Targeted Disruption of Luteinizing Hormone/Human Chorionic Gonadotropin Receptor Gene

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LH/hCG receptors were disrupted by gene targeting in embryonic stem cells. The disruption resulted in infertility in both sexes. The gonads contained no receptor mRNA or receptor protein. Serum LH levels were greatly elevated, and FSH levels were moderately elevated in both sexes; estradiol and progesterone levels decreased but were not totally suppressed in females; testosterone levels were dramatically decreased and estradiol levels moderately elevated in males. The external and internal genitalia were grossly underdeveloped in both sexes. Abnormalities included ambiguous vaginal opening, abdominal testes, micropenis, dramatically decreased weights of the gonads and reproductive tract, arrested follicular growth beyond antral stage, disarray of seminiferous tubules, diminished number and hypotrophy of Leydig cells, and spermatogenic arrest beyond the round spermatid stage. LH/hCG receptor gene disruption had no effect on FSH receptor mRNA levels in ovaries and testes, progesterone receptor (PR) levels in ovaries and androgen receptor (AR) levels in testes. However, it caused a dramatic decrease in StAR and estrogen receptor-α (ERα) mRNA levels and an increase in ERβ mRNA levels in both ovaries and testes. Estradiol and progesterone replacement therapy in females and testosterone replacement in males, to determine whether phenotype and biochemical changes were a consequence of decreased gonadal steroid levels or due to a loss of LH signaling, revealed complete restoration of some and partial restoration of others. Nevertheless, the animals remained infertile. It is anticipated that the LH receptor knockout animals will increase our current understanding of gonadal and nongonadal actions of LH and hCG. (Molecular Endocrinology 15: 184–200, 2001)

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INTRODUCTION

LH from anterior pituitary gland and human (h)CG from human placenta are glycoprotein hormones that belong to a family that includes FSH and TSH (1). LH and hCG also belong to cystine-knot growth factors' family, which includes nerve growth factor, platelet derived growth factor-β, and transforming growth factor-β (2). Members of glycoprotein hormone family are heterodimers of noncovalently bound α- and β-subunits (1). The α-subunit is identical, whereas β-subunits, which specify hormone specificity, are different among these hormones except LH and hCG (1). These two hormones have similar but not identical β-subunits (1). Structural homology between LH and hCG makes them functionally similar (1). The functional similarity comes from the fact that both hormones bind to the same receptors (3, 4). These receptors are single-chain transmembrane glycoproteins that belong to the G protein-coupled receptor family (3, 4). Members of this family have an extracellular hormone binding domain, seven transmembrane spanning regions, and an intracellular region that couple to G proteins (3, 4). The LH/hCG receptor is encoded by a single-copy TATA-less gene (5). It spans more than 70 kbp containing 11 exons and 10 introns (5–7). The first 10 exons encode the extracellular hormone binding domain, and the last exon encodes the rest of the receptor (5–7). The transcription is initiated from multiple sites present 50 to 450 nucleotides upstream from the translation start site (5–7). As a result and also due to differences in polyadenylation and alternate splicing, virtually every LH/hCG receptor-positive tissue/cell contains multiple transcripts (8–10).

In addition to gonads, which contain a high abundance of receptors (1, 11–14), a number of nongonadal tissues (*i.e.* female and male reproductive tract, fetoplacental unit, brain, adrenal zona reticularis, skin, breast, urinary bladder, etc.) also contain low levels of functional LH/hCG receptors (10, 15–22). The gonadal actions of LH and hCG result in an increased synthesis

of steroid hormones in the body, which act on multiple targets, including gonads themselves (1, 11, 13, 14, 23–26). Nongonadal actions of these hormones are diverse and vary with the organ and its physiological state (10, 15–22). There is an unavoidable degree of uncertainty concerning the role of LH *vs.* the role of other hormones in different functions of gonadal and nongonadal tissues. This is due to different hormones acting both sequentially and synergistically. Our long-term goal is to advance current understanding of the total actions of LH and hCG in the body. Toward this goal, we have developed mice in which LH/hCG receptors were completely inactivated by gene targeting in embryonic stem cells. The phenotypes of these animals were characterized, and the effect of steroid hormone replacement therapy on the phenotype reversal was tested.

RESULTS

Generation of LH/hCG Receptor Knockout Mice

A single gene with multiple transcription initiation sites present in the 5′-flanking region encodes multiple transcripts and usually a single protein in virtually every LH/hCG receptor-positive tissue in the body (8–10). To ensure that the gene is completely inactivated, targeting vector was constructed so that a part of the 5′-flanking region containing the promoter region and multiple transcription initiation sites, as well as most of exon 1, would be deleted upon homologous DNA recombination in the host cell. Predicted from the restriction sites in the targeting construct, recombination events in two alleles should give only a 10 kbp fragment by Southern blotting with probe A on genomic DNA digested with *Stu*I. Animals containing one wild-type allele (+/−) should also give an additional 8-kbp fragment. Figure 1B shows that this expectation was met. Further analysis with B or neomycin probes on genomic DNA digested with *Sph*I also confirmed the disruption of LH/hCG receptor gene and integration of neomycin gene into the host cell genome with no rearrangements or extra insertional events (data not shown).

Figure 1C shows that while LH/hCG receptor mRNA could be detected in gonads of +/+ and +/− animals by RT-PCR, it was undetectable in gonads of −/− littermates despite the amplification of glyceraldehyde-3-phosphate dehydrogenase (GAPDH). Analysis of expression changes in genes that are important for gonadal function by semiquantitative RT-PCR demonstrated that targeted disruption of the LH/hCG receptor gene had no effect on FSH receptor mRNA levels in ovaries and testes, progesterone receptor (PR) mRNA levels in ovaries, and androgen receptor (AR) mRNA levels in testes (Fig. 1D). However, it caused a dramatic decrease in steroidogenic acute regulatory protein (StAR) and estrogen receptor-α (ERα) mRNA levels and an increase in ERβ mRNA levels in both ovaries and testes.

Immunostaining for LH/hCG receptor protein revealed high levels in thecal cells, followed by granulosa and luteal cells in the ovaries of +/+ and +/− animals (Fig. 2, a and b). In testes, Leydig cells contain the highest receptor immunostaining (Fig. 2, e and f). In addition, different stages of spermatogenic cells also contained some receptor immunostaining, which is in agreement with earlier studies demonstrating that epididymal and ejaculated sperm contain functional LH/hCG receptors (22, 27, 28). Receptor immunostaining was absent in procedural controls performed on gonads from +/+ animals (Fig. 2, d and h). In contrast to +/+ and +/− animals, gonads of −/− littermates had no detectable receptor immunostaining (Fig. 2, c and g). The lack of receptor protein in the gonads of −/− animals is further confirmed by ligand binding studies demonstrating the absence of ^{125}I-hCG binding in contrast to +/+ and +/− littermates (Fig. 1E). While ovaries contained the same level of binding, testes showed a modest decrease in +/− animals compared with +/+ littermates.

Female and Male Phenotype in LH/hCG Receptor Knockout Animals

The vaginal opening was quite ambiguous in −/− animals (Fig. 3A). The ovaries were small and pale and the reproductive tract was very thin compared with +/+ and +/− littermates at 60 days of age (Fig. 3B). The wet weights of the reproductive tract and ovaries dramatically decreased in −/− animals compared with +/+ and +/− littermates (Fig. 3C).

Null males had abdominal testes and a micropenis with a shorter anogenital distance at 60 days of age compared with +/+ and +/− littermates (Fig. 3D). The size of testes, epididymides, and seminal vesicles dramatically decreased in −/− animals compared with +/+ and +/− littermates (Fig. 3E). The prostates were so hypoplastic that they were barely recognizable even under a dissection microscope and were difficult to dissect from the underlying tissue. The wet weights of testes, epididymides, and seminal vesicles dramatically decreased in −/− animals compared with +/+ and +/− littermates (Fig. 3F).

Gonadal and Nongonadal Morphology in LH/hCG Receptor Knockout Animals

Ovaries of −/− animals contained preantral and antral but no preovulatory follicles or corpora lutea, suggesting a follicular arrest beyond the antral stage (Fig. 4, e and f). The thickness of all uterine layers decreased, and only a few glands were present in endometrium (Fig. 4, k and l). The treatment of these animals with PMSG resulted in a greater number of small antral follicles with no other obvious changes in either ovaries or in the reproductive tract (data not shown).

