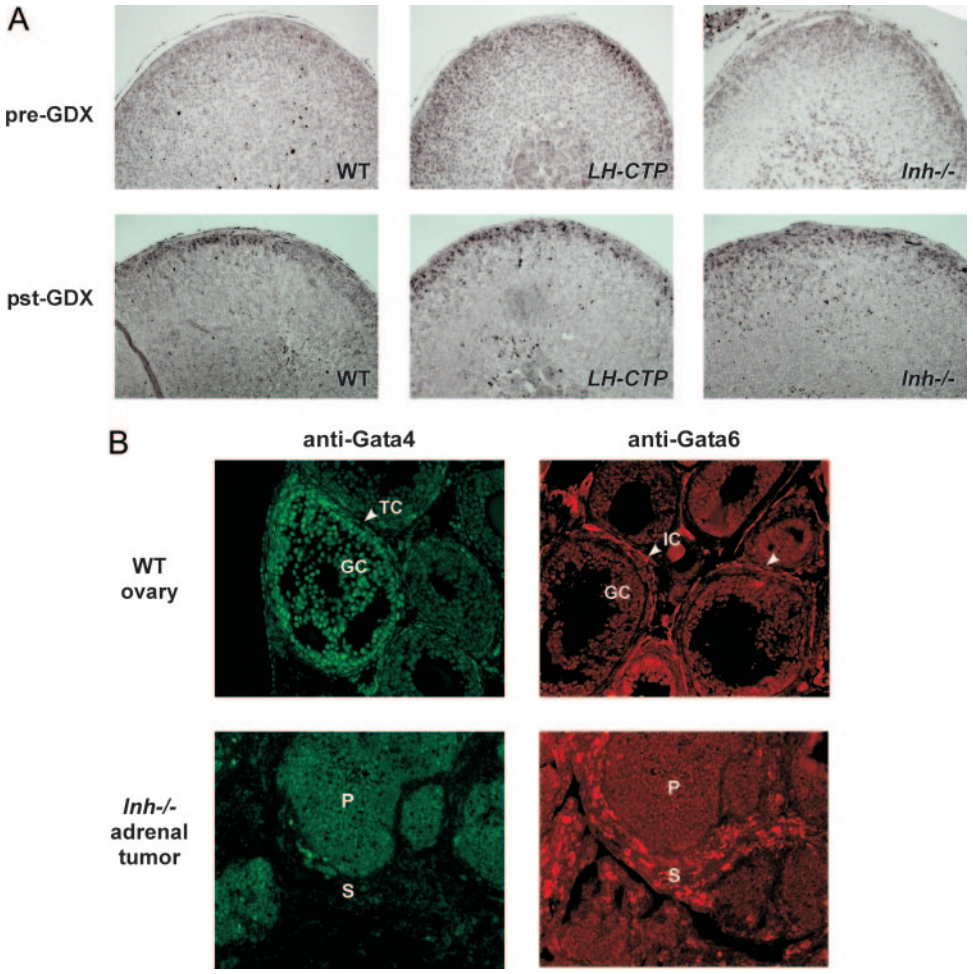

Fig. 5. Gata4 and Gata6 Are Expressed in a Mutually Exclusive Pattern in the Ovarian Follicle and Adrenal Cortex

Immunohistochemical detection of Gata4 and Gata6 was performed in adrenal and ovarian tissue sections. A, Gata4 is not expressed in the adrenal cortex of wild-type (WT) or inhibin-null (*Inha*−/−) mice before gonadectomy (GDX), whereas it is exclusively localized to the subcapsular region of the adrenal cortex in these mice after gonadectomy. Gata4 is similarly detected in LH overexpression (*LH-CTP*) mice after gonadectomy but is also weakly detected before gonadectomy, consistent with a role for gonadotropins in Gata4 transcriptional activation. B, In the ovarian follicle, Gata4 is prominently expressed in both GC and TC of the growing follicle. Gata6 is absent from both GC and TC but is weakly expressed in the ovarian interstitial cells surrounding follicles. Similar to the ovarian follicle, Gata4, but not Gata6, is detected in the proliferating parenchymal tissue of *Inha*−/− adrenocortical tumors. The nonproliferating adrenocortical stroma that surrounds the tumor tissue displays the opposite pattern of expression, being positive for Gata6 but negative for Gata4. IC, Interstitial cell; P, parenchymal; S, stromal.

