Impaired Follicle Development and Infertility in Female Mice Lacking Steroidogenic Factor 1 in Ovarian Granulosa Cells

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ABSTRACT

The nuclear receptor steroidogenic factor 1 (SF-1 [officially designated NR5A1]) is essential for fetal gonadal development, but its roles in postnatal ovarian function are less well defined. Herein, we have extended our analyses of knockout (KO) mice with markedly decreased SF-1 expression in granulosa cells. As described, these SF-1 KO mice had hypoplastic ovaries that contained a decreased number of follicles and lacked corpora lutea. In the present study, we showed that SF-1 KO mice exhibited abnormal estrous cycles, were infertile, and released significantly fewer oocytes in response to a standard superovulation regimen. Moreover, they had blunted induction of plasma estradiol in response to gonadotropins. The granulosa cell-specific SF-1 KO also significantly affected ovarian expression of putative SF-1 target genes. Consistent with their decreased follicle number, these mice had reduced ovarian expression of anti-müllerian hormone (Amh), which correlates with the reserve pool of ovarian follicles, as well as decreased gonadotropin-induced ovarian expression of aromatase (Cyp19a1) and cyclin D2 (CcnD2). In contrast, perhaps because of their abnormal cyclicity, SF-1 KO ovaries had higher basal expression of inhibin-alpha. They also had increased immunoreactivity for genes related to proliferation (CcnD2 and Mki67 [also known as Ki67]) and increased expression of Cdkn1b, also known as p27, which inhibits cyclin-dependent kinases, arguing for a role of SF-1 in granulosa cell proliferation. These findings demonstrate that SF-1 has a key role in female reproduction via essential actions in granulosa cells.

INTRODUCTION

Ovarian folliculogenesis is a complex process that starts during fetal life with the transformation of primordial germ cells into oocytes enclosed within primordial follicles. These primordial follicles sequentially give rise to primary, secondary, preantral, antral, and finally preovulatory (graafian) follicles that release their oocytes in response to the LH surge; the residual follicle cells then form the corpus luteum (CL). Throughout follicle maturation, sex steroids—whose biosynthesis is regulated by pituitary hormones (e.g., gonadotropins) and by growth factors that act in autocrine/paracrine fashion (e.g., insulin-like growth factors)—modulate the differentiation and growth of ovarian cells [1].

Steroidogenic factor 1 (SF-1 [officially designated NR5A1]) is a nuclear receptor that has essential roles in development of the fetal gonads in both sexes [2]. SF-1 acts at multiple levels of the hypothalamic-pituitary-steroidogenic organ axis to regulate expression of genes that are important in steroid biosynthesis and sex differentiation. Knockout (KO) mice lacking SF-1 exhibit multiple endocrine abnormalities, including adrenal and gonadal agenesis, impaired expression of pituitary gonadotropins, and structural abnormalities of the ventromedial hypothalamic nucleus [2].

In both rodent and human ovaries, SF-1 is expressed in multiple somatic cell lineages, including granulosa cells, theca/interstitial cells, and the CL [3–5]. Because multiple ovarian cell types express SF-1, it has been difficult to define specific roles of SF-1 in each compartment. Proposed SF-1 target genes in granulosa cells include the following: anti-müllerian hormone (Amh), which is involved in regulation of follicular growth and development; aromatase (Cyp19a1), which is essential for estrogen biosynthesis by granulosa cells; and inhibin-alpha (Inha), which is important in the control of ovarian hormonal homeostasis [6–9].

In humans, SF-1 mutations have been described in a number of 46,XY subjects who presented with impaired sex differentiation, with or without adrenal insufficiency [10–15]. However, only one 46,XX subject with a heterozygous loss-of-function mutation of SF-1 has been reported, to our knowledge; this individual had adrenal insufficiency but apparently normal ovaries [16]. Thus, the roles of SF-1 in female development and reproduction remain unclear. Moreover, the orphan nuclear receptor LRH-1 (officially designated NR5A2), which is more than 95% identical to SF-1 in its DNA-binding domain, also is expressed in rodent [3, 17] and human [18] granulosa cells, and studies [19, 20] have demonstrated that proposed ovarian SF-1 target genes also can be transactivated by LRH-1. These findings have raised questions about the relative roles of these two NR5A family members in the ovaries.

To explore specific roles of SF-1 within ovarian granulosa cells, we have used the Cre/IoxP strategy to inactivate a conditional SF-1 allele in a cell-specific manner. We previously reported that female mice with granulosa cell-specific KO of SF-1 had hypoplastic ovaries that lacked CLs and failed to exhibit postnatal sex differentiation [21]. In this article, we extend these studies to characterize the ovaries of these mice with respect to fertility, follicle number and distribution, and expression of markers of differentiated ovarian function.