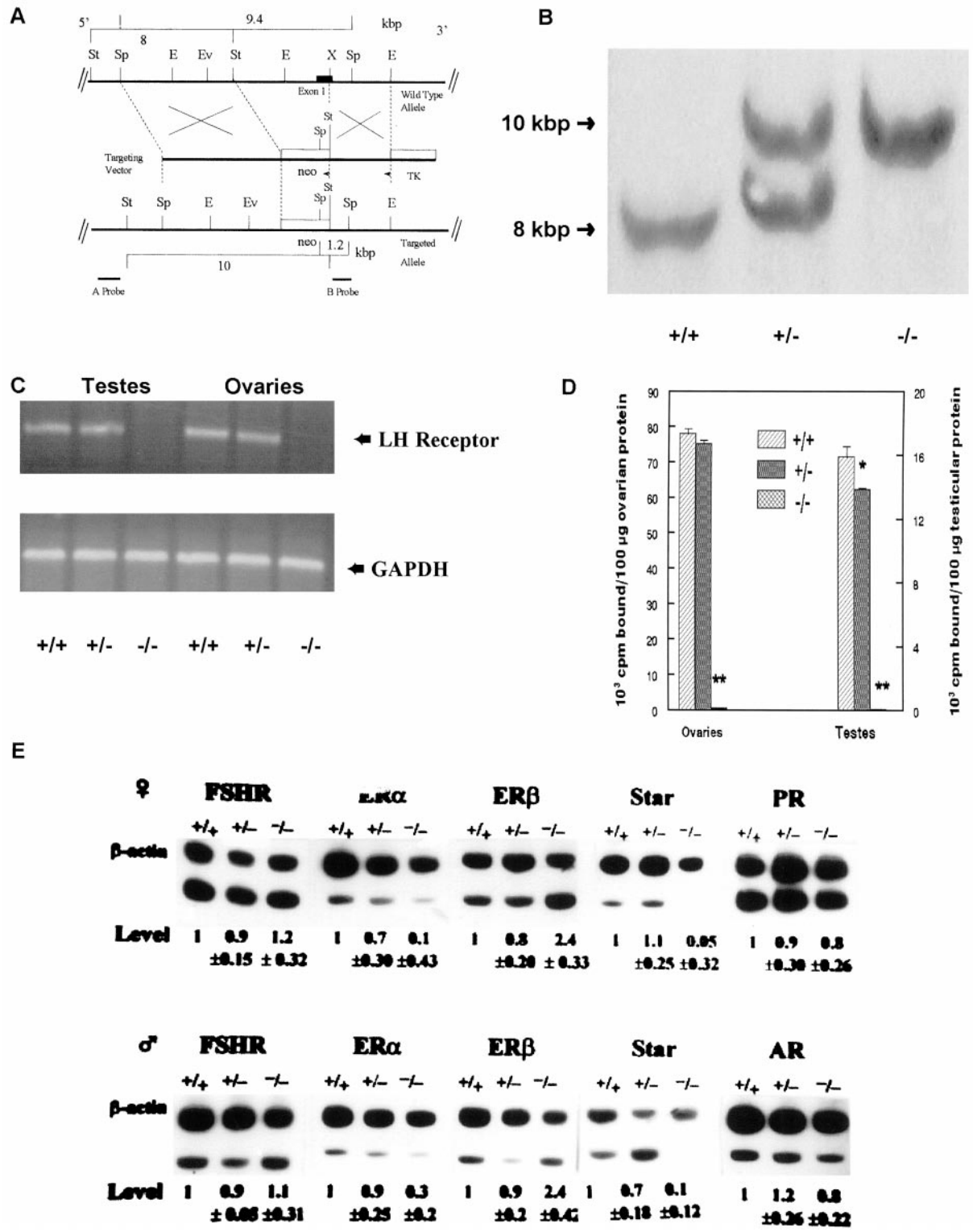

Fig. 1. Generation of LH/hCG Receptor Gene Knockout Mice

A, Construction of a targeting vector. Four kilobase pairs of the LH/hCG receptor gene (St through X) that contained the promoter region and most of exon 1 sequence (*dark bar*) were replaced with the neomycin resistance gene cassette. Two *arrowheads* indicate opposite orientation of neomycin and thymidine kinase genes from transcription of the LH/hCG receptor gene. Two DNA probes designated as A and B were used for screening clones of embryonic stem cells with homologous DNA recombination. Restriction enzyme sites: E, *Eco*RI; Ev, *Eco*RV; Sp, *Sph*I; St, *Stu*I, X, *Xho*I. B, Southern blot analyses of DNA isolated from tail biopsies. DNA was digested with *Stu*I and hybridized with A probe. An 8-kbp wild-type LH/hCG receptor allele and a 10-kbp mutant allele in both +/− and −/− mice are shown. C, Nonquantitative RT-PCR for LH/hCG receptor mRNA. A

Wild-type and +/− animals were indistinguishable in their ovarian (Fig. 4, panels a and b *vs.* panels c and d) and uterine (Fig. 4, panels g and h *vs.* panels i and j) morphology. The presence of corpora lutea indicates the normal progression of follicular growth to ovulation. The uterine wall was thick with normal morphology with numerous glands in endometrium. While both these animal groups were fertile, −/− females were infertile.

The low power histological examination revealed a marked reduction in the seminiferous tubule diameters and a drastic decrease in the Leydig cell number, which were hypotropic in −/− animals compared with +/+ and +/− animals (Fig. 5, panel e *vs.* panels a and c). High-power pictures demonstrate the arrest of spermatogenesis beyond round spermatid stage in homozygous animals (Fig. 5, panel f *vs.* panels b and d).

Epididymal, seminal vesicle, and prostate histology in −/− animals was consistent with marked hypoplasia compared with +/+ and +/− littermates (Fig. 6, panels c, f, i, and l *vs.* panels a, b, d, e, g, h, j, and k). Not only were the epididymal tubule diameters much smaller, they also were completely devoid of sperm, and seminal vesicles and prostates contained fewer acini in −/− animals. The morphology of testes and secondary sex organs was similar between +/+ and +/− males. While both these animal groups were fertile, −/− males were infertile.

Serum Hormone Levels

Serum LH levels were dramatically elevated and FSH levels were moderately elevated in −/− female and male animals compared with +/+ and +/− littermates (Fig. 7, A, B, E, and F). Estradiol and progesterone levels decreased, but were not totally suppressed, in −/− females compared with +/+ and +/− littermates (Fig. 7, C and D). Testosterone levels were dramatically decreased and estradiol levels were moderately increased in −/− males compared with +/+ littermates (Fig. 7, G and H). Hormone levels in +/− were similar to +/+ animals except for a modest elevation in FSH and a decrease in testosterone levels (Fig. 7, G and H).

Effect of Steroid Hormone Replacement Therapy

Twenty one-day estradiol and progesterone replacement therapy of 30-day-old −/− females resulted in normal vaginal development (not shown) but had no effect on ovarian morphology (Fig. 8, a and b). The uterus became thicker; however, the number of endometrial glands remained low (Fig. 8, d *vs.* c). The animals were still infertile.

Twenty one-day testosterone replacement therapy of 30-day-old −/− males resulted in a scrotal descent of testes and growth of the penis (not shown). In addition, therapy caused an enlargement of seminiferous tubule diameters with resumption of spermatogenesis (Fig. 8, f *vs.* e) but failed to restore Leydig cell number or improve hypotrophy. Therapy also improved the morphology of epididymides with enlargement of tubules, which contain spermatozoa, and seminal vesicles and prostate showed increased size and number of acini (Fig. 8, panels h, j, and l *vs.* panels g, I, and k). The size and weight of these organs compared with that of +/+ animals appeared to have been restored, yet the animals remained infertile.

DISCUSSION

LH is important for follicular maturation, ovulation, and increasing the synthesis of ovarian steroid hormones (11–14, 23, 29–32). Males require LH for Leydig cell proliferation and maturation and increasing the synthesis of testosterone, which promotes spermatogenesis and also regulates the function of accessory sex organs (22, 24–26, 33, 34).

Numerous reports from several laboratories demonstrated that LH is also capable of regulating functions of the reproductive tract, brain, skin, mammary glands, adrenal zona reticularis, urinary bladder, cells of immune system, etc. (10, 15–22). Since LH controls the functions of many tissues, it would be important to have either LH receptor-deficient or LH-deficient animal models to further advance current understanding on the total LH actions in the body. The LH receptor deficiency would be a better model than the LH-deficient animal because of the possibility that there might be similar molecule(s) *in vivo* that might interact with the receptors. In any case, LH deficiency through targeted disruption of LH-β subunit gene has not been done.