coincident with loss of Gata6 expression, which is normally found throughout the adult adrenal cortex (16, 29). To determine whether this was also true of *Inha*−/− adrenocortical tumors, we performed immunofluorescent staining for Gata6 and Gata4 in adrenocortical tumors. Similar to studies in other tumor models, we found that Gata6 was excluded from the parenchymal tissue of *Inha*−/− adrenocortical tumors, although it appeared to be retained in the adrenal-derived stromal tissue surrounding these clusters of proliferative tumor cells (Fig. 5B). Parenchymal tumor cells that were Gata6 negative instead expressed Gata4, whereas Gata6-expressing stomal cells did not. These data are supported by quantitative RT-PCR and immunoblot analyses comparing tumor tissue to wild-type adrenal tissue, which show decreases in both Gata6 mRNA and Gata6 protein in tumors, and dramatic increases in the presence of both Gata4 mRNA and protein (Fig. 4, A–C). These data imply that the conserved phenotype among the several mouse models of adrenal tumorigenesis is commonly induced by gonadectomy, despite the disparate genetic mechanisms by which the tumors arise.

The expression pattern of Gata4 and Gata6 may also be relevant with regards to the cellular identity of *Inha*−/− adrenocortical tumors because the rodent ovary displays cell-specific staining for these factors. In the growing follicle, both GC and TC broadly express nuclear Gata4, which can be seen from immunufluorescent staining in ovarian tissue sections, whereas Gata6 expression appears to be tightly restricted to ovarian interstitial cells (Fig. 5B). The fact that the majority of follicular cells express only Gata4—and not Gata6—suggests that not only have *Inha*−/− adrenal tumor cells adopted a mixed follicular identity, but that they have done this at the expense of adopting an adrenocortical identity. This change in cellular identity is additionally demonstrated by the decrease or loss of adrenal-specific gene expression from *Inha*−/− adrenocortical tumors, including the melanocortin-2 receptor (*Mc2r*), 21-hydroxylase (*Cyp21a1*), and aldosterone synthase (*Cyp11b2*) (Fig. 4A). Gata4/Gata6 switching therefore suggests a complete identity change of adrenocortical progenitors toward a gonadal cell fate as opposed to a superimposement of the gonadal fate onto adrenocortical cells.

Gata4 Is Sufficient to Activate the Transcription of Both Theca and Granulosa-Specific Markers in Adrenocortical Cells

The mutually exclusive pattern of gene expression found for Gata4 and Gata6 in the gonad and adrenal suggests that these two closely related transcription factors may interact with one another at the transcriptional level by means of direct or indirect repression. To address this possibility, we stably expressed mouse Gata4 in the mouse Y1 adrenocortical cell line, which does not express endogenous Gata4 at any appreciable level (Fig. 6A). After screening several monoclonal lines of stably transfected cells, we chose a single monoclonal line designated Y1-G4 for further studies. Y1-G4 cells show a dramatic increase in Gata4 mRNA, and display significant levels of Gata4 protein as well (Fig. 6A). Interestingly, these cells also show a 2- to 3-fold decrease in Gata6 mRNA compared with normal Y1 cells, suggesting that Gata4 may repress Gata6 expression, either directly or indirectly. Because the transcriptional repression was minimal and did not grossly affect Gata6 protein levels, however, it is likely that other genetic mechanisms than direct transcriptional repression of the Gata6 promoter account for the mutually exclusive expression of Gata4 and Gata6 in the ovary and in *Inha*−/− adrenocortical tumors.

Y1 cells represent an exclusively adrenocortical cell lineage and are therefore not expected to express transcripts that specifically characterize steroidogenic cells of the gonad (60). Because Gata4 is coordinately expressed with several GC and TC-specific genes in *Inha*−/− adrenocortical tumors and is known to regulate expression of several of these genes in the context of gonadal cells, we asked whether overexpression of Gata4 is sufficient to activate expression of these genes in the context of an adrenocortical cell. To answer this question, we compared the expression of genes that are exclusively found in the ovary between Y1 and Y1-G4 cells.