MATERIALS AND METHODS

Ethics

All experiments involving mice were conducted under protocols approved by the Institutional Animal Care and Use Committee of University of Texas...
CHARACTERIZATION OF MICE LACKING SF-1 IN GRANULOSA CELLS

Generation of Granulosa Cell-Specific SF-1 KO Mice

The Amhr-2/Cre knockin allele [22] was used to drive Cre expression in granulosa cells of the ovary, resulting in mice with granulosa cell-specific KO of SF-1 as previously described [21]. To generate mice with targeted disruption of a conditional SF-1 allele in ovarian granulosa cells, mice heterozygous for the null SF-1 allele (N) and the Amhr-2/Cre knockin allele were crossed with mice homozygous for the conditional floxed SF-1 allele (F) [21]. Mice were maintained at standard temperature and light conditions (12L:12D) and were fed ad libitum with phytoestrogen-free mouse chow (D10001; Research Diets, Inc., New Brunswick, NJ) to reduce possible effects of exogenous estrogens on the ovarian phenotype.

Genotyping was performed by PCR analysis of genomic DNA from tail snip. The primers used to detect Amhr-2/Cre, to distinguish genetic sex, and to delineate the wild-type (WT), N, and F SF-1 alleles have been described [21]. The PCR products were resolved by agarose gel electrophoresis, and product sizes were determined relative to size markers. Mice that were heterozygous for the F allele (F+) and negative for the Amhr-2/Cre allele were designated WT, mice that carried the F and N SF-1 alleles (F/N) but lacked the Amhr-2/Cre allele were designated SF-1 heterozygous (SF-1 Het), and mice with N/SF-1 alleles and positive for the Amhr-2/Cre allele (Cre+/+) were considered KO.

Determination of Estrous Cycle and Fertility

To determine the stages of the estrous cycle, vaginal washes were collected for 21 consecutive days. Estrous phase was defined by the presence of epithelial and cornified enucleated cells within the specimen. To assess fertility, 8- to 10-wk-old SF-1 KO and control females were paired with one young fertile male mouse. Cages were monitored daily, and the number of litters and litter size were recorded.

Stimulation of Granulosa Cells with eCG

Female mice at age 8–12 wk were injected s.c. with 5 IU of eCG (Sigma Chemical Co., St. Louis, MO). Blood samples were drawn, and ovaries were harvested for histological examination or RNA extraction at 48 h after treatment.

Hormone Assays

Mice were anesthetized, and blood was collected by cardiac puncture or retro-orbital bleeding into plastic centrifuge tubes containing EDTA (Sarstedt, Numbrecht, Germany); after centrifugation, the plasma was stored at −20°C until assays were performed. Samples were obtained both basally and at 48 h after eCG injection. Hormone assays for estrogen, progesterone, and testosterone were performed at the Oregon National Primate Research Center, Oregon Health & Science University, Beaverton, on extracted serum samples. The intraassay coefficients of variation were less than 10% for all hormone assays. Assays for plasma LH and FSH were performed by the Ligand Assay and Analysis Core Laboratory, University of Virginia Health Science Center, Charlottesville.

Histology and Immunohistochemistry

After euthanasia, the ovaries and uterus were removed, cleaned of fat and mesentery, blotted on filter paper, and weighed. The tissues were then fixed overnight at 4°C in 4% paraformaldehyde and then embedded in paraffin. Sections (7 μm) were cut and stained by standard protocols with hematoxylin-eosin or used for immunohistochemical analyses.

Sections used for immunohistochemistry were washed with PBS and boiled for 20 min in 10 mM sodium citrate (pH 6.0) for antigen retrieval. Section were then incubated overnight at 4°C with primary antibodies, including the following at the indicated dilutions: goat anti-AMH (sc-6886, 1:5000 dilution; Santa Cruz Biochemicals, Santa Cruz, CA), rabbit anti-SF-1 (1:10,000 dilution; a gift from Professor Ken Morohoshi), rabbit anti-CYP11A1 (AB1244, 1:5000 dilution; Chemicon International, Inc., Temecula, CA), mouse anti-InHA (MCA951ST, 1:500 dilution; Serotec Ltd., Oxford, U.K.), rabbit anti-CYP19A1 (1:200 dilution; a gift from Professor Shinji Hayashi), rabbit anti-Ki67 (PRO229, 1:200 dilution; YLEM, Rome, Italy), rabbit anti-cyclin D2 (sc-1811, 1:200 dilution; Santa Cruz Biochemicals), and rabbit anti-p27 (sc-528, 1:2000 dilution; Santa Cruz Biochemicals). After washing, sections were incubated with secondary antibodies (Vectorstain Elite ABC kit; Vector, Burlingame, CA) for 1 h at room temperature, washed, and developed.