In the past, investigators have used the approach of LH deprivation to investigate the importance of its actions in reproduction (24, 35–39). Although this approach gave valuable insights, questions remained on how much residual LH is left in circulation to act.

predicted 437-bp LH/hCG receptor fragment was amplified from total RNA of +/+ and +/− mice gonads. This fragment was not detected in −/− mice gonads despite amplification of GAPDH mRNA. D, Specific ^{125}I-hCG binding to ovaries and testes. *, $P < 0.05$; and **, $P < 0.0001$, compared with corresponding wild-type littermates. The data presented were the means ± SEs of measurements on three animals. E, Semiquantitative RT-PCR for FSHR, ERα, ERβ, PR, StAR, and AR mRNAs in mouse gonads. The relative abundance of mRNA levels in +/+, +/−, and −/− mice gonads was determined by scanning the density of each band and expressing as ratios with β-actin. The ratio for each mRNA species in +/+ mice was set at 1 for calculating changes in +/− and −/− littermates. The blots presented are representative and the fold changes (means ± SEs) were calculated from three independent experiments.

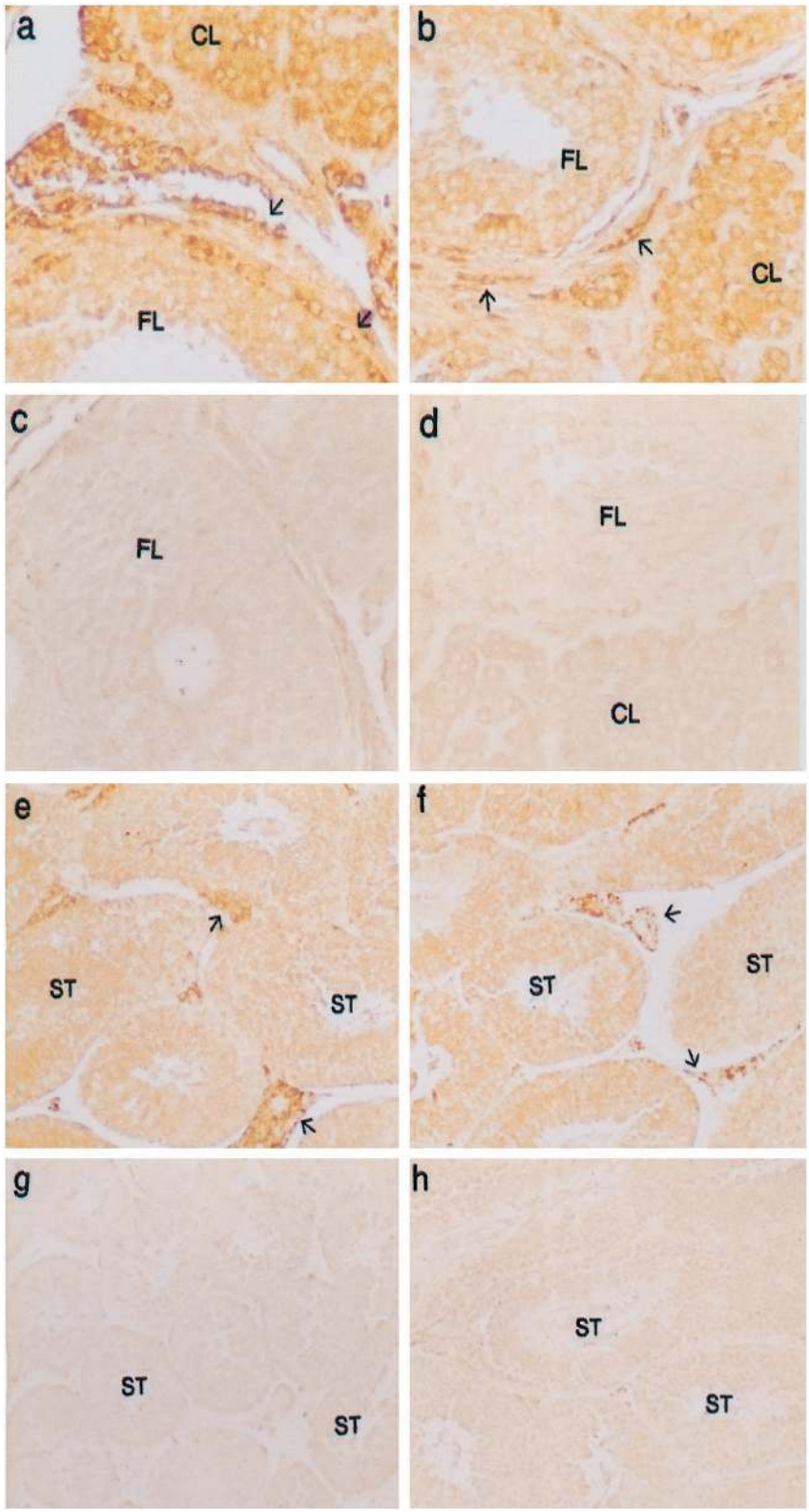

Fig. 2. Immunocytochemistry for LH/hCG Receptors in Ovaries (a, b, c, and d) and Testes (e, f, g, and h) from +/+ (a, d, e, and h), +/− (b and f), and −/− (c and g) Mice

Arrows show strong receptor immunostaining in theca and Leydig cells. Moderate to low receptor immunostaining is also seen in granulosa and luteal cells, spermatogonia, and spermatocytes. Panels d and h are immunostaining controls in which receptor antibody was preabsorbed with excess receptor peptide. FL, Follicle; CL, corpus luteum; ST, seminiferous tubule. Magnification, 300×.

We have inactivated LH receptors by gene targeting in embryonic stem cells. Gonads of these animals had no receptor transcripts or receptor protein. Despite the fact that LH regulates a wide variety of body functions, there was no evidence of increased mortality during embryogenesis or during pre- or postnatal life through at least day 60. This should not be a surprise considering the fact that lethality generally results from inactivation of genes that are required for formation of vital body organs. LH receptor disruption, however, may have affected the quality of life (*i.e*. behavior changes, feeding problems, weakened immune system, skeletal changes, etc.).

LH receptor gene inactivation had no effect on FSH receptor mRNA levels in either ovaries or testes, PR mRNA levels in ovaries, and AR mRNA levels in testes. ERα and StAR mRNA levels decreased and ERβ mRNA levels increased in both ovaries and testes, indicating that, either directly or indirectly, LH maintains ERα and StAR and inhibits ERβ mRNA levels. It is unlikely that gonadal cell type changes could account for all mRNA changes in $-/-$ animals. Reciprocal ER changes in LH receptor knockout animals is consistent with a concept that the two ERs may have different roles in regulating gonadal functions (40, 41). Neither cholesterol side-chain cleavage enzyme nor CYP17 mRNA levels were determined in ovaries or testes of $-/-$ animals.

The LH receptor knockout females were about 50% heavier with a lot of visceral fat at 60 days of age compared with $+/+$ and $+/-$ littermates. Males also showed a lot of visceral fat at a later age ($\sim$120 days), but were lighter which could be due to decreased muscle mass and bone density. Whether these and other changes in LH receptor knockout animals would affect their health and longevity is not known. ERKO and ARKO females were also reported to be heavier with an accumulation of body fat (40, 42). However, it is not known whether the distribution pattern or type of fat would be the same between these and LH receptor knockout animals.

Animals with only one functioning LH receptor allele ($+/-$) were indistinguishable from $+/+$ littermates except for a slight reduction in testicular ^{125}I-hCG binding, moderate reduction in serum testosterone levels, and a modest increase in serum FSH levels in both sexes. Obviously, none of these changes have affected their fertility or litter size; however, whether these would be affected as the animals grow older is not known.

LH receptor knockout animals have normal genitalia except that they were hypoplastic, suggesting that LH signaling is not required for early gonadal and reproductive tract differentiation. LH-independent synthesis of testosterone by early fetal Leydig and Mullerian inhibitory substance by Sertoli cells may allow their differentiation (43, 44). After they are formed, LH signaling seems to be required for their continued development through pre- and postnatal life.

Null mice showed several external and internal phenotypic defects at 60 days of age. For example, female animals are acyclic and had an ambiguous vaginal opening, and their ovaries and reproductive tract were smaller and lighter. Ovarian histology indicated an arrest in folliculogenesis beyond the antral stage, which reaffirms that follicular growth through this stage is independent of LH signaling. The reproductive tract was underdeveloped with a decreased thickness of all uterine layers and few glands in the endometrium.

Null males had a micropenis and abdominal testes. The size and weight of the testes dramatically decreased. The seminiferous tubules were in disarray, their diameters decreased, and spermatogenesis was arrested beyond round spermatid stage. Scarce intertubular connective tissue contained only a few hypotropic Leydig cells, which appeared to be fetal type by the lack of 11β-hydroxysteroid dehydrogenase immunostaining. This indicates that LH signaling is required for adult, but not fetal, type Leydig cell development. All accessory sex organs were small to rudimentary with a dramatic decrease in weight.