In their endogenous state, Y1 cells fail to express *Cyp17*, although—like the normal adrenal cortex—they do express other TC-specific genes including *Set*. Y1-G4 cells express comparable levels of *Set*, but unlike Y1 cells, they also express *Cyp17* (Fig. 6B). All of the GC-specific genes we tested—including *Cyp19*, *Esr2*, *Amh*, and *Inhbb*—follow the same pattern as *Cyp17* and are absent from Y1 cells, but are expressed in Y1-G4 cells (Fig. 6B). Expression of both sets of genes in this system is clearly independent of gonadotropins because neither *Lhr* nor *Fshr* can be detected in Y1 or Y1-G4 cells. These data indicate that Gata4 expression likely lies downstream of gonadotropins and is sufficient to drive expression of several ovary-specific genes in an adrenocortical cell line, most importantly those required to stimulate estrogen biosynthesis. In contrast to the loss of adrenal markers in *Inha*−/− adrenocortical tumors, which demonstrate a complete switch in Gata6/Gata4 expression, the Y1-G4 cells retain expression of Gata6 (Fig. 6A) and adrenal-specific markers such as *Mc2r* (data not shown). This limited data set therefore implies that both the loss of Gata6 and concomitant gain of Gata4 are required for phenotypic switching between adrenal and gonadal cellular identity.

DISCUSSION

Previous reports have demonstrated that adrenocortical tumors from *Inha*−/− mice produce high levels of

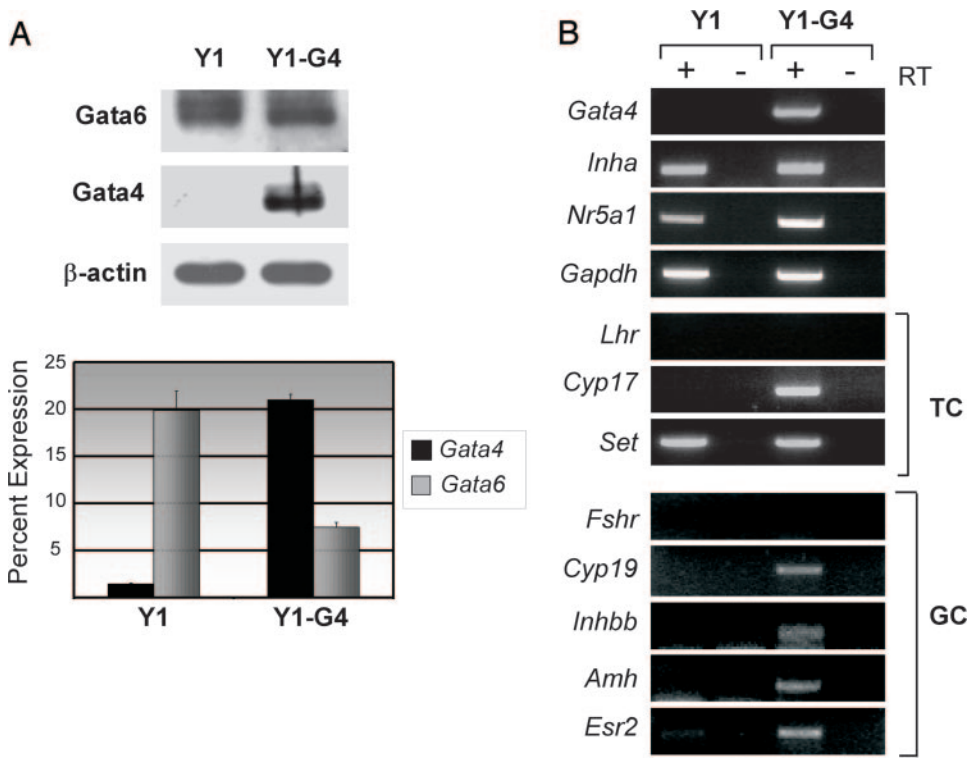

Fig. 6. Ectopic Expression of Gata4 in an Adrenocortical Cell Line Is Sufficient to Induce Gonad-Specific Gene Expression

The complete coding sequence for mouse *Gata4* was subcloned into pCDNA3 and was subsequently linearized and transfected into Y1 adrenocortical cells. Stable monoclonal transfectants were selected by G418 treatment and expanded over a 6-wk period. Several stable lines were tested for expression by immunoblotting for Gata4. A, A single clone from this group was selected for further studies based on its high expression of Gata4 protein compared with parental Y1 cells. Consistent with their identity as adrenocortical cells, Y1 cells express significant levels of Gata6 protein. Gata6 expression is retained in Y1-G4 cells, although expression of *Gata6* is slightly suppressed at the transcriptional level, as seen by quantitative RT-PCR detection. B, Expression patterns of markers for both the TC and GC identities were compared in Y1 and Y1-G4 cells. As expected, both cell lines express Sf1 (*Nr5a1*) and *Inha*, which mark steroidogenic cells of the adrenal and ovary, and the housekeeping control *Gapdh*. Y1 cells do not express the enzymes necessary for estrogen biosynthesis; however, overexpression of Gata4 in Y1-G4 cells leads to ectopic activation of both *Cyp17* and *Cyp19*. Similar to what we observed in whole adrenal tissues, other markers of the theca identity such as *Set* are expressed in both Y1 and Y1-G4 cells. GC-specific markers, however, were only expressed in Y1-G4 cells, indicating that GATA4 is sufficient to activate at least part of the GC identity in the context of Sf1 expression. Interestingly, neither *Lhr* nor *Fshr* was induced in Y1 cells by stable GATA4 expression, consistent with data that suggests gonadotropins lie upstream of *Gata4* expression in the gonad. RT, Reverse transcription.