Negara controls were prepared by omitting the primary and/or secondary antibody. No staining above the background was detected. Sections from at least three mice in each genotype were processed in parallel for comparison of immunostaining, and immunohistochemical studies were repeated three to five times to ensure reproducibility of results.

RNA Isolation and Quantitative RT-PCR Analysis of Gene Expression

RNA was extracted from frozen ovaries using TRIzol reagent (Invitrogen, Carlsbad, CA) according to the manufacturer’s protocol. All RNA samples were purified using RNeasy Mini Kit (Qiagen, Studio City, CA). Reverse transcription was performed with 1 μg of RNA using 250 U of Superscript II reverse transcriptase (Invitrogen) and random primers (Invitrogen). Ten microliters was used for each reaction. Quantitative PCR (qPCR) was performed on the ABI Prism 7900HT sequence detection system (Applied Biosystems, Foster City, CA) using commercially purchased primers (Invitrogen) with SYBR Green master mix (Applied Biosystems). All primers sets were validated to ensure efficient amplification of a single product before use in assays. Relative mRNA levels were calculated using the comparative ΔΔCT method (Applied Biosystems user bulletin 2). Comparisons were made with samples from mice harvested from WT ovaries using cyclophilin as the reference gene; expression levels in basal samples from WT ovaries were set to 1.

Primer pairs used for qPCR assays were designed as follows: SF-1 (Nr5a1) (forward), 5′ CTTTATCGGCTGAGAATT 3′; SF-1 (Nr5a1) (reverse), 5′ CCAGGTCTCTGTCGTCGCA 3′; LHR-1 (Nr5a2) (forward), 5′ TGGGAAAG-GAGGAACTCTTT 3′; LHR-1 (Nr5a2) (reverse), 5′ CGGACTCAG-GAGGTTGGTTGAA 3′; Cyp19a1 (forward), 5′ GGGAAACATTGCCCTTCTC 3′; Cyp19a1 (reverse), 5′ CAACCTTT-CACCTGTCGAA 3′; Inha (forward), 5′ ATGTCTCCCGACG-TATCTCTTTCTC 3′; Inha (reverse), 5′ CGTATGTGGTGGATGCGCGGA 3′; Cyp11a1 (forward), 5′ CGTACACTTCCTCTATCGG 3′; Cyp11a1 (reverse), 5′ CTTTCCTTCCAGACCTGAC 3′; Cnd2 (forward), 5′ ACACCGCAACCTGTTGAAGC 3′; Cnd2 (reverse), 5′ GCAAGTTCC-CACCTGCTTTA 3′; Anh (forward), 5′ GCAGTTGCTAGTCCTACATC 3′; Anh (reverse), 5′ TCATCCGGTGAAACACC GG 3′; Fshr (forward), 5′ CCTTCAGAGTGATTTCCGT 3′; Fshr (reverse), 5′ AGCAATTGGCTGTTATGAGG 3′; Lhcg (forward) 5′ CTGAAAACTTGCCTCAG 3′; Lhcg (reverse) 5′ AATCTGAAATCCAGC-CACTG 3′; cyclophilin (Ppiα) (forward), 5′ TGGGAGACACCAAGAGA-GACA 3′; cyclophilin (Ppiα) (reverse), 5′ TGGCGAGTCGACAGATTGAT 3′.

Morphological Classification and Quantitation of Follicles

Ovaries were sectioned at 6 μm and stained with hematoxylin-eosin. The follicles in each ovary were counted serially in every tenth section through the entire ovary. Only healthy nonatretic follicles with visible oocyte nuclei were scored. Follicles were classified as primordial, primary, small preantral, or antral. Primordial follicles had a compact oocyte surrounded by a single layer of flattened (fusiform) granulosa cells, primary follicles had an enlarged oocyte surrounded by a single layer of cuboidal granulosa cells, and small preantral follicles had an enlarged oocyte surrounded by three or four layers of granulosa cells. Follicles with five or more layers of granulosa cells were considered antral follicles.

Oocyte Counts after Superovulation

Mice aged 8–12 wk were treated s.c. with 5 IU of eCG (Sigma Chemical Co.), followed 48 h later by 5 IU s.c. of hCG (Sigma Chemical Co.). Mice were killed at 16–18 h after hCG treatment, and the oocytes were harvested from the oviducts and incubated in PBS with 10% BSA. After treatment with hyaluronidase (300 μg/ml; Sigma Chemical Co.) for 10 min at 37°C, oocytes were collected and counted manually under a Nikon microscope (Nikon, Natick MA).

