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Ovarian Pathology and High Incidence of Sex Cord Tumors in Follitropin Receptor Knockout (FORKO) Mice

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In this investigation we describe our observations of the status of the aging ovary in mice with disruption of the receptor for FSH. Knockout mice at 3–5 months of age are acyclic and sterile, with very small, underdeveloped ovaries. Thus, they exhibit hypergonadotropic-hypogonadism with high levels of circulating FSH similar to the postmenopausal state in women. By 12 months more than 92% of these animals developed various kinds of ovarian pathology, including neoplasms of sex cord-stromal type as well as cysts. Interestingly, the majority of tumors were located in the right ovary, with the contralateral ovary remaining unaffected but atrophic. The ovary from heterozygotes also showed pathology after 15 months. None of the age-matched wild-type mice that remained fertile developed any sign of ovarian tumors. Circulating LH and FSH levels were increased in follitropin receptor knockout mice and remained severalfold higher in tumor-bearing animals. The histological appearances of ovarian tumors were similar to the pathology observed in some types of sex cord-stromal neoplasms in women. The tumor burden caused weight loss and cachexia in follitropin receptor knockout mice. Based on these characteristics as well as the high incidence of ovarian pathology in the aging mutant, we propose that the loss of the FSH receptor signaling mechanisms predispose the ovary to molecular and structural changes leading to tumor formation. Hence, in the intact and fertile animal, FSH receptor signaling offers a protective mechanism that is lost upon reproductive senescence (menopause in women). Further studies are warranted in this genetic model to explore the molecular changes underlying the development of ovarian neoplasia. (*Endocrinology* 142: 3673–3684, 2001)

OVARIAN CANCER IS the most lethal class of neoplasia among all gynecological diseases (1). It is the fourth leading cause of cancer among North American women after lung, breast, and colon cancers. It is also the most common cause of death from gynecological malignancies (52%). More frequently, ovarian cancer exists as germ cell and epithelial cell tumors and with low incidence of tumors from the sex cord-stromal compartment (2). Sex cord-stromal tumors made up of granulosa/thecal cells, Sertoli cells, or Leydig cells represent approximately 10% of all ovarian neoplasms and affect all age groups (3). Sex cord-stromal tumors although less frequent compared with other ovarian neoplasms, are of interest partly because of their hormonal nature. Some of these tumors behave as low grade malignancies in women, with long-term survival, whereas others become large due to their very aggressive nature and subsequently rupture causing extraovarian spread (2, 3).

In general, the only risk factor to be clearly recognized for ovarian cancer is the inherited germline mutations in the BRCA1 or BRCA2 genes, but uninterrupted ovulation has been postulated to be a contributing factor (4). The molecular changes that induce various ovarian tumors are ill defined at present. There is also no reliable and general diagnostic screening method available for ovarian cancer. Some studies have linked granulosa cell tumors of the ovary to the actions of pituitary gonadotropins, FSH, and LH. A causal connection is often suspected, because as the concentrations of gonadotropins rise in the early menopausal years the incidence of ovarian cancer also increases, to become quite prominent during postmenopause (5). Some investigations in animals have shown a relation between chronic and abnormal gonadotropin exposure and the development of granulosa cell tumors. For instance, targeted overexpression of the LH β-subunit in transgenic mice causing high circulating levels of LH at an early age results in polycystic ovaries and ovarian tumors inducing infertility in female mice (6). Mice deficient in the inhibin α-subunit gene, a partner in the heterodimeric ovarian protein inhibin belonging to the TGFβ family, also develop granulosa and/or Sertoli cell tumors (7). Besides a possible tumor suppressor role for inhibin α, the accompanying rise in activin levels that elevate FSH in the circulation may contribute to tumor formation. Whether the propensity of the ovary to progress toward a cancerous state increases after stimulation by gonadotropins remains highly controversial. Thus, arguments that incessant ovulation as induced during infertility treatments may or may not predispose the ovaries to cancer are mired in extreme controversy (8, 9). Some reports that found no abnormal elevation of serum LH and FSH levels of granulosa tumor patients (10–12) at the time of diagnosis suggest that the hypothesis of excess gonadotropins driving tumorigenesis might be simplistic.

Upon binding to structurally related, but distinct, receptors in the ovary, the two gonadotropins FSH and LH cause a cascade of events leading to stimulation of gonadal growth,

Abbreviations: FORKO, Follitropin receptor knockout; 3βHSD, 3β-hydroxysteroid dehydrogenase; MIS, Müllerian inhibiting substance.

differentiation, and steroidogenesis (13, 14). As ovarian function requires a perfect interaction between FSH and its receptor, disruption in any manner is likely to cause various reproductive deficits. For instance, an inactivating point mutation in extracellular domain of the FSH receptor gene converting Ala^{189} to Val causes absolute infertility due to primary amenorrhea in Finnish women (15). This mutation, however, is not present in infertile women of other backgrounds. Recently a case of compound heterozygotic mutation of the FSH receptor gene provoking a partial loss of function of the receptor has been described in a woman with secondary amenorrhea (16).

Given these considerations, it was logical to assume that the FSH receptor might be a good candidate for activating mutations that could alter the growth and function of ovarian tumors. Reports of finding binding sites for FSH, albeit low in cells derived from human granulosa cell and thecal cell tumors (17, 18), have been interpreted as suggesting that FSH may influence the growth and activity of sex cord-stromal tumors. Although several studies have looked for mutations of the FSH receptor that may lead to tumor initiation, none has been found (19). The single report by Gromoll *et al.* (20) that the $Asp^{567}Gly$ substitution in a man led to constitutive activation also remains in question (21).

To understand the biology of FSH receptor-dependent processes in the ovary, we have produced mice lacking FSH receptor(s) (22) by using homologous recombination. The resulting mutant female or FORKO mice are sterile despite very high levels of FSH (22). In continuing our investigations on these mutants, we have now found for the first time that essentially all mutant females analyzed after 12 months of age demonstrated macro- or microscopic evidence of ovarian tumors with various pathologies. This strongly suggests that removal of the FSH receptor induces major perturbations in the gonadotropin signal transduction pathway(s) and internal milieu of the ovary leading to the development of tumors in mutants.

Based on the high incidence of tumor appearance in the ovary of aging FORKO mice, we propose that the lack of the beneficial effects of FSH receptor signaling in association with other confounding factors might predispose the ovarian structures to assume a variety of pathologies. In fact, in the dysfunctional ovary after menopause in women there is a natural loss of functional FSH receptor. We believe that a better understanding of the molecular changes that underlie ovarian pathogenesis during aging could be gained by studying models such as the FORKO mouse.

Materials and Methods

Animals

The studies described in this report were performed according to accepted and approved guidelines of the institutional animal care committee. The FORKO mice were established as previously described (22). Animals were housed under controlled temperature and constant light (12 h of light, 12 h of darkness), with food and water provided *ad libitum*. The female mice used in this experiment were derived by breeding F_2 generation heterozygotes on the sv129 background. They were genotyped by PCR according to methods we have described recently (23, 24). Age-matched mutants and wild-type mice were compared in each experiment. Aging heterozygous females were also included in some analyses.