estrogen, and that these tumors both respond to and require pituitary gonadotropins for their initiation and subsequent growth (37, 38). In this study, we show that the cellular phenotype of these tumors is not limited to the expression of estrogenic enzymes and includes an array of genes that are normally restricted to the gonad. This array includes genes that are specific markers of distinct somatic cell types of the ovarian follicle, suggesting that a global change in cellular identity has occurred in *Inha*$-/-$ adrenal tumor cells. The global change in cell identity from an adrenocortical to gonadal phenotype in tumors is centrally characterized by loss of Gata6 expression and transcriptional activation of Gata4 expression, which is normally restricted to steroid-producing cells of the gonad. Gata4 not only characterizes the aberrant specification of gonadal cells in the adrenal but is also a vital mediator of this process, as evidenced by the sufficiency of Gata4 to drive expression of a large panel of gonad-restricted genes in an adrenocortical cell line.

Both the expression of Gata4 and the broader gonadal phenotype of Gata4-positive cells in the adrenal cortex have important implications for the mechanism of inhibin action in the adrenal. The inhibin-α gene—which is genetically disrupted in *Inha*$-/-$ mice—contains specific Gata4 binding sites within its promoter, and has been shown to be directly activated by Gata4 in several *in vitro* studies (27). This transcriptional interaction is critical in the context of ovarian follicles, where Gata4 mediates a negative feedback loop that is initiated by gonadotropins and ends with the production of bioactive inhibin from follicular GC. Release of inhibin from the follicle serves two critical roles in the control of follicular growth: 1) endocrine negative feedback to the pituitary gonadotropes to down-reg-

ulate activin-stimulated production of FSH, and 2) autocrine/paracrine feedback to follicular GC to directly inhibit cellular growth (61). Whereas the first of these two roles has been well understood for some time, the second role is more recently inferred from mouse genetic models, which have shown that simple overexpression of FSH is not sufficient to stimulate gonadal tumorigenesis (49). Precisely how inhibin prevents cellular growth in the gonad is unclear at this time, although recent studies have indicated that inhibin can interfere with multiple TGFβ family signaling pathways, suggesting that the mechanism may be more complex than previously thought (62, 63). Whatever the mechanism may be, however, it is clear that inhibin is absolutely required to directly prevent overgrowth of follicular cells. The fact that *Inha*−/− adrenal tumor cells have essentially adopted the identity of follicular cells strongly suggests that the tumor suppressive mechanism inhibin employs is conserved between these tissues.

Within the adrenal cortex, Gata4 expression occurs only in the context of chronic gonadotropin signaling and is restricted to a narrow subset of cells. The spatial localization of Gata4 to the subcapsular region of the adrenal cortex by immunofluorescence is of particular interest because all cellular proliferation in the adrenal cortex is restricted to the same subcapsular zone. Because the adrenal cortex continuously renews all of its distinct zonal compartments from subcapsular tissue, it is commonly thought that this zone contains a distinct population of cells that are both pluripotent and self-renewing—key hallmarks of a somatic stem cell (58, 59). Although it is unclear from our data precisely what happens to Gata4-expressing cells in the wild-type adrenal cortex, their spatial restriction to the subcapsular zone suggests two possibilities: 1) These cells represent a transient cell type that is respecified as a normal adrenal cell upon migration out of the subcapsular zone, or 2) they are eliminated by apoptosis to prevent improper respecification of the adrenal cortex. We favor the latter hypothesis, as the analogous Gata4 expressing cells of the ovarian follicle are eventually eliminated by apoptosis during atresia or after complete luteinization, although more studies are needed to confirm this hypothesis.