Statistical Analysis

Data are given as mean ± SEM. One-way ANOVA was applied to compare means among the groups. Appropriate post hoc, pairwise multiple comparisons were performed (least significant difference test) when applicable. The SPSS version 14.0 software package (SPSS Inc., Chicago, IL) was used for the statistical analysis, and P ≤ 0.05 was considered significant.

RESULTS

Disrupted Estrous Cycle in SF-1 KO Mice

Estrous cycles in adult female mice were assessed in vaginal smears collected for 21 consecutive days. The WT and SF-1...
Het mice had cyclical estrous cycles that lasted 4–5 days and progressed consecutively through the expected changes in cell content, with an estrous phase of approximately 2 days (Table 1). In contrast, the granulosa-cell-specific SF-1 KO mice had markedly disordered estrous cycles that ranged from irregularly prolonged cycles (50%) to complete acyclicity (50%).

Fertility and Oocyte Yield after Induced Ovulation

We also evaluated fertility by mating adult female granulosa-cell-specific SF-1 KO mice with young WT male mice. In longer than 1 yr of controlled observation, no SF-1 KO mice delivered any pups; this contrasts with WT mice, which averaged 9 ± 1 litters per year, with 6.3 ± 1.8 pups per litter. However, we found in the uterus of one SF-1 KO mice a single embryo at early stage of development.

To determine if the infertility was due to a primary ovarian defect or was secondary to changes at other levels of the hypothalamic-pituitary-gonadal axis, we used a standard superovulation protocol and counted the number of oocytes released into the oviducts. As shown in Figure 1, WT females ovulated an average of 35.3 ± 2.9 oocytes per female, while SF-1 Het females ovulated an average of 23.3 ± 5.1 oocytes per female; a marked decrease was seen in the number of oocytes collected from mice with granulosa-cell-specific KO of SF-1 (1.0 ± 0.7 oocytes per female). Most (80%) SF-1 KO females had no oocytes retrieved from the oviducts; two mice released three and four oocytes each, some of which were in immature stages of development (P < 0.001 compared with WT and SF-1 Het mice). These data show that SF-1 KO mice exhibit an impaired or absent response to gonadotropin-induced superovulation. The number of oocytes retrieved also differed significantly between SF-1 Het and WT mice (P < 0.01), suggesting that SF-1 haploinsufficiency diminishes oocyte production to some degree without impairing fertility.

**Ovarian Weight and Morphology**

We next examined ovarian size and histology. Ovaries from SF-1 KO mice (1.79 ± 0.15 mg, n = 12) weighed significantly less than those from SF-1 Het mice (5.05 ± 0.50 mg, n = 6; P < 0.01) and from WT mice (7.39 ± 0.52 mg, n = 12; P < 0.001). The ovaries of adult WT and SF-1 Het mice contained follicles at all stages of development, including CLs (Fig. 2 and data not shown). In contrast and consistent with our previous study [21], sections of SF-1 KO ovaries showed the expected range of follicles up to the preovulatory stage but had no CL, suggesting a defect in the final steps of folliculogenesis and/or ovulation. Moreover, careful quantitation in serial sections of adult SF-1 KO ovaries revealed a reduction in the total number of follicles.

**FIG. 1.** Oocyte yields after gonadotropin-induced ovulation in 8- to 12-wk-old WT, SF-1 Het, and SF-1 KO mice. All mice were treated with eCG (5 IU), followed 48 h later with hCG (5 IU). Oocytes were harvested from the oviducts and counted at approximately 16 h after hCG treatment. Each data point represents the oocyte yield of an individual mouse of the respective genotypes. The horizontal line represents the mean value of each group. The numbers of mice studied for each genotype were WT (n = 8), SF-1 Het (n = 6), and SF-1 KO (n = 7).

**TABLE 1.** Vaginal cytology.*

| Genotype (n) | Estrous cycles over 21 days | Estrous phase length (days) |
|--------------|-----------------------------|----------------------------|
| WT (6)       | 4.33 ± 0.21                 | 2.17 ± 0.17                |
| SF-1 Het (6) | 4.50 ± 0.67                 | 1.70 ± 0.33                |
| SF-1 KO (8)  | 0.75 ± 0.31<sup>a</sup>     | 1.25 ± 0.18                |

* Values are expressed as means ± SEM. <sup>a</sup> The values correspond only to the SF-1 KO females that exhibited an estrous phase.