Histological analysis

Animals were exsanguinated during ether anesthesia, and all internal organs were examined for visual signs of abnormalities. The ovaries were cleaned of extraneous tissue for weighing and then fixed in 10% formalin at room temperature for 16 h. All tissues were embedded in paraffin, sectioned, and stained with hematoxylin and eosin according to standard histological procedures that we have used in previous studies (24). Classification of ovarian pathology including tumor type was performed according to the descriptions provided in the atlas on basic histopathology (25) and pathology of the female genital tract (26).

Immunohistochemistry

Immunohistochemistry of the following antigens was performed according to established procedure (24) using antibodies from different sources. Thin sections were processed for immunostaining using the ImmunoCruz Staining System (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) according to the supplier's instructions. The 3β-hydroxysteroid dehydrogenase (3βHSD) antibody was provided by Dr. A. H. Payne (Stanford University, Palo Alto, CA). This rabbit antiserum produced against the recombinant mouse 3βHSD1 protein was used at a dilution of 1:750. Dr. Payne also supplied the antibody to porcine P450c17 enzyme that was used at 1:600 dilution. Purified antibody against human placental aromatase (used at 1:500 dilution) was a gift from Dr. N. Harada (Toyoake, Japan). An antibody to the N-terminal peptide of inhibin α-subunit was given to us by Dr. B. D. Schanbacher (formerly of USDA, Clay Center, NE). This reacts with all forms of inhibin and was used at a dilution of 1:1000. Goat antibodies to the transcription factor GATA 4 and Müllerian inhibiting substance (MIS) obtained from Santa Cruz Biotechnology, Inc., were used at 1:200 dilution. The corresponding rabbit second antibody was used for subsequent processing. Antigen retrieval procedure was performed for localization of inhibin, GATA 4, and MIS.

Plasma and pituitary gonadotropins

Plasma LH and FSH were measured by RIA using kits provided by the National Hormone and Pituitary Program (courtesy of Dr. A. F. Parlow, University of California-Los Angeles, Torrance, CA). As none of the FORKO females show cycles, plasma samples were collected at random, but usually in the morning before noon. Although the wild-type mice continue to cycle, we did not make any distinction with regard to the stage of the cycle for collection of samples. These were also obtained randomly. For estimating pituitary hormone content by the same RIA, each frozen tissue was homogenized with an extraction buffer (0.2 M glycine-NaOH, pH 9.0) containing 5 mM benzamidine hydrochloride. The supernatant obtained after centrifugation at $13{,}000 \times g$ was frozen until used for RIA. The inter- and intraassay variations were 5–8% for both RIAs. The hormone contents are expressed in terms of the respective rat pituitary hormone standards provided in the kit.

Steroid hormone profile

The two major ovarian steroid hormones (estradiol and testosterone) in serum samples of 12-month-old females (wild-type and FORKO) were estimated by solid phase RIAs (24) using commercially available kits (Diagnostic Products, Los Angeles, CA). Where pertinent, statistical significance was calculated for all comparisons by performing one-way ANOVA.

Results

Ovarian steroid hormones

We have previously reported that the circulating levels of these hormones in infertile FORKO mice at 4–5 months of age were completely different from those in wild-type mice (24). Plasma estrogen levels were very low, accounting for greater than 95% reduction. However, in marked contrast to estrogen, testosterone that acts as the precursor for estrogen synthesis had increased about 10-fold, indicating that failure

of estrogen production was probably due to a lack of aromatase activation (24). Plasma estrogen levels measured again by the same technique in 3- and 12- to 15-month-old FORKO females were significantly lower than those in the corresponding +/+ females (Table 1). Circulating testosterone levels were higher in the 1-yr-old mutants (~2-fold) compared with those in the +/+ females of comparable age; however, they were not as exaggerated as in the 3-month-old (Table 1) or 4- to 5-month-old FORKO mice (24).

Plasma and pituitary gonadotropins

Circulating levels of both FSH and LH were elevated in FORKO mice at all the three ages (Fig. 1). At 12+ months of age the plasma levels (per ml) of both FSH and LH in FORKO mice had increased about 3- to 4-fold compared to those in age-matched wild-type females. Although plasma LH in 12- to 15-month-old FORKO females declined significantly ($P < 0.01$) from the 7 month level, it was still higher ($P < 0.05$) than that in the corresponding age group +/+ mice. The pituitary FSH content was higher at 3 months and continuously increased with age in FORKO mice as well as in +/+ animals. The highest FSH levels were found in pituitary of 12- to 15-month-old mice. The pituitary content of LH that became different in FORKO only at the seventh month remained high at 1 yr.

TABLE 1. Plasma levels of estradiol and testosterone in FORKO female mice

Genotype	Age (months)	Estradiol (pg/ml)	Testosterone (ng/dl)
Wild-type	3	17.7 ± 3.4 (8)	2.0 ± 0.2 (6)
	7	14.6 ± 2.4 (7)	6.84 ± 0.7 (6)
	12–15	5.6 ± 1.8 (10)	14.3 ± 1.6 (6)
FORKO	3	1.5 ± 0.3[b] (6)	27.3 ± 2.2[b] (6)
	7	1.4 ± 0.2[b] (8)	32.3 ± 4.3[b] (6)
	12–15	1.9 ± 0.5[a] (20)	30.3 ± 2.8[a] (22)

Randomly cycling adult mice (3, 7, and 12–15 months of age) were used. The levels of hormones determined by respective RIAs are shown. Values are the mean ± SEM. The number of animals is in *parentheses*.

[a] Means significantly different from the control (wild-type) group by one-way ANOVA, $P < 0.05$.

[b] Means significantly different from the control (wild-type) group by one-way ANOVA, $P < 0.005$.

Ovarian morphology

We examined the histology of the reproductive tracts of 25 FORKO and 15 wild-type littermates, ranging from 12–15 months of age. Remarkably, this careful examination of FORKO ovaries revealed ovarian pathology of varying degree. Some typical examples are described in this report. Between 12 and 15 months of age nearly every null mutant (23 of 25) developed ovarian tumors, and 2 females had large ovarian cysts. Thus, in total, 92% of the 1-yr or older knockout females displayed significant ovarian pathology. On the contrary, none of wild-type age-matched littermates (n = 15) showed any sign of ovarian tumorigenesis at this age. Interestingly, we did not observe morphological or histological signs of ovarian tumors in FORKO or wild-type female mice between 2–5 months of age. However, intermediate stages between 7–11 months were not examined in the current study.

