Normal Prenatal but Arrested Postnatal Sexual Development of Luteinizing Hormone Receptor Knockout (LuRKO) Mice

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To study further the role of gonadotropins in reproductive functions, we generated mice with LH receptor (LHR) knockout (LuRKO) by inactivating, through homologous recombination, exon 11 on the LHR gene. LuRKO males and females were born phenotypically normal, with testes, ovaries, and genital structures indistinguishable from their wild-type (WT) littermates. Postnatally, testicular growth and descent, and external genital and accessory sex organ maturation, were blocked in LuRKO males, and their spermatogenesis was arrested at the round spermatid stage. The number and size of Leydig cells were dramatically reduced. LuRKO females also displayed underdeveloped external genitalia and uteri postnatally, and their age of vaginal opening was delayed by 5–7 days. The (−/−) ovaries were smaller, and histological analysis revealed follicles up to the early antral stage, but no preovulatory follicles or corpora lutea. Reduced gonadal sex hormone production was found in each sex, as was also reflected by the suppressed accessory sex organ weights and elevated gonadotropin levels. Completion of meiosis of testicular germ cells in the LuRKO males differs from other hypogonadotropic/cryptorchid mouse models, suggesting a role for FSH in this process. In females, FSH appears to stimulate developing follicles from the preantral to early antral stage, and LH is the stimulus beyond this stage. Hence, in each sex, the intrauterine sex differentiation is independent of LH action, but it has a crucial role postnatally for attaining sexual maturity. The LuRKO mouse is a close phenocopy of recently characterized human patients with inactivating LHR mutations, although the lack of pseudohermaphroditism in LuRKO males suggests that the intrauterine sex differentiation in this species is not dependent on LH action. (Molecular Endocrinology 15: 172–183, 2001)

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INTRODUCTION

The two gonadotropins, LH and FSH, have a key role in the differentiation and maturation of mammalian sexual organs and functions. After identification of genes for the gonadotropin subunits and gonadotropin receptors (R), their mutations have been discovered in males and females with various types of hypogonadism (for a review, see Ref. 1). Mutations of the FSHβ subunit gene cause infertility with arrested follicular maturation in women and azoospermia in men. Inactivating FSHR mutations in women cause the same phenotype as the ligand mutations, but in men the phenotype is milder with only variable impairment of spermatogenesis (1). Knockout models for both FSHβ and FSHR have been produced (2–4), and they display complete phenocopies of the human FSHR mutations. A discrepant finding is the azoospermia detected in the two men so far described with FSHβ mutation (5, 6), which is not found in the receptor or ligand knockout mice produced (2–4) or in men with an inactivating FSHR mutation (7). Hence, the necessity of FSH for spermatogenesis still remains controversial.

The consequences of inactivation of LH action also remains to be clarified. Only a single man with LHβ mutation has been reported (8); he presented with normal sexual differentiation at birth but total lack of postnatal sexual development. No women with such a mutation have yet been described. Neither are there knockout models for LHβ or LH receptor (LHR). More is known about consequences of inactivating LHR mutations in man (1). Depending on completeness of the

receptor inactivation, men present with pseudohermaphroditism ranging from mild micropenis and hypospadias to complete sex reversal. The phenotype in women is milder, including only anovulatory infertility.

In our exploration of the consequences of inactivation of gonadotropin action, we concluded that a knockout mouse model for the LHR would be a logical next step. It is known that LH stimulates Leydig cell differentiation and steroidogenesis in the postnatal testis, but its role in the fetal period is controversial (9). In the ovary, LH stimulates theca cell androgen production, triggers ovulation, and stimulates estrogen and progesterone production of corpus luteum. LH actions in early stages of female development are unlikely, because LHRs appear in the ovary only postnatally (10). In addition, there are recent findings on LHR expression and LH actions in extragonadal organs (11, 12). In addition to the expected phenotype of hypogonadism of the LH receptor knockout (LuRKO) mice, the developmental and possible extragonadal findings were especially hard to predict. Likewise, it was interesting to see, to what extent FSH alone, in the absence of LH action, was able to support gonadal function. We report here the phenotypes of male and female mice with targeted disruption of the LHR gene.

RESULTS

Gene Targeting

The purpose of the gene targeting was to eliminate exon 11 of the LHR gene (see Fig. 1). After electroporation of the targeting construct (Fig. 1A) into ES cells, and after drug selection, 250 surviving colonies were picked and screened by PCR. Positive clones were further confirmed by Southern blots. Five of the 250 clones gave a 2.3-kb fragment in PCR analysis, and bands with expected size of 11 kb for the wild-type (WT) allele and 9 kb for the targeted allele in Southern blot, indicating that homologous recombination had occurred (Fig. 1, B and C). Three of these cell lines (C30, C76 and C101) were injected into blastocysts, yielding nine male chimeras. The male chimeras were bred with C57BL/6 females, and germline transmission (F1 offspring) was obtained with three of them. Heterozygous mice were fertile and viable. Intercrossing the F1 heterozygotes yielded F2 progeny including 43 LHR WT (+/+) (22 female, 21 male), 86 heterozygous (+/−) (32 female, 53 male), and 37 homozygous (−/−) mice (16 female, 21 male), in agreement with the expected Mendelian mode of inheritance.

Phenotype of the Animals

Homozygous LuRKO mice of both sexes were born phenotypically normal. Testes of the newborn males were similar in size and microscopic appearance (Fig. 2) as those of WT littermates, and their intraabdominal location adjacent to the urinary bladder was the same as in WT males. Likewise, the internal genitalia revealed under stereomicroscopy no difference between (+/+), (+/−), and (−/−) males (data not shown). From about 3 weeks' postnatal age the male LuRKO mice were significantly lighter than (+/+) or (+/−) mice of the same age, and at 45 days this weight difference reached 30% (results not shown). No clear weight differences were found between (−/−), (+/−), and (+/+) females up to 7 weeks of age (data not shown). The LuRKO males could be distinguished from their normal male littermates by external examination after 30–35 days by their small penis, short anogenital distance, and underdeveloped scrotum. At the age of 7 weeks, their testes were very small [(+/+), 94 ± 6.9; (+/−), 91 ± 3.1; (−/−), 17 ± 0.77 mg; mean ± SEM, n = 8–10] and located in the abdominal cavity adjacent to the urinary bladder. The accessory reproductive organs (seminal vesicles, epididymides, and prostates) were macroscopically invisible (Fig. 2A). Histology of the (−/−) testes showed that seminiferous tubules were narrower and spermatogenesis was arrested at the round spermatid stage (Fig. 2). The numbers and sizes of Leydig cells appeared dramatically decreased as compared with (+/+) testes (Fig. 2).