**TABLE 2.** Follicle counts in 21-day-old mice.*

| Genotype (n) | Primordial | Primary | Small preantral | Antral | No. of growing follicles | No. of all follicles |
|--------------|------------|---------|-----------------|--------|--------------------------|---------------------|
| WT (n = 3)   | 233 ± 6.36 | 259 ± 45.5 | 42.0 ± 9.45<sup>b</sup> | 16.3 ± 8.45 | 0.60 ± 0.60<sup>b</sup> | 333 ± 17.9          |
| SF-1 Het (n = 3) | 44.0 ± 9.02 | 35.0 ± 7.77 | 5.60 ± 1.33<sup>b</sup> | 4.73 16.3 | 6.60 ± 1.66<sup>b</sup> | 333 ± 17.9          |
| SF-1 KO (n = 5) | 33.7 ± 1.20 | 41.7 ± 4.37 | 6.36 259 | 14.7 12.8 | 45.5 42.0 | 333 ± 17.9 |

* Values are expressed as means ± SEM; n values correspond to one ovary per genotype. <sup>a</sup> P < 0.05 between SF-1 KO and WT. <sup>b</sup> P < 0.05 for comparison between SF-1 KO and SF-1 Het.
decreased reserve of follicles before puberty that then persists into adulthood.

Abnormal Ovarian Steroidogenesis and Gonadotropin Levels in SF-1 KO Mice

We next evaluated plasma levels of ovarian steroid hormones and gonadotropins (Table 3), including E2, progesterone, testosterone, FSH, and LH. Unexpectedly, the basal levels of the ovarian steroid hormones in the granulosa cell-specific SF-1 KO mice did not differ significantly from WT and SF-1 Het levels at the diestrous phase. The normal testosterone level was consistent with the known role of theca cells in ovarian androgen production; these cells do not express Amhr-2/Cre, and SF-1 should remain functionally intact. We further hypothesized that the apparently normal circulating E2 levels reflected not only ovarian E2 production but also E2 produced by other sites of aromatase expression such as adipocytes. Although the LH levels did not differ significantly among the three groups, plasma FSH was increased significantly in SF-1 KO mice relative to WT and SF-1 Het mice ($P < 0.01$).

In an effort to uncover defects in ovarian E2 production, we measured the steroidogenic response to eCG stimulation. Female mice at age 8–12 wk were treated with eCG, and plasma samples were collected 48 h later. As summarized in Table 3, plasma E2 levels were stimulated by eCG in WT mice, whereas plasma progesterone levels were unaffected. Similar responses were seen in SF-1 Het mice. In contrast, the eCG-stimulated increase in E2 levels was markedly blunted in mice with granulosa cell-specific KO of SF-1 ($P < 0.001$ vs. WT and SF-1 Het mice). This relative defect in ovarian estrogen

| Hormones          | Basal        | 48 h eCG     |
|-------------------|--------------|--------------|
|                   | WT SF-1 Het  | SF-1 KO      |
| Estradiol (pg/ml) | 15.2 ± 1.62  | 12.9 ± 1.95  |
|                   | 14.8 ± 1.59  | 14.8 ± 1.59  |
|                   | 39.5 ± 4.70  | 32.2 ± 2.83  |
|                   | 15.8 ± 2.86  | 14.3 ± 1.71  |
| Progesterone (ng/ml) | 6.02 ± 0.96 | 6.02 ± 1.64  |
|                   | 5.29 ± 1.61  | 6.02 ± 1.64  |
|                   | 5.29 ± 1.61  | 6.02 ± 1.64  |
|                   | 13.3 ± 1.03  | 13.3 ± 1.03  |
|                   | 13.4 ± 1.69  | 13.4 ± 1.69  |
|                   | 13.4 ± 1.69  | 13.4 ± 1.69  |
| Testosterone (ng/ml) | 12.5 ± 2.66 | 12.5 ± 2.66  |
|                   | 13.3 ± 1.03  | 13.3 ± 1.03  |
|                   | 13.4 ± 1.69  | 13.4 ± 1.69  |
|                   | 13.4 ± 1.69  | 13.4 ± 1.69  |
| LH (ng/ml)        | 0.56 ± 0.39  | 0.29 ± 0.13  |
|                   | 0.72 ± 0.28  | 0.72 ± 0.28  |
|                   | 0.72 ± 0.28  | 0.72 ± 0.28  |
|                   | 0.72 ± 0.28  | 0.72 ± 0.28  |
| FSH (ng/ml)       | 3.57 ± 0.60  | 6.06 ± 1.96  |
|                   | 18.9 ± 5.02  | 18.9 ± 5.02  |
|                   | 18.9 ± 5.02  | 18.9 ± 5.02  |

* Values are expressed as means ± SEM and the numbers of samples are indicated in parentheses.

$^a P < 0.05$ for comparison between SF-1 KO and WT.