Serum LH levels are markedly elevated in $-/-$ animals, which could be due to a loss of estradiol (female) and testosterone (male) negative feedback and/or loss of negative LH feedback on its own secretion through decreased hypothalamic GnRH levels in both sexes (45–47). Moderate elevation of serum FSH levels could be due to decreased gonadal inhibin secretion in both sexes. Continued steroid synthesis in growing follicles through the antral stage under FSH influence may have prevented a greater decrease than was noted in estradiol and progesterone levels. The androgen precursor for this continued estradiol synthesis may come from LH-independent basal synthesis by theca with small amounts made by granulosa cells. Serum testosterone levels were undetectable in $-/-$ females as they were in $+/+$ littermates. Levels in $-/-$ males, on the other hand, dramatically decreased, which reflects Leydig cell hypoplasia and hypotrophy. The low androgen levels seen in these animals could be coming from these few remaining Leydig cells or from their adrenals. The moderate increase in estradiol levels in $-/-$ males could be due to elevated FSH levels driving increased Sertoli cell synthesis and/or decreased estradiol metabolism (48). It is unlikely, however, that they came from aromatization in adipose tissue because androgen precursor levels were not elevated.

We used hormone replacement therapy to determine whether phenotype and biochemical changes were due to decreased gonadal steroid hormone levels, which in females were not totally suppressed, or they resulted from loss of LH signaling. If they were due solely to decreased steroid-hormone levels, then their restoration should correct them. Lack of reversal of ovarian morphology is consistent with a concept that only LH, not estradiol, progesterone or FSH, can stimulate follicular growth beyond the antral stage and induce ovulation. In relation to biochemical changes,

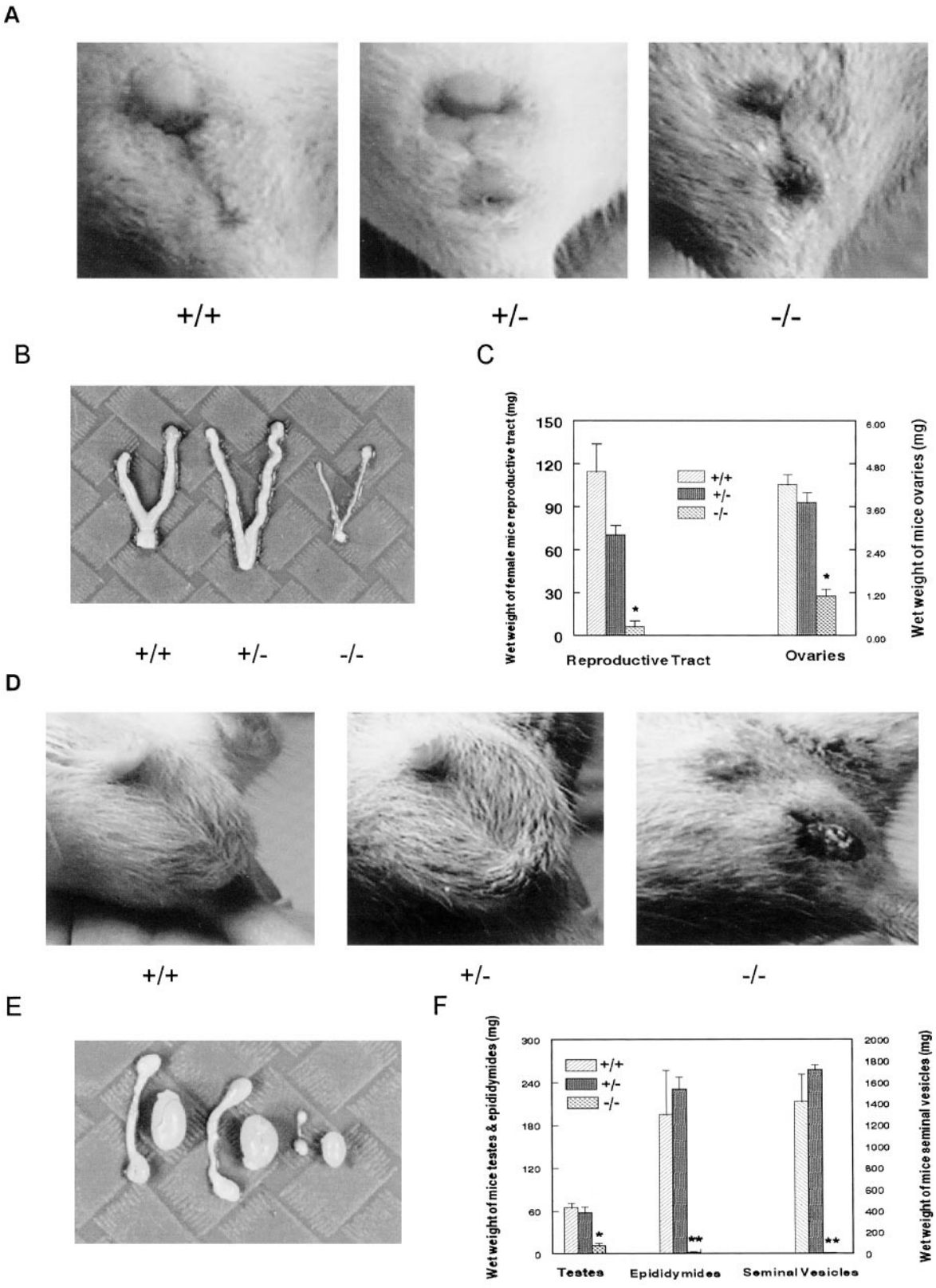

Fig. 3. Female and Male Phenotype in LH/hCG Receptor Gene Knockout Mice

A, Appearance of external female genitalia. Vaginal orifice was difficult to detect in −/− compared with +/+ and +/− littermates. B, Gross appearance of ovaries and reproductive tract. Only one ovary is shown; the other was removed for analysis. Ovaries were small and pale, and the reproductive tract was thin in −/− mice compared with +/+ and +/− littermates. C, Wet weight of reproductive tract and ovaries. *, $P < 0.001$ compared with corresponding tissues from +/+ and +/− littermates. The

only ERα and StAR decreases were reversed, whereas the ERβ increase was not reversed, suggesting that LH signaling was inhibitory and is required to maintain normal ovarian ERβ levels. Since there was no resumption of follicular growth beyond the antral stage and anovulation, $-/-$ animals placed on estradiol and progesterone replacement therapy remained infertile.

Testosterone replacement therapy resulted in the testes descent into the scrotum, suggesting that it is androgen dependent. The penis grew but it remained small compared with $+/+$ animals, suggesting that it is not completely dependent on androgens. The decrease in testicular ERα and StAR and increase in ERβ mRNA levels were not reversed by testosterone replacement therapy, suggesting that LH signaling may be required for their reversal. Neither hyperplasia nor hypotrophy of Leydig cell was corrected. The therapy increased the diameter of seminiferous tubules and the resumption of spermatogenesis. Although sperm number remained relatively low, they were motile. The morphology of other accessory sex organs was improved. Despite these changes, treated $-/-$ males remained infertile even after the length of testosterone replacement therapy was increased to 42 days. There could be many reasons (behavioral and ejaculatory problems) for continued infertility in these animals, which we are now beginning to investigate.

Anovulatory phenotype was seen not only in LH receptor but also in FSH receptor (49, 50), FSHβ (51), ERα (40, 41), ERα/ERβ (41), aromatase (42), PR (52), COX-2 (53), cyclin D2 (54), p27 (Kipl) (55), and glycoprotein hormone α-subunit (56) knockout animals. Various degrees of spermatogenic failure that did or did not affect fertility have been reported after the disruption or overexpression of several genes (40, 42, 49, 57–69). These findings indicate that ovulation and spermatogenesis are the end results of a series of molecular changes controlled by a number of different factors, and disruption of any one of them can result in ovulation and spermatogenic failure.

Targeted disruption of the LH receptor gene resulted in a loss of receptors from uterus, oviduct, brain, skin, mammary gland, urinary bladder, etc. If the phenotype and biochemical changes in nongonadal tissues of LH receptor knockout animals were due solely to decreased steroid hormone levels, which in the case of females were not totally suppressed, then their restoration should correct them. We have obtained data on a few tissues and are in the process of obtaining the rest on the other tissues. As of now, we found that the vagina was underdeveloped in $-/-$ mice but became normal after estradiol and progesterone replacement therapy. This suggests that vaginal development is ovarian steroid hormone dependent. However, although the vaginal cytology seemed to be improved, the number of leukocytes remained markedly low, suggesting that something other than ovarian steroid hormones is required for this reversal. Whether LH signaling is the answer is not known. The reversal of uterine morphology, except the endometrial gland number, suggests that perhaps LH actions may be required for complete gland restoration. Uterine ERβ, but not ERα mRNA, decreased in LH receptor knockout animals. This decrease could not be reversed by estradiol and progesterone-replacement therapy, suggesting that LH may also be required for the reversal of ERβ decrease. Thus, we are beginning to get a sense that ovarian steroid hormones alone may not be adequate to maintain complete phenotype of nongonadal tissues. Thus, LH receptor knockout animals will be useful in further investigating the importance of LH signaling in nongonadal tissues.