In female (−/−) mice, the age of vaginal opening was delayed to 35–38 days, compared with 30–32 days in WT mice. Internally, the ovaries were about 50% reduced in size, and the uteri were significantly thinner (Fig. 3A). Ovarian histology at the age of 7 and 12 weeks showed in (−/−) mice presence of follicles up to the early antral stage, but no preovulatory follicles or corpora lutea (Fig. 3). No apparent differences were seen in the thickness of the theca cell layers surrounding the developing follicles. The uterine histology showed thinning of all cell layers and absence of glandular structures (Fig. 3). Comparison of estrous cycles of the (+/+) and (−/−) mice at the age of 12 weeks showed the cyclic periodicity with recognizable estrus every 4 days in (+/+) mice, but not in (−/−) mice.

No differences were found between (+/−) and (+/+) mice in any of the parameters studied.

Analysis of the Disrupted LHR Gene

The targeting construct was designed so that the pGKneo insert would replace exon 11, which encodes the transmembrane and cytoplasmic receptor domains, and part of the 3′-extracellular domain, thus preventing the formation of full-length functional LHR capable of anchoring to the plasma membrane and of signal transduction. However, it is possible that the remaining fragment of the LHR gene could be transcribed into truncated forms of LHR mRNA. We performed RT-PCR analysis with different primer pairs on testicular and ovarian RNA

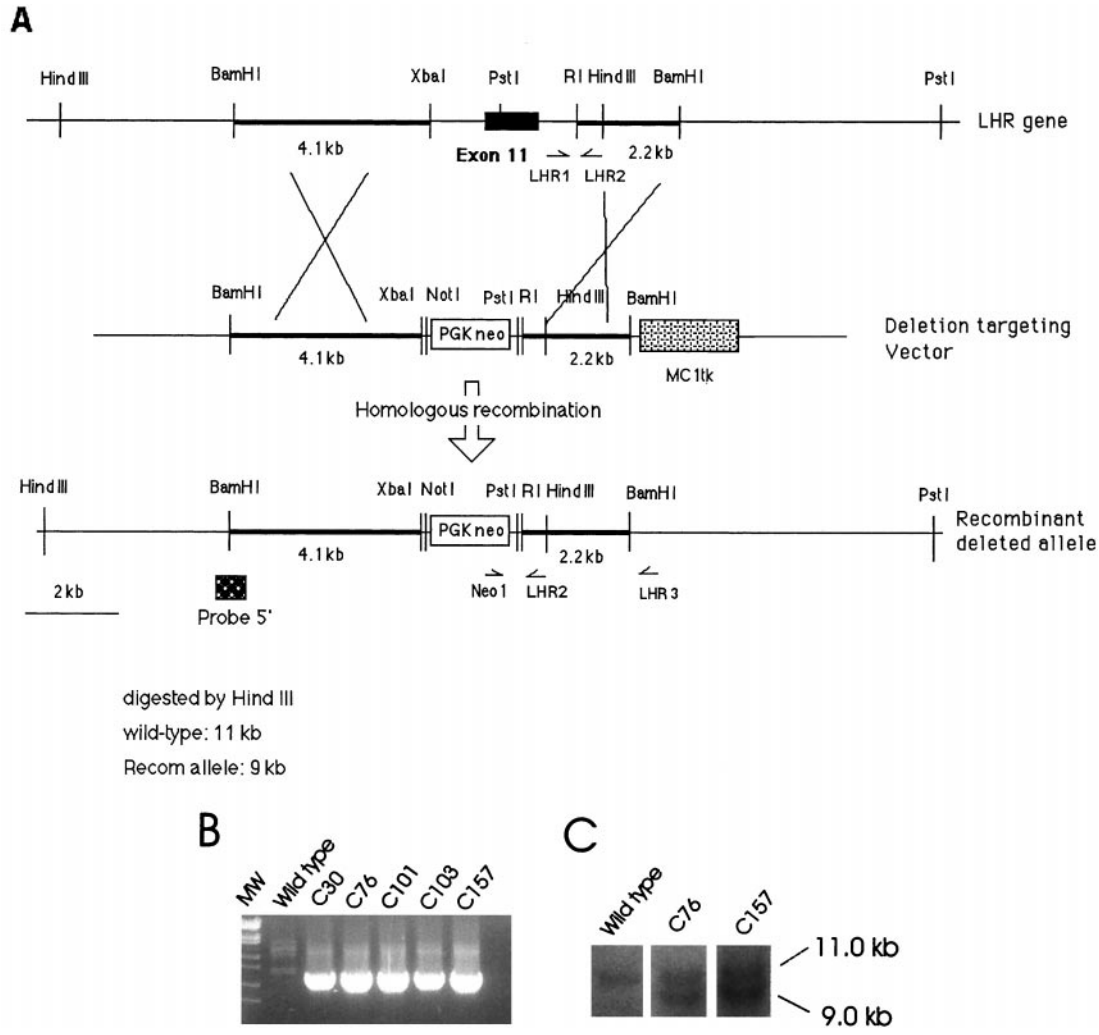

Fig. 1. Targeted Disruption of the LHR Gene

A, The replacement targeting vector to delete exon 11 and part of intron 10 of the LHR gene. The approximate locations of the PCR primers used to screen for homologous recombinants and phenotype are shown (*arrows*) with the original and predicted structure of the gene after homologous recombination. B, Positive ES clones were identified as homologous recombinations by PCR screen using the primers (Neo1 and LHR3) shown in panel A. C, Representative of genomic DNA isolated from two ES clones with homologous recombination and one WT ES clone of cells, digested with *Hin*dIII, and analyzed by Southern hybridization. The presence of 9- and 11-kb bands indicates homologous recombination.

from LuRKO and WT mice. Using primer pair LHRm1 and 2 (specific for the extracellular domain of LHR) an amplicon of 412 bp was detected from (+/+), (+/−), and (−/−) testes and ovaries (Fig. 4). Using primer pair LHRm3 and 4 (specific for transmembrane and cytoplasmic domains), a 359-bp band was found in (+/+) and (+/−) mouse testes and ovaries, but not in those of (−/−) mice (Fig. 4). Northern hybridization analysis of testicular RNA by using a cRNA probe specific for extracellular domain of LHR revealed four major transcripts of LHR mRNA with sizes of 6.9, 2.6, 1.7, and 1.2 kb in the (+/+) and (+/−) mice, but only one band of 1.2 kb in the (−/−) mice. When using the cRNA probe specific for the transmembrane domain of LHR, two major bands with sizes of 6.9 and 2.6 kb were present in the (+/+) and (+/−) mice, but no hybridization was observed in the (−/−) samples (data not shown). Hence, the LuRKO mice do not synthesize any mRNA encoding the full-length functional LHR.

hCG Binding

To confirm whether the LHR-deficient mice express functional or truncated LHR, radioligand receptor assay was carried out using both testicular membranes and detergent-solubilized testicular homogenates. The results indicated that (+/+) and (+/−) mice expressed high levels of specific [^{125}I]iodo-hCG binding in both testis membranes and detergent-solubilized extracts, whereas none was detected in either samples from the (−/−) mouse testes (Fig. 5).