$^b P < 0.05$ for comparison between SF-1 KO and SF-1 Het.
biosynthesis in these mice is consistent with their increased basal levels of FSH; both of these factors suggest a primary gonadal defect secondary to the absence of SF-1 that eventuates in premature ovarian failure. However, because large antral and preovulatory follicles are the major source of E2, we cannot exclude a contribution of the decreased number of these follicles toward the impaired eCG-stimulated synthesis of E2.

**Uterine Morphology in SF-1 KO Mice**

As a biomarker of E2 levels, we examined uterine weight and histology. At age 8–10 wk, uterine weights in WT mice (151 ± 10 mg, n = 12) and SF-1 Het mice (155 ± 12 mg, n = 6) did not differ significantly; mice of both genotypes had significantly increased uterine weights relative to SF-1 KO mice (48.8 ± 7.0 mg, n = 12; P < 0.001 vs. both groups). The uterine histology of WT and SF-1 Het mice revealed thick myometrial and stromal layers, ample endometrial glands, and multiple layers of luminal epithelial cells (Fig. 3, B and C). In contrast, the uterine histology of SF-1 KO mice showed reductions in epithelial, myometrial, and stromal layers. In particular, the glandular elements of the endometrial layer were less complex, with only a few scattered glands detected in the stroma. These findings collectively indicate that ovarian E2 production is inadequate to stimulate normal uterine differentiation in the granulosa cell-specific SF-1 KO mice.

**Quantitative PCR Analysis of Basal and eCG-Induced Gene Expression in WT, SF-1 Het, and Granulosa Cell-Specific SF-1 KO Mice**

The FSH-induced differentiation of ovarian granulosa cells during folliculogenesis activates a complex pattern of gene expression. To examine the effect of the granulosa cell-specific SF-1 KO during these processes and to permit more precise quantitation, we used qPCR assays to evaluate gene expression in the ovaries of 8- to 12-wk-old WT, SF-1 Het, and SF-1 KO mice before and after stimulation with eCG (Fig. 4).

Expression levels for many of the genes analyzed did not differ significantly among these mice. However, several differences are notable. Basal expression of SF-1 was decreased in SF-1 KO samples relative to WT samples (P < 0.05). This is consistent with the immunohistochemical analyses shown in Figure 2 and the fact that our inactivation strategy targets SF-1 expression in a significant population of ovarian cells. Following gonadotropin stimulation, SF-1 expression in RNA from whole ovaries of WT and SF-1 KO mice did not differ significantly, perhaps reflecting gonadotropin-induced SF-1 expression in the theca cells. In contrast, basal expression of LRH-1 in SF-1 KO ovaries was increased significantly relative to WT levels (P ≤ 0.05) and nonsignificantly relative to SF-1 Het levels; similar responses to gonadotropins were seen in each group. This finding may reflect a compensatory mechanism within granulosa cells to maintain function in the absence of SF-1.

The gene whose expression was most severely impaired by the SF-1 KO was anti-müllerian hormone (Amh). This well-characterized SF-1 target gene normally is expressed exclusively in granulosa cells, where it is proposed to have an important role in the recruitment of ovarian follicles [23]. SF-1 KO ovaries had significantly decreased basal and gonadotropin-stimulated expression of Amh, strongly supporting the role of SF-1 in Amh expression in vivo.

Another gene whose expression was affected by the SF-1 KO is inhibin alpha (Inha). This target gene of SF-1 is induced by FSH and has been implicated as a negative regulator of stromal proliferation in the ovary [24]. As expected, both WT and SF-1 Het mice showed significant induction of Inha expression after eCG treatment. In contrast, SF-1 KO mice showed increased basal expression of Inha but—unlike WT and SF-1 Het mice—did not show increased expression in
This increased basal expression of Inha, coupled with high FSH levels, suggests that the negative regulation of FSH by ovarian inhibin is lost in SF-1 KO mice, a typical phenomenon of aging.

The expression of other genes in SF-1 KO mice differed significantly only after gonadotropin stimulation. For example, gonadotropin-stimulated expression of aromatase (Cyp19a1), which is selectively expressed in granulosa cells [7], was significantly decreased in SF-1 KO mice relative to SF-1 Het mice (P = 0.05) and was slightly reduced relative to WT mice. This is consistent with the proposed key role of SF-1 in aromatase expression and the impaired E2 production in granulosa cell-specific SF-1 KO mice in response to gonadotropins (Table 3). The expression of Cyp11a1 was increased in the SF-1 KO mice after eCG injection compared with WT and SF-1 Het mice. These data were in agreement with the results of the immunohistochemical analyses (discussed herein). The expressions of FSH receptor (Fshr) and LH receptor (Lhcgr) were also analyzed [25, 26]. No significant differences in Fshr expression were shown among the three groups. Instead, significantly higher expression of Lhcgr at the basal level (P = 0.02 vs. WT mice and P = 0.014 vs. SF-1 Het mice) and exaggerated increase after eCG injection compared with controls (P = 0.047 vs. WT mice) were seen in SF-1 KO mice. Similarly, the expression of cyclin D2, a marker of granulosa cell proliferation that normally is induced by FSH [27, 28], was significantly lower in SF-1 KO mice only after eCG treatment (P = 0.02 vs. WT mice). These data suggest underlying impairment in the proliferation of SF-1-deficient ovarian granulosa cells following gonadotropin stimulation (Fig. 4).