Since LH and FSH are indispensable for gonadal regulation, it is of interest to compare the consequences of inactivating one *vs.* the other receptor gene. It turns out that the main similarity was that females in both cases were infertile (49, 57). The main difference was that FSH receptor knockout males had partial spermatogenic failure, reduced fertility, and normal to reduced development of accessory sex organs compared with complete spermatogenic failure, infertility and hypoplastic accessory sex organs in LH receptor knockout males (49, 57, 69). The other notable differences were that the folliculogenesis was arrested at the preantral stage and ovarian ERα and ERβ were unaffected in FSH receptor knockout animals compared with arrest beyond the antral stage, a decrease in ovarian ERα, and an increase in ERβ mRNA levels in LH receptor knockout animals (49, 50, 57). These findings suggest that, while FSH and LH signaling are equally important for female fertility, LH signaling is more important in maintaining male fertility through initiating spermatogenesis via androgens. A dramatic decrease in testosterone levels in LH receptor-inactivated animals compared with FSH receptor-inactivated animals may explain male phenotype differences.

Experimental LH receptor inactivation and naturally occurring inactivating human LH receptor mutations should be expected to give rise to similar phenotypes. Thus, it is of interest to compare similarities and differences between them. The shared similarity was infertility in both sexes (70–74). The phenotype of internal male genitalia and histology was quite similar in

data presented were the means ± SEs of measurements on three animals. D, Appearance of external male genitalia. Null mice had micropenis and no scrotal testes compared with $+/+$ or $+/-$ littermates. E, Gross appearance of testes and accessory glands. Testes were small and the size of epididymides, seminal vesicles, and prostate dramatically decreased in $-/-$ mice compared with $+/+$ or $+/-$ littermates. Prostate could not even be recognized by the naked eye and was difficult to dissect from the underlying tissue. F, Wet weight of testes and accessory glands. *, $P < 0.01$; and **, $P < 0.001$, compared with corresponding tissues from $+/+$ and $+/-$ littermates. The data presented were the means ± SEs of measurements on three animals.

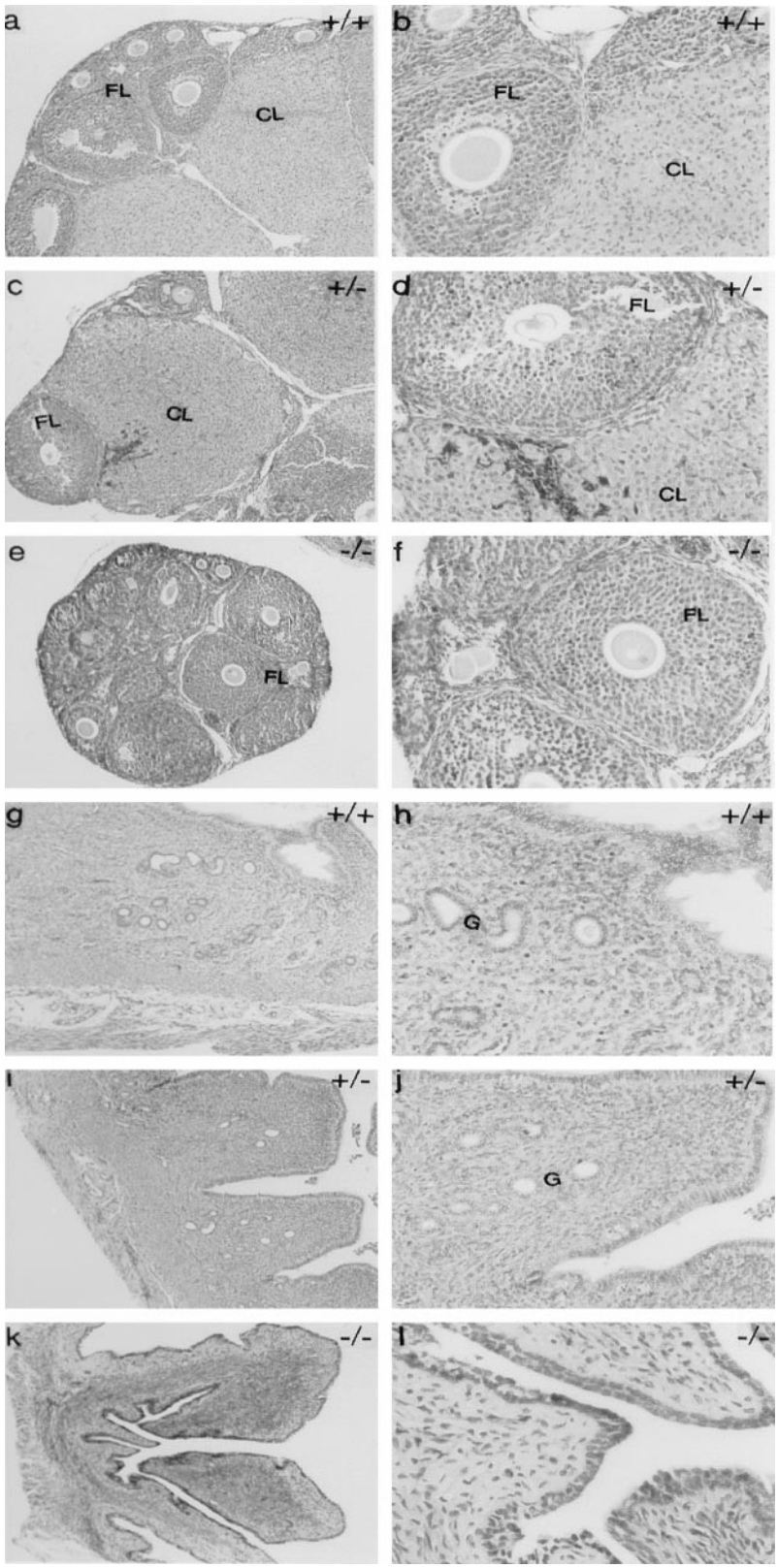

Fig. 4. Histology of Ovaries (a–f) and Uteri (g–l)

Preantral and antral follicles, but not preovulatory follicles or corpora lutea, were present in ovaries (e and f) and very few glands present in the endometrium (k and l) of $-/-$ mice. Wild-type and $+/-$ mice were indistinguishable in their ovarian (a, b, c, and d) and uterine (g, h, I, and j) morphology. CL, Corpus luteum; FL, follicle; G, endometrial gland. Magnification: panels a, c, e, g, i, and k, 60×; panels b, d, f, h, j, and l, 300×.