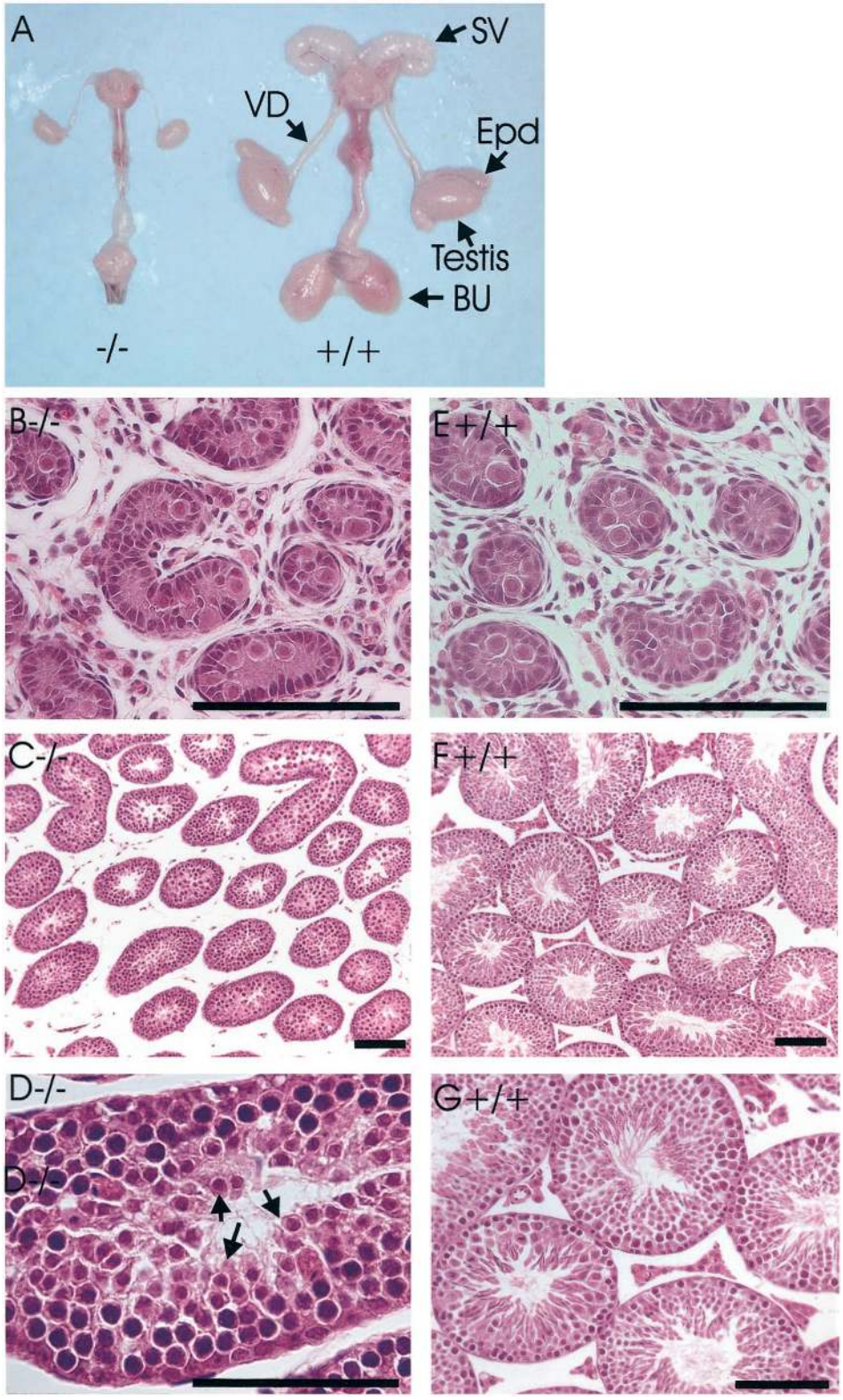

Fig. 2. Morphology and Histology of the Testes of Control (+/+) and Homozygous (−/−) LuRKO Male Mice

A, Testes and accessory sex organs of a (−/−) and a (+/+) littermate. VD, Vas deferens; SV, seminal vesicle; Epd, epididymis; BU, bulbo-urethral gland. B and E**,** Testicular histology of a 1-day-old (−/−) and (+/+) mouse. C and F, Testicular histology of a 45-day-old (−/−) and (+/+) mouse. D and G, As in panels C and F, at higher magnification. *Arrows* indicate round spermatids. The *bar* in panels B–G is 100 μm.