Collectively, these results suggest that the ovaries in granulosa cell-specific SF-1 KO mice do not respond properly to eCG stimulation both in terms of expression of certain critical genes (e.g., aromatase, Amh, and cyclin D2) and in terms of estrogen biosynthesis. This inability presumably reflects a primary ovarian defect that results from the absence of SF-1 expression in the granulosa cells.

**Immunohistochemical Analyses in Granulosa Cell-Specific SF-1 KO Mice**

We next used immunohistochemical analyses to evaluate ovarian expression of other genes of interest. In WT mice, AMH was expressed at high levels in granulosa cells of primary, secondary, and small preantral follicles, while ovaries in SF-1 KO mice contained only a few primary and secondary follicles that expressed AMH (Fig. 5). Also, CYP19A1 levels
in WT mice seemed to be higher in granulosa cells of growing follicles compared with those in SF-1 KO mice. Although WT mice expressed CYP11A1 only in the theca cells and the CLs, ovaries from SF-1 KO mice also contained some large follicles that ectopically expressed CYP11A1 in granulosa cells before and after hormone treatment. This expression may reflect efforts to compensate for the absence of estrogen production in SF-1 KO mice and suggests that CYP11A1 transcription can be induced by SF-1-independent mechanisms under certain circumstances. In contrast to these other putative SF-1 target genes, the qPCR data suggested that basal expression of Inha was actually increased in SF-1 KO mice (Fig. 4). Consistent with this, SF-1 KO mice seemed to have higher expression of INHA in the immunohistochemical analyses (Fig. 5). Thus, these studies largely confirmed the conclusions drawn from the qPCR analyses of gene expression.
Evidence for Decreased Granulosa Cell Proliferation in Granulosa Cell-Specific SF-1 KO Mice

The reduced number of follicles and ovarian size in the granulosa cell-specific SF-1 KO mice suggested that these mice might have defects in cell proliferation in the ovaries. Therefore, we extended our immunohistochemistry analyses to examine the expression of CCND2 (cyclin D2), MKI67 (Ki67), and CDKN1B (p27). As shown in Figure 6, granulosa cell-specific KO of SF-1 was associated with considerably decreased expression of cyclin D2 and MKI67, while there was increased expression of CDKN1B in the granulosa cells of growing follicles. These findings suggest that impaired cell proliferation is an important component of ovarian hypoplasia seen in these mice.

DISCUSSION

Using the Cre/loxP system, we studied the roles of SF-1 in ovarian physiology in vivo, focusing specifically on its functions in granulosa cells. Consistent with our initial description [21], the granulosa cell-specific inactivation of SF-1 caused marked postnatal gonadal defects, including ovarian hypoplasia, altered uterine differentiation, and defects in both estrogen biosynthesis and fertility. Although these findings do not exclude important roles of LRH-1 in ovarian function, they define essential roles of SF-1 in granulosa cell function that cannot be replaced by LRH-1. In most respects, SF-1 Het mice exhibited no reproductive phenotype; they had mild ovarian hypoplasia with normal ovarian function and approximately 65% of the normal number of oocytes harvested after superovulation. Moreover, the gene expression profiles in SF-1 Het mice generally paralleled those in WT controls both basally and after eCG treatment, indicating that the presence of one SF-1 allele suffices for largely normal ovarian function.

SF-1 KO mice exhibited markedly reduced ovarian size, with a significantly decreased number of growing follicles and the absence of CLs (Fig. 2), suggesting that they are impaired in terminal stages of follicle differentiation and/or ovulation. A careful inspection of ovarian histology in young adult SF-1 KO mice showed a paucity of primary and secondary follicles, which led us to evaluate the number of follicles just before the initiation of puberty. At age 21 days, SF-1 KO mice already had a decreased number of follicles (Table 2). These data again suggest that SF-1 has important roles in ovarian development and follicular maturation. This defect was not apparent during fetal development of SF-1 KO ovaries up to Embryonic Stage 16.5 [21].