Between 3–5 months of age all null female mutants examined demonstrated the characteristic underdeveloped phenotype, with follicular development blocked before antrum formation, without any corpora lutea. Thus, there was

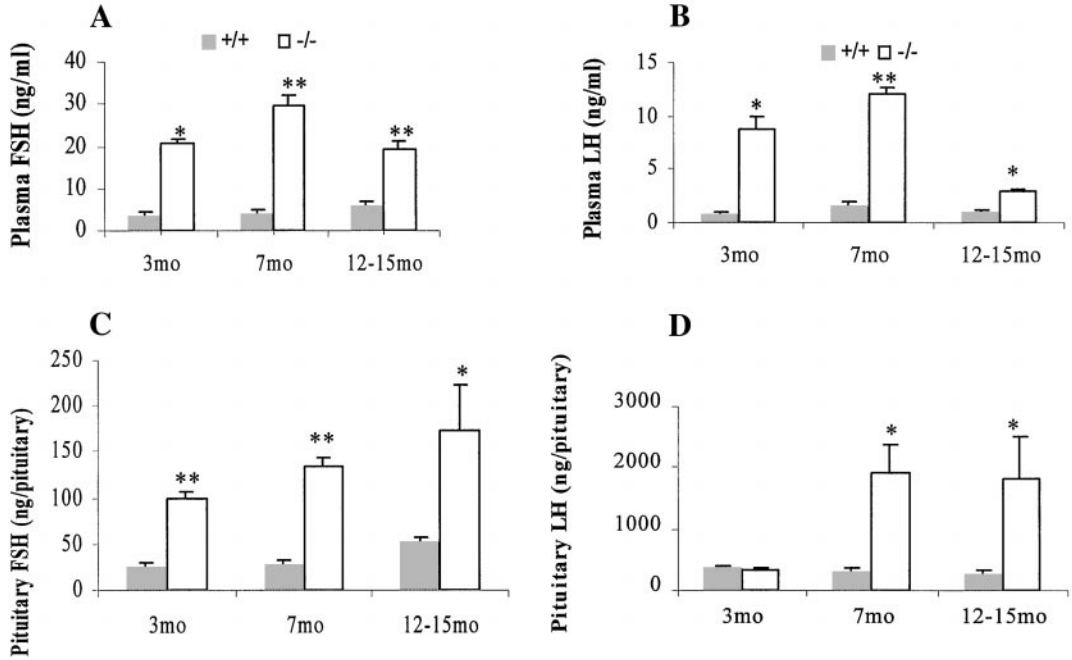
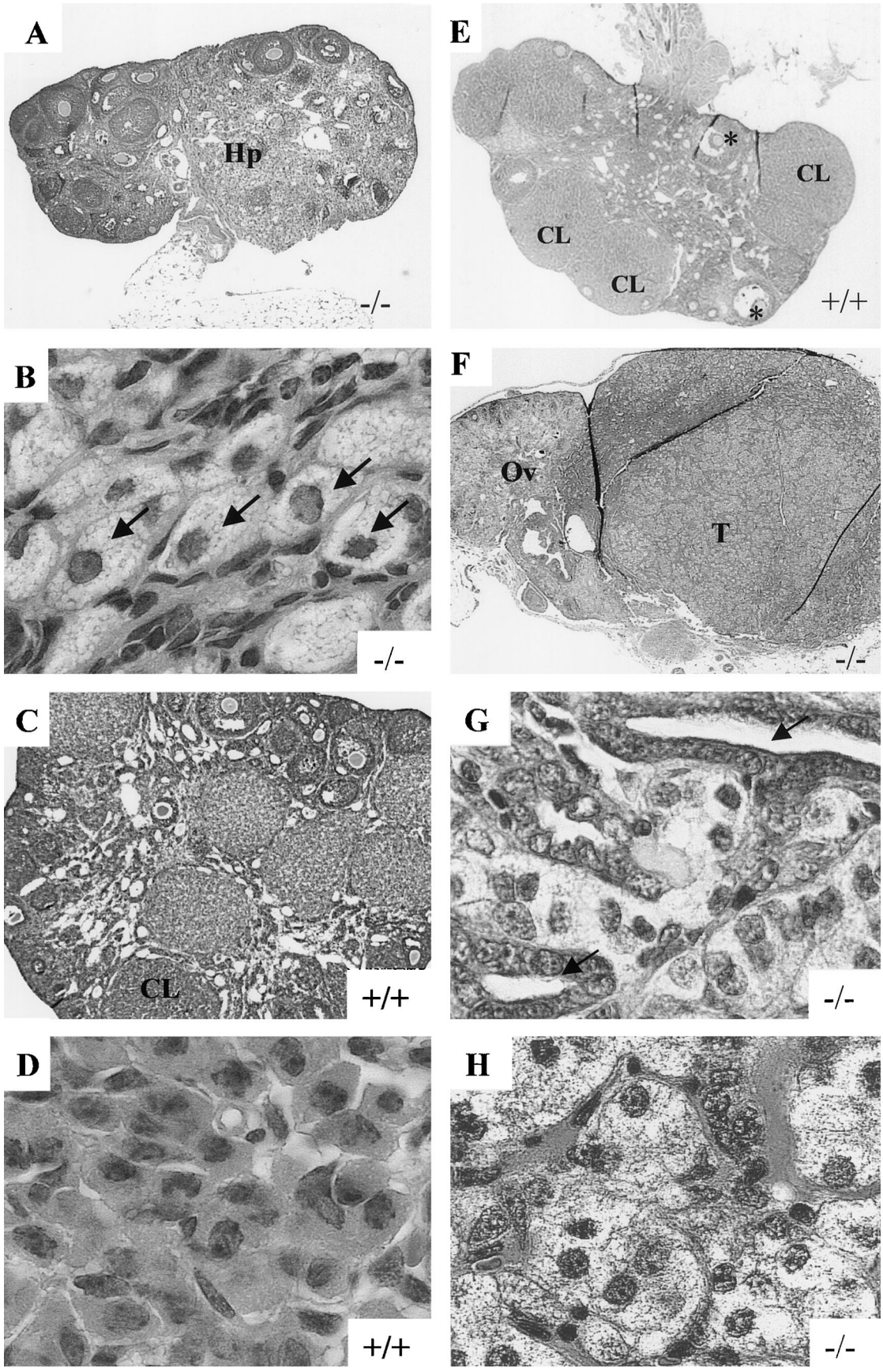


FIG. 1. Gonadotropins in wild-type and FORKO female mice. Plasma levels and pituitary content of FSH and LH at three different ages [indicated in months (mo) on the *x*-axis] were determined by RIA as described in *Materials and Methods*. FSH and LH values are expressed as nanogram equivalents of the respective mouse reference preparations AFP-5308D and AFP-5306A. There were 6–16 samples for each group and genotype. *, $P < 0.05$; **, $P < 0.005$.



no ovulation in FORKO mice, in agreement with our previous reports (22, 24). In those few follicles that had any semblance of antrum there was atresia (not shown). Hyperplasia was noted in the interstitial tissue in the middle of the FORKO ovary (Fig. 2A). Another interesting characteristic of the 3- to 5-month-old FORKO ovary was the presence of large islands of polygonal cells with a round central nucleus and abundant cytoplasm-containing lipid droplets (Fig. 2B). These cells with hyperplastic appearance have the features of luteinized cells that are steroidogenically active, consistent with the high testosterone values in the circulation at 3–5 months (24) (Table 1). In contrast to the FORKO mice, 3- to 5-month-old wild-type mice do not have such exaggerated luteinization (Fig. 2, C and D).