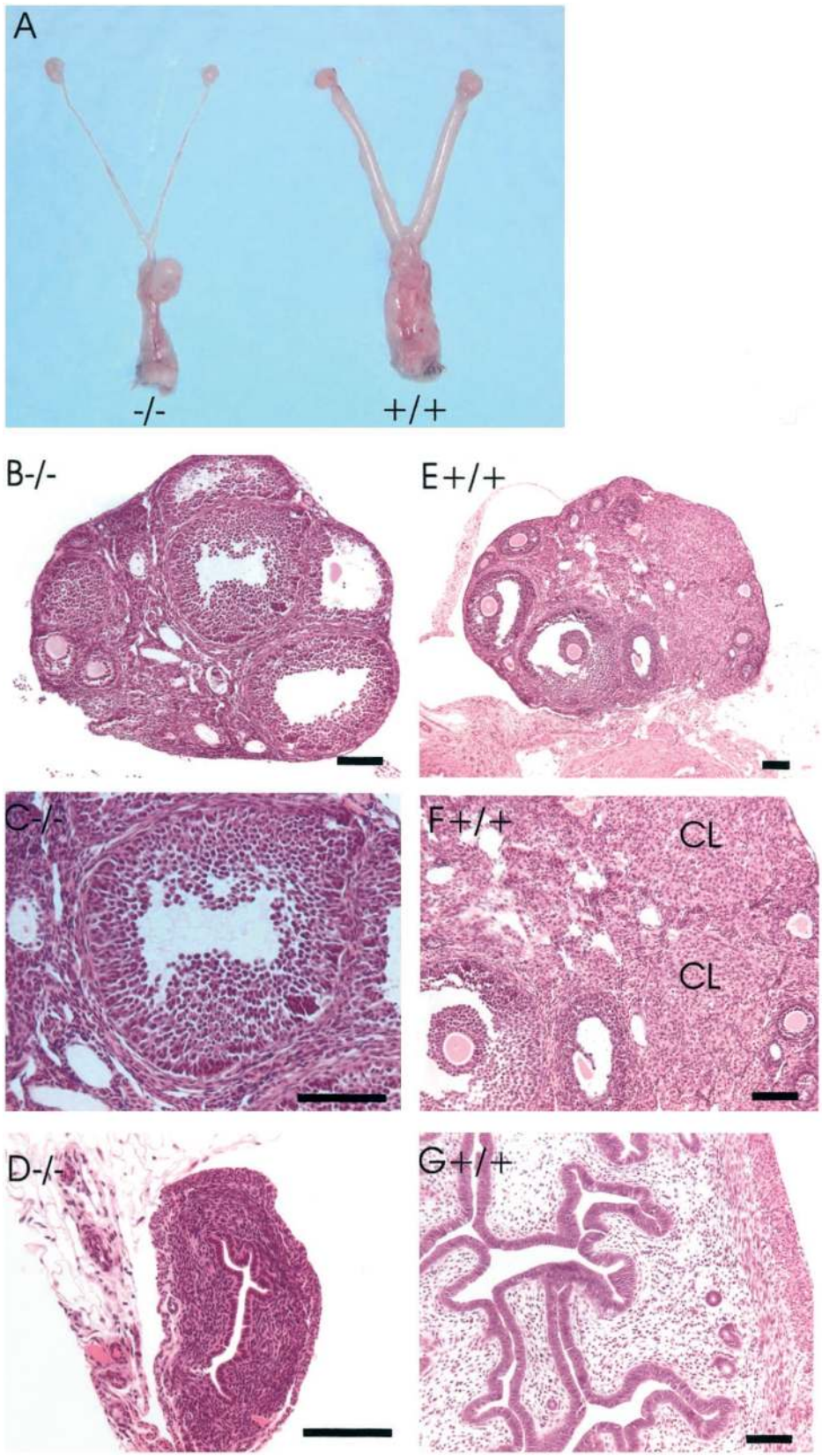

Fig. 3. Morphology and Histology of Ovaries and Genital Organs of Control (+/+) and Homozygous (−/−) LuRKO Female Mice

A, Ovaries, uteri, and vagina of a (−/−) and a (+/+) littermate. B and E, Ovarian histology of a 7-week-old (−/−) and (+/+) mouse. C and F, As in panels B and E, at higher magnification. D and G, Uterine histology of a (−/−) and (+/+) mouse. CL, Corpus luteum. The *bar* in panels B–G is 100 μm.

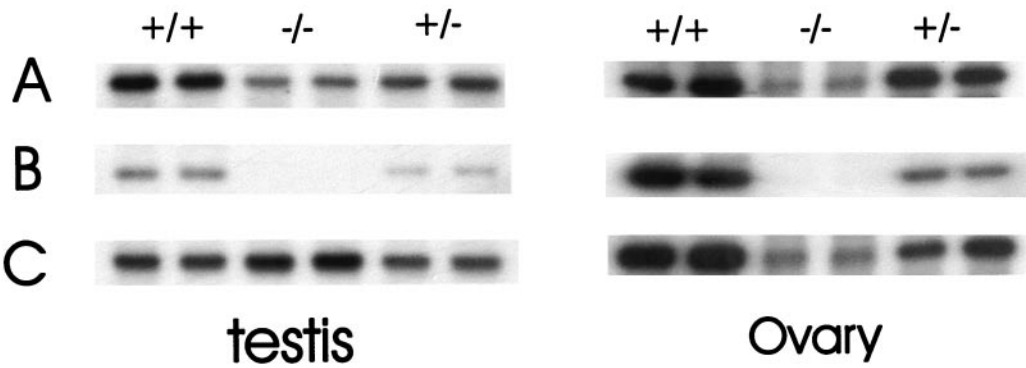

Fig. 4. RT-PCR Detection of LHR mRNA in WT (+/+), Heterozygous (+/−), and LHR Deficient (−/−) Mice Testis and Ovary

A, PCR products generated by using a primer pair specific for the extracellular receptor domain. B, PCR products generated by using a primer pair specific for the transmembrane, cytoplasmic, and 3′-part of extracellular domains. C, PCR products using primers specific for β-actin mRNA.

Analysis of Cholesterol Side-Chain Cleavage P450 ($P450_{scc}$) and Cytochrome P-450 17-Hydroxylase (P450 17-OH) Gene Expressions

To further characterize the potential residual activities of steroidogenesis of Leydig and theca cells of the LuRKO mice, we analyzed the expression of the P450scc and P450 17-OH mRNAs. P450scc, converting cholesterol to pregnenolone, is a key rate-limiting step in Leydig cell testosterone biosynthesis. Northern blot analysis indicated that P450scc mRNA was dramatically decreased (>20 fold) in LuRKO, compared with WT testes (Fig. 6). P450 17-OH is a theca cell-specific enzyme and necessary for their androgen production. In the LHR-deficient ovaries, the P450 17-OH mRNA was decreased by 12-fold as compared with the expression of WT ovaries (Fig. 6). These data provide evidence that the LHR gene deletion profoundly, although not totally, reduces the steroidogenic activities of the Leydig and theca cells.

Gonadotropin and Steroid Assays

Gonadotropin assays (Fig. 7) indicated that serum LH levels were dramatically increased in (−/−) males and females, whereas there were no significant difference of pituitary LH levels between the (+/+), (+/−), and (−/−) mice. Serum FSH levels were elevated, and pituitary FSH levels were decreased in (−/−) males and females as compared with (+/−) and (+/+) males and females. Serum and testicular testosterone levels of (−/−) males were significantly decreased (Fig. 8) as compared with (+/+) and (+/−) males. However, the former levels were somewhat higher than we detect in castrated mouse circulation (13), indicating that testicular steroidogenesis was not totally abolished by LHR inactivation. Likewise, the ovarian estradiol concentration was significantly decreased in (−/−) female mice compared with (+/+) and (+/−) females (Fig. 8). Similar suppression of ovarian progesterone was observed in the LuRKO females (data not shown).

The small seminal vesicles and prostates of the (−/−) males, and thin uteri of the (−/−) females, provide further functional evidence for long-term suppressed sex steroid production in LuRKO mice.