We also evaluated ovarian function by determining the plasma levels of ovarian steroid hormones both basally and after stimulation with gonadotropins (Table 3). The basal E2 level in SF-1 KO mice did not differ significantly from the levels in WT and SF-1 Het mice, but marked impairment was noted in the response to eCG. Moreover, basal plasma FSH revealed a significantly increased concentration in SF-1 KO mice compared with mice of other genotypes. These data together indicate that ovarian estrogen production is defective in mice with granulosa cell-specific KO of SF-1. Although developing ovarian follicles are the main source of E2, other tissues (e.g., adipose tissue) can produce estrogen. The normal
basal E2 level in SF-1 KO mice may derive from these other sources.

Studies [29, 30] have demonstrated that AMH—which is produced by granulosa cells of the growing follicles—suppresses development of the primordial follicles. Thus, the FSH-dependent recruitment of small antral follicles in Amh KO mice was increased, and premature exhaustion of the primordial follicle reserve occurred [31]. SF-1 has a key role in Amh transcription [32]; consistent with this, Amh transcripts were decreased in our qPCR analyses, and only a few follicles contained immunoreactive AMH (Fig. 5). Therefore, the decreased levels of AMH in the granulosa cell-specific SF-1 KO mice may contribute to the diminished follicle number observed.

Similarly, the reduced induction of Cyp19a1 expression in ovarian granulosa cells seen in the qPCR and immunohistochemical analyses likely contributes to the deficient E2 production. The hypoplastic uteri with impaired glandular differentiation were consistent with estrogen deficiency in the granulosa cell-specific SF-1 KO mice. We cannot exclude the possibility that these ovaries had few follicles and therefore had lower capability to produce E2 after gonadotropin induction.

Studies [7, 33] in female aromatase KO mice showed severely impaired E2 synthesis and follicle development, resulting in defective ovulation. The phenotype described herein resembles several aspects of aromatase KO mice but apparently was less pronounced in terms of hormone deficiencies and gonadal morphology. The attenuated phenotype in mice with granulosa cell-specific KO of SF-1 likely arises because they lack SF-1 and CYP19A1 only in ovarian granulosa cells, whereas aromatase KO mice lack CYP19A1 expression in all tissues. By avoiding any exogenous source of estrogen with a soy-free diet [34], we showed that our mice can still produce E2 but not at levels sufficient to induce ovulation. Moreover, we found that the ovarian hemorrhagic cysts previously described [21] occurred in only a few of these mice fed a phytoestrogen-free diet.

As a proposed target gene of SF-1 in ovarian cells, we also examined inhibin-alpha expression in the granulosa cell-specific SF-1 KO mice. This gene is transcriptionally activated by FSH in ovarian granulosa cells during follicular growth [24]. Transient transfection assays using the inhibin-alpha promoter showed that both SF-1 and LRH-1 can increase inhibin-alpha promoter activity [20, 35]. A dynamic association/dissociation model has been proposed to explain the interactions of NR5A members and the inhibin-alpha promoter: at baseline, SF-1 is bound to the inhibin-alpha promoter, but FSH induces LRH-1 to replace SF-1 [20]. In our investigations, increased basal expression of Inha was detected in SF-1 KO mice, localizing to granulosa and theca/interstitial cells. Therefore, we hypothesize that LRH-1 can replace SF-1 to induce basal Inha expression in granulosa cells. However, LRH-1 alone was unable to increase Inha expression after gonadotropin stimulation, as revealed by the reduced Inha transcript levels in SF-1 KO mice. These findings, together with the elevated FSH concentration, suggest that adult ovarian granulosa cells of SF-1 KO mice are unable to respond appropriately in terms of gene expression and steroid biosynthesis. A similar defect is seen in aging mice where the negative regulation of FSH by ovarian inhibin is lost, supporting an accelerated ovarian senescence phenotype in these mice. Chronically elevated expression of inhibin-alpha was reported in ovaries of acyclic aging female rats, and increased expression of INHA has been associated with lack of ovulation and loss of estrous cyclicity due to loss of estrogen secretion [36]. The extent to which altered expression of Inha contributes to the phenotype seen in the granulosa cell-specific SF-1 KO mice remains to be determined.

The impaired induction of cyclin D2 transcripts by eCG, the lower immunoreactivity for cyclin D2 and MKI67, and the intense signal of CDKN1B suggest that SF-1 KO mice have an underlying defect in granulosa cell proliferation and follicle growth [27, 37]. Thus, changes in proliferation may be a major factor in their phenotype. We previously showed that male mice with Leydig cell-specific SF-1 KO had impaired somatic cell proliferation during fetal development [21], and evidence from other studies [38, 39] strongly implicates SF-1 as a key regulator of adrenocortical proliferation. By analogy, it is likely that SF-1 regulates other genes involved in granulosa cell proliferation; the identification of these genes will be an important target for future studies.

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