The majority of the examined tumors (17 of 23) were characterized by their unilateral appearance and were confined exclusively to the right ovary (74%). In addition, cysts that developed in 2 mice were confined to the right ovaries. There were 4 exceptions when only the left ovary had tumors (17%). Thus, in 91% of the mutants neoplasia was confined to a single ovary. In only 2 animals were both ovaries affected (9%). In most cases the size of the tumor overshadowed the remaining small part of the affected ovary. The mean weight of the right ovary with tumor or cyst was 36 ± 11.4 mg. The mean weight of the left affected ovary was 24.7 ± 9.1 mg. The nonaffected ovary in tumor-bearing FORKO mice was very small, weighing only 2.3 ± 1.5 mg. Thus, the affected ovary was enlarged 12–16 times ($P < 0.01$) in about 7–10 months. The mean ovarian weight (total) of both nonaffected ovaries in 12- to 15-month-old FORKO females was 3.4 mg. This is significantly smaller ($P < 0.005$) compared with the ovarian weight (13.13 ± 0.9 mg) in wild-type littermates at the age of 12–15 months.

Morphological examination also revealed that ovaries containing tumors had solid or solid and cystic consistency and yellowish-white color. Two FORKO females had huge balloon-like right ovaries filled with a large amount of liquid. Ovaries from two null mutants remained atrophic and did not have any macroscopic signs of tumorigenesis or cysts.

Pathology and histological appearance

The histological examination of FORKO ovaries revealed the presence of tumors in 23 mutants. Among these we were able to identify sex cord-stromal tumors of the Sertoli-Leydig cell type based on their appearance (Fig. 2, E–H). In most cases, as shown in the example (Fig. 2F), the small ovarian component was overshadowed by the tumor. These tumors showed a variable proportion of Sertoli and Leydig type cells. Some were characterized by an equal admixture of tubular structures (Sertoli cells) and vacuolated plump stromal cells (Leydig cells; Fig. 2G). Others were almost devoid of the tubular element, consisting almost entirely of clusters of Leydig type cells (Fig. 2H). Occasionally there was a focal microfollicular change in the neoplastic cells, reminiscent of granulosa cells, a feature sometimes described in Sertoli-Leydig cell tumors of the human ovary (26).

The cysts encountered in a few of the ovaries were lined by a columnar epithelium with occasional vacuoles, with subjacent stroma containing vacuolated plump cells (Fig. 3, A and B). The epithelial cells ranged from a single cell-lining population to a stratified cell population thrown into papillary folds. One cyst showed a transition from a simple columnar epithelium to a stratified epithelium with a papillary configuration (Fig. 3, C and D). It is interesting to note that these cysts resemble the retiform type of Sertoli-Leydig cell tumors used in describing the pathology of some ovarian cancer (2).

By 12 months of age, nonaffected ovaries of null mutants contained neither oocytes nor any clearly recognizable follicles (Fig. 3, E and F). All of these structures had disappeared. However, there was widespread luteinization of the interstitial tissue with the characteristic indication of steroidogenically active cells. We also observed multiple areas of calcification within the nonaffected ovaries, possibly caused by destruction of follicular structures by this age (Fig. 3F).

Body weight and cachexia

As reported recently (24) all FORKO females develop obese tendencies at 3 months of age. This condition increased progressively (Fig. 4) up to about 10 months, after which there was a steady decline. At the time of death, the tumor-bearing animals were definitely smaller, with a reduction in body weight by about 50% compared with their peak at 10 months. By about 15 months they were leaner than wild-type littermates, exhibiting signs of cachexia that is normally associated with advanced stages of neoplasia. In addition, there were signs of splenomegaly and anemia, as indicated by the pale extremities of the mutants. These changes were not evident in any of the control wild-type mice.

Hyperplasia in heterozygous mice

As we recently found that heterozygous FORKO mutants undergo early reproductive senescence (24), we were

FIG. 2. Ovarian histology of 3-month-old female mice (A–D) and appearance of sex cord-stromal tumors in older females (E–H). A, Section of a 3-month-old FORKO ovary showing preantral follicles with hyperplasia (Hp) of interstitial tissue in the middle of the ovary. Note the absence of corpus luteum (magnification, ×25). B, Stroma in the FORKO ovary enclosing large islands of polygonal cells containing lipid droplets (*arrows*; magnification, ×315). C, A wild-type ovary at 3 months containing follicles at different stages of development and corpora lutea (CL; magnification, ×25). D, A high power magnification of the stroma of a representative wild-type female (magnification, ×315). E–H, Ovaries from FORKO females between 12 and 15 months of age displaying sex cord-stromal tumors. E, Wild-type ovary from 13-month-old female mouse containing antral follicles (*asterisk*) and corpora lutea (CL; magnification, ×12.5). F, A representative ovary from null mutant showing Sertoli-Leydig cell tumor (T). Note that the remnant of the ovary (Ov) is very small, with no identifiable follicular structure compared with the large tumor (magnification, ×12.5). G, The common feature of Sertoli cell tumor is the occurrence of tubular-like structures (*arrows*) and vacuolated plump stromal, Leydig-like cells. Note hollow rounded or ovoid tubules (*arrows*) lined by a single layer of cuboidal or low columnar cells with clear cytoplasm and basal nuclei. The tubules are separated by Leydig type cells in the intervening stroma (magnification, ×500). H, Sertoli-Leydig cell tumor lacking the tubular element and consisting completely of clusters of Leydig cells with abundant cytoplasm (magnification, ×500).