In contrast to wild-type adrenal cells, *Inha*−/− adrenal cells that express Gata4 grow and proliferate outside of the subcapsular zone, forming adrenocortical tumors with a distinct ovarian identity. Because Gata4 expression and adrenal tumorigenesis parallel one another in *Inha*−/− mice, it seems likely that tumor cells initially arise as Gata4 expressing cells in the subcapsular zone—as in the normal adrenal—but fail to be eliminated or properly respecified upon exit from the subcapsular zone due to the absence of inhibin. The improper specification and survival of these cells therefore requires two convergent events in a single cell type: 1) chronic exposure to elevated levels of gonadotropins and 2) the loss of inhibin. Because the gonadal phenotype of adrenocortical tumor cells requires that they must arise from a developmentally plastic adrenocortical cell, these data suggest the exciting possibility that *Inha*−/− tumors are actually composed of improperly specified adrenocortical stem cells that proliferate continuously due to loss of growth control.

With respect to tumor phenotype, the *Inha*−/− mouse adrenocortical tumor model demonstrates striking similarities to other mouse models of adrenocortical tumorigenesis, including strains of mice that bear the $SF1^{S172}$ allele (*e.g.* CE, DBA/2J, C3H, and BALB/c) and inhibin-α promoter/ simian virus 40 T-antigen mice (Inhα/Tag) (32–34). All of these models share a common requirement for gonadectomy-induced tumor initiation, as well aberrant expression of Gata4 and production of sex steroids—specifically estrogens. A closer examination, however, shows that these adrenocortical tumor models can be further separated into two distinct groups, namely 1) those that bear the $SF1^{S172}$ allele and 2) the *Inha*−/− and Inhα/Tag mice. These groups differ in that only the *Inha*−/− and Inhα/Tag mice develop gonadal tumors in the absence of gonadectomy, whereas mice that have the $SF1^{S172}$ allele do not. In addition, mice bearing the $SF1^{S172}$ allele display a distinct pattern of adrenal tumor progression that is not reproduced in the *Inha*−/− and Inhα/Tag mice. In this pattern, adrenocortical tumors develop from a distinct population of subcapsular, nonsteroidogenic, spindle-shaped cells (A-cells) that subsequently give rise to large, lipid-filled steroidogenic cells (B-cells) that compose the actual tumor (33). Tumors in *Inha*−/− and Inhα/Tag mice appear to be histologically distinct from either A- or B-cells and arise as a single population of epitheloid cells that most resemble sex steroid-producing cells of the human fetal adrenal or adrenocortical blastomas (64). Whereas adrenocortical tumors in the *Inha*−/− model were not previously associated with the subcapsular zone of the adrenal, we provide evidence here that cells from this zone give rise to the tumors in *Inha*−/− mice based on their common expression of Gata4. These data support the notion that all sex-steroid producing adrenocortical tumors in the various models discussed above arise from a common pluripotent progenitor cell in the subcapsular region of the cortex, despite the divergence in molecular events that lead to loss of cellular growth regulation in these tumors.

We propose that the many similarities between *Inha*−/− and Inhα/Tag mice exist because loss of cellular growth regulation occurs at the same time during cellular differentiation in these two models. In both cases, adrenal tumor onset is stochastic after gonadectomy and likely requires the ability of adrenal stem/progenitor cells to respond to LH. Because LH has been previously reported to drive the expression of its own receptor (LHR), it is possible that responsiveness of adrenal stem/progenitor cells to LH increases progressively under chronic LH stimulation (57). As these cells gradually increase LHR expression,

they may eventually reach a threshold at which key transcriptional regulators of the gonadal fate—including Gata4—are induced. Although FSH is normally the key regulator of Gata4 expression, our data above show that LH can activate Gata4 expression in responsive cells, albeit more weakly. This assertion is supported by genetic data from *Inha*−/− mice crossed to FSH-deficient mice, which still develop tumors, although more slowly than *Inha*−/− mice (49). Once Gata4 is induced, one of its transcriptional targets is the inhibin α promoter. As discussed above, inhibin acts as a suppressor of cellular growth by both direct and indirect mechanisms, and in its absence, improperly specified adrenal stem/progenitor cells progress to form tumors. Although Inhα/Tag mice contain normal expression of inhibin α, they also begin to drive expression of tumorigenic T-antigen at the same time inhibin α expression is activated (34). Studies with cells derived from transgenic mice that express temperature-sensitive T-antigen (the H-2Kb-tsA58 immortomouse) show that activation of T-antigen essentially immortalizes cells in their present differentiated state, and therefore the Inhα/Tag adrenal cells become immortalized in the same state of abnormal differentiation as the cells that do not express inhibin (65). As such, we would suggest that the *Inha*−/− and Inhα/Tag mice represent complementary models of the same tumorigenic process, driven by disparate mechanisms of cellular growth control.