DISCUSSION

Targeted disruption of exon 11 of the LHR gene resulted in complete loss of LHR binding in both intact cells and detergent-solubilized extracts from the LHR-deficient mouse testes. RT-PCR analyses demonstrated the absence of exon 11-specific mRNA sequences in testes and ovaries, whereas those encoding the extracellular domain of the receptor were transcribed. Since the extracellular part of the receptor is unable to anchor to the plasma membrane and does not have the structures needed for signal transduction (14), it cannot exert regulatory functions alone even if secreted into the extracellular space. Admittedly, modulatory effects of truncated LHR forms on function of the full-length receptor have been proposed (15), but in the absence of the latter these are not possible in the LHR-deficient mice. Furthermore, our data showed that no ligand binding was found in both intact cells and detergent-solubilized extracts from the LHR-deficient mouse testes. Previous studies have demonstrated that [^{125}I]iodo-hCG binding could detect truncated LHR species in detergent-solubilized extracts (14). For this reason, the knockout model produced represents total inactivation of LHR function.

Interestingly, the sexual differentiation and gonadal histology of female and male LuRKO mice were indistinguishable from WT littermates at birth. In females, this is not surprising, since the early female sex differentiation is known to be independent of ovarian function (16), and gonadotropin receptors occur in rodent ovaries only several days after birth (17). Although fetal testicular testosterone synthesis is crucial for male sexual differentiation, the LuRKO mice provide, for the first time, direct evidence that specific elimination of

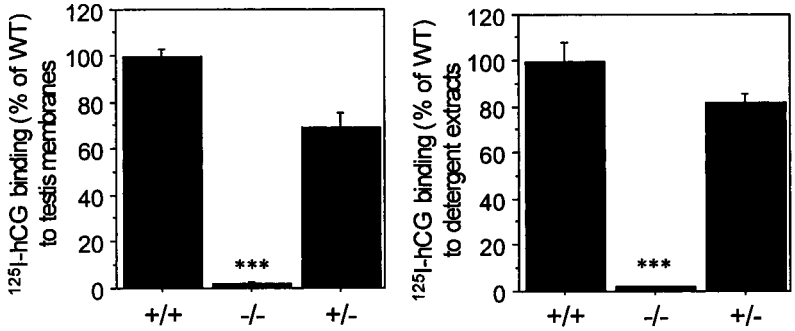

Fig. 5. Specific [^{125}I]iodo-hCG Binding in Control (+/+), Heterozygous (+/−), and LHR-Deficient (−/−) Adult Mice (7 weeks old) to Testis Membranes (*left panel*) and Detergent Extracts (*right panel*)

The mean binding measured in (+/+) testes was assigned a value of 100%. Each *bar* is the mean ± SEM of three samples. The binding in (−/−) samples was indistinguishable from zero, and it differed from (+/+) samples at $P < 0.001$.

LH action does not hamper this function. This has been suggested indirectly by earlier findings on normal intrauterine masculinization of gonadotropin-deficient *hpg* (18) and common α-subunit knockout (19) mice, and on unmeasurable levels of LH in rat fetal circulation at the time of the sharpest increase in fetal testicular testosterone production (20).

The testes of the LuRKO mice weighed about 17 mg, which were about 18% of that of WT testes, but 5-fold more than those of the *hpg* mice (18, 21). This increased weight over *hpg* mice can be ascribed to the elevated FSH action in the LuRKO mice, because this gonadotropin is known to stimulate Sertoli cell proliferation in the neonatal testis (22–24). While the spermatogenesis of *hpg* and common α-subunit knockout mice proceeds up to the diplotene stage (18, 19), some tubules of the LuRKO mice show round spermatids, *i.e.* completion of meiosis. This is supported by the findings that progression of spermatogenesis from spermatocytes to spermatids can be stimulated after hypophysectomy by either FSH or androgen (25). However, both the intraabdominal location and insufficient testosterone production of the LuRKO testes offset further progression of spermatogenesis. Interestingly, it is typical for experimental cryptorchidism with undisturbed Leydig cell testosterone production (26), including the recently developed Insl3 knockout mouse (27), that they, despite normal androgen levels, lack postmeiotic germ cells. This raises the possibility that normal intratesticular testosterone concentration in the abdominal temperature is deleterious to spermatogenesis, as seems to occur during the recovery of spermatogenesis after cytotoxic or radiation insults (28).

Concerning testicular descent, no difference was found at birth in the location of the testes, adjacent to the urinary bladder, between the (−/−) and (+/+) mice. Hence, the lack of LH stimulation *in utero* did not hamper the first transabdominal phase of testicular descent, known to be dependent on both androgen and Insl3 (29).

The ovaries of the LuRKO mice were reduced in size and histological analysis revealed follicles up to the early antral stage, but no preovulatory follicles or corpora lutea. The ovaries of *hpg* mice have follicles up to the preantral stage (18), which indicates that FSH action, present in LuRKO mice, has a distinct effect on progression of preantral follicles to the early antral stage. Correspondingly, the lack of preovulatory follicles and corpora lutea indicates that the very last steps of follicular maturation, as well as ovulation, do not occur without LH action. Another intriguing feature of the LuRKO ovaries was the apparent normal thickness of thecal cell layers surrounding the follicles. Hence, although theca cells are a target of LH action, their survival is apparently not dependent on LH, which observation can also be made in *hpg* ovaries (18). However, theca cell androgen production, to provide substrate for granulosa cell estrogen production, is LH dependent. Therefore, the defective estrogenization of LuRKO females was expected, as demonstrated by their low ovarian estradiol level, delayed sexual maturation, and hypoplastic uteri.

As an indicator of Leydig cell steroidogenic activity (30, 31), we measured the mRNA level of $P450_{scc}$ in the LuRKO testes. The low but detectable level of expression indicates that low constitutive expression of this enzyme is possible in the absence of LH action in the precursor Leydig cells detected in the LuRKO testes. In accordance, the serum testosterone levels in the LuRKO males were slightly higher than measured by us in orchidectomized mice (13). However, the physiological significance of this residual androgen production is unlikely in view of the lack of postnatal sexual development of the LuRKO males. Likewise, low but detectable levels of P450 17-OH mRNA, a marker of theca cell steroidogenesis (32), was detected in the LuRKO ovaries, indicating that this enzyme is also expressed constitutively at low levels. This finding, together with the well developed theca cells and low but detectable estradiol level of the LuRKO ovaries, explains the delayed vaginal opening of the LuRKO females.