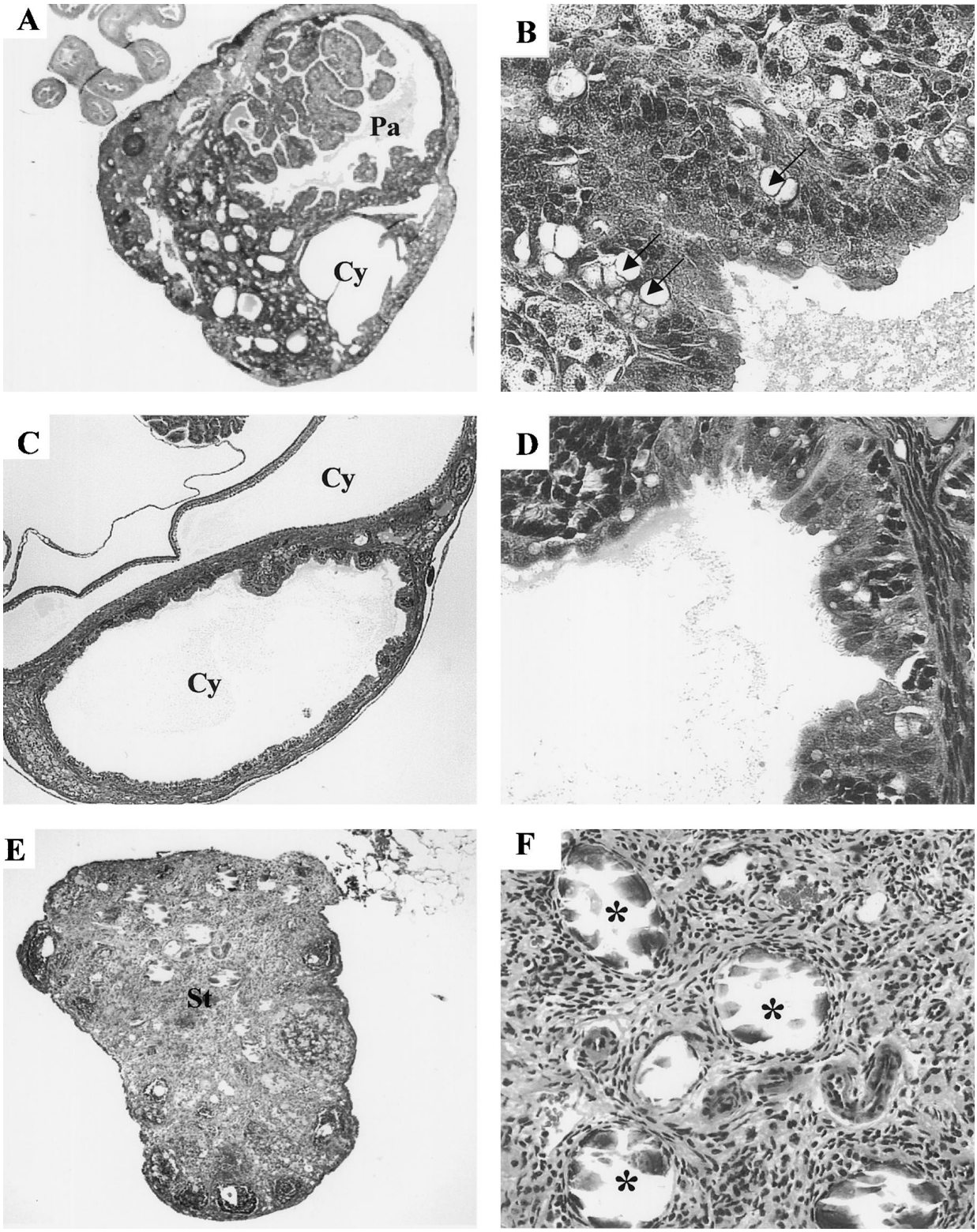


FIG. 3. Occurrence of the retiform type of Sertoli-Leydig cell tumors in ovaries of affected aging FORKO mice (12–15 months) and the structure of the nonaffected ovaries from aging FORKO mice. A, Ovarian section showing a retiform tumor containing large intracystic papillae (Pa; magnification, ×6.25). B, Papillary-like structures revealed at higher magnification. Note the accumulation of fat droplets intra- and extracellularly (*arrows*) with subjacent stroma containing vacuolated plump cells (magnification, ×200). C, Example of a cyst (Cy) showing the transition from a simple columnar epithelium to a stratified epithelium with a papillary configuration (magnification, ×25). D, Example of the epithelium lining the cystic space being stratified (magnification, ×200). E, Section showing complete loss of follicular structures with multiple regions of calcification (St, stroma; magnification, ×25). F, High power magnification showing areas of calcium deposition in the FORKO ovary (*asterisks*; magnification, ×100).

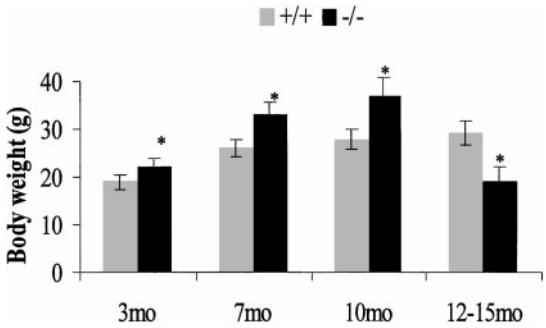


FIG. 4. Difference in body weights at different ages. Wild-type and FORKO female mice were weighed (mean ± SEM) at 3, 7, 10, and 12–15 months of age. The following numbers of mice were used: 3 months, 21 wild-type and 27 FORKO mice; 7 months, 19 wild-type and 21 FORKO mice; 10 months, 20 wild-type and 24 FORKO mice; and 12–15 months, 15 wild-type and 25 FORKO mice. *, $P < 0.05$, statistically significant difference from wild-type females.

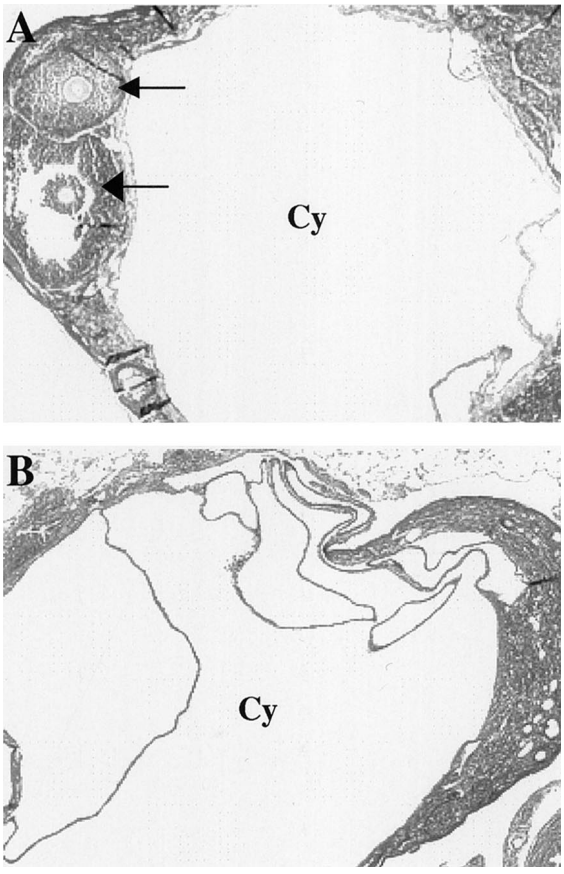


FIG. 5. Pathology of the ovary in aging +/− females. Ovaries from 15-month-old heterozygous mice characterized by the presence of large cysts. A and B, Ovaries obtained from two virgin heterozygotes containing one huge cyst filled with serous fluid. Note that the few follicles (*arrows*) that are present are squeezed out close to the periphery of the ovarian structure (magnification, ×6.25).

also interested in examining these animals at 15 months of age. In the limited number of animals (n = 12) that we studied, there was also evidence of ovarian pathology. This included cysts (Fig. 5) many months after ovulation had ceased, indicating that loss of one FSH receptor allele might also create imbalances sufficient to produce pathological changes upon aging.