The suggestion that adrenocortical tumors in *Inha*−/− and Inhα/Tag mice arise from improperly specified adrenocortical stem/progenitor cells is also supported by analogous human conditions. Several cases of thecal metaplasia of the adrenal (adrenal luteomas) have been reported in post-menopausal women who, due to decreased ovarian estrogen output, have chronically elevated levels of gonadotropins (66–68). Similar to the tumors found in *Inha*−/− mice, these tumors depend on LH for growth and maintenance, and produce sex steroids rather than other adrenal hormones. In addition, a subset of human adrenocortical tumors has been reported to demonstrate a genetic switch from the predominant expression of Gata6, which is normally found in the cortex, to Gata4, which is normally absent from the cortex (29). Although this report did not correlate this switch to steroidogenic output or tumor phenotype, the presence of Gata4 strongly suggests that these tumors may have adopted a more gonadal fate. Other reports detail gonadotropin-responsive adrenocortical tumors that illicitly express LHR, although in these cases initiation of the tumor was not likely due to elevated gonadotropins and resulted in tumors that produced normal adrenal steroids and not estrogens (69–71).

Although the human cases noted above support the presence of a plastic cell population in the adrenal, case reports on patients affected with congenital adrenal hyperplasia suggest that than an analogous, pluripotent population of cells might also exist in the gonad. Patients suffering from congenital adrenal hyperplasia lack the ability to produce adrenal cortisol, and therefore have chronically elevated ACTH levels due to the lack of negative feedback regulation on pituitary corticotrope cells. Years of exposure to elevated levels of ACTH in these patients often results in the acquisition of hormonally active adrenal tissue in the gonad termed adrenal rests (72, 73). Although some studies have attributed the existence of these rests to adrenocortical metastasis, this seems unlikely because the adrenal overgrowth in these patients does not represent transformed cancer tissue, but rather benign hyperplasia. We suggest instead that the gonad also contains a population of plastic somatic cells, which are able to adopt an ectopic adrenocortical identity under chronic stimulation from pituitary ACTH. The possibility that both the adrenal and gonad contain populations of cells able to transdifferentiate into cells of the opposite tissue is particularly credible given their common developmental origin in the urogenital ridge. Formal identification and isolation of these hypothetical cells has not yet been accomplished, however, and will likely be an area of increasing interest given the therapeutic potential these cells hold for various degenerative conditions of the adrenal and gonad.

MATERIALS AND METHODS

Experimental Animals

All experiments involving animals were performed in accordance with institutionally approved and current animal care guidelines from University of Michigan Committee on Use and Care of Animals. Generation and genotyping of mice with a targeted deletion of the α-inhibin gene (*Inha*−/−) and mice harboring the bLH-βCTP transgene (*LH-CTP*) under the control of the bovine LH α-subunit promoter have been described previously (35, 56). Surgical gonadectomies were performed on mice at the age of 3–5 wk and mice were euthanized at 20 wk or more after gonadectomy as indicated. Only female mice were used for this study because gonadectomized female *Inha*−/− mice develop adrenocortical tumors more rapidly than their male counterparts.

RT-PCR and Real-Time PCR

Tissues were removed, cleaned, and snap frozen as above. Frozen tissues were lysed in Trizol reagent using an electric tissue homogenizer, and total RNA was collected according to the manufacturer's recommended protocol. In the case of cultured cells, the total cell pellet was washed once with PBS, and then directly harvested in Trizol reagent with vigorous pipetting to homogenize the cellular lysates. Total RNA was treated with deoxyribonuclease (Ambion, Austin, TX) to remove any residual genomic DNA, and was quantified by UV spectrometry. One microgram of total RNA was used to synthesize cDNA using the *iScript* kit (Bio-Rad, Hercules, CA) according to the manufacturer's recommended protocol. The final cDNA product was purified and eluted in 50 μl of Tris-EDTA buffer using PCR purification columns (QIAGEN, Valencia, CA). One microliter of this product was used as template for all subsequent semiquantitative PCRs. Primer sequences and thermocycling conditions for each gene are shown in Table 1. Amplified products were resolved on 1–2%