Both female and male LuRKO mice represent close phenocopies of the respective human mutation (1). LHR inactivation in males causes pseudohermaphroditism of varying severity. The most severe forms present with female genitals, absence of uterus, low testosterone, and high LH. The milder forms, with par-

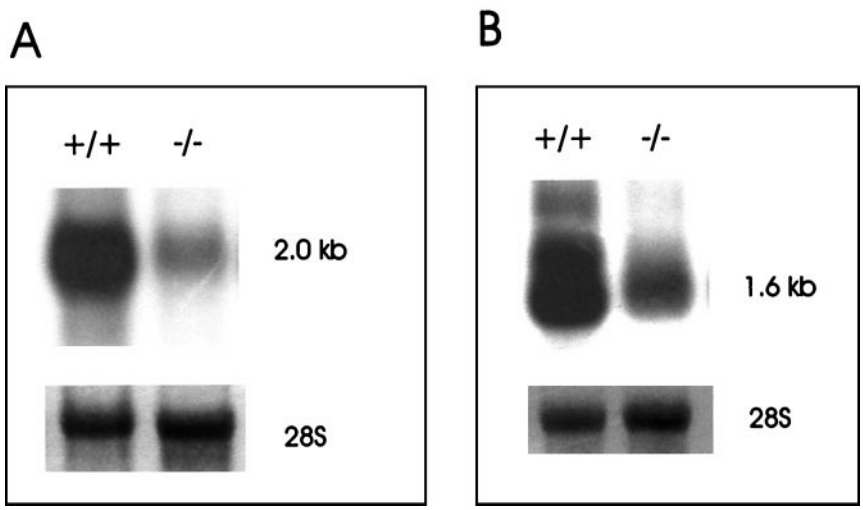

Fig. 6. Analysis of Gene Expressions in Control (+/+) and LHR-Deficient (−/−) Mice

A, Northern blot analysis of $P450_{scc}$ gene expression in testis samples. B, Northern blot analysis of P450 17-OH gene expression in ovarian samples. The *lower panels* are the 28S ribosomal RNA loading controls.

tial LHR inactivation, display a broader array of phenotypes ranging from micropenis to hypospadias. The severity of the phenotype has been shown to correlate with the degree of LHR inactivation (1). Although the receptor inactivation in LuRKO mice is total, the male phenotype is less dramatic than in connection with similar human mutations. This indicates that the gonadotropin- independent component of fetal Leydig cell androgen production is more prominent in the mouse than in the human. The presence of chorionic gonadotropin (hCG) in human fetal circulation may explain the difference. Regulation of the critical process of testosterone- dependent male sexual differentiation needs, in addition to pituitary LH, a backup mechanism, which is hCG in the human (33) and testicular paracrine regulation (20) in the rodent.

The female phenotype of LuRKO mice is even closer to that of the inactivating human LHR mutations (1). Affected women have normal primary and secondary sex characteristics, increased gonadotropins, and low estrogen and progesterone production. Likewise, suppressed but not absent estrogen production of the LuRKO mice is reflected by the presence of granulosa cells in their ovaries, delayed vaginal opening, and hypoplastic uteri. Ovarian histology demonstrates follicles at early stages of development, but no preovulatory follicles or corpora lutea, in both women with LHR inactivation and in LuRKO mice. These observations in the human and mouse support the view that LH is essential for normal estrogen production and ovulation, whereas follicular development is initially independent of gonadotropins and, in its final stages, is dependent on FSH and LH.

In conclusion, the LuRKO mouse allows us to identify directly the specific LH- dependent steps of male and female sexual differentiation and adult gonadal functions. It is a close phenocopy of completely inactivating mutations of the human LHR gene, and it provides a valuable tool for experimental studies of pathogenesis of this condition. Although all effects characterized in the present study were concerned with development and function of gonads and sex organs, the LuRKO model also helps us to explore the putative and recently documented extragonadal actions of LH (11, 12), which most notably apply to the tumorigenic effects observed with this hormone (34, 35).

MATERIALS AND METHODS

Genomic Clone Isolation

A 9.8-kb genomic *Bam*HI/*Bam*HI fragment on the mouse LHR gene, containing exon 11, 4.7 kb of the flanking intron 10 sequences and 4.0 kb of flanking 3′-sequences was isolated from a mouse ES129/SvJ BAC library (Genome Systems, St. Louis, MO), using a cDNA probe specific for exon 11 of the rat LHR gene (36) (Fig. 1). Sequencing confirmed validity of the genomic fragment.

Targeting Vector Construction

The targeting plasmids pKO Scramber913, pKO SelectNeo V800, and pKO SelectTK V830 were purchased from Lexicon Genetics, Inc. (The Woodlands, TX). Briefly, the targeting vector contained a 4.2-kb *Bam*HI-*Xba*I fragment as the 5′-homology region and a 2.2-kb *Eco*RI-*Bam*HI fragment representing the 3′- homology region, a positive selection marker (the PGK-Neo expression cassette), and an MC1-tk (thymidine kinase) expression cassette (Fig. 1).

Embryonic Stem (ES) Cell Culture

The ES cell line, AB.2.2-prime ES cells, was purchased from Lexicon Genetics, Inc. (The Woodlands, TX) and cultured on neomycin-resistant primary embryonic fibroblast feeder layers irradiated with 3000 rads. Ten million cells were electroporated (500 μF, 240 V) with 30 μg of linearized targeting construct. After electroporation, the surviving cells were plated on 100-mm di-