Immunohistochemistry

Based on the idea that Sertoli-Leydig cell tumors are often androgen producing, we became interested in assessing their steroidogenic capacity by focusing on some of the enzymes involved in the process. To assess this as well as other parameters in a large number of sections and relate the changes to cellular morphology, we stained them for a number of antigens as functional markers. These included the steroidogenic enzymes 3βHSD, aromatase, and P450c17, inhibin (as recognized by the anti inhibin α peptide antiserum), the transcription factor GATA 4, and MIS. Representative staining patterns of ovaries of aging FORKO and wild-type mice are shown in Figs. 6 and 7. There was an intense staining for 3βHSD in cells resembling Leydig cells in neoplastic ovaries containing Sertoli-Leydig cell tumors (*arrows* in Fig. 6B) compared with that in +/+ ovary of the same age (Fig. 6A). Similarly, P450c17 was localized in these cells (Fig. 6D). In contrast to this, it may be noted that the tubular structures lined with Sertoli-like cells did not express 3βHSD (Fig. 6B, *asterisks*) or the P450c17 (Fig. 6D, *asterisks*), indicating differences in steroidogenic activities of these two types of cells in the tumors. Aromatase staining was weak in all FORKO ovaries (Fig. 6F) compared with that in wild-type mice (Fig. 6E), suggesting that expression of this enzyme had decreased in the aging FORKO ovary. Interestingly, this is in contrast to the situation previously reported for the 3- to 5-month-old FORKO ovary. At this age there was no difference in mRNA (RT-PCR) or protein, as verified by Western blot analysis of the ovaries of FORKO and wild-type mice (24). In ovaries collected at random from 1-yr-old wild-type females there were many antral follicles with granulosa cells that intensely stained for the inhibin α peptide that was tested in our study (Fig. 6G). In older FORKO ovaries (Fig. 6H) there were no such structures, although some weakly stained cells were present. When we compared the total content of aromatase and inhibin by Western blots, both were drastically reduced in the aging FORKO ovaries (data not shown).

MIS was weakly expressed in some granulosa cells of a small number (one or two) of follicles in 1-yr-old +/+ females (Fig. 7, A and B), whereas the expression of this protein was much stronger in the aging FORKO ovary. In the 1-yr-old atrophic FORKO ovary that had not yet developed tumors there were multiple foci of MIS expression. From the example shown (Fig. 7, C and D), intense and localized expression of MIS in what was previously a degenerating follicle is clearly evident. In the FORKO ovary with tumor (Fig. 7, E and F) there was a more generalized expression of MIS. Like MIS, the transcription factor GATA 4 was also selectively expressed only in the granulosa cells of a few large follicles (Fig. 7, G and H) of the 1-yr-old +/+ ovary. In Fig. 7, I and J, are depicted two examples of the altered expression of this factor in the aging FORKO ovaries with tumors. In both cases expression was confined to Sertoli-type cells that were present in the tubular-like structures.

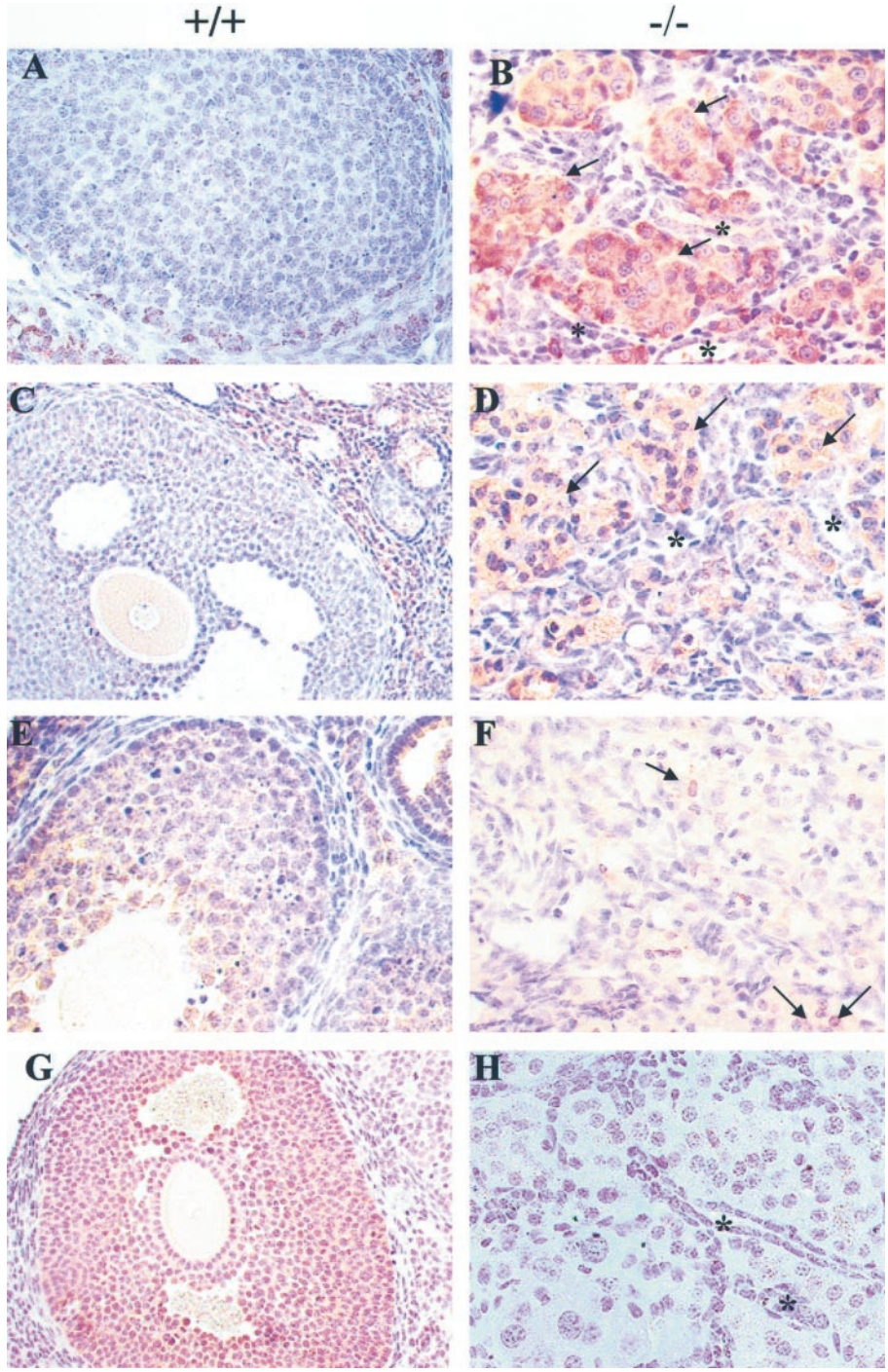


FIG. 6. Expression of steroidogenic enzymes and inhibin α-subunit in ovaries from 12- to 15-month-old wild-type (+/+) and FORKO (−/−) mice. A, Immunoexpression of 3βHSD in wild-type ovary was confined to the stroma and thecal cells surrounding the follicles. The granulosa cell compartment was immunonegative (×200). B, In ovaries from FORKO females, the presence of strongly 3βHSD-immunopositive Leydig-like cells (*arrows*) was observed in ovary containing Sertoli-Leydig cell tumor. Note that the tubule-like structures (denoted by *asterisks*) are completely immunonegative (×200). For clarity we have not identified all positive and negative cells. C, Expression of 17α-hydroxylase was found only in the thecal layer and interstitial stroma of the +/+ ovary, with no immunopositive cells observed in the granulosa cells (×100). D, In the −/− ovary, groups of 17α-hydroxylase-immunopositive cells are observed throughout the tumorigenic tissue confined to Leydig-like cells (*arrows*). The tubule-like structures (*asterisks*) were completely immunonegative (×200). Only some of these typical structures are identified. E, In ovaries from wild-type females, expression of aromatase was confined to granulosa cell compartment of big follicles (×200). F, In the aged FORKO (−/−) ovary, very few cells randomly distributed within the ovarian tissue (*arrows*) expressed aromatase (×200). G, Inhibin α-subunit was most abundant in large antral follicles of wild-type ovary (×100). H, Sertoli-Leydig tumors in mutant ovaries are immunonegative for inhibin α-subunit (×200).