Table 1. Primer Sequences for Semiquantitative and Quantitative RT-PCR Analysis

Gene/Transcript Name	Abbreviation	Sense Primer	Antisense Primer	Annealing Temperature (C)
Inhibin-α	*Inha*	GGT GAA GGC TCT ATT CCT AGA TG	GAT GAT AGC ACC AGA AGA TCT AG	56
Steroidogenic factor-1	*Nr5a1*	GCA TTA CAC GTG CAC CGA G	GGC TCT AGT TGC AGC AGC TG	60
Glyceraldehyde phosphate dehydrogenase	*Gapdh*	GTG GCC CCT GGT GAG TGG	CAG GCT ATG GCT TGA TGA GG	60
LH receptor	*Lhr*	CTG CTG TGC TTT CAG GAA TTT GCC	ACC CTA AGG AAG GCA TAG CCC ATA	60
$P450_{c17\alpha[\rho]}$	*Cyp17*	CAA CTG CAG TGA TTG TCG GTC	GGT CTG TAT GGT AGT CAG TAT CG	55
Progesterone receptor	*Pr*	TGT CAC TAT GGC GTG CTT ACC TGT	TCA TGC CCT GCA TAG ACC ACA TCA	60
Se. translocation	*Set*	AAG CTG GAG GAC AAG TCG GC	CAA GCA GTG CAG ACA CTT GTG GAT	60
Relaxin-like Factor	*Rlf*	TGA CAG TCT GGG TTC TGT GAG TGT	GAA TTG CTG TGA GTT GGA GGC AGT	60
Aminopeptidase-N	*Anpep*	CAA GAA AGT GGT GGC TAC AAC GCA	ACC ATT GGC TGA GAC GGA GCT TAT	60
Relaxin	*Rlx*	AGT GGA TGG ACG GAT TCA TTC GGA	AAC ACA GGT GCG TGT TGT AGC TCT	64
FSH receptor	*Fshr*	AAG GTC TAT TCC CTG CCC AAC CAT	CTG GGT TCA TCA TCT ACG AGA GAG	57
$P450_{aromatase}$	*Cyp19*	GAC ACA TCA TGC TGG ACA CC	CAA AGC CAA AAG GCT GAA AG	55
Inhibin/activin-βA	*Inhba*	GAG ATC GTA GAG GCT GTC AAG	CAC TTC TGC ACG CTC CAC TAC	56
Inhibin/activin-βB	*Inhbb*	GGT GAA GAG ACA CAT CTT GAG C	CCT GTT CTT GGA AGT ACA CCT TG	56
Mullerian-inhibiting substance	*Amh*	GGT CTG AAC AGC TAT GAG TAT GCC	CTC TGC TTG GTT GAA GGG TTA AG	56
Estrogen receptor-β	*Esr2*	TGA ACT ACA GTG TTC CCA GCA GCA	ATA ATC ACT GCA GAC GGC GCA GAA	60
Oxytocin	*Oxt*	TTG CTG CCT GCT TGG CTT ACT	AAG GCA GGT AGT TCT CCT CCT GG	64
GATA binding protein-4	*Gata4*	ATT CTA GTT CTT CTC TGC CTC GT	TGC CTT CTG AGA AGT CAT CAA AC	60
GATA binding protein-6	*Gata6*	TGT ACC AGA CCC TCG CC	CAG ACC AGG GCC AGA GC	56
Melanocortin-2 receptor	*Mc2r*	TTC TCA GCA CCA CAA ATG ATT C	TCT TTG TGT GGA AGG ATC TGG	60
21-hydroxylase	*Cyp21a1*	CTG CTT CAC CAC CCT GAG A	AGC TGC ATT CGG TTC CTG T	60
Aldosterone synthase	*Cyp11b2*	AGA AGT TGC ACC AGG TGG AGA GTA	TGG TGA CAG CAC ATT TCG GTT CAG	60
GATA binding protein-4 (q-PCR)	*Gata4*	AGA TGG GAC GGG ACA CTA CCT	ACA GCG TGG TGG TGG TAG TCT	60
GATA binding protein-6 (q-PCR)	*Gata6*	CAA AAG CTT GCT CCG GTA ACA	GGT CGC TTG TGT AGA AGG AGA AG	60
Acidic ribosomal phosphoprotein (q-PCR)	*Arbp*	CGT GAT GCC CAG GGA AGA	TCC CAC AAT GAA GCA TTT TGG	60

q-PCR, Quantitative PCR.

agarose gels and detected with ethidium bromide staining on a UV lamp.

For quantitative, real-time PCR analysis of mRNA transcript abundance, PCRs were made up using a 2× SYBR Green PCR mastermix (Applied Biosystems, Foster City, CA) along with gene-specific primers, and thermocycling was performed in the *ABI 7300* thermocycler system (Applied Biosystems). Each quantitative measurement was normalized to Rox dye as an internal standard and performed in triplicate. Transcript abundance was normalized to the average cycle threshold value of mouse acidic ribosomal phosphoprotein (*Arbp*) for each sample. For mRNA quantitation directly from adrenal or tumor tissue, measurements were performed with an $n \geq 4$, with *n* being the number of individual tissue samples from different animals.