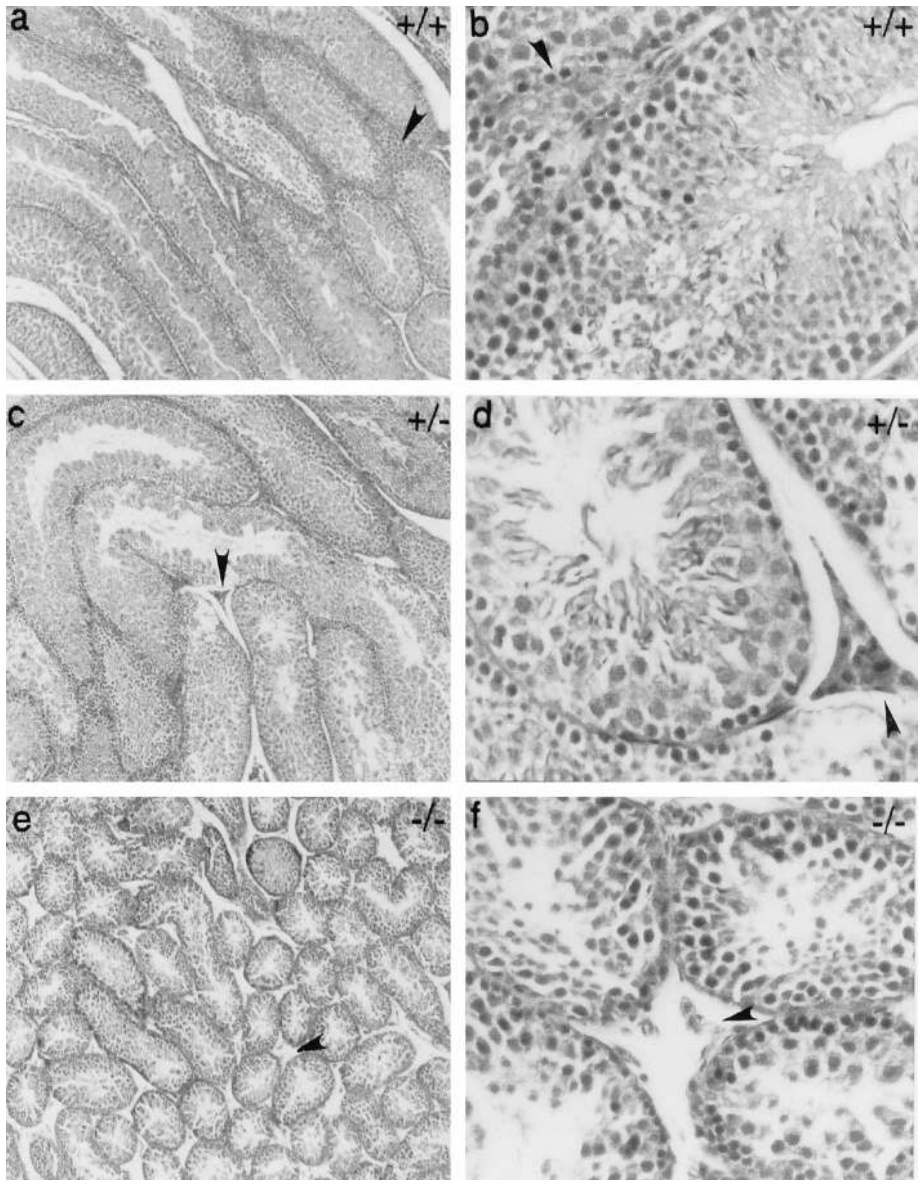

Fig. 5. Histology of Testes

Null mice contained fewer hypotrophic Leydig cells, and sperm were absent in seminiferous tubules compared with +/+ and +/− littermates, in which sperm were indistinguishable. *Arrowheads* indicate Leydig cells. Magnification: panels a, c, and e, 60×; panels b, d, and f, 300×.

both cases. The differences were that affected men have external female genitalia (except breast development), which were not seen in LH receptor knockout animals. Affected women presumably had normal external and internal genitalia, both of which were dramatically underdeveloped in LH receptor knockout mice. Some of these differences could reflect developmental differences between mice and humans, while others may reflect partial instead of complete inactivating human mutations (75, 76).

In summary, LH/hCG receptor gene disruption resulted in female and male infertility with several external and internal phenotypic defects and gene expression changes in gonads. Infertility could not be reversed by hormone replacement therapy even though some of the phenotypic defects were corrected. The LH receptor-disrupted animals are anticipated to increase our current understanding of gonadal and nongonadal actions of LH and hCG.

MATERIALS AND METHODS

Construction of the Targeting Vector

Based on the published sequence of 2.2 kbp 5′-region of the mouse LH/hCG receptor gene (77), a 199-bp DNA fragment (131–329 bp) was designed as a probe to screen a mouse

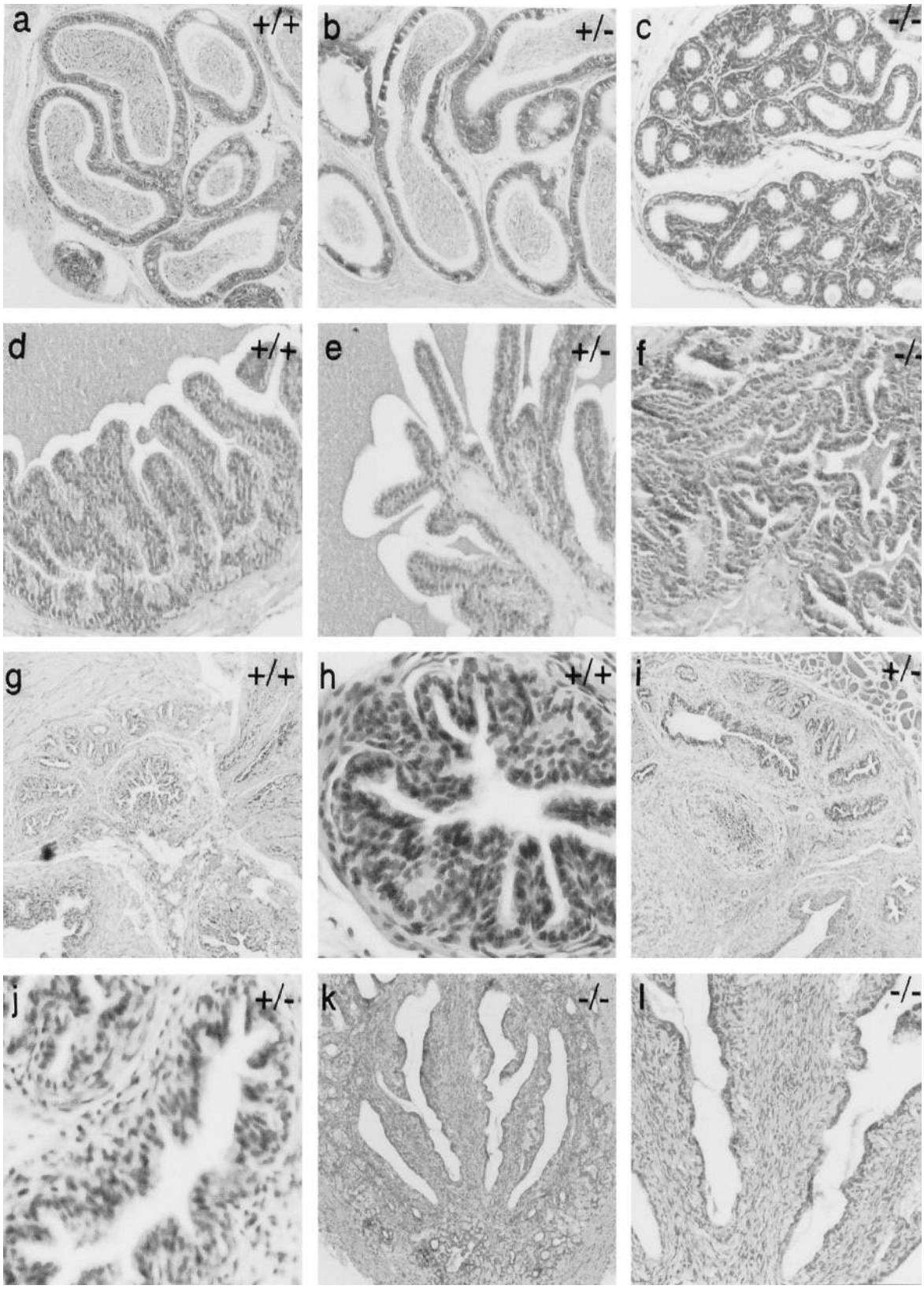

Fig. 6. Histology of Epididymides (a–c), Seminal Vesicles (d–f), and Prostates (g–l)

All the accessory organs of −/− mice were hypoplastic compared with +/+ and +/− littermates in which accessory organs were indistinguishable. Magnification: panels a–f, g, i, and k, 60×; h, j, and l, 300×.