Discussion

Among all gynecological cancers, tumors of ovarian origin account for approximately 25% (1) of the cases. The poor prognosis in ovarian cancer is mainly due to the lack of sensitive tests for detecting the early stage of the disease. Quite often it causes only vague symptoms that go unnoticed until the tumor is advanced, and the prognosis is poor. At the time of diagnosis about 70% of the patients have cancer cells that have already spread to the pelvic and abdominal viscera or developed distant metastasis (stage III/IV) (4). Although the incidence of sex cord-stromal tumors in humans is only 10% of all ovarian cancers, their ability to metastasize to extrapelvic organs and their tendency for late recurrence after surgery makes them very important for study, especially in animal models.

To investigate the consequences of the disruption of the FSH receptor signaling on reproductive function as well as long-term implications for changes in gonadal structures, we generated homozygous mutant mice that lack the FSH receptor. As noted previously (22), these animals are infertile

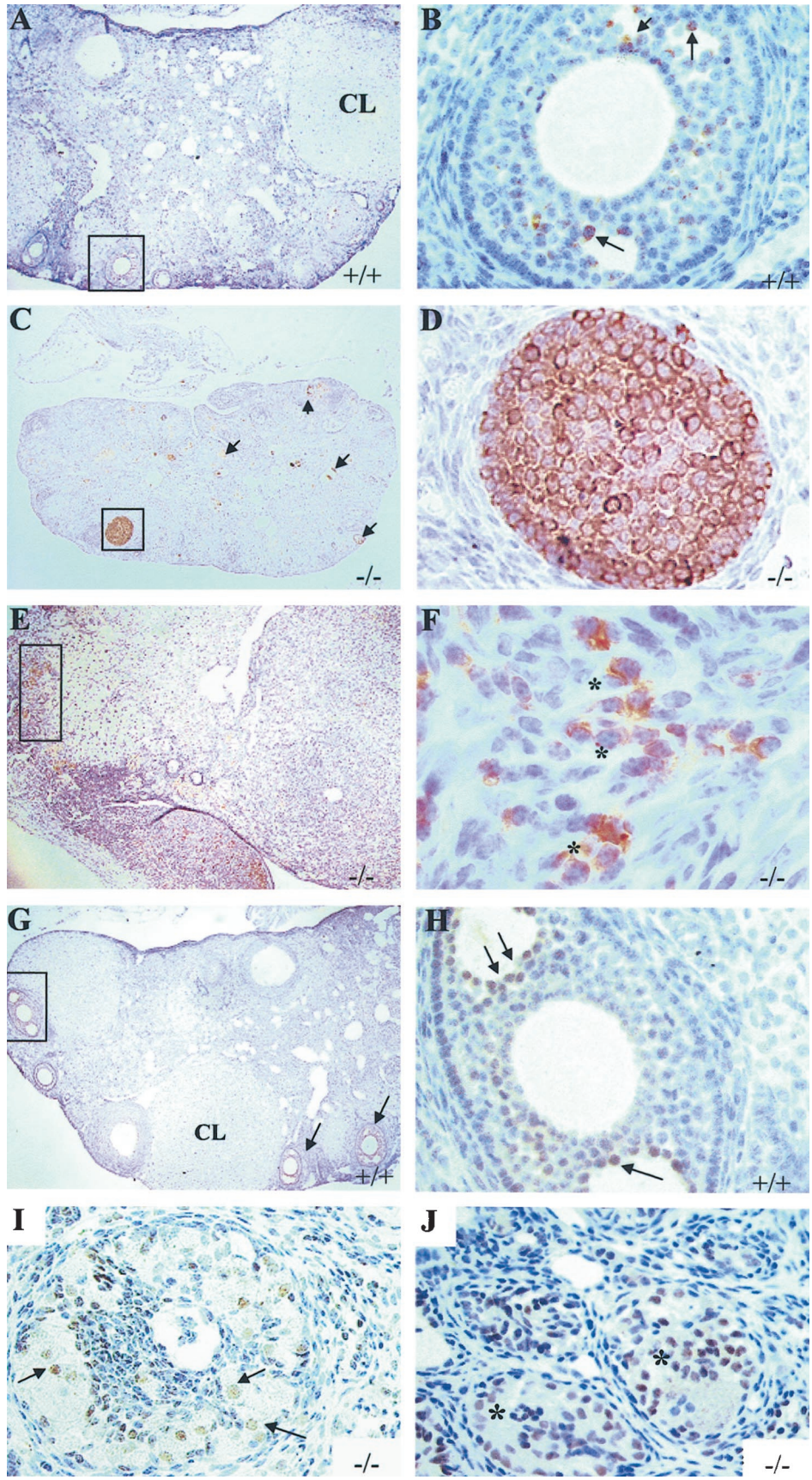


FIG. 7. Expression of MIS (A–F) and GATA-4 (G–J) in ovaries from 12- to 15-month-old wild-type (+/+) and FORKO (−/−) mice. A and B, Ovary of wild-type (+/+) females expressed very low levels of MIS (*arrows* in B) within small follicles (×25 and ×200 for A and B, respectively); B is an enlargement of the *boxed area* in A. CL, Corpus luteum. C and D, Mutant ovaries. This mutant atrophic ovary that had not yet developed the tumor expressed high levels of MIS in structures resembling the remnants of follicles (*arrows*; ×25). The *boxed structure* in C is shown at higher magnification (×200) in D. Note the intense positive staining, whereas others outside the boundary are negative. E and F, MIS in the tumor-bearing ovary. Strong immunopositive staining for MIS was detected in ovaries from −/− mice with Sertoli-Leydig tumors. F is an enlargement (×200) of the box in E (×25). The expression of MIS was localized to Sertoli tubule compartment (*asterisks* in F). G and H, Expression of GATA 4 in 1-yr-old +/+ mice. Granulosa cells in developing follicles from +/+ ovaries were strongly immunopositive for GATA-4 (*arrows*) follicles (×25 and ×200, respectively; CL, corpus luteum). I and J, Mutant ovaries with tumor. Two types of structures are shown at ×200. GATA-4-immunopositive cells (*arrows* in I and *asterisks* in J) were observed in Sertoli cell-like tubules. In I, note that the follicle with degenerating granulosa cells in the middle is reshaping itself into what appears to be several tubule-like structures. This change is more advanced in J, showing a different FORKO ovary.

and have no reproductive cycles (24, 27). Our results recorded here clearly suggest that ablation of the FSH receptor causes development of gonadal tumors in aging female mutants. Considering the normal life span of about 3 yr for mice, the ovarian tumors were discovered quite early, by about 1 yr. The exact stage at which the tumors begin to appear has not been pinpointed, as this requires more detailed examination of the mutants between 5 and 12 months. It is quite likely that the hormonal imbalances may have altered the expression of regulatory genes in a manner that led to the appearance of large tumors. It was interesting to find that the loss of FSH receptor caused various types of Sertoli-Leydig cell tumors and cysts. The retiform type tumors described in women (2) also occur in FORKO mice. As these tumors are apparently very aggressive in women compared with nonretiform Sertoli-Leydig cell types, the prognosis becomes very poor (2). Similarly, FORKO mice with such tumors were also among those that became anemic and lost body weight.