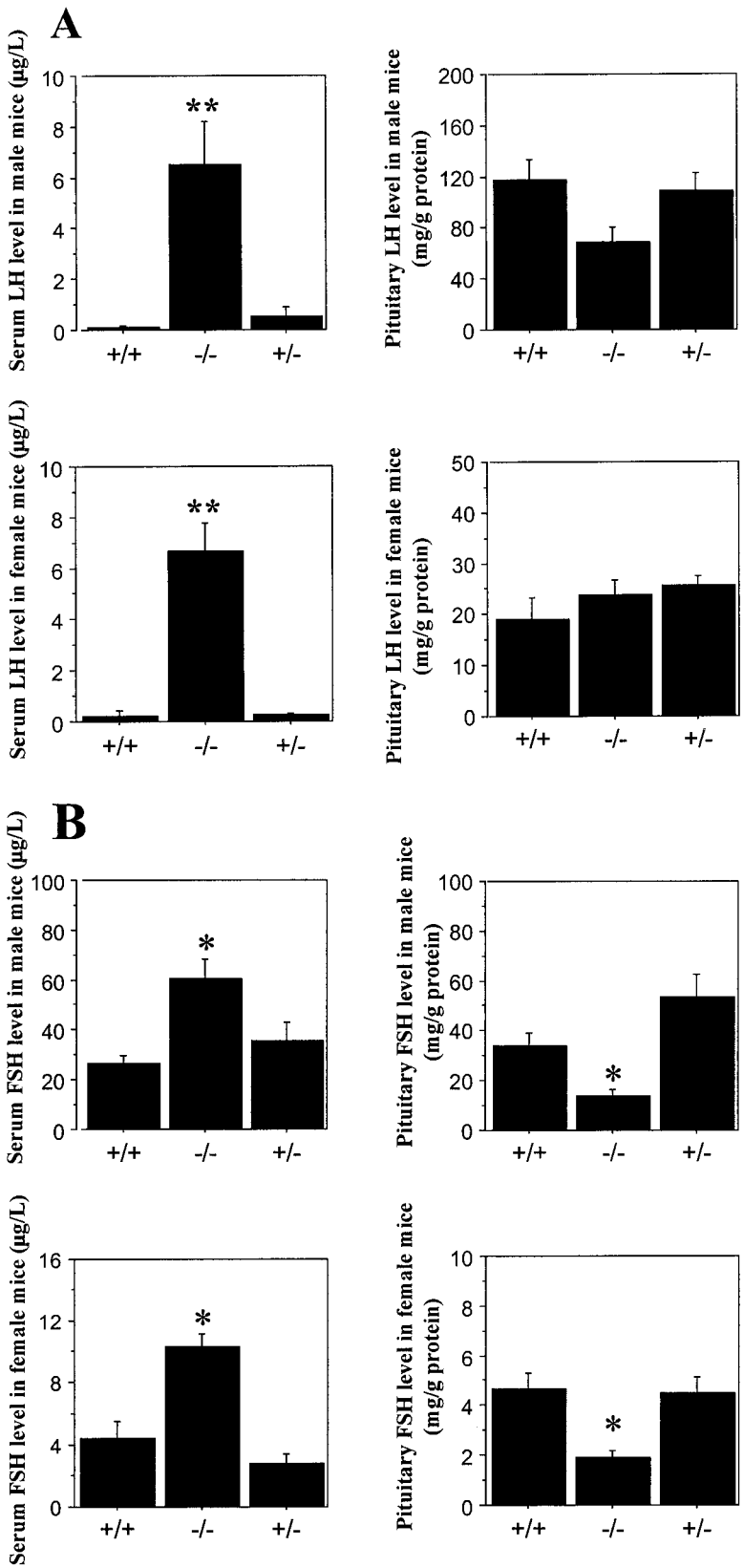

Fig. 7. Gonadotropin Levels in WT (+/+), Heterozygous (+/−), and LHR-Deficient (−/−) Adult Mice

A, Serum and pituitary LH levels of 7-week-old male and female mice. B, Serum and pituitary FSH levels from 7-week-old male and female mice. *, $P < 0.05$; and **, $P < 0.01$, compared with (+/+) and (+/−) groups. n = 6–10 mice per group.

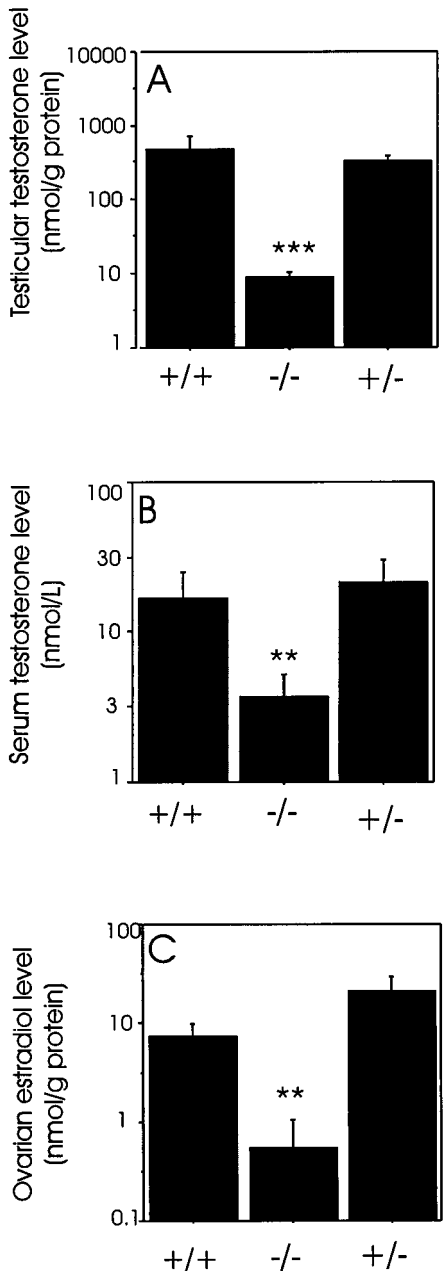

Fig. 8. Testosterone and Estradiol Levels in WT (+/+), Heterozygous (+/−), and LHR-Deficient (−/−) Adult Mice

Testicular (A) and serum testosterone (B) levels of 7-week-old male mice. C, Ovarian estradiol level of 7-week-old female mice. **, $P < 0.01$; and ***, $P < 0.001$, compared with (+/+) and (+/−) groups. n = 6–10 mice per group.

ameter culture dishes and exposed to G418 (Sigma) at 300 mg/liter and 1 μM of ganciclovir (Hoffman-LaRoche Inc., Basle, Switzerland) for 9–10 days. Colonies were picked into 24-well plates and grown for 5–6 days, when about one eighth of a colony was replated onto 24-well plates for genomic DNA extraction, with the remainder being frozen at −80 C.

Screening of Targeting Clones

DNA was isolated from each individual clone and screened by PCR, which produced a 2.3-kb amplicon with a primer pair corresponding to the 3′-end of pGKneo (Neo1, 5′-GGGCTC-TATGGCTTCTGAGGCGGA-3′) and to the flanking 3′ end of exon 11 (LHR3, 5′-TCTCAGGGAGGATTTGGGTATGG-3′) (Fig. 1A). Correct targeting of the ES cells was further confirmed by Southern hybridization analysis of *Hin*dIII- digested ES cell genomic DNA and a probe specific for the flanking sequence of intron 10; the expected band from unmodified LHR was 11 kb in size and 9 kb for the deleted LHR gene.

Mouse Breeding

The targeted ES cells were injected into blastocysts from C57BL/6J females and implanted into pseudopregnant mothers to proceed to term. Chimeras were identified by coat color, and males were bred to C57BL/6J females to test germline transmission. Genotyping of the mice was carried out by using PCR on genomic DNA with primer pairs for the WT allele (LHR1, 5′-TCTGGGGATCTTGGAAATGA-3′; LHR2, 5′-CACCTTGACACCTGGAGT-3′) and for the targeted allele (Neo1-LHR2) (Fig. 1). Tail DNA from F1 offspring with agouti coat color was screened by PCR with primer pair Neo1 and LHR2. F2 offspring and the subsequent generations were screened by PCR using primer pairs for detecting presence of the targeted LHR gene and the WT gene.

All mice were handled in accordance with the institutional animal care policy of the University of Turku.

Histological Analysis

Testes, ovaries, epididymides, seminal vesicles, uteri, and pituitary glands were removed, fixed in 4% paraformaldehyde at 4 C for 4–14 h, dehydrated, and embedded in paraffin, and sectioned at 5 μm thickness. Sections were stained with Harris hematoxylin and eosin (BDH Ltd., Poole, UK). The reproducibility of all the morphological data was verified by similar findings in at least three different animals.