Immunoblotting

Ovaries and adrenals were removed, cleaned of surrounding adipose and connective tissue under a microscope in PBS, and immediately snap frozen in liquid nitrogen. Protein lysates were collected by briefly sonicating frozen tissues in lysis buffer (40 mM HEPES, 120 mM sodium chloride, 10 mM sodium pyrophosphate, 10 mM sodium glycerophosphate, 1 mM EDTA, 50 mM sodium fluoride, 0.5 mM sodium orthovanadate, 1% Triton X-100) containing protease inhibitor cocktail (Sigma, St. Louis, MO), followed by 1-h rotation at 4 C to solublize proteins. Soluble protein was collected from centrifuged total lysates and quantified by Bradford assay. SDS-PAGE was performed on 9–10% polyacrylamide Tris-glycine gels loaded with 20–40 μg of protein per sample, and separated proteins were transferred to nitrocellulose membranes using a semidry transfer unit. After transfer, membranes were blocked 1 h in 4% nonfat dry milk in TBS/0.1% Tween 20 (TBST), and then incubated overnight at 4 C with primary rabbit antibodies against Gata4 (1:1000; Santa Cruz Biotechnology, Inc., Santa Cruz, CA), Gata6 (1:1000; Santa Cruz), $P450_{aromatase}$ (1:1000; Abcam, Cambridge, MA), or Lhr (1:1000; Sigma), or primary mouse antibodies against β-actin (1:5000; Sigma) or Sry (Abcam; 1:1000). The next day, membranes were washed three times for 5–10 min in TBST, and then incubated with horseradish peroxidase-labeled goat antirabbit or antimouse secondary antibody for 1 h at room temperature. After three more washes in TBST, membranes were incubated for 2–5 min in West Dura ECL reagent (Pierce, Rockford, IL), and then exposed to film for detection.

Immunohistochemistry

Ovaries and adrenals were collected at indicated ages and treated as prepared for staining as above, except that final rehydration steps were performed in Tris-buffered saline (TBS, pH 7.5) instead of PBS. Antigen retrieval was performed by boiling rehydrated sections in 1 mM EDTA or 10

mM sodium citrate for 15 min, followed by two washes in TBS at room temperature. Tissue sections were then blocked in antibody diluent solution (TBS, 5% BSA, 0.1% Tween 20) containing 5% normal goat serum for 30 min, and incubated with anti-LHR (1:200; Sigma), anti-MIS (1:50; Abcam), anti-Gata4 or anti-Gata6 primary antibodies (1:50; Santa Cruz) overnight at 4 C in a hydration chamber. The next day, sections were washed three times for 5 min in TBS and then exposed to biotinylated secondary antibodies for 30 min at room temperature. When costaining for multiple epitopes was performed, direct fluorophore-coupled secondary antibodies were used. After three wash steps, sections were incubated with streptavidin-fluorescein isothiocyanate or streptavidin-rhodamine for 15 min at room temperature to fluorescently label stained tissue. Sections were finally washed three times more with TBS, nuclear costained with 4′,6-diamidino-2-phenylindole, and coverslipped with aqueous mounting media (Biomeda, Foster City, CA). Immunohistochemistry performed on adrenal sections was detected using the Vectastain ABC kit (Vector Laboratories, Burlingame, CA) and diaminobenzidine staining due to the high autofluorescent background of the adrenal cortex. Brightfield and fluorescent images were captured with an RT-Slider digital camera and Spot (version 4.6) software (Diagnostic Instruments, Sterling Heights, MI).

Y1 Cell Culture and Stable Transfection

Y1 mouse adrenocortical cells obtained from ATCC (Manassas, VA) were maintained in DMEM supplemented with 7.5% horse serum, 2.5% bovine serum, penicillin, streptomycin, and fungizone (Invitrogen, Carlsbad, CA). The coding sequence for mouse Gata4 was subcloned into pCDNA3, linearized by restriction digest, and transfected by calcium phosphate precipitation into Y1 cells. Stable transfectants were selected with G418 for 2–4 wk and then subcultured individually to 24-well plates. Stable monoclonal lines that were successfully expanded to 10-cm plates were screened for Gata4 expression by immunoblotting as described above. A single high expressing clone designated Y1-G4 was used for subsequent experiments to analyze the expression of various gonadal transcripts by RT-PCR as described above.

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