Confinement of the tumors to the right ovary in a majority of the aging FORKO females is interesting and worthy of note. In a minority of the mutants tumors were also induced in the left or both ovaries. We are not aware of reports of such selective localization of ovarian tumors in other types of transgenic mice (6, 7, 28, 29) or in women with ovarian cancer (4). Although we have no rational explanation at the present time for this intriguing anatomical confinement of the tumor in our mutant mice, attention might be drawn to reports that note differences in the sympathetic innervation of the right and left ovary (30). Whether these had any bearing on tumor localization in the mutants can only be a matter of speculation. Further studies are needed to explore this question and other possibilities.

The potential involvement of pituitary gonadotropins, LH and FSH, in ovarian tumorigenesis has been under investigation for a long time. It has been noted that the incidence of ovarian neoplasms rises around the onset of menopause, accompanied by high levels of plasma gonadotropins (4, 31). Although a causal link between ovarian stimulation in women undergoing *in vitro* fertilization and tumor development remains controversial (4, 8, 9, 32–34), the precise mechanisms remain unknown. Experimental studies in transgenic animals tend to draw an apparent correlation between serum gonadotropins and tumor development due to overexpression of LH (6). That this does not occur in mice of all genotypes further suggests that there are other modifying genetic factors that could be important in particular backgrounds (29), situations that might also occur in women of different races. The 2- or 3-fold elevation of FSH levels in inhibin α-deficient mice that causes gonadal stromal tumors (7, 35) has been suggested to stimulate the development and/or progression of the tumors. On the contrary, attempts to induce granulosa cell tumors in BALB/c mice with exogenous PMSG, a biological surrogate hormone for pituitary FSH, have been unsuccessful (36). Exogenous gonadotropin treatment for 180 d of hypogonadal (*hpg/hpg*) mice, deficient in GnRH and lacking serum FSH and LH, also did not cause ovarian tumors (37). In addition, the recent report in transgenic mice overexpressing FSH has shown that elevated FSH levels alone do not directly cause gonadal tumors (38).

As sex cord-stromal tumors in women are known to have steroidogenic capabilities and apparent binding sites for LH and FSH (17, 18), several different groups have actually searched for mutations in the FSH receptor gene in granulosa cell tumors to test the implications of such changes in ovarian tumor induction. As these attempts have not been successful (19, 39), other possibilities must be considered to explain the findings. Our observations reported here in the FORKO mouse model suggest that it may be the loss of receptor function that triggers the abnormality leading to certain ovarian tumors. As nearly all (92%) of our aging FORKO mice developed ovarian pathology, it is reasonable for us to propose a beneficial role of the FSH receptor's signaling function in protecting the ovary from developing tumors. Therefore, it would be instructive to identify these protective (tumor suppressors?) genes that are influenced by FSH receptor signaling. A lack of such effects, as happens in the postmenopausal state in women, may predispose the ovary to tumors at later stages in life, especially if other confounding factors are also present. Indeed, infertility has been suggested as a significant risk factor for various gynecological cancers (40). It should be noted again that in the FORKO mice ovulation is completely suppressed (22, 24, 27). Other findings, such as increased angiogenesis after loss of ovarian function (41) in menopause, may also support our proposition of the FSH receptor system's protective effect on the ovary. The appearance of ovarian pathology in our +/− female mice that follows early reproductive senescence is noteworthy and requires more detailed investigations, as this experimental paradigm may indeed duplicate menopausal conditions in some manner. Whether there was a complete loss of FSH receptor function at this stage in the +/− female has not yet been ascertained. Nevertheless, these mice could provide an interesting model to examine many issues related to menopause and the appearance of ovarian tumors.

It seems likely that removing the FSH receptor from the scene of action causes alterations in several important genes. For example, the plasma level of inhibin α that showed some tendency to decrease at 4 months as reported in one study (27) could be further reduced upon aging, as shown by our immunohistochemical analysis of the FORKO ovary at 1 yr. Although we have not measured the dimeric inhibin in the present study, it is reasonable to assume that levels would be low in the circulation, as total expression itself is extremely weak in the FORKO ovary. This would in some way be consistent with the report of loss of inhibin α-subunit gene in transgenic mice, strongly predisposing the ovary to granulosa cell tumors (7). Interestingly, as double homozygous mutant mice that lack both FSHβ and inhibin α-subunit develop slow-growing ovarian neoplasm (38), tumor suppressors of the TGFβ family may also be important.

The mechanisms underlying tumor development in aging FORKO females are not clear at present. The elevated LH levels in the FORKO mice could contribute to ovarian pathology, as was apparently the case in LH-overexpressing transgenic mice (6). Even though circulating FSH remained high at all times, this hormone could not function in FORKO mice, as all receptors had been ablated. Other changes that include parameters such as increased concentration of plasma testosterone and the presence of large islands of

polygonal cells with abundant cytoplasm containing lipid droplets and high intensity of 3βHSD and P450c17 (features of steroidogenic cells) in the ovarian stroma may also have played a part in the induction of pathology. Ovarian androgens synthesized in response to LH action may be among the signals for induction of tumors (42), and FORKO mice have high levels of circulating testosterone at 3–5 months (24) and also at the time of tumor detection. However, because of the fact that the +/− mouse that does not show such high levels of testosterone (24) also develops ovarian pathology later in life after reproductive senescence, we may infer that other mechanisms must also be involved. Regardless of the underlying mechanisms it is remarkable that the granulosa cells of the mutant ovary acquire the capacity to reorganize themselves into tubular-like structures. The immunohistochemical evidence of expression of two Sertoli cell markers, namely MIS and GATA 4, supports these conclusions. In this context it is interesting to point out that a similar redifferentiation of the granulosa cells into Sertoli cells has been reported in female mice that lacked both ERα and ERβ (43). Although these animals have high estrogen levels, the hormone is nonfunctional due to lack of the two deleted nuclear receptors. However, in our FORKO mutants the circulating estrogen ligand level became extremely low (or absent), but the expression of both ERα and ERβ was unaltered at the age of 3–5 months.

In conclusion, we observed that the loss of FSH receptor signaling results in ovarian tumor development of aging mutant mice. Our results provide the first *in vivo* evidence that the complete elimination of FSH receptor is involved in the induction of gonadal tumorigenesis. We believe that further studies in this animal model may provide valuable insights into the molecular mechanisms of this insidious disease in women. The induction of such varied pathology caused by the loss of a single receptor might provide a new perspective in understanding why ovarian tumors are precipitated during menopause in women. Based on the present studies we hypothesize that the loss of FSH receptor expression/signaling that occurs naturally in all women at the time of menopause predisposes the ovary to neoplasia, a condition that might be exacerbated in combination with other confounding (genetic or environmental?) factors.

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