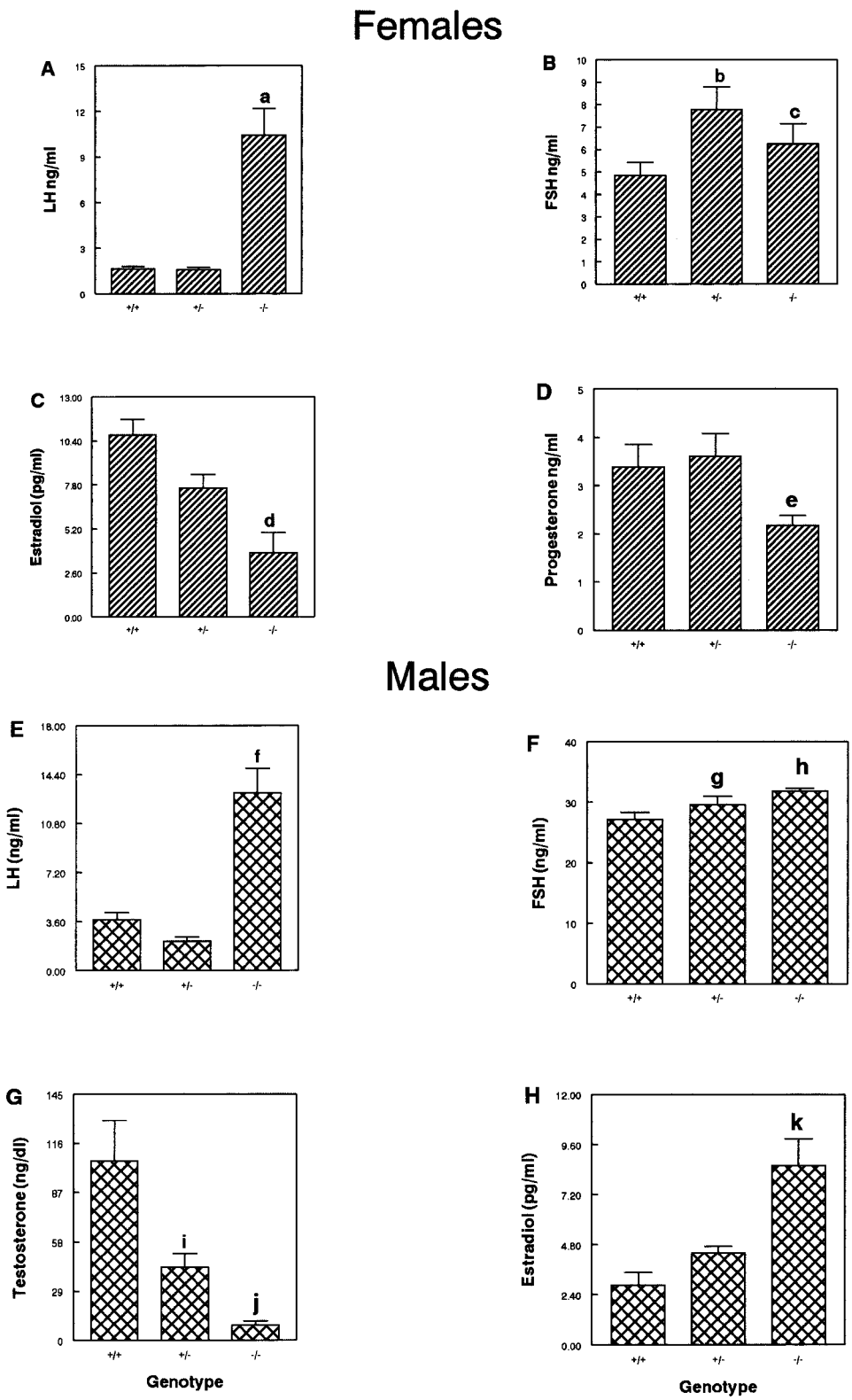

Fig. 7. Serum Hormone Levels of Gonadotropins and Steroid Hormones

a and f, [−/−] *vs.* [+/+] and [+/−] at $P < 0.0001$; b and g, [+/−] *vs.* [+/+] at $P < 0.05$; c and h, [−/−] *vs.* [+/+] and [+/−] at $P < 0.05$; d and j, [−/−] *vs.* [+/+] at $P < 0.05$; e and k, [−/−] *vs.* [+/+] and [+/−] at $P < 0.05$; i, [+/−] *vs.* [+/+] at $P < 0.01$. The data presented were the means ± SEs of measurements on four to eight animals in each group.

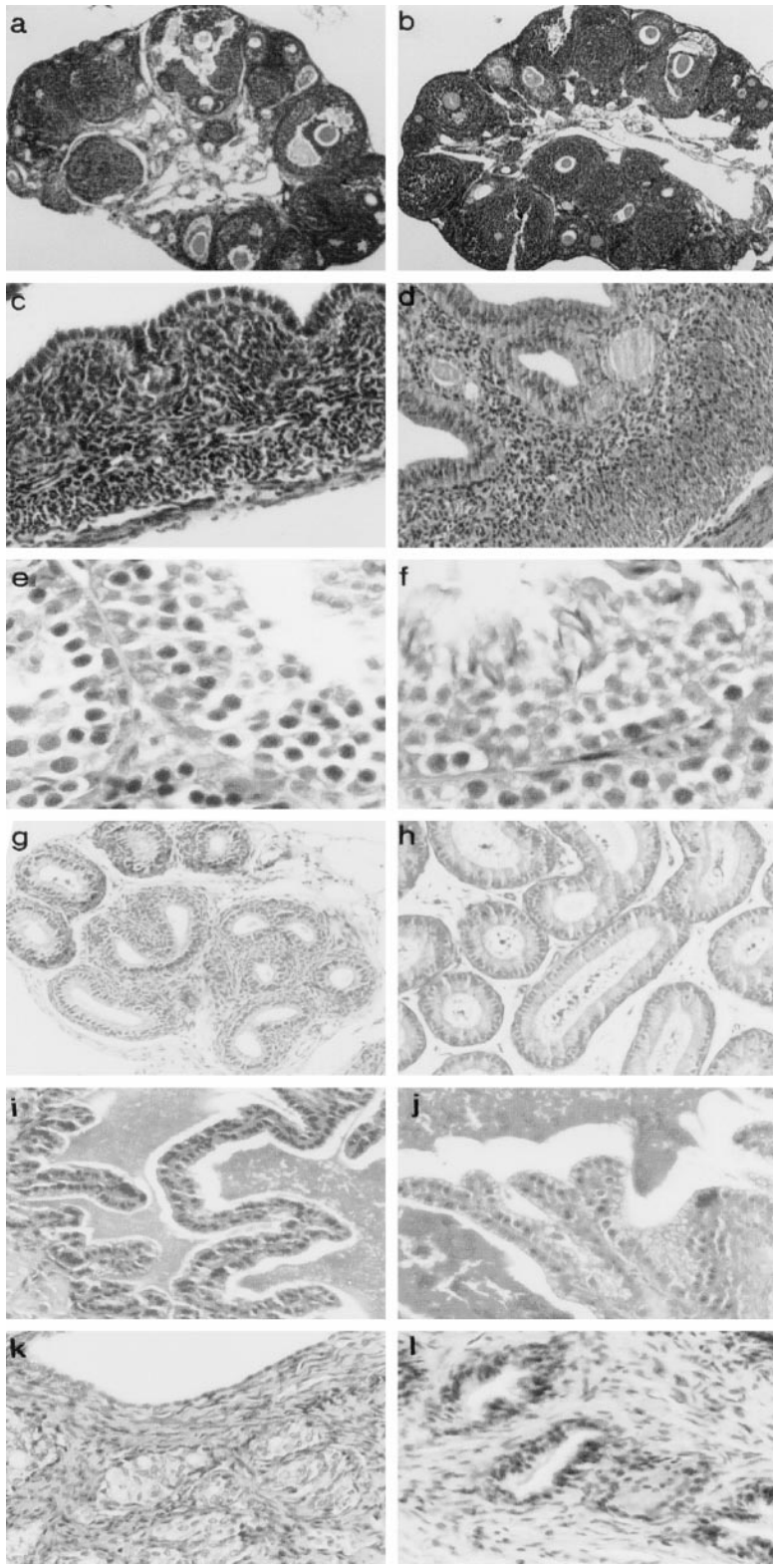

Fig. 8. Histology of Ovaries (a and b), Uteri (c and d), Testes (e and f), Epididymides (g and h), Seminal Vesicles (i and j), and Prostates (k and l) in Estrogen and Progesterone (b and d), Testosterone (f, h, j, and l) Replaced and Placebo-Treated (a, c, e, g, i, and k) −/− Mice

Hormone replacement therapy had improved uterine, testicular, epididymal, seminal vesicle, and prostate morphology. However, the number of endometrial glands remained low and the ovarian morphology was unaffected. There was a resumption of spermatogenesis, but the sperm numbers remained low. Germ cells in both testes and epididymis showed high pyknosis. Magnification: a and b, 60×; c, d, and g–l, 300×; e and f, 800×.

ES-129/OLA P1 genomic DNA library (Genome Systems, St. Louis, MO). The DNA fragment containing about 10.1 kbp of the 5′-region, 0.5 kbp of the first exon, and 2.5 kbp of the first intron sequence have been identified. A pPNT vector (a gift from Dr. Colin Funk, University of Pennsylvania, Philadelphia, PA) that contains PGKneomycin (PGKneo) and PGKthymdine kinase (PGKtk) cassettes, separated and flanked by a number of unique cloning sites, was used to construct the LH/hCG receptor targeting vector. PGKneo served to insert DNA fragment for disruption of the LH/hCG receptor gene and also as a positive selection marker. PGKtk was included for negative selection marker. A 4.6-kbp fragment of the 5′- region of the LH/hCG receptor gene was subcloned downstream of PGKneo cassette and a 2.5-kbp fragment containing only 21 bp of exon 1 and a part of intron 1 of the LH/hCG receptor gene was inserted between PGKneo and PGKtk gene cassettes. The orientation of PGKneo and PGKtk gene cassettes was in the opposite direction of the LH/hCG receptor gene to avoid any possibility of fake activation of the LH/hCG receptor gene by PGKneo promoter (Fig. 1). The predicted targeted recombination event would replace the 5′-flanking region and most of the exon 1 sequence (4 kbp) of the LH/hCG receptor gene with PGKneo sequence (1.8 kbp).