RNA Isolation and Analyses

RNA isolation and RT-PCR were carried out as previously reported (37). RNA was extracted by the single-step method (38). cDNA was generated by reverse transcriptase (RT) from 2 μg of testicular or ovarian RNA, using avian myeloblastosis virus (AMV)-RT with random hexamers (Promega Corp., Madison, WI), in a final volume of 25 μl. Subsequent PCR analysis was performed on 3 μl of the cDNA and 0.1 μl of [^{32}P]-CTP (~400 Ci/mmol; Amersham Pharmacia Biotech, Aylesbury, UK), and the PCR products were analyzed by electrophoresis on 1.4% agarose gels. The oligonucleotide primers used for RT-PCR were designed according to the published cDNA sequences of mouse LHR (39). PCR amplification with the primer pair LHRm1 and 2 (LHRm1, 5′-TGAACCCGGTGCTTTTACAA-3′; LHRm2, 5′-CGTGGCGAT-GAGCGTCTGAATG-3′), specific for the extracellular domain of LHR, yields a 412-bp fragment. With the primer pair LHRm3 and 4 (LHRm3, 5′-ATCGCCACGTCATCCTACT-CACTG-3′; LHRm4, 5′AGCCAAATCAACACCCTAAG-3′), specific for exon 11 of LHR, a 359- bp amplicon is produced.

Northern Hybridization Analysis

Ten or five micrograms of total RNA from testis and ovary were resolved on 1.2% formaldehyde denaturing agarose gel and transferred onto nylon membrane (Hybond-XL, Amersham Pharmacia Biotech). Prehybridization and hybridization were performed as previously described (37). Briefly, the filters were prehybridized for at least 4 h at 65 C in a solution containing 50% formamide, 3 × SCC (1 × SCC = 150 mM NaCl and 15 mM sodium citrate, pH 7.0), 5 × Denhardt' solution, 1% SDS. Hybridization was carried out at 66 C

overnight in the same solution after adding the [^{32}P]-labeled cRNA probe. After hybridization, the membranes were washed twice with 1 × SCC and 0.1% SDS at 65C for 30 min each time and twice with 0.1 SCC and 0.1% SDS at 66 C for 30 min each time. The membranes were exposed to x-ray film (Kodak XAR-5, Eastman Kodak Co., Rochester, NY) at −70 C for 1–3 days. The molecular sizes of the mRNA species were estimated by comparison with mobility of the 18S and 28S ribosomal RNAs. The [^{32}P]-labeled cRNAs were synthesized using a Riboprobe synthesis II kit (Promega Corp.), [^{32}P]UTP (Amersham Pharmacia Biotech), and the corresponding cDNA templates. For generation of $P450_{scc}$ riboprobe, a template composed of a fragment of rat $P450_{scc}$ cDNA (spanning bp 186–695), subcloned into T vector under T7 RNA polymerase promoter, was used (40). Antisense cRNA probe for P450 17-OH mRNA analysis was produced using as template a fragment of mouse P450 17-OH cDNA (spanning bp 55–616) subcloned into T vector under the T7 RNA polymerase promoter (41). For the LHR cRNA probes, the cDNAs used as templates corresponded to bases 441–849 of extracellular domain of rat LHR cDNA (36), and to bases of 1,002–1,461 of transmembrane domain of mouse LHR cDNA (36), respectively.

hCG Binding Assay

Testicular LHR binding was measured as previously reported (42, 43). Briefly, a piece of testis tissue was homogenized with an Ultra-Turrax 18/10 homogenizer in Dulbecco's PBS + 0.1% BSA (0.5 mg tissue/ml). Highly purified hCG (NIH CR-125; 13,000 IU/mg) was radioiodinated using a solid phase lactoperoxidase method. One-hundred-microliter aliquots of testicular homogenate were incubated in triplicate at room temperature for 18 h, in the presence of a saturating concentration (150,000 cpm; ~ 3 ng) of [^{125}I]iodo-hCG. Nonspecific binding was assessed in the presence of a 1,000-fold excess of unlabeled hCG (Pregnyl, Organon, Oss, The Netherlands). The centrifugation step used to separate bound and free hormone (1000 × *g*, 30 min at 4 C) precipitates only membrane-bound receptors (42).

To measure hCG binding to detergent-solubilized (*i.e.* membrane-bound and soluble) receptors, the testes were homogenized in ice-cold buffer A (150 mM NaCl, 20 mM HEPES, pH 7.4) containing 20% glycerol, 1% Nonidet P-40 (NP-40), and protease inhibitor cocktail (Sigma), and incubated on ice for 30 min. After centrifugation at 13,000 rpm for 30 min at 4 C, the supernatant was used for the ligand binding assay. The binding reaction was carried out as above, except that the incubation was overnight at 4 C. Free and bound [^{125}I]iodo-hCG were separated by precipitating the samples with polyethylene glycol (mol wt 8,000). Each tube received 0.2 ml of 5 g/liter solution of bovine γ-globulin in buffer A and 0.5 ml of 30% (wt/vol) polyethylene glycol in buffer A. After incubation at 4 C for 10 min, the samples were pelleted at 2,800 rpm for 30 min, and supernatants were removed. Pellets were resuspended in 0.9 ml of buffer A containing 0.1% NP-40 and 20% glycerol. After addition of 0.5 ml of polyethylene glycol, the tubes were mixed, incubated at 4C for 10 min, and centrifuged again. The supernatants were aspirated, and the pellets were counted in a γ-spectrometer. Nonspecific binding was determined in these measurements as above.

Hormone Measurements

Serum and pituitary LH and FSH levels were determined by immunofluorometric assays as earlier described (44, 45). Intratesticular testosterone and ovarian estradiol and progesterone were determined by homogenizing one (−/−) testis and a weighed portion (approximately half) of one (+/+) or (+/−) testis in 0.5 ml PBS, and a pair of ovaries in 0.2 ml PBS. One hundred microliters of the gonadal homogenates or serum were extracted twice in 2 ml diethyl ether and evaporated to dryness overnight in a fume hood. After reconstitution into PBS, testosterone and progesterone were measured by standard RIAs. Estradiol level was measured by a DELFIA Estradiol kit (Wallac, Inc., Turku, Finland) according to the manufacturer's instruction. Protein concentrations in homogenates were measured using the Bradford method (46).

Statistical Analysis

The Statview program (Windows version 4.57; Abacus Concepts Inc., Berkeley, CA) was used for ANOVA and *t* tests. Significance was set as $P < 0.05$. The values are presented as mean ± SE.

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