Generation of LH/hCG Receptor Knockout Mice

The targeting vector was linearized at a unique *Not*I site that lies outside the homologous sequences. Thirty micrograms of linearized DNA were electroporated into 1×10^7 of 129/SVJ embryonic stem (ES) cells (Genome Systems)at 185 V and 500 mF (BTX ECM-600, Genetronics, Inc., San Diego, CA). Then, ES cells were grown on a feeder layer of mitomycin-inactivated mouse embryonic fibroblasts (Genome Systems) and selected in medium containing 350 μg/ml G418 and 2 mM ganciclovir (a generous gift from Roche Products Ltd, Welwyn Garden City, Herfordshire, UK). A total of 264 doubly-resistant ES clones was genotyped by digesting 10 μg of their genomic DNA with *Stu*I or *Sph*I and Southern blotting with [^{32}P]-labeled 5′- (A probe), 3′- (B probe), or neomycin probes (Fig. 1). Southern blotting with all three probes confirmed the disruption of LH/hCG receptor gene and the integration of neomycin gene into host genome.

Chimeric mice with ES cells carrying the disrupted LH/hCG receptor allele were generated by microinjection of 3.5-day-old C57BL/6 blastocysts, which were transferred into the uteri of pseudopregnant recipient mice (University of Cincinnati Gene Targeted Mouse Service Center, Cincinnati, OH). Chimeric animals were mated with C57BL/6 (Taconic Farms, Inc., Germantown, NY) or 129/SVJ (Charles River Laboratories, Inc. Wilmington, MA) partners. Agouti offspring were genotyped by digesting 10 μg of their tail genomic DNA with *Stu*I and/or *Sph*I and Southern blotting with [^{32}P]-labeled A or B probes. Although the results of DNA digestion with *Stu*I and hybridization with A probe are presented, predicted DNA fragments (wild-type, 9.4 kbp, and targeted allele, 1.2 kbp) were obtained when digested with *Sph*I and Southern blotted with B probe. The use of neomycin probe on *Sph*I-digested DNA showed no wild-type fragment and targeted allele fragments of 5.9 and 1.2 kbp.

Male and female +/− animals were crossed to obtain −/− mice, who were also genotyped by Southern blotting with the same probes. The crossing of +/− animals had no obvious effect on litter size. Among the litter, approximately 25% were +/+, 50% were +/−, and 25% were −/−, indicating that there was no increased intrauterine mortality among −/− fetuses.

All animals were housed in rooms with 12-h light, 12-h dark cycles with free access to food and water. Estrous cycles were monitored by daily vaginal smears and +/− and +/+ animals were killed on the day of proestrus. The developmental status of external and internal genitalia was determined at 60 days of age. At least 48 +/+, 48 +/−, and 79 −/− animals were used in these studies. Included in the count of −/− animals were 20 animals that were placed on hormone replacement therapy.

RT-PCR

A nonquantitative procedure was used for detection of LH/hCG receptor mRNA, and a semiquantitative procedure was used for mRNAs of the others (78). For both, total RNA was isolated using a single-step acid guanidinium thiocyanate-chloroform extraction method. Master Amp RT-PCR kits (Epicentre Technologies Corp., Madison, WI) were used for cDNA synthesis and amplification. Briefly, cDNA was synthesized from 5 μg RNA using 3′-primer of mouse LH/hCG receptor cDNA (765 to 785 bp, 5′-AGTGAGTAGGATGACGTGGCG-3′). The cDNA was then amplified for 40 cycles with 5′-LH/hCG receptor primer (347 to 367 bp, 5′-CCTGCTATACATT-GAACCCGG-3′). The RNA was also amplified using housekeeping gene GAPDH primers to verify the integrity of isolated RNA samples.

For semiquantitative RT-PCR, 2 μg of total RNA were reverse transcribed into cDNA with oligo dT primer and AMV reverse transcriptase (Invitrogen, San Diego, CA). The cDNA was then coamplified with β-actin primers, [^{32}P]-dCTP and one of the following primer sets (top strand is 5′-primer and bottom strand is 3′-primer). All PCR primers were designed from published mouse sequences using a Designer PCR computer program (Research Genetics, Inc., Huntsville, AL) and synthesized by Operon Technologies Inc. (Alameda, CA). Optimal conditions and a PCR cycle number for each set of primers were predetermined to ensure that coamplification was within linear range.

ERα: 5′-CACATTCCTTCCTTCCGTCTTA-3′
and 5′-TCGGGGTAGTTGAACACAGTG-3′

ERβ: 5′-ACCAGGACTTACTGCTGAATGC-3′
and 5′-GTAGGAATGCGAAACGAGTTGA-3′

PR: 5′-TCTACCCGCCATACCTCAACT-3′
and 5′-CTTACGACCTCCAAGGACCAT-3′

Androgen receptor (AR): 5′-ATGGGACCTTGGATGGAG-AA-3′
and 5′-CCCTGCTTCATAACATTTCCG-3′

FSH receptor (FSHR): 5′-TTGTGGTCATCTGTGGTTGC-T-3′
and 5′-GCCAAACTTGCTCATCAGGA-3′

StAR: 5′-GGAACCCAAATGTCAAGGAG-3′ and 5′
and CTGAGCAGCCAAGTGAGTTTAG-3′

PCR products were resolved by electrophoresis in agarose (nonquantitative) or polyacrylamide gels (semiquantitative), and both ethidium bromide staining (nonquantitative) and autoradiography (semiquantitative) identified the bands. Intensities of the bands were quantified by a Z-gel Scanning System (Zaxis Inc., Hudson, OH) and expressed as ratios with β-actin.

Immunocytochemistry

This procedure was performed by an avidin-biotin immunoperoxidase method (45, 79). The polyclonal LH/hCG receptor antibody raised against a synthetic N terminus amino acid sequence of 15–38, kindly provided by Dr. Patrick Roche, at the Mayo Clinic (Rochester, MN), was used at 1:500 dilution. Preabsorption of the receptor antibody with excess receptor peptide and omission or substitution of unabsorbed receptor antibody with normal rabbit serum were used for immunostaining controls.

Ligand Binding Assays

Unlabeled hCG (CR-127 from the National Hormone and Pituitary Program supported by NIDDK, NICHHD, and US Department of Agriculture) was radioiodinated by the lactoperoxidase technique (80). One hundred microgram protein

aliquots of gonadal homogenates were incubated for 2 h at 38 C with 1×10^5 cpm [^{125}I]hCG in the presence or absence of 5 μg/ml of unlabeled hCG. Receptor-bound [^{125}I]hCG was separated from free hormone by centrifugation for 20 min at 5,000 $\times$ *g,* and the radioactivity in the pellets was counted.

Hormone Assays

Mice were anesthetized with ether and exsanguinated by cardiac puncture. Sera were separated and stored at −80 C until assayed. LH, FSH, estradiol, progesterone, and testosterone levels were measured in duplicate using immuno- or RIA kits [LH and FSH kits from Amersham Pharmacia Biotech (Arlington Heights, IL) and estradiol, progesterone, and testosterone kits from Diagnostic Products, (Los Angeles, CA)]. All assays were performed according to procedures provided by the manufacturers. The inter- and intraassay coefficients of variations were within 5–15%.

Histological Analysis

Tissues were fixed in 10% formalin overnight and embedded in paraffin. Then, 5 μm thick tissue sections were cut and stained with hematoxylin and eosin and examined under bright-field microscopy and photographed.

Hormone Replacement Therapy

Twenty-one day release 3-mm pellets (Innovative Research of America, Sarasota, FL) were implanted subcutaneously at the back of the neck of 30-day-old −/− animals with a precision trocar. The pellets integrate principles of diffusion, erosion, and concentration gradient, resulting in a biodegradable matrix that effectively and continuously releases the hormones. Testosterone (5 mg) pellets were used in males, and pellets containing 0.1 mg 17β-estradiol and 5 mg progesterone were used in females. Placebo pellets were implanted into control −/− animals. The animals were killed at the end of 21 days and tissues were removed. The serum hormone levels were in the physiological range in hormone-replaced animals.

Statistical Analysis

The data presented are the means ± SEs. ANOVA and Duncan's multiple range tests were used for determination of significant difference (81).

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